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Interaction of enrofloxacin with breast cancer resistance protein (BCRP/ABCG2): influence of flavonoids and role in milk secretion in sheep

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The ATP-binding cassette (ABC) transporter breast cancer resistance protein (BCRP)/ABCG2 is a high-capacity efflux transporter with wide substrate specificity located in apical membranes of epithelia, which is involved in drug availability. BCRP is responsible for the active secretion of clinically and toxicologically important substrates to milk. The present study shows BCRP expression in sheep and cow by immunoblotting with MAb (BXP-53). Vanadate-sensitive ATPase activity with specific BCRP substrates and inhibitors was measured in bovine mammary gland homogenates. To assess the role of BCRP in ruminant mammary gland we tested the fluoroquinolone enrofloxacin (ENRO). In polarized cell lines, ENRO was transported by Bcrp1/BCRP with secretory/absorptive ratios of 6.5 and 2 respectively. The efflux was blocked by the BCRP inhibitor Ko143. ENRO pharmacokinetics in plasma and milk was studied in sheep after co-administration of drug (2.5 mg/kg, i.v.) and genistein (0.8 mg/kg, i.m.) or albendazole sulfoxide (2 mg/kg, i.v) as BCRP inhibitors. Concomitant administration of BCRP inhibitors with ENRO had no significant effect on the plasma disposition kinetics of ENRO but decreased ENRO concentrations in milk.

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INTRODUCTION

The ATP-binding cassette (ABC) transporter breast cancer resistance protein (BCRP)/ABCG2 is a high-capacity efflux transporter with broad substrate specificity. In human tissues BCRP has been located in a variety of normal tissues and on the apical side of ducts and lobules in the breast (Maliepaard *et al.*, 2001; Jonker *et al.*, 2005). The tissue distribution of BCRP overlaps extensively with that of P-glycoprotein, which suggests a similar role in substrate transport (Schinkel & Jonker, 2003). BCRP/Bcrp1 affects the intestinal uptake of drugs administered orally and their hepatobiliary excretion (Allen *et al.*, 2002; Mizuno *et al.*, 2003). In addition, BCRP is responsible for the active secretion of clinically and toxicologically important substrates to milk (Jonker *et al.*, 2005).

In veterinary therapy anti-parasitic drugs such as ivermectin, selamectin, moxidectin or oxfendazole and albendazole sulfoxide (ABZSO) are transported by P-glycoprotein or BCRP/ABCG2 (Laffont *et al.*, 2002; Griffin *et al.*, 2005; Merino *et al.*, 2005a). Their inhibition increases drug bioavailability in sheep (Dupuy *et al.*, 2003; Merino *et al.*, 2003).

BCRP presence in ruminants could affect the efflux of hydrophobic toxins and drugs, including their active secretion to milk and a reduction in the withdrawal time of the drug milk residues. Recently, it has been shown that polyphenols, which are present in vegetables and plants, interact with BCRP (Cooray *et al.*, 2004; Zhang *et al.*, 2004). These compounds are present in forage of ruminants (in tannins, gossypol, fagopyrine) (Broderick, 1995). Enrofloxacin (ENRO) is a synthetic antibacterial agent of the fluoroquinolone family used in both human and veterinary medicine. ENRO is partly de-ethylated *in vivo* to ciprofloxacin, which is also pharmacologically active, and a recently known BCRP substrate (Merino *et al.*, 2006). Both ENRO and ciprofloxacin are excreted in milk by ruminants (Kaartinen *et al.*, 1995; Haritova *et al.*, 2003; Sooud, 2003). Maximum residue limits for ENRO (as sum of ENRO and ciprofloxacin) in milk is 100 μg/kg (Commission Regulation EC No. 1181/2002).

Here we examine the polarized canine kidney cell line MDCK-II and its subclones transduced with murine Bcrp1 and human BCRP cDNA cell lines, to test the possible role of murine Bcrp1 and human BCRP in the transport of ENRO *in vitro*. The influence of flavonoids was assessed by inhibition of ENRO

transport in the transduced cultures. Expression of BCRP in the mammary gland of lactating cow and sheep was analyzed by Western blot and also by measuring vanadate-sensitive ATPase activity. The pharmacokinetics of ENRO in plasma and milk was examined after administration of the drug to sheep in combination with ABZSO or genistein.

MATERIALS AND METHODS

Reagents and drugs

Mouse monoclonal anti-BCRP (BXP-53), and biotinylated horse-antimouse antibody, were obtained from MONOSAN® SANBIO b.v. (Uden, The Netherlands). Polyclonal rabbit anti-rat immunoglobulin G/horseradish peroxidase (IgG/HRP) was purchased from DakoCytomation A/S (Glostrup, Denmark). Enrofloxacin (ENRO) was obtained from Bayer AG (Leverkusen, Germany) (Baytril 5% solution). Albendazole sulfoxide (ABZSO) was obtained from Microsules Argentina SA (Buenos Aires, Argentina; ricobendazole 15%. ABZSO is not registered in Europe and its only use is experimental. Genistein and daizcein were purchased from Sigma Chemical Co. (Steinheim, Germany); Ko143, an analog of fumitremorgin C, has been described as a powerful inhibitor of the ABCG2-mediated multidrug resistance and was used as 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 BCRP- and murine Bcrp1-transduced MDCK-II subclones were as described previously (Jonker *et al.*, 2000; Pavek *et al.*, 2005). The MDCK-II cells and transduced subclones were kindly supplied by Dr A.H. Schinkel from the Netherlands Cancer Institute. The cells were cultured in Dulbecco's modified Eagle's medium containing glutamax (Life Technologies, Inc., Rockville, MD, USA) 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 3–4 days for subculturing.

Transport assays

Transport assays were carried out as described (Merino *et al.*, 2005a), with minor modifications. Cells were seeded on microporous membrane filters (3.0 μm pore size, 24 mm diameter; Transwell 3414; Costar, Corning, NY, USA) at a density of 1.0×10^6 cells per well. Cells were grown for 3 days, and medium was replaced daily. Transepithelial resistance was measured in each well using a Millicell ERS ohmmeter (Millipore, Bedford, MA, USA); 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 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. The experiment was started ($t = 0$) by replacing the medium in either the apical (AP), or basolateral (BL) compartment with fresh Optimem medium, either with or without 1 μM Ko143 and containing 10 μM ENRO. Cells were incubated at 37 °C in 5% CO_2 and 100-μL aliquots were taken at $t = 2$ and 4 h, and stored at −20 °C until the time of analysis. The appearance of ENRO in the opposite compartment was measured by high-performance liquid chromatography (HPLC) as described below, and results are showed as the fraction of total ENRO added at the beginning of the experiment.

