AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

PHARMACOLOGY

Role of the Abcg2 Transporter in Secretion into Milk of the Anthelmintic Clorsulon: Interaction with Ivermectin

Esther Blanco-Paniagua,[a,b] Laura Álvarez-Fernández,[a,b] Andrea Rodríguez-Alonso,[b] Alicia Millán-Garcia,[a,b] Ana I. Álvarez,[a,b] Gracia Merino[a,b]

[a]Departamento de Ciencias Biomédicas-Fisiología, Facultad de Veterinaria, Universidad de León, León, Spain
[b]Instituto de Desarrollo Ganadero y Sanidad Animal (INDEGSAL), Universidad de León, León, Spain

ABSTRACT Clorsulon is a benzenesulfonamide drug that is effective in treating helminthic zoonoses such as fascioliasis. When used in combination with the macrocyclic lactone ivermectin, it provides high broad-spectrum antiparasitic efficacy. The safety and efficacy of clorsulon should be studied by considering several factors such as drug-drug interactions mediated by ATP-binding cassette (ABC) transporters due to their potential effects on the pharmacokinetics and drug secretion into milk. The aim of this work was to determine the role of ABC transporter G2 (ABCG2) in clorsulon secretion into milk and the effect of ivermectin, a known ABCG2 inhibitor, on this process. Using *in vitro* transepithelial assays with cells transduced with murine Abcg2 and human ABCG2, we report that clorsulon was transported *in vitro* by both transporter variants and that ivermectin inhibited its transport mediated by murine Abcg2 and human ABCG2. Wild-type and Abcg2$^{-/-}$ lactating female mice were used to carry out *in vivo* assays. The milk concentration and the milk-to-plasma ratio were higher in wild-type mice than in Abcg2$^{-/-}$ mice after clorsulon administration, showing that clorsulon is actively secreted into milk by Abcg2. The interaction of ivermectin in this process was shown after the coadministration of clorsulon and ivermectin to wild-type and Abcg2$^{-/-}$ lactating female mice. Treatment with ivermectin had no effect on the plasma concentrations of clorsulon, but the milk concentrations and milk-to-plasma ratios of clorsulon decreased in comparison to those with treatment without ivermectin, only in wild-type animals. Consequently, the coadministration of clorsulon and ivermectin reduces clorsulon secretion into milk due to drug-drug interactions mediated by ABCG2.

KEYWORDS ABCG2, clorsulon, substrate, milk, ivermectin, ABC transporters

Helminthic infections such as fascioliasis, which affects a wide range of domestic and wild animals and is caused by foodborne trematodes, produce significant economic losses in the livestock sector through losses of milk and meat yields. Noteworthy, not only animals but also humans can be affected by these infections (1, 2). Chemotherapy based on anthelmintics is essential for parasitic control, and their unsuitable use led to the serious problem of anthelmintic resistance (3, 4). The increase in the above-mentioned phenomenon has prompted the study of new strategies to slow its development, and the use of anthelmintic combinations has been described as one of them (4–6).

Clorsulon is a benzenesulfonamide antiparasitic used for treatment against adult liver flukes (7, 8), although it has limited efficacy against immature stages of flukes in domestic animals (9, 10). Moreover, clorsulon has been tested *in vitro* against *Echinococcus* spp. that cause helminthic zoonoses in humans, albeit the results are controversial (11, 12). Furthermore, and according to the principles of drug repurposing, it was recently proposed as an alternative for the treatment of schistosomiasis (13). Clorsulon is marketed in combination with the macrocyclic lactone ivermectin as a broad-spectrum anthelmintic formulation thanks to the association of a nematicide and a flukicide. This combination is

Address correspondence to Gracia Merino, gmerp@unileon.es.

The authors declare no conflict of interest.

Received 20 January 2023
Returned for modification 5 March 2023
Accepted 30 March 2023
Published 20 April 2023

effective, including against anthelmintic-resistant flukes, in sheep, rats, and cattle (14–17). Clorsulon is well absorbed and eliminated via the urinary tract without being metabolized (12, 18, 19).

