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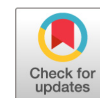

Secretion into Milk of the Main Metabolites of the Anthelmintic Albendazole Is Mediated by the ABCG2/BCRP Transporter

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ABSTRACT Albendazole (ABZ) is an anthelmintic with a broad-spectrum activity, widely used in human and veterinary medicine. ABZ is metabolized in all mammalian species to albendazole sulfoxide (ABZSO), albendazole sulfone ($ABZSO_2$) and albendazole 2-aminosulphone ($ABZSO_2$-NH_2). ABZSO and $ABZSO_2$ are the main metabolites detected in plasma and all three are detected in milk. The ATP-binding cassette transporter G2 (ABCG2) is an efflux transporter that is involved in the active secretion of several compounds into milk. Previous studies have reported that ABZSO was *in vitro* transported by ABCG2. The aim of this work is to correlate the *in vitro* interaction between ABCG2 and the other ABZ metabolites with their secretion into milk by this transporter. Using *in vitro* transepithelial assays with cells transduced with murine Abcg2 and human ABCG2, we show that $ABZSO_2$ and $ABZSO_2$-NH_2 are *in vitro* substrates of both. *In vivo* assays carried out with wild-type and $Abcg2^{-/-}$ lactating female mice demonstrated that secretion into milk of these ABZ metabolites was mediated by Abcg2. Milk concentrations and milk-to-plasma ratio were higher in wild-type compared to $Abcg2^{-/-}$ mice for all the metabolites tested. We conclude that ABZ metabolites are undoubtedly *in vitro* substrates of ABCG2 and actively secreted into milk by ABCG2.

KEYWORDS ABCG2, albendazole, metabolites, substrates, milk, knockout mice, milk secretion

ABZ is a benzimidazole drug with a broad-spectrum anthelmintic activity, commonly used in human and veterinary medicine (1). It is effective against lungworms, gastrointestinal nematodes, tapeworms (*Echinococcosis* spp.), and liver flukes (*Fasciola hepatica)* (2). In humans, it is widely used against soil-transmitted helminths, which are responsible for high diseases burdens and are still endemic in some countries (3, 4). It also is the election drug in programs to eliminate lymphatic filariasis (5). Deworming, with anthelmintic drugs such as ABZ, is extensively recommended in women in reproductive age, including pregnant and lactating women, who are infected with hookworm which causes malabsorption of nutrients, loss of appetite, chronic blood loss and iron deficiency anemia (4). Recent studies have reported antitumor activity of ABZ (6–9). This drug is well tolerated in humans but some minor to moderate adverse effects such as headaches, fever and gastrointestinal upset have been reported (5).

ABZ is metabolized in all mammalian species studied (10). After its oral administration, it is absorbed from the intestinal lumen and metabolized in gut and liver by oxidation to ABZSO followed by further oxidation to $ABZSO_2$, and finally by deacetylation of carbamate group to $ABZSO_2$-NH_2 (2, 11–13) (Fig. S1). In most cases, ABZSO and $ABZSO_2$ are the main metabolites detected in plasma and urine; the parent drug, ABZ, is not detected in plasma (2, 14). With regard to anthelmintic activity, ABZSO has been reported to be active, whereas in the case of $ABZSO_2$ there are contradictory studies

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(15–18). The sum of ABZSO, $ABZSO_2$ and $ABZSO_2$-NH_2 is used as a marker residue in milk, kidney, liver, fat, and muscle from livestock (19). Regarding the transfer of drugs into milk, the ATP-binding cassette (ABC) transporter ABCG2 is an important and widely described mechanism. The ABCG2 protein behaves like a pump that extrudes a broad range of xenotoxins from cells due to its expression at the apical membrane of epithelial cells in several organsm, such as intestine, kidney, liver, brain, testicles, among others (20–22), limiting drug accumulation in cells and modulating absorption, distribution and elimination. Moreover, ABCG2 is located in the apical membrane of alveolar epithelial cells in the lactating mammary gland (23) and is the only ABC transport involved in active secretion of its substrates into milk (24). Several natural compounds (25, 26), carcinogens, antitumoral (27), antibiotic (28, 29), anti-inflammatory (30, 31), hypertensive (32), and antiparasitic drugs (33) have been reported to be actively secreted into milk by ABCG2.

ABCG2 *in vitro* interaction with ABZ and ABZSO has been shown in preceding studies. ABZSO was efficiently transported by murine Abcg2 and moderately by human ABCG2 (34). However, the *in vitro* interaction of $ABZSO_2$ and $ABZSO_2$-NH_2 with ABCG2 using ABCG2-transduced cells and its correlation with the *in vivo* effect of ABCG2 on active secretion of these ABZ metabolites into milk using Abcg2$^{-/-}$ mice have not yet been investigated and are the main aims of our study.