The percentage of inhibition of ENRO transport with flavonoids genistein and daidzein (50 μM) was calculated according to the following method (Merino *et al.*, 2005a; Pavek *et al.*, 2005).

$$\text{Inhibition (\%)} = \frac{\begin{array}{c}\text{BA/AB at 4 h with tested flavonoid}\\ -\text{BA/AB at 4 h without inhibitor}\end{array}}{\begin{array}{c}\text{BA/AB at 4 h with Ko143}\\ -\text{BA/AB at 4 h without inhibitor}\end{array}}.$$

Accumulation assays

In vitro accumulation assays were carried out as described previously (Pavek *et al.*, 2005). In brief, 5 μM mitoxantrone (MXR) was used as fluorescent substrate and ENRO was used as inhibitor (200 μM). Cells were cultured in 12-well plates (20×10^3 cells/well) in complete medium for 36 h to subconfluence. Medium was aspirated, and cells were preincubated in prewarmed Optimem medium with or without ENRO for 60 min before adding 5 μM MXR. The mixture was incubated for 1 h at 37 °C. Cells were washed in ice-cold phosphate-buffered saline (PBS) and trypsinized. Collected cells were sedimented by centrifugation and resuspended in PBS with 2.5% of fetal calf serum. Relative cellular accumulation of MXR was determined by flow cytometry using a FACSCalibur cytometer. Fluorescence of accumulated substrate in populations of at least 5000 cells was quantified from histogram plots using the median of fluorescence (MF). BCRP inhibition increases accumulation of MXR in Bcrp1/BCRP-transduced cells and thus increases MF. Possible background fluorescence from fluoroquinolone was checked in appropriate channels, but was found to be negligible in all cases. Flow cytometry data were processed and analyzed using WinMDI ver.2.8 software [TSRI (The Scripps Research Institute) da Jolla, CA, USA].

BCRP-Western blot analyses in cow and sheep

Merino ewes weighing 44 ± 8 kg obtained from the University research farm and lactating Friesian cows weighing 330 ± 10 kg from the local slaughterhouse were used. The mammary gland from lactating and nonlactating ewes, and mammary gland from lactating cows were collected and rapidly frozen in nitrogen liquid.

The Western blot analyses followed Rocchi *et al.* (2000). Samples (0.5 g) were homogenized in 5 mL of buffer containing 1 M Tris, 1 M sucrose and 0.5 M ethylenediaminetetraacetic acid, and centrifuged for 15 min at 3000 ***g*** at 4 °C. The membranes were sedimented by centrifugation at 100 000 ***g*** for 30 min. The pellet was resuspended in 0.5 mL of homogenization buffer and protein concentration was measured by Bradford assay. Protein was fractionated on 8% polyacrylamide gels with a 5% stacking gel and subsequently transferred electrophoretically to nitrocellulose membranes. Proteins were hybridized using BXP-53 and HRP-conjugated rat anti-mouse IgG, and were detected by chemiluminescence ECL. The public domain Scion Image Beta 4.02 Win (Scion Corporation, Frederick, MD, USA) was used to measure OD values.

Membrane ATPase activity was measured by colorimetric detection of inorganic phosphate liberation as described by Janvilisry *et al.* (2003) in homogenates of cow mammary gland. Membranes were prepared according to Maliepaard *et al.* (2001). Briefly, tissue samples were homogenized in a Polytron Homogenizer in 50 mM Tris (pH 7.0), containing 50 mM mannitol, 2 mM ethyleneglycoltetraacetic acid (EGTA) and protease inhibitor cocktail (Roche Diagnostics, S.L., Barcelona, Spain). The homogenate was initially centrifuged at 500 ***g*** for 10 min. The supernatant was then centrifuged for 60 min at 100 000 ***g***, and the pellet was resuspended in homogenization buffer at a protein concentration of 8–10 μg/mL. All procedures were carried out at 4 °C, and the membranes were stored at −80 °C. The reaction mixture of ATPase activity contained 50 mM Mops-Tris (pH 7.0), 1 M KCl, 0.1 M dithiothreitol, 100 mM EGTA-Tris (pH 7.0), 0.1 M Na-azide, 10 mM ouabain and 7 μg membrane protein. The reaction was initiated by the addition of 5 mM MgATP and the plate was incubated at 37 °C for 40 min. The vanadate-sensitive fraction was determined in the presence of 1.2 mM Na-orthovanadate (difference of the two values, without and with Na-orthovanadate, means the vanadate-sensitive component). The reaction was stopped by the addition of sodium dodecyl sulfate 5%. The detection reagent contained 35 mM ammonium molybdate and 10% of ascorbic acid. Absorbance was measured at 630 nm.

Pharmacokinetic studies

Twelve lactating Assaf-breed sheep (3–4 months in lactation) aged 2–3 years and weighing 40–45 kg were used. They were regularly milked twice daily. The animals were parasite free and for 3 weeks before administration of the drugs, they were fed on the same diet as that given during the experiment (a mixture of dehydrated alfalfa and dehydrated maize). Drinking water was available *ad libitum*. The experimental design was based on Sooud (2003); animals were divided into three groups of four. The first group received a single dose of 2.5 mg/kg b.w. of ENRO through the left jugular vein. The second and third groups were injected with genistein (0.8 mg/kg, i.m.) and ABZSO (2 mg/kg, i.v.) co-administered with ENRO at the same dose (2.5 mg/kg). Blood samples were collected from the jugular vein not used for drug administration before and 10, 20, 30, 45, 60 min and 2, 4, 6, 8, 10, 12 and 24 h thereafter. The plasma was separated by centrifugation at 1200 ***g*** for 15 min and stored at −20 °C until analysis. Milk samples were collected after complete milking of the gland before each treatment and at intervals of 30, 60 min and 2, 4, 6, 8, 10, 12 and 24 h thereafter. Samples were stored at −20 °C until analysis.

Calculation of the P_{app} of ENRO across MDCK cells

The apparent permeability coefficients (P_{app}) across MDCKII parent, m-Bcrp1MDCKII and H-BCRP MDCKII cell monolayers in both AP-to-BL ($P_{app\ A\text{-}B}$) and BL-to-AP ($P_{app\ B\text{-}A}$) directions were calculated as follows:

$$P_{app} = \frac{\Delta Q}{\Delta t}\frac{1}{AC_0},$$

where $\Delta Q/\Delta t$ is the rate of ENRO appearing in the receiver chamber, which was obtained as the slope of the regression line on the transport-time profile of ENRO across the cell monolayers; C_0 is the initial concentration of ENRO loaded in the donor chamber; A is the cell monolayer surface area (4.67 cm^2).