Despite its benefits, anthelmintic therapy in veterinary medicine is closely related to the unwanted disposition of drug residues in animal-derived food such as milk, which is harmful to public health (20). Concerning clorsulon and milk, the presence of residues of the clorsulon parent drug is used as a marker to establish the maximum residue limit in milk (16 μg/mL), and a withdrawal period in combination with ivermectin of 66 days has been established for bovine milk (21–24). Despite this disadvantage, at present, there is a lack of authorized products for the treatment of immature-fluke infections in animals producing milk for human consumption, and the availability of an adequate range of products for the treatment of fascioliasis, a highly debilitating disease, is essential in order to avoid the unnecessary suffering of the animals (21). In this context, the study of potential mechanisms or factors that affect the safety of anthelmintic therapeutics, including the appearance of residues in milk and drug-drug interactions, is of the utmost relevance in order to develop new treatment strategies. The activity of the ATP-binding cassette (ABC) transporter breast cancer resistance protein (BCRP/ABCG2) is one of the main factors that affect the pattern of excretion of several compounds at the mammary gland level, which determines the appearance of drug residues in milk (25).

The ABCG2 transporter is expressed at the apical membrane of epithelial cells in relevant organs such as the intestine, kidney, liver, brain, and testicles, among others, and it is a pump that extrudes a broad range of compounds. Consequently, it is involved in pharmacokinetic processes modulating drug absorption, distribution, and elimination along with limiting drug accumulation in cells (26–28). Furthermore, ABCG2 is the only ABC transporter implicated in the active secretion of drugs into milk (25), which is attributable to its induced expression in the apical membrane of alveolar epithelial cells in the lactating mammary gland (29). Several studies have reported that the coadministration of drugs that interact with the ABCG2 transporter affects the drug pharmacokinetics and patterns of excretion into milk (30–34).

Subsequently, this study aimed to determine if the antiparasitic clorsulon is an *in vitro* substrate for murine Abcg2 and human ABCG2 as well as to analyze the involvement of this transporter in the secretion of clorsulon into milk. Besides, in this study, the effect of the macrocyclic lactone ivermectin, a known ABCG2 inhibitor (35), on this process was assessed.

RESULTS

***In vitro* transport of clorsulon: inhibition by ivermectin.** Transcellular transport assays using parental Madin-Darby canine kidney II (MDCK-II) cells and murine Abcg2- and human ABCG2-transduced MDCK-II cells were carried out to prove whether clorsulon is an *in vitro* substrate of ABCG2. Cell lines were grown to a confluent polarized monolayer, and the efflux transport of clorsulon at 10 μM was determined.

For the MDCK-II parental cells, the apically and basolaterally directed translocation of clorsulon were similar (Fig. 1A). However, increases in the apically directed translocation of clorsulon in murine Abcg2 (Fig. 1B)- and human ABCG2 (Fig. 1C)-transduced MDCK-II cells were observed. The relative efflux transport ratio for clorsulon in murine Abcg2-transduced cells was significantly higher (2.20 $\pm$ 0.13) than that in the parental cells (1.05 $\pm$ 0.08) ($P = 0.0002$) (Table 1). Similarly, for human ABCG2-transduced cells (Fig. 1C), significant differences in the relative efflux transport ratios were also found between human ABCG2-transduced cells and the parental cells (1.63 $\pm$ 0.17 and 1.05 $\pm$ 0.08, respectively [$P = 0.005$]) (Table 1). The selective participation of ABCG2 transport was confirmed using a specific inhibitor of ABCG2, Ko143 (Fig. 1D to F). In the presence of Ko143, the efflux transport ratios in murine Abcg2- and human ABCG2-transduced cells were similar to those in the parental MDCK-II cells (Table 1). These outcomes showed that clorsulon is an *in vitro* substrate for murine Abcg2 and human ABGC2.