RESULTS

***In vitro* transport of ABZ metabolites $ABZSO_2$ and $ABZSO_2$-NH_2.** To determine whether ABZ metabolites are efficiently transported *in vitro* by ABCG2, we used Madin-Darby Canine Kidney (MDCK-II) cell line and its subclones transduced with murine Abcg2 and human ABCG2 to conduct transepithelial transport assays. The parental and subclones cell lines were grown to confluent polarized monolayers, and vectorial transport of tested drugs at 5 μM across the monolayers was determined. As stated before, ABZSO has been previously tested *in vitro* using murine and human subclones cell lines, being a substrate of both (34).

For $ABZSO_2$, the outcome obtained in the MDCK-II parental cells for apical and basal translocation was similar (Fig. 1A and Table 1). Nevertheless, basal to apical transport in cells transduced with murine Abcg2 (Fig. 1B) was higher than apical to basal transport, with an efflux ratio significantly higher (5.47 $\pm$ 0.32) than in the parental cells (0.97 $\pm$ 0.08; $P \leq 0.05$) (Fig. 1A). When human ABCG2-transduced cells were used (Fig. 1C), the difference with parental cells in apically directed translocation was lower compared to apical directional transport in the case of murine cells. A significant difference between the efflux ratio obtained for human ABCG2-transduced cells and for parental cells was observed (1.35 $\pm$ 0.16 vs 0.97 $\pm$ 0.08; $P \leq 0.05$). To confirm that this effect is caused by ABCG2, the specific ABCG2 inhibitor Ko143 was used (35) (Fig. 1D to F), causing a similar efflux ratio in the transduced cells compared to the MDCK-II parental cell line (Fig. 1A).

In the same way, for $ABZSO_2$-NH_2, the apical and basolateral translocations in MDCK-II parental cells were similar (Fig. 2A). Apical directional transport in murine Abcg2 (Fig. 2B) and human ABCG2-transduced cells (Fig. 2C) was higher (efflux ratio of 4.48 $\pm$ 0.53 and 3.58 $\pm$ 0.79; respectively) than in parental cells (Fig. 2A), showing in both cases, a significant difference in efflux ratio compared to parental cells (1.02 $\pm$ 0.12; $P \leq 0.05$). Similarly, the ABCG2 inhibitor Ko143 was used (Fig. 2D to F) to confirm the Abcg2 specific transport effect. The results also show a similar efflux ratio between murine and human subclones compared to the MDCK-II parental cell line (Fig. 2A) with the use of Ko143. From this, it is evident that $ABZSO_2$ and $ABZSO_2$-NH_2 are *in vitro* substrates of murine Abcg2 and human ABCG2.

Secretion of ABZ metabolites into milk in Abcg2$^{-/-}$ and wild-type female mice. To determine whether Abcg2 is involved in the secretion of ABZ metabolites into milk, Abcg2$^{-/-}$ and wild-type lactating female mice were used. Intravenous (i.v.) administration of 2 mg/kg of tested compounds was made, and blood and milk samples were collected 30 min after administration.