HPLC analysis

The conditions for HPLC analysis of ENRO were modified according to published methods (Idowu & Peggins, 2004; Marazuela & Moreno-Bondi, 2004). Ten microliters of a 12.5 μg/mL oxfendazole solution was added to each 100-μL aliquot of sample, as an internal standard in a 1.5 mL reaction tube. The mixture was vigorously vortexed, and 300 μL of dichloromethane was added. Samples were shaken for 60 sec, and the organic and water phases were separated by centrifugation at 5000 ***g*** for 5 min, then evaporated to dryness under a nitrogen stream. The samples were resuspended in 100 μL of methanol and injected into the HPLC column. Samples from the transport assays were not processed and 100 μL of the culture media was injected into the HPLC column. The system consisted of a Waters 600 pump, a Waters 717 plus autosampler and a Waters 2487 UV detector (Waters Corporation, Milford, MA, USA). Samples were separated on a reversed-phase column (AQUA™ C18 polar encapped, 5-μm particle size, 250 × 4.6 mm; Phenomenex, Torrance, CA, USA). The composition of the mobile phase was 25 mM orthophosphoric acid (pH 3.0)/acetonitrile (77:23). The flow rate of the mobile phase was set to 1.0 mL/min. UV absorbance was measured at 278 nm. Peak area ratios (fluoroquinolone/oxfendazole) were used for comparison with the standard curve. The integration was performed using the software Millennium^{32} (Waters). Standard samples in the appropriate drug-free matrix were prepared at a concentration ranging from 0.08 to 10 μg/mL.

Pharmacokinetic calculations and statistical analyses

The peak concentration (C_{max}) and time-peak concentrations (T_{max}) were read from the plotted concentration–time curve for each animal. The area under the plasma concentration–time

curves (*AUC*) from time zero to the moment at which the concentration (C_p) was no longer measurable was calculated using the linear trapezoidal rule. The mean residence time (*MRT*) was calculated by the linear trapezoidal rule without extrapolation to infinity, using the formula:

$$MRT = \frac{AUMC}{AUC},$$

where *AUMC* is the mean area under the momentum curve. The results are presented as the mean ± standard deviations. Statistical analysis was performed by one-way analysis of variance (ANOVA), using the LSD test ($P < 0.05$).

RESULTS

Western blot and ATPase activity

A broad band at about 72 kDa was observed using the monoclonal antibody BXP-53 reactive against mammalian BCRP, showing the BCRP expression and its relative abundance in mammary glands of lactating cows and lactating and nonlactating sheep (Fig. 1). As shown Fig. 1, BCRP in bovine mammary glands migrated at a lower molecular mass (around 60 kDa), which could be due to the glycosylation of membrane proteins.

Figure 2 shows the effects of various compounds on vanadate-sensitive ATPase activity in membrane homogenates of the mammary gland of the lactating cows. The addition of MXR stimulated ATPase activity significantly. Prazosin stimulated the activity in crude homogenates of mammary gland membranes, but the measured ATPase activity was lower. Both increased activities were inhibited in the presence of Ko143 5 μM. The measured ATPase activity stimulated by MXR was inhibited by genistein and quercitin (Fig. 2).

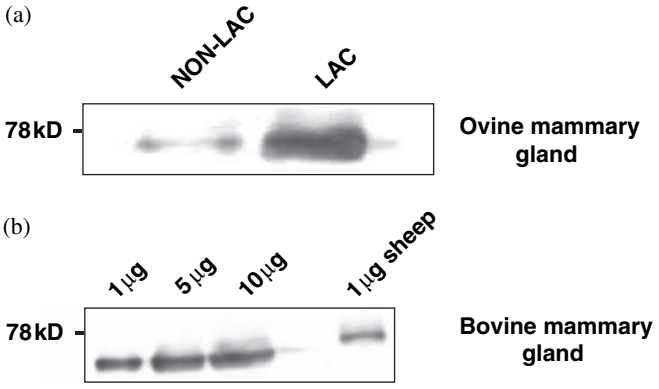

Fig. 1. Western blot analyses of the expression and quantification of BCRP (BXP-53) in ovine and bovine crude membrane fractions. (a) Mammary gland of lactating and nonlactating ewes, each lane contained 50 μg of membrane protein (mp); (b) mammary gland from lactating cows with different amounts of protein.

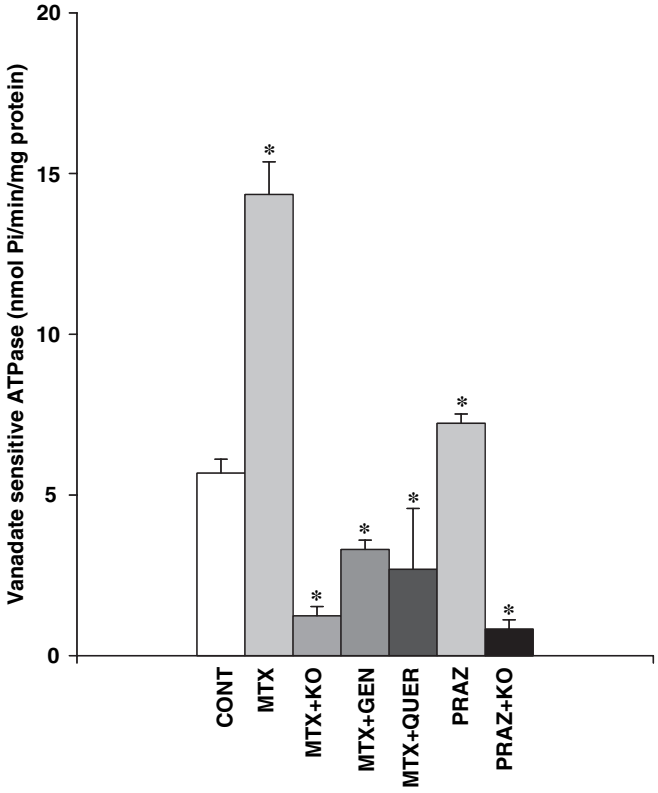

Fig. 2. Reversing effect of the BCRP inhibitor Ko143 (5 μM) (KO) on the vanadate-sensitive ATPase activity of substrates of BCRP: mitoxantrone (MXT) (50 μM) and prazosin (PRAZ) (50 μM). Reversing effects of the flavonoids genistein (GEN) (10 μM) and quercitin (QUER) (10 μM) on mitoxantrone (MXT) (50 μM)-induced activity. Control (CONT) values show the activity measured in absence of added compounds. Data points indicate the mean ± SD values of three measurements, performed in two different membrane preparations. *Difference from control group, $P < 0.05$.