To evaluate the effect of ivermectin on the Abcg2/ABCG2-mediated transport of clorsulon, ivermectin at 10 μM was added instead of Ko143 (Fig. 1G to I). For both murine Abcg2

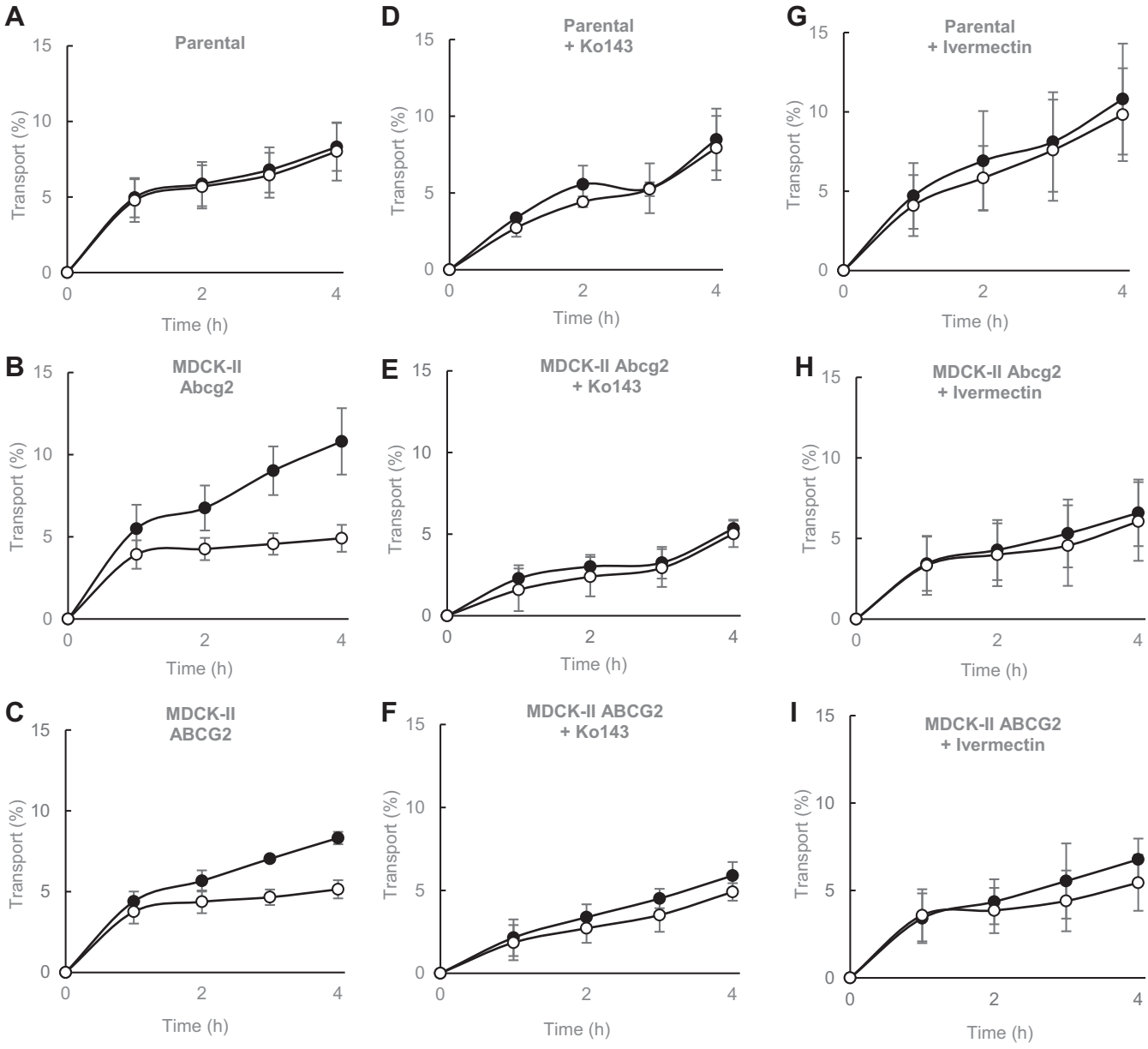

FIG 1 Transepithelial transport assays of clorsulon at 10 μM in the parental MDCK-II cell line and its subclones transduced with murine Abcg2 and human ABCG2 in the absence of inhibitors (A to C) and in the presence of the ABCG2 inhibitors Ko143 (1 μM) (D to F) and ivermectin (10 μM) (G to I). The assay was started by replacing the medium in either the apical or the basolateral compartment with fresh transport medium containing 10 μM clorsulon with or without Ko143 at 1 μM or ivermectin at 10 μM. Aliquots were taken from the opposite side at 1, 2, 3, and 4 h and measured by HPLC. The presence of clorsulon in the opposite compartment was related to the total drug added at the beginning of the assay. Results are presented as the means ± SD. ●, basolateral-to-apical transport; ○, apical-to-basolateral transport ($n \geq 3$).

(Fig. 1H)- and human ABCG2 (Fig. 1I)-transduced cells, apically directed translocation was inhibited in the presence of ivermectin compared to clorsulon treatment alone. As a result, the efflux transport ratios were significantly decreased from 2.20 ± 0.13 (without ivermectin) to 1.13 ± 0.16 (with ivermectin) in murine Abcg2-transduced cells ($P = 0.00003$) (Table 1) and from 1.63 ± 0.17 (without ivermectin) to 1.28 ± 0.17 (with ivermectin) in human ABCG2-transduced cells ($P = 0.033$) (Table 1). Moreover, the efflux transport ratios of each cell line in the presence of ivermectin did not show significant