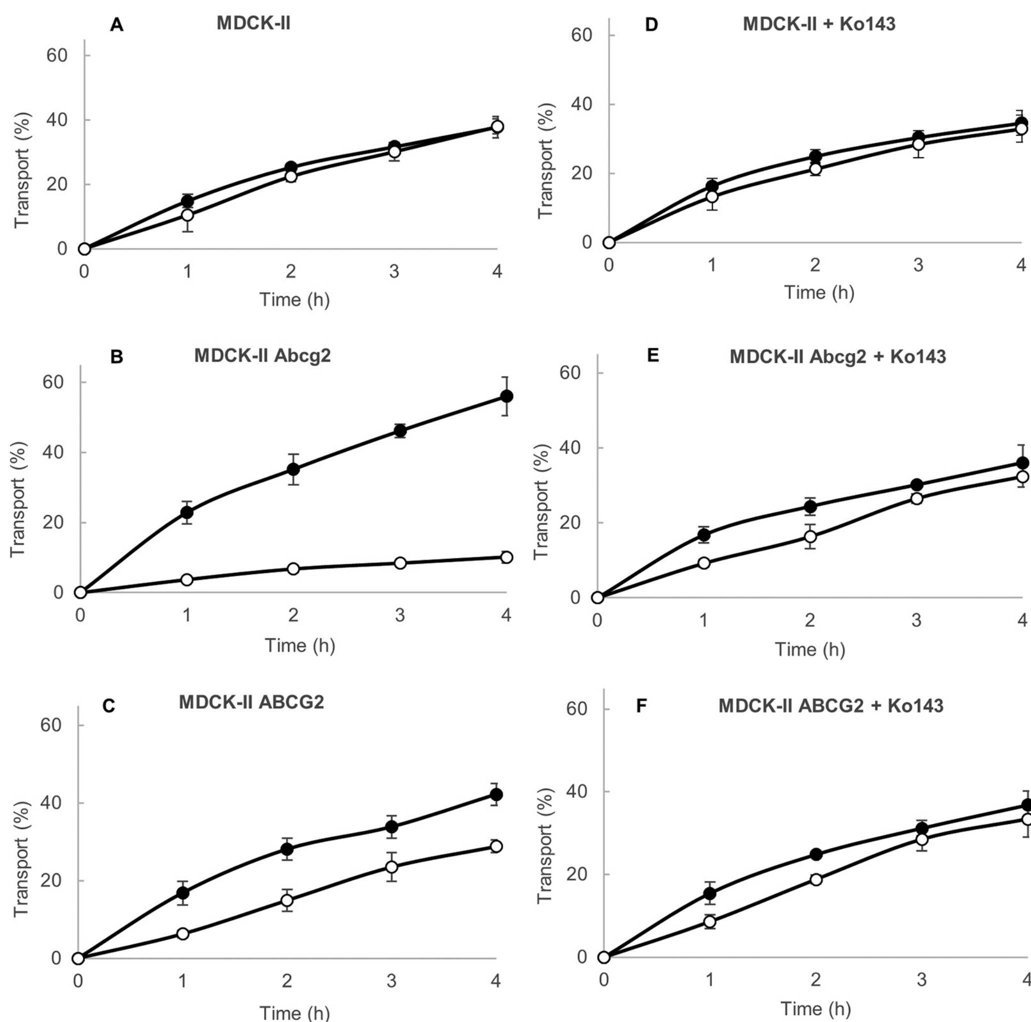

FIG 1 Transcellular transport assay of $ABZSO_2$ (5 μM) with or without Ko143 (ABCG2 inhibitor) in parental MDCK-II cells (A and D, respectively) and MDCK-II cells transduced with murine Abcg2 (B and E, respectively) and with human ABCG2 (C and F, respectively). The assay was started by changing the medium in apical or basolateral compartment with fresh culture medium with or without Ko143 at 1 μM and 5 μM $ABZSO_2$. The appearance of $ABZSO_2$ in the opposite compartment measured by HPLC, was related to the total drug added at the beginning of the experiment. The mean and error bars indicate S.D. (●) transport from basal to the apical compartment; (○) transport from apical to the basal compartment (n = 3–6).

After ABZSO administration (Fig. 3A), a similar concentration of ABZSO was obtained in plasma from wild-type and Abcg2$^{-/-}$ mice (1.95 $\pm$ 0.29 μg/mL versus 2.11 $\pm$ 0.41 μg/mL). In contrast to plasma, milk concentration of ABZSO was higher in wild-type than in Abcg2$^{-/-}$ mice (2.19 $\pm$ 0.33 μg/mL versus 1.83 $\pm$ 0.21 μg/mL; $P \leq 0.05$). Moreover, the milk-to-plasma ratio of ABZSO in wild-type was 1.3-fold higher compared to Abcg2$^{-/-}$ mice (1.13 $\pm$ 0.14 μg/mL versus 0.89 $\pm$ 0.17 μg/mL; $P \leq 0.05$). $ABZSO_2$ was detected in plasma and milk at very low levels and no differences were found between both types of mice (data not shown).

Likewise, similar assays were carried out by administrating $ABZSO_2$ and $ABZSO_2$-NH_2. After $ABZSO_2$ administration (Fig. 3B), plasma concentrations of $ABZSO_2$ were similar in wild-type and Abcg2$^{-/-}$ mice (1.25 $\pm$ 0.46 μg/mL versus 1.30 $\pm$ 0.41 μg/mL). However, milk concentration of $ABZSO_2$ was also higher in wild-type than in Abcg2$^{-/-}$ mice (1.78 $\pm$ 0.50 μg/mL versus 1.34 $\pm$ 0.41 μg/mL; $P \leq 0.05$). Therefore, the milk-to-plasma ratio of $ABZSO_2$ in wild-type was 1.4-fold higher compared to Abcg2$^{-/-}$ mice (1.52 $\pm$ 0.49 μg/mL versus 1.09 $\pm$ 0.32 μg/mL; $P \leq 0.05$). In this case, $ABZSO_2$-NH_2 was

TABLE 1 Apparent permeability (Papp) coefficients for transepithelial transport of $ABZSO_2$ (5 μM) and $ABZSO_2$-NH_2 (5 μM) with or without the inhibitor Ko143 (1 μM)[a]

Drug	Subclones[b]	BL-AP, x10^{-5} cm/s (Papp B-A)	AP-BL, x10^{-5} cm/s (Papp A-B)	Efflux ratio Papp B-A / Papp A-B
$ABZSO_2$	MDCK-II	1.27 ± 0.36	1.32 ± 0.37	0.97 ± 0.08
	MDCK-II Abcg2	1.94 ± 0.54	0.36 ± 0.11	5.47 ± 0.32 *
	MDCK-II ABCG2	1.35 ± 0.29	1.02 ± 0.26	1.35 ± 0.16 *
$ABZSO_2$	MDCK-II	0.99 ± 0.08	0.96 ± 0.11	1.03 ± 0.10
+	MDCK-II Abcg2	1.03 ± 0.08	0.98 ± 0.08	1.04 ± 0.07
Ko143	MDCK-II ABCG2	1.08 ± 0.06	1.04 ± 0.12	1.04 ± 0.10
$ABZSO_2$ -NH_2	MDCK-II	0.38 ± 0.14	0.37 ± 0.12	1.02 ± 0.12
	MDCK-II Abcg2	0.70 ± 0.14	0.16 ± 0.03	4.48 ± 0.53 *
	MDCK-II ABCG2	0.50 ± 0.05	0.14 ± 0.02	3.58 ± 0.79 *
$ABZSO_2$-NH_2	MDCK-II	0.28 ± 0.07	0.31 ± 0.08	0.92 ± 0.1
+	MDCK-II Abcg2	0.28 ± 0.08	0.28 ± 0.07	1.01 ± 0.06
Ko143	MDCK-II ABCG2	0.28 ± 0.08	0.27 ± 0.08	1.05 ± 0.06