In vitro transport of ENRO

The parental and transduced cell lines were grown to confluent polarized monolayers on porous membrane filters, and vectorial transport of the fluoroquinolone (10 μM) across the monolayers was determined. In the MDCK-II parental cell line, apically and basolaterally directed translocation were similar (Fig. 3a). In the Bcrp1-transduced MDCK-II cell lines, apically directed translocation was highly increased and basolaterally directed translocation drastically decreased (Fig. 3b). When the selective Bcrp1 inhibitor Ko143 was used the Bcrp1/BCRP-mediated transport was completely inhibited (Fig. 3c,e), resulting in a vectorial translocation pattern equal to that of the MDCK-II parental cell line. When the human BCRP-transduced cell line was used, the efficiency in the directional transport was lower than in the case of the murine Bcrp1-transduced cell line (Fig. 3d). These results show highly efficient transport of ENRO by murine Bcrp1 and moderate transport by human BCRP. The ratio of the P_{app} coefficients (Table 1) corresponding to m-Bcrp1 was 4.1 ± 1.1 ($P < 0.05$) and to H-BCRP was 1.5 ± 0.3 ($P < 0.05$). In addition, the bidirectional transport of ENRO was assessed in

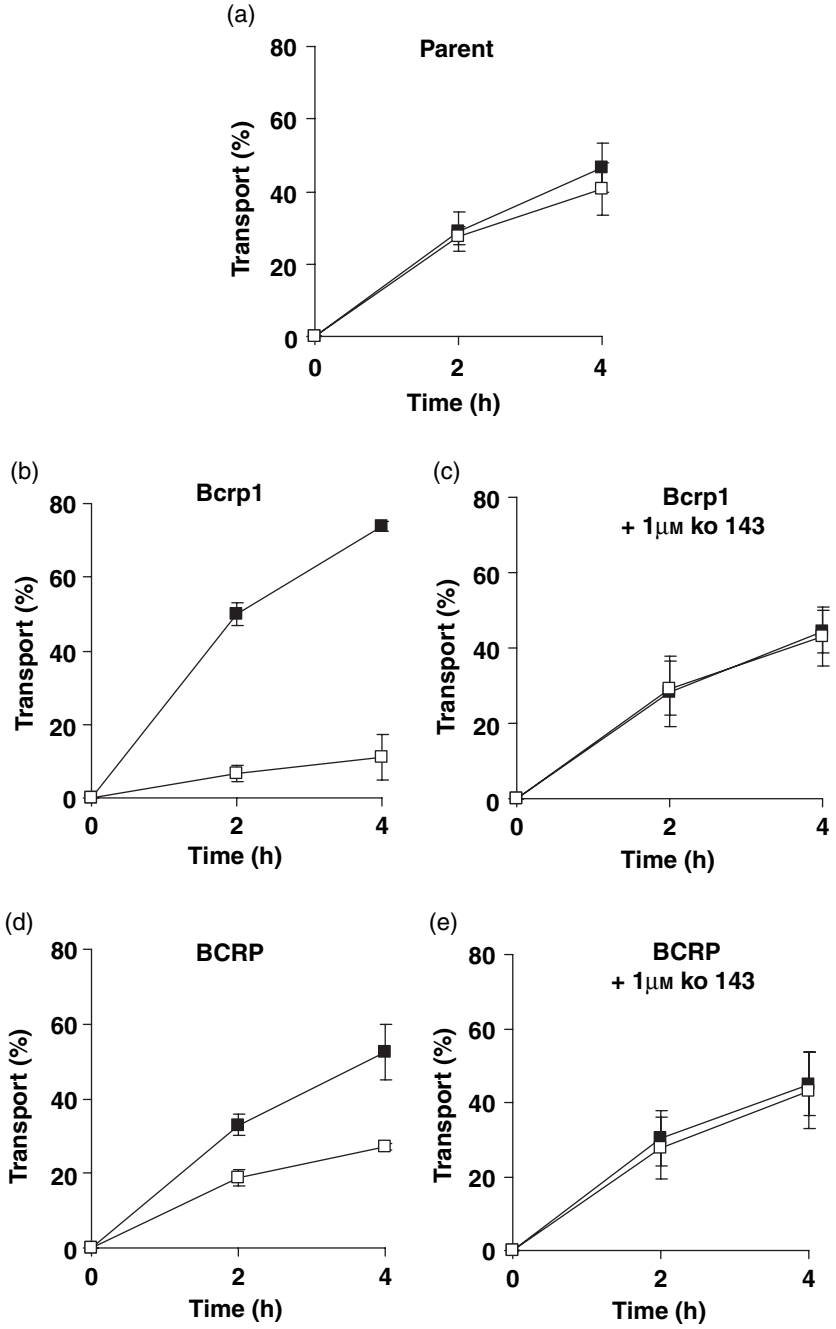

Fig. 3. Transepithelial transport of enrofloxacin (10 μM) in MDCKII (parent) (a), MDCKII-Bcrp1 (b) and MDCKII-BCRP (d), monolayers. The experiment was started with the addition of enrofloxacin to one compartment (basolateral or apical). After 2 and 4 h, the percentage of drug appearing in the opposite compartment was measured by HPLC and plotted. BCRP inhibitor Ko143 (c, e) was present as indicated. Results are the mean values; error bars (sometimes smaller than the symbols) indicate the standard deviations ($n = 3$). Closed squares: translocation from the basolateral to the apical compartment; open squares: translocation from the apical to the basolateral compartment.

the presence of the flavonoids genistein and daidzein. ENRO bidirectional transport was drastically decreased by 50 μM genistein; the inhibition values calculated using the previous formula were 92.71 ± 7.9% and 96.27 ± 0.8% in human BCRP, and murine Bcrp1 cells, respectively. The inhibition values of daidzein (50 μM) were 78.94 ± 0.75% and 90.02 ± 1.19% in human BCRP, and murine Bcrp1 cells, respectively.

Mitoxantrone accumulation studies

In order to further characterize the interactions of the ENRO with Bcrp1/BCRP, we tested the ability of these compounds to reverse the reduced MXR accumulation in murine Bcrp1- and BCRP-expressing cell lines, in flow cytometry experiments (Fig. 4). BCRP inhibition with Ko143 increased the accumulation of MXR in Bcrp1- and BCRP-transduced cells, and thus increased the MF. The inhibitory effect of ENRO on BCRP-mediated transport was confirmed, demonstrating inhibition (40%) by ENRO (200 μM) of human BCRP-mediated MXR transport. No inhibition was found in murine Bcrp1 (Fig. 4).