differences compared to Ko143 treatment. Concisely, clorsulon transport was inhibited in the presence of ivermectin, leading to inhibition similar to that attained with the specific ABCG2 inhibitor Ko143. These results reveal that ivermectin affects the Abcg2/ABCG2 transport of clorsulon, acting as an inhibitor.

TABLE 1 Percentage of transport toward the apical or basolateral compartment and relative transport efflux ratios at 4 h for parental MDCK-II cells and murine Abcg2- and human ABCG2-transduced cells lines in the presence of clorsulon and clorsulon with the ABCG2 inhibitor Ko143 or ivermectin[c]

Treatment and cell line	Mean % transport ± SD		Mean BL-AP/AP-BL ratio ± SD
	BL-AP	AP-BL	
Clorsulon (10 μM)			
MDCK-II parental	8.31 ± 1.58	8.01 ± 1.93	1.05 ± 0.08
MDCK-II Abcg2	10.81 ± 2.02	4.91 ± 0.82	2.20 ± 0.13[a]
MDCK-II ABCG2	8.32 ± 0.38	5.15 ± 0.56	1.63 ± 0.17[a]
Clorsulon (10 μM) + Ko143 (1 μM)			
MDCK-II parental	8.48 ± 2.01	7.93 ± 2.09	1.07 ± 0.03
MDCK-II Abcg2	5.34 ± 0.54	5.02 ± 0.81	1.07 ± 0.13[b]
MDCK-II ABCG2	5.89 ± 0.82	4.91 ± 0.53	1.20 ± 0.08[b]
Clorsulon (10 μM) + ivermectin (10 μM)			
MDCK-II parental	10.81 ± 3.50	9.82 ± 2.92	1.11 ± 0.21
MDCK-II Abcg2	6.59 ± 2.07	6.05 ± 2.44	1.13 ± 0.16[b]
MDCK-II ABCG2	6.78 ± 1.19	5.44 ± 1.60	1.28 ± 0.17[b]

[a]Significant difference in the transport ratio compared to that for the parental MDCK-II cells ($P \leq 0.05$).
[b]Significant difference in the transport ratio for each cell line compared to that with treatment with clorsulon alone ($P \leq 0.05$).
[c]Results are presented as the means ± SD ($n \geq 3$). BL-AP, basolateral-to-apical transport; AP-BL, apical-to-basolateral transport.