[a]Results are expressed as mean values and standard deviations from at least three experiments. *, Significant differences from parental group (MDCK-II), $P \leq 0.05$.
[b]AP-BL: apical to basal, BL-AP: basal to apical. Abcg2: murine Abcg2, ABCG2: human ABCG2.

detected in milk and plasma at low levels and no differences were found between both types of mice.

Finally, after administration of $ABZSO_2$-NH_2 (Fig. 3C), wild-type and $Abcg2^{-/-}$ plasma concentrations were not different (0.43 ± 0.12 μg/mL versus 0.48 ± 0.08 μg/mL). Nonetheless, there were differences in milk concentrations between wild-type and $Abcg2^{-/-}$ mice (2.62 ± 0.79 μg/mL versus 1.45 ± 0.34 μg/mL; $P \leq 0.05$). The milk-to-plasma ratio of $ABZSO_2$ -NH_2 in wild-type was 2.2-fold higher compared to $Abcg2^{-/-}$ mice (6.60 ± 2.61 μg/mL versus 3.00 ± 0.25 μg/mL; $P \leq 0.05$).

These results show that ABCG2 is clearly involved in the active secretion of ABZ metabolites into milk.

DISCUSSION

Widely validated *in vitro-in vivo* correlation approaches have shown the *in vitro* interaction between ABCG2 and ABZ metabolites and the *in vivo* role of Abcg2 in the secretion of these compounds into milk.

In vitro transepithelial assays using MDCK-II cells transduced with murine Abcg2 and human ABCG2 show that $ABZSO_2$ (Fig. 1) and $ABZSO_2$-NH_2 (Fig. 2) are *in vitro* substrates of murine Abcg2 and human ABCG2, and that they are both efficiently transported by murine Abcg2 (efflux ratio of 5.47 ± 0.32 for $ABZSO_2$ and 4.48 ± 0.53 for $ABZSO_2$-NH_2). However, $ABZSO_2$ is moderately transported by human ABCG2 (efflux ratio of 1.35 ± 0.16) compared to $ABZSO_2$-NH_2 (efflux ratio of 3.58 ± 0.79). This difference in efficiency of transport between murine and human has been previously shown in other tested drugs. A difference in the affinity/selectivity of murine Abcg2 and human ABCG2 for substrates could be a possibility (31, 34, 36). In this regard, the concentration used in the present study (5 μM) is similar to the *in vivo* plasma concentrations achieved in rats and in livestock after therapeutic dosing (2, 11, 13, 37, 38).

ABZSO has been described in preceding studies as an *in vitro* substrate of murine Abcg2 and human ABCG2, but ABZ has not been found to be an *in vitro* Abcg2 substrate (34). Interactions with ABCG2 are closely related to physicochemical properties of drugs, especially hydrophobicity (39). In our case, ABZ is metabolized to more hydrophilic metabolites (2), ABZSO, $ABZSO_2$ and $ABZSO_2$-NH_2, which are efficiently transported by ABCG2, in contrast to ABZ. In addition, ABZSO, $ABZSO_2$ and $ABZSO_2$-NH_2, described as ABCG2 substrates, have a lower octanol-water partition coefficient compared to ABZ (Table S1). Other benzimidazoles previously identified as substrates of ABCG2, such as oxfendazole or pantoprazole, with transport ratios of around 6 (34, 40), have similar octanol-water partition coefficients. However, ABCG2 is inhibited by more