Plasma and milk ENRO pharmacokinetics

The mean plasma concentrations in sheep are shown in Fig. 5. Pharmacokinetic parameters of ENRO in plasma and milk are shown in Table 2. The noncompartmental analyses did not show

Table 1. P_{app} values for transepithelial transport of enrofloxacin (ENRO) (10 μM) across cells monolayers with or without the inhibitor Ko143 (1 μM)

	AP-to-BL ($\times 10^{-6}$ cm/sec)	BL-to-AP ($\times 10^{-6}$ cm/sec)	Ratio BL-to-AP/ AP-to-BL
ENRO			
MDCKII	14.9 ± 2.1	13.4 ± 2.9	0.9 ± 0.1
m-Bcrp1	5.8 ± 2.4	23.5 ± 3.6	4.1 ± 1.1*
H-BCRP	10.5 ± 3.2	15.4 ± 3.2	1.5 ± 0.3*
ENRO Ko143			
MDCKII	12.4 ± 2.5	12.7 ± 1.8	1.0 ± 0.1
m-Bcrp1	12.1 ± 2.7	12.5 ± 2.0	1.0 ± 0.1
H-BCRP	12.2 ± 3.2	12.7 ± 2.9	1.0 ± 0.1

Results are expressed as mean values and standard deviations from at least three experiments.
*Difference from parental group (MDCKII), $P < 0.05$.

Table 2. Mean (±SD) pharmacokinetic parameters of enrofloxacin (ENRO) in plasma and milk after i.v. administration at dosage of 2.5 mg/kg in sheep co-administered with genistein (GEN) (0.8 mg/kg, i.m.) and albendazole sulfoxide (ABZSO) (2 mg/kg, i.v.)

	ENRO	ENRO + GEN	ENRO + ABZSO
Plasma			
AUC (μg·h/mL)	13.65 ± 3.50	9.33 ± 3.45	10 ± 3.3
C_{max} (μg/mL)	4.27 ± 0.91	4.4 ± 1.01	3.76 ± 0.55
MRT (h)	4.75 ± 1.46	3.06 ± 0.89	3.26 ± 0.38
Milk			
AUC (μg·h/mL)	10.17 ± 2.41	6.4 ± 2.15*	6.3 ± 0.6*
C_{max} (μg/mL)	1.6 ± 0.496	1.66 ± 0.321	1.33 ± 1.523
T_{max} (h)	2.5 ± 1.5	0.83 ± 0.28	1.33 ± 0.57
MRT (h)	10.2 ± 2.42	10.76 ± 2.89	10.56 ± 1.85
AUC (milk/plasma)	0.75 ± 0.08	0.69 ± 0.04	0.67 ± 0.22

*Difference from control group, $P < 0.05$.

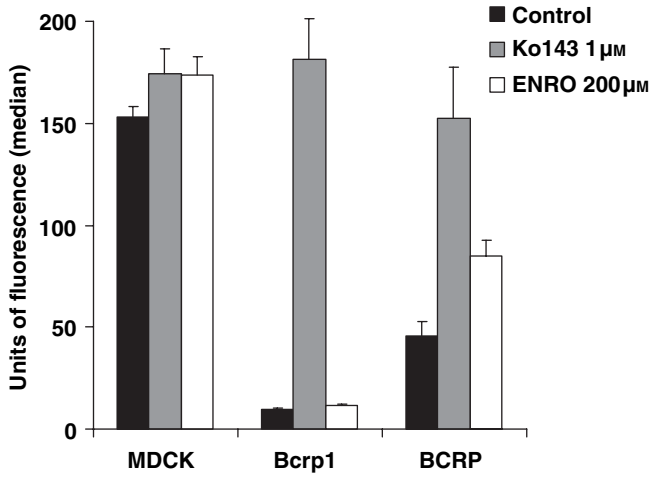

Fig. 4. Effect of enrofloxacin on accumulation of mitoxantrone in parent MDCKII cells and in their BCRP- and Bcrp1-transduced derivatives, preincubated with or without Ko143 or enrofloxacin at the indicated concentration. Results are expressed as mean values; the error bars indicate the standard deviations from at least three experiments.

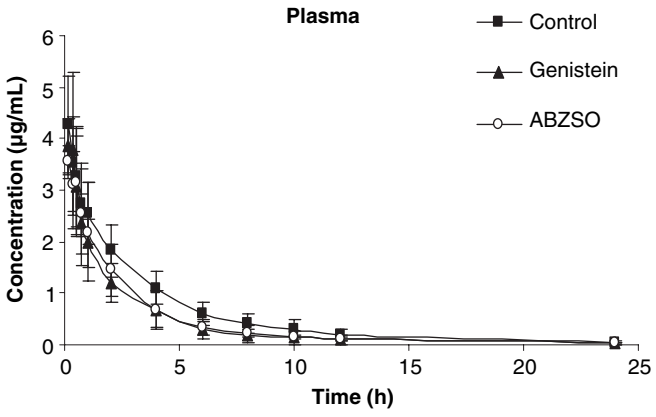

Fig. 5. Mean (±SD) plasma concentration of enrofloxacin in sheep after i.v. administration at dosage of 2.5 mg/kg in sheep co-administered with albendazole sulfoxide (2 mg/kg, i.v.) and genistein (0.8 mg/kg, i.m.).

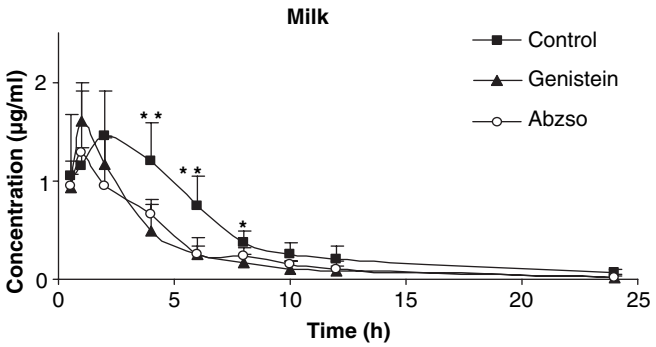

Fig. 6. Mean (±SD) milk concentration of enrofloxacin in sheep after i.v. administration at dosage of 2.5 mg/kg in sheep co-administered with albendazole sulfoxide (i.v. 2 mg/kg) and genistein (0.8 mg/kg, i.m.). **Control difference in relation to genistein and albendazole sulfoxide (ABZSO) groups, $P < 0.05$; *control difference in relation to genistein group $P < 0.05$.

significant differences. The curve of the mean serum concentration after i.v. administration in sheep in all experiments showed an initial rapid phase corresponding to the distribution of the drug and a second phase with slower elimination. ENRO *MRT* (h) value of 4.75 ± 1.46 was not significantly modified after co-administration of ABZSO (3.26 ± 0.38 h) and genistein (3.06 ± 0.89 h). The secretion of ENRO into the milk was significantly decreased following the co-administration of the BCRP inhibitors. Figure 6 shows that ENRO milk concentration decreased significantly after genistein administration from 2 to 8 h after the drug injection. Following ABZSO co-administration, ENRO levels significantly decreased from 2 to 6 h. The corresponding *AUC* (trapezoidal rule) values in Table 2 showed a significant reduction in milk values compared with the controls (6.4 ± 2.15 and 6.3 ± 0.6 vs. 10.17 ± 2.41 $P < 0.05$). However the *AUC* milk/plasma ratio was not different in any experimental group. The presence of inhibitors slightly lowered T_{max} value in milk (Fig. 6 and Table 2). The milk excretion of ENRO as a percentage of total administered dose was calculated considering ENRO concentration and volume of milk at each

time, it was about 0.25% without significant differences between the experimental groups.