Secretion of clorsulon into milk in Abcg2$^{-/-}$ and wild-type mice: interaction with ivermectin. To assess whether Abcg2 is involved in the active secretion of clorsulon into milk as well as whether ivermectin has any effect on the secretion of clorsulon into milk mediated by Abcg2, clorsulon (5 mg/kg of body weight) was intravenously (i.v.) administered to lactating wild-type and Abcg2$^{-/-}$ female mice, with or without ivermectin administration (0.5 mg/kg of body weight) intraperitoneally (i.p.) 10 min prior to the i.v. administration of clorsulon. After 30 min of clorsulon administration, milk and plasma were collected.

No differences were found in the plasma concentrations of clorsulon between wild-type and Abcg2$^{-/-}$ mice for the treatments with clorsulon alone (3.09 ± 1.29 μg/mL and 3.81 ± 1.65 μg/mL, respectively) (Fig. 2A) and with the combination of clorsulon and ivermectin (3.71 ± 1.24 μg/mL and 4.76 ± 1.32 μg/mL, respectively) (Fig. 2A). Additionally, no differences in the plasma concentrations were found when treatments with and without ivermectin were compared in both types of mice.

After the administration of clorsulon alone, milk concentrations (Fig. 2B) were 1.6-fold higher in wild-type mice than in Abcg2$^{-/-}$ mice (2.56 ± 0.75 μg/mL versus 1.61 ± 0.65 μg/mL [$P = 0.01$]). In the same way, the milk-to-plasma ratio of clorsulon (Fig. 2C) was significantly higher in wild-type than in Abcg2$^{-/-}$ mice (1.01 ± 0.61 μg/mL versus 0.51 ± 0.36 μg/mL [$P = 0.028$]). These outcomes show that Abcg2 is involved in the active secretion of clorsulon into milk.

Furthermore, when clorsulon was administered with ivermectin, the milk concentrations of clorsulon (Fig. 2B) were 1.7-fold lower (1.47 ± 0.45 μg/mL) than those with clorsulon alone in wild-type animals (2.56 ± 0.75 μg/mL) ($P = 0.016$). Likewise, the milk-to-plasma ratio in wild-type mice treated with ivermectin (Fig. 2C) was significantly lower than that in animals treated with clorsulon alone (0.43 ± 0.15 μg/mL and 1.01 ± 0.61 μg/mL, respectively [$P = 0.028$]). No differences in the milk concentrations (Fig. 2B) or milk-to-plasma ratios (Fig. 2C) of clorsulon were found between treatments with and without ivermectin in Abcg2$^{-/-}$ mice.

Our results reveal that the coadministration of clorsulon with ivermectin efficiently inhibits the Abcg2-mediated secretion of clorsulon into milk, diminishing the milk levels of clorsulon in wild-type animals to levels similar to those in Abcg2$^{-/-}$ mice.

DISCUSSION

Unfortunately, the necessary use of anthelmintic drugs has led to the serious problem of anthelmintic resistance, which consequently affects animal health and