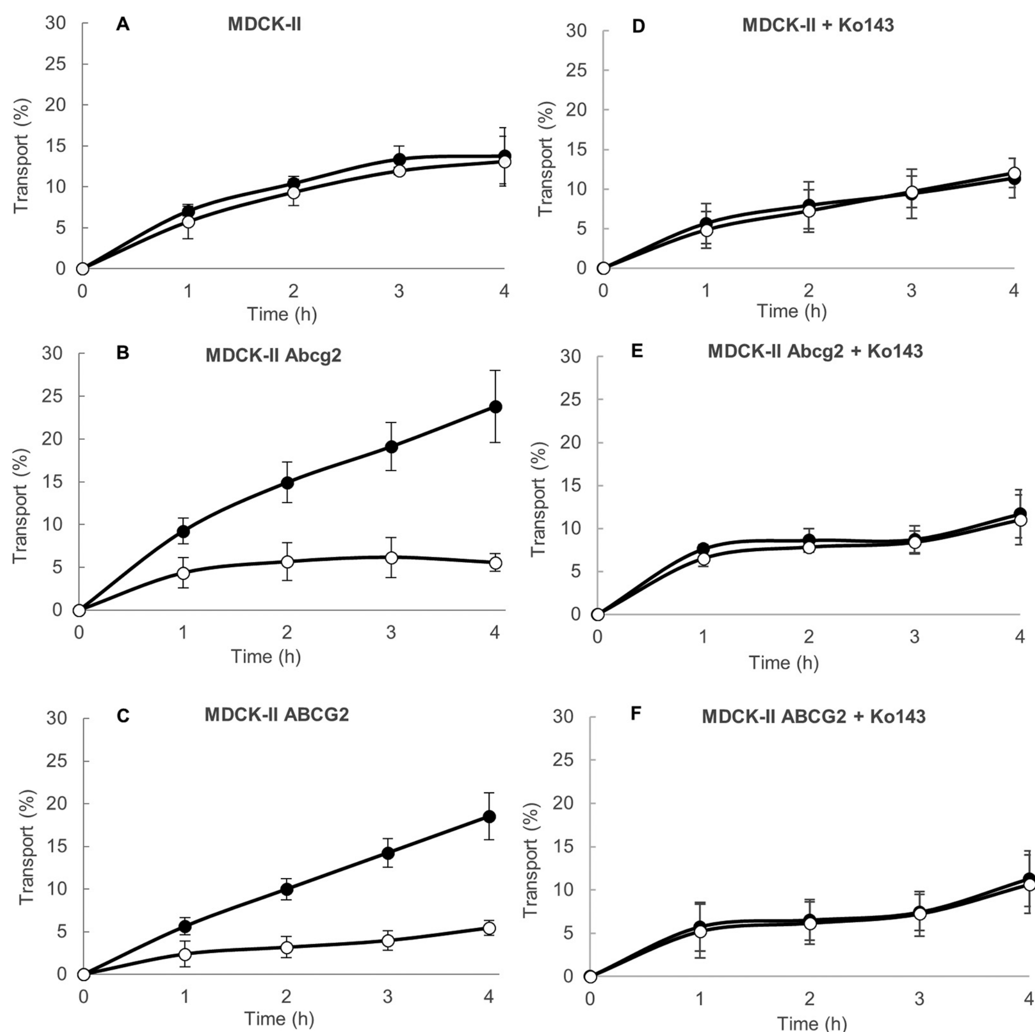

FIG 2 Transcellular transport of $ABZSO_2$-NH_2 (5 μM) with or without Ko143 (ABCG2 inhibitor) in parental MDCK-II cells (A and D, respectively) and MDCK-II cells transduced with murine Abcg2 (B and E, respectively) and with human ABCG2 (C and F, respectively). The assay was started by changing the medium in apical or basolateral compartment with fresh culture medium with or without Ko143 at 1 μM and 5 μM $ABZSO_2$-NH_2. The appearance of $ABZSO_2$-NH_2 in the opposite compartment measured by HPLC was related to the total drug added at the beginning of the experiment. The mean and error bars indicate S.D. (●) transport from basal to the apical compartment; (○) transport from apical to the basal compartment (*n* = 3–7).

hydrophobic benzimidazoles with higher lipid-water partition coefficients such as triclabendazole metabolites, with inhibitory potencies between 40 and 55% (41). Furthermore, substrate binding with ABCG2 transporter increases with the number of hydrogen bond acceptors (HBAs) (42) and, in our case, ABZSO and $ABZSO_2$ have one more HBA than ABZ and the same as oxfendazole (Table S1).

In vivo assays with lactating $Abcg2^{-/-}$ and wild-type lactating female mice were carried out to determine whether Abcg2 is involved in the secretion of ABZ metabolites into milk and whether the drug levels in milk could be affected by Abcg2. The dose chosen was 2 mg/kg because milk concentrations achieved with this dose were similar to those in ovine milk in a former study (43). Our results show that after i.v. administration of ABZSO (Fig. 3A), $ABZSO_2$ (Fig. 3B) and $ABZSO_2$-NH_2 (Fig. 3C), milk levels and milk-to-plasma ratios were higher in wild-type compared to $Abcg2^{-/-}$ mice. Pilot attempts to administer the parent drug ABZ failed to show differences in milk levels and milk-to-plasma ratios for metabolites between both types of mice (data not shown), probably due to the difficulty in obtaining the appropriate parameter settings,