DISCUSSION

The BCRP expression in mammary glands of lactating and nonlactating ewes agrees with the results reported by Jonker *et al.* (2005) in cow. BCRP expression was high in the mammary gland of the lactating cow (1, 5, and 10 μg of protein, Fig. 1). As shown in Fig. 1, bovine BCRP migrated at a lower molecular mass (around 60 kDa), which could be due to the degree of glycosylation of membrane proteins present. However, glycosylation appears to be unnecessary for the drug transport function of ABCG2/BCRP (Ozvegy *et al.*, 2001; Diop & Hrycyna, 2005).

The presence of this protein in the mammary glands of ruminants may be related to the presence of xenobiotic residues in milk. This is important as withdrawal periods can be shortened as a consequence of interference with the efflux transporters. Our results point to high BCRP expression in ovine and bovine mammary glands. The effects of specific substrates and inhibitors of BCRP on vanadate-sensitive ATPase activity in the crude homogenates of bovine mammary gland emphasize the function of BCRP in the tissue (Fig. 2). The basal ATPase activity in our mammary gland preparation was only 9–10% of that in Sf9 cells reported by Ozvegy *et al.* (2001), and could be a consequence of the unpurified tissue (crude homogenates) used in this study. The addition of MXR, a well-established substrate drug for ABCG2/BCRP (Miyake *et al.*, 1999), stimulated ATPase activity. Prazosin, a vasodilatation agent actively extruded from different multidrug-resistant cells by BCRP (Litman *et al.*, 2000), stimulated the ATPase of mammary gland membranes, but in this case the effect on activity was lower. Ko143, the potent BCRP/Bcrp1 inhibitor used to test the function of BCRP (Allen *et al.*, 2002; Merino *et al.*, 2005a), inhibited ATPase activity at lower concentration in mammary gland membranes. The flavonoids genistein and quercitin showed a clear BCRP interaction in homogenates from mammary gland through ATPase activity.

Study of the role of efflux proteins in veterinary therapeutic is incipient (Laffont *et al.*, 2002). Antiparasitic agents such as selamectin and ivermectin are substrates and inhibitors of P-glycoprotein (Griffin *et al.*, 2005). Some reports establish a link between benzimidazoles (anthelmintics) and ABC transporters (Nare *et al.*, 1994; Merino *et al.*, 2005a). Merino *et al.* (2005a) reported that ABZSO and oxfendazole sulfoxide derivatives of the methylcarbamate benzimidazoles, albendazole and fenbendazole respectively, were efficiently transported by murine Bcrp1, and moderately transported by human BCRP, but not by P-glycoprotein or MRP2. Previous research supported that other fluoroquinolones are subjected to active efflux (Griffiths *et al.*, 1994; Rabbaa *et al.*, 1996).

Our results (Fig. 3), showed that ENRO was a good Bcrp1/BCRP substrate with ratios of $P_{app\ A\text{-}B}/P_{app\ B\text{-}A}$ of 4.1 ± 1.1, 1.5 ± 0.3, and 0.9 ± 0.1 in murine Bcrp1, human BCRP, and parent MDCKII. The inhibitory effect of ENRO on BCRP-mediated transport was confirmed by flow cytometry experiments demonstrating inhibition (40%), by ENRO of human BCRP-mediated MXR transport (Fig. 4). No inhibition was found in murine Bcrp1. Variance in efficiency of transport between the murine and human homologues could be due to differences in the affinity/selectivity for substrates, as has been hypothesized (Mizuno *et al.*, 2004) and/or to an effectively lower level of the human protein in the line used, as also suggested by the nearly fivefold reduction in MXR accumulation in these cells (see Fig. 4). The bidirectional transport of ENRO was determined with the flavonoids genistein and daidzein. ENRO transport in human BCRP and murine Bcrp1 cells was drastically decreased by 50 mM genistein, with similar levels of inhibition by daidzein (see Results section). Flavonoids, including isoflavones (genistein, daidzein, apigenin and luteolin) reverse BCRP-mediated drug resistance (Cooray *et al.*, 2004; Imai *et al.*, 2004; Zhang *et al.*, 2004). Isoflavones such as genistein, daidzein, apigenin and luteolin are present in soybean (Zhang & Morris, 2003). Some of these compounds are present in forage of ruminants; furthermore, it has been reported that flavonoids are present in bovine milk (Antignac *et al.*, 2003). Dupuy *et al.* (2003) reported that the flavonoid quercitin increased the plasma moxidectin bioavailability as quercitin diminishes the biliary and intestinal secretions of moxidectin due to a P-glycoprotein involvement.

Following intravenous administration, ENRO data in different ruminant species were explained by a two-compartmental model (Mengozzi *et al.*, 1996; Bregante *et al.*, 1999; Haritova *et al.*, 2003; Sooud, 2003). ENRO as well as other fluoroquinolones including ciprofloxacin penetrated rapidly and extensively from blood into milk (Aramayona *et al.*, 1996; Chung *et al.*, 2002). Although most drugs enter milk by passive diffusion, some drugs are actively transported into milk. The BCRP transporter plays an important role in the secretion of xenotoxins and drugs such as nitrofurantoine into the milk (Jonker *et al.*, 2005; Merino *et al.*, 2005b). Drugs actively transported into milk showed higher milk-to-plasma (M/P) ratios than those predicted by diffusion model (Gerk *et al.*, 2001). Our data, based on the HPLC analyses, showed that ENRO concentration in milk occurs in parallel to that in plasma. These results are according to Kaartinen *et al.* (1995), but some authors (Haritova *et al.*, 2003; Sooud, 2003) report high values of the ratio *AUC* milk/*AUC* serum for ENRO in goats and sheep. Such results are based on antimicrobial activity and higher concentrations of antibacterial drugs such as ENRO can be measured using this assay (Mckellar *et al.*, 1999). Haritova *et al.* (2003) suggest that the high values of ENRO in milk could be explained by the low degree of binding of the drug to serum proteins and the presence of the lipophilic drug in the serum in the non-ionic form. On the other hand, our low milk concentration of ENRO could be due to transporter saturation in the mammary gland. Recently we found that ciprofloxacin, the major active ENRO metabolite in sheep acts as BCRP substrate in the same cells; in addition in bcrp knockout mice, plasma levels were increased and the M/P ratio was twofold higher in wild-type mice (Merino *et al.*, 2006). Unfortunately, in the present work, ciprofloxacin levels could not be accurately quantified. Comparative pharmacokinetics of ENRO