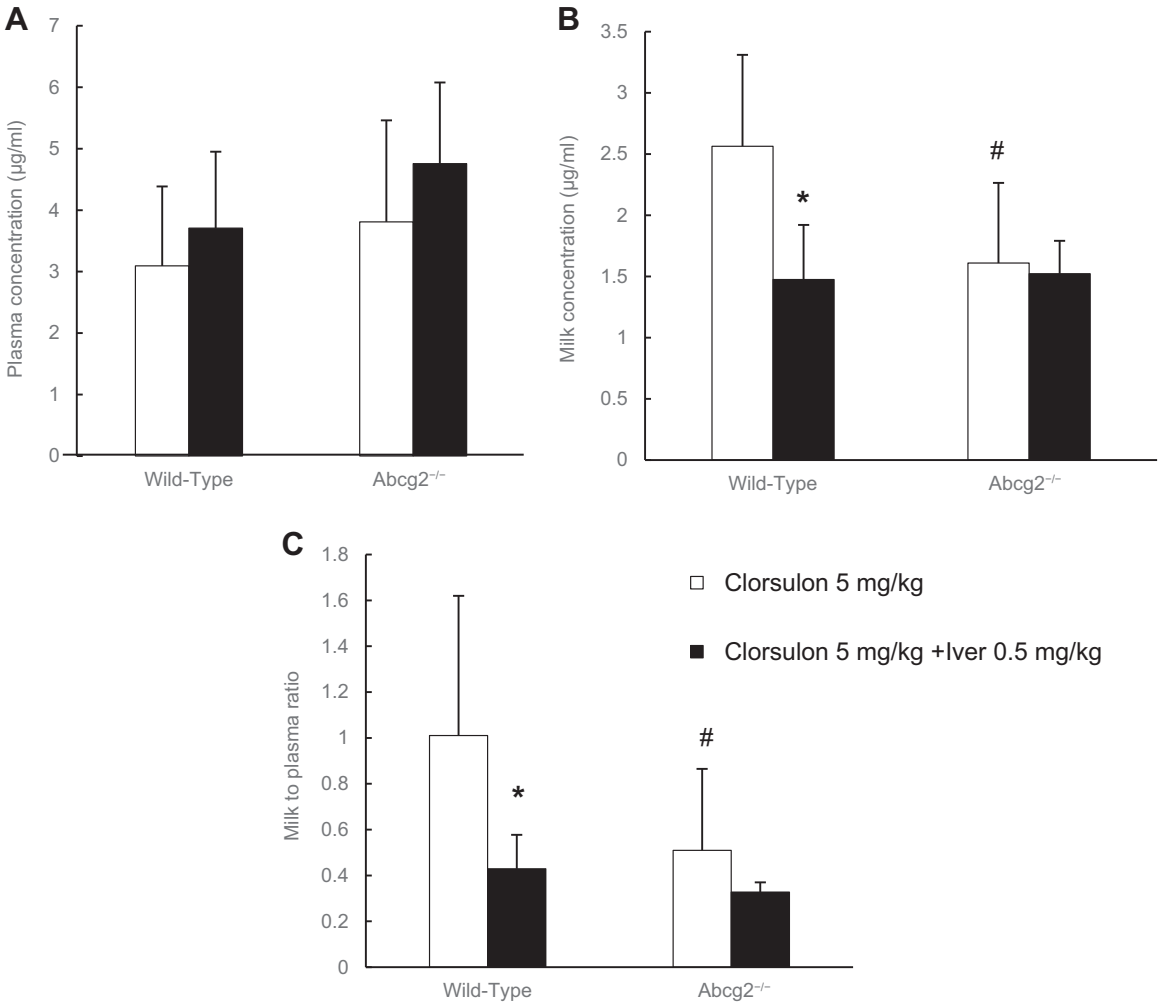

FIG 2 *In vivo* effect of Abcg2 and ivermectin on the secretion of clorsulon into milk. (A) Plasma concentrations of clorsulon in wild-type and Abcg2$^{-/-}$ lactating females. (B) Milk concentrations of clorsulon in wild-type and Abcg2$^{-/-}$ lactating females. (C) Milk-to-plasma ratios of clorsulon in wild-type and Abcg2$^{-/-}$ lactating females. White columns represent the i.v. administration of clorsulon (5 mg/kg). Black columns represent the i.p. administration of ivermectin (0.5 mg/kg) 10 min prior to the i.v. administration of clorsulon (5 mg/kg). Milk and plasma were collected 30 min after clorsulon administration, and concentrations were determined by HPLC. Results are presented as the means ± SD (n = 5 to 12). #, $P \leq 0.05$ (significant differences between wild-type and Abcg2$^{-/-}$ mice); *, $P \leq 0.05$ (significant differences between treatment with clorsulon alone and treatment with clorsulon and ivermectin).