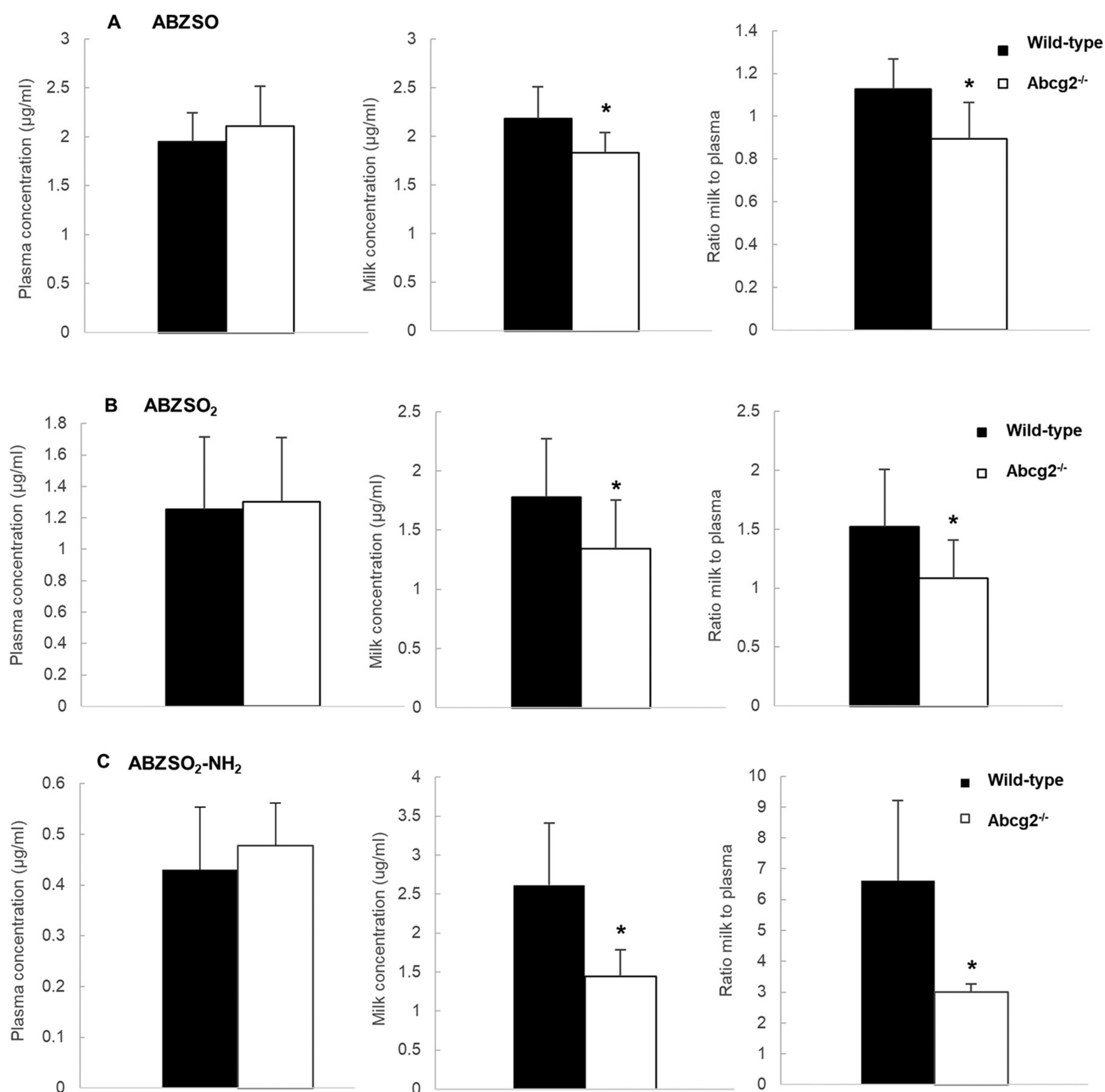

FIG 3 (A) Plasma and milk concentration and milk-to-plasma ratio of ABZSO in wild-type and $Abcg2^{-/-}$ lactating females after i.v. administration at a dose of 2 mg/kg. (B) Plasma and milk concentration and milk-to-plasma ratio of $ABZSO_2$ in wild-type and $Abcg2^{-/-}$ lactating females after i.v. administration at a dose of 2 mg/kg. (C) Plasma and milk concentration and milk-to-plasma ratio of $ABZSO_2$-NH_2 in wild-type and $Abcg2^{-/-}$ lactating females after i.v. administration at a dose of 2 mg/kg. Milk and plasma were collected 30 min after administration, and metabolite concentrations were determined by HPLC. Results are means ± SD (n = 4–11). (*) $P \leq 0.05$ significant differences between both groups of mice.

including ABZ metabolism, for ABCG2 interaction in these kinds of assays. Future experiments on target species are needed. In fact, we cannot discard those changes in administration route, dose rate and sampling points may alter the final outcome.

Most drugs pass into milk from blood by passive diffusion, and the milk-to-plasma ratio can be affected by the composition of the milk or by the physicochemical properties of the drug. However, drugs actively transported into milk by ABCG2 present higher milk-to-plasma ratios than predicted by diffusion, usually higher than 1 (44–46). In fact, in these experiments the milk-to-plasma ratios pointed to a specific role for ABCG2 in transport because in all cases the ratio was higher than 1 in the presence of the transporter (Fig. 3). It should be noted that the milk-to-plasma ratio in wild-type mice for $ABZSO_2$-NH_2 is the highest (6.60 ± 2.61) in all the drugs tested, despite its *in vitro* ratio transport in murine-Abcg2 transduced cells being the lowest (4.34 ± 0.68, Fig. 2B) compared to ABZSO, higher than 10 (34), and $ABZSO_2$ (5.59 ± 0.40, Fig. 1B).