in some mammal species suggest a differing rate of drug metabolism or a differing extent of ENRO renal elimination in the species studied (Bregante *et al.*, 1999). Different factors, such as co-administered drugs or natural products (Zhang & Morris, 2003; Cooray *et al.*, 2004) gender (Merino *et al.*, 2005b) or genetic polymorphisms can modify the expression and function of BCRP. Cohen-Zinder *et al.* (2005) have published that fat and protein content and quantity of milk production were found to correlate with a polymorphism in BCRP (Y581S) in Holstein cattle.

This study supports a BCRP-mediated efflux of ENRO as the drug acts as a substrate and inhibitor of BCRP/brcp1 *in vitro* (MCDK cultures) and because of the significant inhibition of ENRO milk secretion by genistein and ABZSO.

Consumers demand protection against the inadvertent transfer of drugs and other substances into milk. The potential role of BCRP inhibitors in the transference of drug residues to milk should be established. This work demonstrates for the first time that flavonoid co-administration modified the *in vivo* milk secretion of BCRP substrates, such as ENRO, in sheep.

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REFERENCES

Allen, J.D., van Loevezijn, A., Lakhai, J.M., der Valk, M., van Tellingen, O., Reid, G., Schellens, J.H.M., Koomen, G. J. & 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. *Molecular Cancer Therapeutics*, **1**, 417–425.

Antignac, J.P., Cariou, R., Le Bizec, B., Cravedi, J.P. & Andre, F. (2003) Identification of phytoestrogens in bovine milk using liquid chromatography/electrospray tandem mass spectrometry. *Rapid Communication in Mass Spectrometry*, **17,** 1256–1264.

Aramayona, J.J., Mora, J., Fraile, L., García, M.A., Abadía, A. R. & Bregante, A. (1996) Penetration of enrofloxacin and ciprofloxacin into breast milk, and pharmacokinetics of the drugs in lactating rabbits and neonatal offspring. *American Journal of Veterinary Research*, **4,** 1040–1043.

Bregante, M. A., Saez, P., Aramayona, J.J., Fraile, L., García. M.A. & Solans, C. (1999) Comparative pharmacokinetics of enrofloxacin in mice rats, rabbits, sheep, and cows. *American Journal of Veterinary Research*, **9,** 1040–1043.

Broderick, G.A. (1995) Desirable characteristics of forage legumes for improving protein utilization in ruminants. *Journal of Animal Science*, **73,** 2760–2773.

Chung, A.M., Reed, M.D. & Blumer, J.F. (2002) Antibiotics and breastfeeding. *Pediatric Drugs*, **4,** 817–837.

Cohen-Zinder, M., Seroussi, E., Larkin, D.M. *et al.* (2005) Identification of a missense mutation in the bovine ABCG2 gene with a major effect on the QTL on chromosome 6 affecting milk yield and composition in Holstein cattle. *Genome Research*, **15,** 936–944.

Cooray, H.C., Janvilisri, T., van Veen, H.W., Hladky, S.B. & Barrand, M.A. 2004, Interaction of the breast cancer resistance protein with plant polyphenols. *Biochemical and Biophysical Research Communications*, **271,** 269–275.

Diop, N.K. & Hrycyna, C.A (2005) N-linked glycosylation of the human ABC transporter ABCG2 on the Asparagine 596 is not essential for the expression, transport activity or trafficking to the plasma membrane. *Biochemistry*, **44,** 5420–5429.

Dupuy, J., Larrieu, G., Sutra, J.F., Lespine, A. & Alvinerie, M. (2003) Enhancement of moxidectin bioavailability in lamb by a natural flavonoid: quercitin. *Veterinary Parasitology*, **112,** 337–347.

Gerk, P.M., , C.Y., Paxton, E.W., Moscow, J.A. & Mcnamara, PJ. (2001) Interactions between cimetidine, nitrofurantoin and probenecid active transport into rat milk. *The Journal of Pharmacology and Experimental Therapeutics*, **296,** 175–180.

Griffin, J., Fletcher, N., Clemence, R., Blanchflower, S. & Brayden, D.J. (2005) Selamectin is a potent substrate and inhibitor of human and canine P-glycoprotein. *Journal of Veterinary Pharmacology and Therapeutics*, **28,** 257–265.

Griffiths, NM, Hirst, BH & Simmons, NL (1994) Active intestinal secretion of the fluoroquinolone antibacterials ciprofloxacin, norfloxacin and pefloxacin; a common secretory pathway? *The Journal of Pharmacology and Experimental Therapeutics*, **269,** 496–502.

Haritova, A., Lashev, L & Pashov, D. (2003) Pharmacokinetics of enrofloxacin in lactating sheep. *Research in Veterinary Science*, **74,** 241–245.

Idowu, O. R. & Peggins, J.O. (2004) Simple, rapid determination of enrofloxacin and ciprofloxacin in bovine milk and plasma by high-performance liquid chromatography with fluorescence detection. *Journal of Pharmaceutical and Biomedical Analyses*, **35,** 143–153.

Imai, Y., Tsukahara, S., Asada, S. & Sugimoto, Y. (2004) Phytoestrogens/flavonoids reverse breast cancer resistance protein/ABCG2-mediated multidrug resistance. *Cancer Research*, **64,** 4346–4352.

Janvilisry, T., Venter, H., Shashi, S., Reuter, G, Balakrishnan, L. & van Veen, H.W. (2003) Sterol transport by human breast cancer resistance protein (ABCG2) expressed in *Lactococcus lactis*. *Journal of Biological Chemistry*, **278,** 20645–20651.

Jonker, J.W., Smit, J.W., Brinkhuis, R.F., Maliepaard, M., Beijnen, J.H., Schellens, J.H.M. & Schinkel, A.H. (2000) Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. *Journal of the National Cancer Institute*, **92,** 1651–1656.

Jonker, J.W., Merino, G., Musters, S., Van Herwaarden, A.E., Bolscher, E., Wagenaar, E., Mesman, E., Dale, T.C. & Schinkel, A.H. (2005) The breast cancer resistance protein BCRP (ABCG2) concentrates drug and carcinogenic xenotoxins into milk. *Nature Medicine*, **11,** 127–129.

Kaartinen, L., Salonen, M., Allí, L. & Pyörälä, S. (1995) Pharmacokinetics of enrofloxacin after single, intramuscular and subcutaneous injections in lactating cows. *Journal of Veterinary Pharmacology and Therapeutics*, **18,** 357–362.

Laffont, C.M., Toutain, P.L., Alvinerie, M. & Bousquet-Mélou, A. (2002) Intestinal secretion is a major route for parent ivermectin elimination in rat. *Drug Metabolism and Disposition*, **30,** 626–630.

Litman, T., Brangi, M., Hudson, E., Fetch, P., Abati, A., Ross, D.D., Miyake, K., Resau, J.H. & Bates, S.E. (2000) The multidrug-resistance phenotype associated with overexpression of the new ABC half transporter MXR (ABCG2). *Journal of Cell Science*, **113,** 2011–2021.

Maliepaard, M., Scheffer, G.L., Faneyte, I.F., Van Gastelen, M.A., Pijnenborg, A.C.L.M., Schinkel, A.H., Van de Vijve, M.J., Scheper, R.J. & Schellens, J.H.M. (2001) Subcellular localization and distribution of the breast cancer resistance protein in normal humans tissues. *Cancer Research*, **61,** 3458–3464.

Marazuela, M.D. & Moreno-Bondi, M.C. (2004) Multiresidue determination of fluoroquinolones in milk by column liquid chromatography with fluorescence and ultraviolet absorbance detection. *Journal of Chromatography A*, **1034,** 25–32.

Mckellar, Q., Gibson, I., Monteiro, A. & Bregante, M. (1999) Pharmacokinetics of enrofloxacin and danofloxacin in plasma inflammmatory exudate, and bronchial secretions of calves following subcutaneous administration. *Antimicrobial Agents and Chemotherapy*, **48,** 1988–1992.

Mengozzi, G., Intorre, L., Bertini, S. & Soldani, G. (1996) Pharmacokinetics of enrofloxacin and its metabolite ciprofloxacin after intravenous and intramuscular administration in sheep. *American Journal of Veterinary Research*, **7,** 1040–1043.

Merino, G., Molina, A.J., García, J.L., Pulido, M.M., Prieto, J.G. & Alvarez, A.I (2003) Intestinal elimination of albendazole sulfoxide: pharmacokinetic effects of inhibitors. *International Journal of Pharmaceutics*, **263,** 123–132.

Merino, G., Jonker, J.W., Wagenaar, E., Pulido, M.M., Molina, A.J., Alvarez, A.I. & Schinkel, A.H. (2005a) Transport of anthelmintic benzimidazole drugs by BCRP (BCRP/ABCG2). *Drug Metabolism and Disposition*, **33,** 614–618.

Merino, G., Jonker, J.W., Wagenaar, E., van Herwaarden, A.E. & Schinkel, A.H. (2005b). The breast cancer resistance protein (BCRP/ABCG2) affects pharmacokinetics, hepatobiliary excretion and milk secretion of the antibiotic nitrofurantoin. *Molecular Pharmacology*, **67,** 1758–1764.

Merino, G., Alvarez, A.I, Pulido, M.M., Molina, A.J., Schinkel, A.H. & Prieto, J.G. (2006) Breast cancer resistance protein (BCRP/ABCG2) transports fluoroquinolone antibiotics and affects their oral availability, pharmacokinetics, and milk secretion. *Drug Metabolism and Disposition*, **34,** 690–695.

Miyake, K., Mickley, L., Litman, T., Zhan, Z., Robey, R., Cristensen, B., Brangi, M., Greenberger, L., Dean, M., Fojo, T. & Bates, S.E. (1999) Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Research*, **59,** 8–13.

Mizuno, N., Niwa, T., Yotsumoto, Y. & Sugiyama, Y. (2003) Impact of drug transporter studies on drug discovery and development. *Pharmacological Reviews*, **55,** 425–461.

Mizuno, N., Suzuki, M., Kusuhara, H., Suzuki, H., Takeuchi, K., Niwa, T., Jonker, J.W & Sugiyama, Y. (2004) Impaired renal excretion of 6-hidroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040) sulfate in breast cancer resistance protein (BCRP1/ABCG2) knockout mice. *Drug Metabolism and Disposition*, **32,** 898–901.

Nare, B., Liu, Z., Prichard, R.K. & Georges, E. (1994) Benzimidazoles, potent anti-mitotic drugs: substrates for the P-glycoprotein transporter in multidrug-resitant cells. *Biochemical Pharmacology*, **48,** 2215–2222.

Ozvegy, O., Litman, T., Szakacs, G., Nagy, Z., Bates, S.E., Varadi, A. & Sarkadi, B. (2001) Functional characterization of human multidrug transporter, ABCG2, expressed in insect cells. *Biochemical and Biophysical Research Communications*, **285,** 111–117.

Pavek, P., Merino, G., Wagenaar, E., Bolscher, E., Novotna, M., Jonker, J.W. & Schinkel, A.H. (2005) Human breast cancer resistance protein (BCRP/ABCG2): interactions with steroid drugs, hormones, the dietary carcinogen PhIP, and transport of cimetidine. *Journal of Pharmacology and Experimental Therapeutics*, **312,** 144–152.

Rabbaa, L., Dautrey, S., Colas-Linhart, N., Carbon, C. & Farinotti, R. (1996) Intestinal elimination of ofloxacin enantiomers in the rat: evidence of a carrier-mediated process. *Antimicrobial Agents and Chemotherapy*, **36,** 2506–2511.

Rocchi, E., Hodjakov, A.K., Volk, E.L., Yang, C-H., Litman, T., Bates, S.E. & Schneider, E. (2000) The product of the ABC half-transporter gene ABCG2 (BCRP/MXR/ABCP) is expressed in the plasma membrane. *Biochemical and Biophysical Research Communications*, **271,** 42–46.

Schinkel, A.H. & Jonker, J.W. (2003) Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Advanced Drug Delivery Reviews*, **55,** 3–29.

Sooud, K.A. (2003) Influence of albendazole on the disposition kinetics and milk antimicrobial equivalent activity of enrofloxacin in lactating goats. *Pharmacological Research*, **48,** 389–395.

Zhang, S. & Morris, M.E. (2003) Effects of the flavonoids biochanin A, morin, phloretin, and silymarin on P-glycoprotein-mediated transport. *Journal of Pharmacology and Experimental Therapeutics*, **304,** 1258–1267.

Zhang, S., Yang, X. & Morris, M.E. (2004) Combined effects of multiple flavonoids on breast cancer resistance protein (ABCG2)-mediated transport. *Pharmaceutical Research*, **21,** 1263–1273.