Probably, in this case, passive diffusion or another transport mechanism (24) play an important role in its transfer into milk, since the milk-to-plasma ratio is also higher than 1 in the Abcg2 $^{-/-}$ mice (3.00 $\pm$ 0.25).

Regarding plasma levels, no significant differences were noted at the doses and collection times tested in female mice (Fig. 3). Comparable results have been reported for other ABCG2 substrates such as danofloxacin (47), ciprofloxacin (36), flunixin and its metabolite (30), and meloxicam (31) between wild-type and Abcg2$^{-/-}$ lactating female mice. A sex-dependent effect of ABCG2-mediated transport has been reported (48), so a systemic effect of Abcg2 cannot be ruled out in other experimental settings. In fact, sex dimorphism in plasma pharmacokinetics of ABZ metabolites has been reported in humans (49).

The role of ABCG2 in ABZ metabolite secretion into milk may have significant consequences in human and veterinary medicine, although this needs to be proven. In veterinary medicine, helminth infections are the main factor cause of significant problems and losses in livestock, and chemotherapy with anthelmintics is essential for parasite control (50, 51). Despite the benefits, drug therapy in dairy cows constitutes a public health and food-safety issue owing to the unwanted disposition of drug residue in milk. It is essential to prevent unacceptable levels of residues from those medicines entering the food chain within a welfare-friendly livestock industry (52). To protect consumers from the presence of risky concentrations of ABZ and its metabolite residues, which are potentially embryotoxic and teratogenic, maximum residue limits have been established at 100 μg/kg for the milk of all ruminants, and withdrawal periods of 3 days (10–12, 19, 43, 53). ABZ metabolites have been reported in routine milk samples from dairy farms that produce and supply milk to the markets and dairy food producers (54). Although levels do not exceed the limits, any change in ABCG2 activity may affect this outcome. However, further *in vivo* studies are needed to confirm this hypothesis.

There are several factors that could modify the expression and function of ABCG2, such as co-administration of drugs and dietary compounds (45, 47, 55–57). ABCG2 polymorphisms such as the bovine Y581S have been associated with changes in transfer of ABCG2 substrates into milk (28–30), thus providing evidence that genetic factors can alter drug concentrations in milk and consequently drug exposure to dairy consumers.

In conclusion, our results support the fact that ABCG2 is clearly involved in the active *in vitro* transport of ABZ metabolites by both murine and human variants. In addition, we demonstrate the crucial role of Abcg2 in the secretion into milk of ABZ metabolites using Abcg2$^{-/-}$ mice.

MATERIALS AND METHODS

Reagents and drugs. ABZ metabolites were purchased from LGC Standards (Teddington, Middlesex, UK). Lucifer Yellow, danofloxacin, and oxfendazole were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ko143 was acquired from Tocris (Bristol, UK). For *in vivo* studies, isoflurane (Isovet) was obtained from B. Braun VetCare (Barcelona, Spain) and oxytocin (Facilpart) from SYVA (León, Spain). All the other compounds used were reagent grade and were available from commercial sources.

Cell cultures. The polarized cell line Madin-Darby Canine Kidney (MDCK-II) was used in the transport assays. Murine Abcg2 and human ABCG2-transduced subclones were provided by Dr. A.H. Schinkel from the Netherlands Cancer Institute (Amsterdam, The Netherlands). Culture conditions have been previously reported (20). Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% mixture of antibiotics (penicillin and streptomycin) and 10% fetal calf serum at 37°C in the presence of 5% CO_2. Cells were trypsinized every 3 to 4 days for subculturing.

Transport assays. Transport assays were carried out as previously described by Merino et al. (58) with minor variations. 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 $\times$ 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 in both compartments, apical and basal, was replaced with 2 mL of Opti-MEM medium (Invitrogen, Carlsbad, CA), with or without 1 μM Ko143. The experiment began by replacing the medium on both sides with fresh Opti-MEM medium, with or without 1 μM Ko143 and 5 μM $ABZSO_2$ or $ABZSO_2$-NH_2. Cells were incubated at 37°C in 5% CO_2 and 100 μL aliquots were taken at 1, 2 and 3 h on the opposite side where drugs were added; this volume was replaced with fresh medium. Finally, 600 μL aliquots were taken at 4 h on both sides of the well. Aliquots were stored at –20°C until analysis by high performance liquid chromatography (HPLC) as described below.

The appearance of ABZ metabolites in the opposite compartment was related to the total drug added at the beginning of the experiment. At the beginning and the end of the experiment, transepithelial resistance was measured to check the tightness of the monolayer using Millicell ERS (Millipore

Burlington, MA). Lastly, at the end of the experiment, confluence of the monolayer was also measured with Lucifer Yellow permeability assays (33). Transport proficiency of these cells is constantly checked by testing a typical ABCG2 substrate like danofloxacin (47).

The apparent permeability (Papp) coefficient across MDCK-II parent, MDCK-II Abcg2 and MDCK-II ABCG2 cells monolayers in both apical to basal (AP-BL) (Papp A-B) and basal to apical (BL-AP) (Papp B-A) directions were calculated using following equation:

$$\mathrm{Papp} = \frac{\Delta Q}{\Delta t}\frac{1}{ACo}$$

Where $\Delta Q/\Delta t$ is the rate of corresponding ABZ metabolite appearing in the receiver chamber, which was obtained as the slope of the regression line on the transport-time profile of ABZ metabolite across the cell monolayers; C_0 is the initial concentration of drug; A is the cell monolayer surface area (4.67 cm^2). The efflux ratio is the Papp B-A/Papp A-B quotient.

Animals. Mice were housed and handled according to institutional and ARRIVE guidelines complying with European legislation (2010/63/EU). Experimental procedures were approved by the Animal Care and Use Committee of the University of León and the Junta de Castilla y León (ULE_011_2019). Animals used were lactating female Abcg2$^{-/-}$ and wild–type mice, all were $>$ 99% FVB genetic background between 8 and 17 weeks of age. Animals, generated (59) and kindly provided by Dr. A. H. Schinkel (The Netherlands Cancer Institute), were kept in a controlled temperature environment with 12 h of light and 12 h of darkness, and received a standard diet and water *ad libitum*.

For milk secretion experiments, pups of approximately 10 days old were separated from their mothers 4 h before starting the experiment. To stimulate milk secretion, 200 μL of oxytocin (1 IU/mL) was administered subcutaneously to lactating mice 10 min before sample collection. ABZSO, $ABZSO_2$ or $ABZSO_2$-NH_2 (2 mg/kg) were administered in the tail vein to wild–type and Abcg2$^{-/-}$ lactating female mice as a solution of 10% ethanol, 40% PEG400, and 50% saline. Intravenous administration consisted of 150 μL of solution per 30 g of body weight. Blood was collected 30 min after administration from the retro-orbital sinus under anesthesia with isoflurane, and then milk was collected from the mammary glands by pressing around the nipple using capillaries. Heparinized blood samples were centrifuged immediately at 3000 *g* for 15 min to obtain plasma. Finally, animals were sacrificed by cervical dislocation. Milk and plasma were stored at –20°C until the HPLC analysis. Four to 11 animals were used for each group of mice.

HPLC analysis. The conditions for HPLC analysis of ABZ metabolites were based on a previously described method (10, 34, 38) with minor modifications. To each 100 μL aliquots of milk and plasma, 10 μL of internal standard (oxfendazole 10 μg/mL) and 100 μL of acetonitrile were added in a 1.5 mL reaction tube. The mixture was vortexed horizontally for 15 min and then the samples were centrifuged at 6000 *g* for 6 min at 4°C. The supernatant was collected and evaporated to dryness under N_2 at 40°C. Samples were resuspended in 100 μL of cold methanol (Merck, Darmstadt, Germany) and injected into the HPLC system. Samples from the transport assays were not processed, and 100 μL of the culture media was directly injected into the HPLC system. The chromatographic system used in samples analysis consisted of a Waters 2695 separation module and a Waters 2998 UV photodiode array detector. Separation was performed on a reversed-phase column (4 mm particle size, 250 $\times$ 4.6 mm, Max-RP 80 Å, Phenomenex, Torrance, CA, USA). The mobile phase used was ammonium acetate 0.025 M pH 5: acetonitrile (76:24). The flow rate of the mobile phase was set to 0.8 mL/min and UV absorbance was measured at 292 nm.

For culture samples, standard samples of $ABZSO_2$ and $ABZSO_2$-NH_2 for calibration curves were prepared at concentrations of 0.039–10 μg/mL, with coefficients of correlation $>$0.99. Precision coefficients of variation were $<$15%, and accuracy values were $<$20%. LOD (limit of detection) and LOQ (limit of quantification) were calculated as described by Taverniers et al. (60). LOQ was 0.006–0.018 μg/mL and the LOD 0.002–0.008 μg/mL for cell culture samples.

For milk and plasma samples, standard samples of ABZSO, $ABZSO_2$ and $ABZSO_2$-NH_2 for calibration curves were prepared at concentrations of 0.156–10 μg/mL for milk and 0.078–10 μg/mL for plasma, with coefficients of correlation $>$0.98. Precision coefficients of variation were $<$15%, and accuracy values were $<$20%. LOQ was 0.102–0.155 μg/mL and LOD 0.038–0.06 μg/mL for milk samples and LOQ was 0.077–0.118 μg/mL and LOD 0.033–0.047 μg/mL for plasma samples.

Statistical analysis. The SPSS Statistics software v. 26.0 (IBM, Armonk, New York, NY, USA) was used for the statistical analysis. Comparisons between groups were made using Student's *t* test and Mann-Whitney U test for normal or not normally distributed variables, respectively. *P* value $\leq$ 0.05 indicates that the differences were statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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