production. Combination therapy with anthelmintics has been proposed as a strategy to slow the development of resistance, even when strong resistance to one component of the combination is reported (4). The rationale behind the use of drug coadministration is based on the lower degree of resistance to multiple drugs than with single treatments (6); for example, it has been described that the combination of nematicides like ivermectin and flukicides such as clorsulon improves efficacy, broadens the spectrum of anthelmintic activity, and limits the emergence of resistance (3). Several other combinations of antiparasitics have been tested against soil-transmitted helminth infections to increase drug efficiency and avoid resistance; for example, albendazole plus ivermectin and tribendimidine plus ivermectin have revealed a broad spectrum of activity against these infections (36). Therefore, combination therapy can be used in order to modulate drug efficacy, which may be useful for the reversal of resistance in chemotherapy (37, 38). However, the use of drug combinations may lead to drug interactions that should be carefully assessed in order to investigate the potential role of ABC transporters, which have been recognized as important mechanisms for clinically relevant drug-drug interactions (38). In this study, the *in vitro* and *in vivo* interactions of clorsulon with ABCG2 and the effect of ivermectin on these processes have been investigated.

In vitro transcellular transport assays using MDCK-II cells transduced with murine Abcg2 showed that clorsulon is effectively transported by murine Abcg2 (Fig. 1B) and human ABCG2 (Fig. 1C). This is the first time that an antiparasitic drug from the benzenesulfonamide group has ever been reported as an ABCG2 substrate. Previously, it was described that other antiparasitics show *in vitro* interactions with ABCG2, mainly benzimidazole drugs like albendazole sulfoxide, oxfendazole (39), and pantoprazole (40), which were reported to be *in vitro* ABCG2 substrates with transport ratios of $\geq$6. Recently, albendazole metabolites, albendazole sulfone and albendazole amino-sulfone, were also described as *in vitro* substrates of murine Abcg2 and human ABCG2 (41) with transport ratios of around 4 and 2, respectively, similar to our transport ratios with clorsulon (Fig. 1B and C).

In contrast, other antiparasitic drugs have been characterized as *in vitro* inhibitors of murine Abcg2 and human ABCG2, such as selamectin (35) or triclabendazole metabolites (37). Moreover, ivermectin was previously reported to show inhibitory potencies of around 36% for murine Abcg2 and 95% for human ABGC2 at 50 μM (35). In addition, 50% inhibitory concentration (IC_{50}) values in the range of 1 to 1.5 μM for human ABCG2 were described previously (42). As mentioned above, the combination of clorsulon and ivermectin is commonly used and marketed (3, 43), so we also evaluated *in vitro* drug-drug interactions between clorsulon and ivermectin by conducting a transcellular transport assay in the presence of ivermectin (10 μM) in MDCK-II cells transduced with murine Abcg2 and human ABCG2. We showed that ivermectin at 10 μM inhibits the ABCG2-mediated transport of clorsulon in murine Abcg2 (Fig. 1H)- and human ABCG2 (Fig. 1I)-transduced cells to the same extent, resulting in efflux ratios similar to those of the parental cells (Table 1). Previous *in vitro* studies have reported that ivermectin can block ABCG2-mediated transcellular transport using albendazole sulfoxide (44) or danofloxacin (31) as the substrate.

After treatments with antiparasitic drugs, the presence of residues in edible products such as milk constitutes one of the main hazards for public health (38, 45). In such a manner, interactions with ABCG2 are gaining clinical importance. ABCG2 is the main factor involved in the active secretion of numerous compounds into milk, leading to important clinical and toxicological consequences (25). Therefore, the influence of Abcg2 on clorsulon secretion into milk was also evaluated in our study. The doses used (5 mg/kg) were chosen based on the results of previous studies with clorsulon in rodents (46, 47). We clearly showed that clorsulon is actively secreted into milk by Abcg2, as indicated by the higher milk concentrations (Fig. 2B) and milk-to-plasma ratios (Fig. 2C) of clorsulon in wild-type mice than in Abcg2$^{-/-}$ mice. Secretion into milk mediated by Abcg2 has been previously reported for other drugs of different classes such as antitumoral (48), antibiotic (31, 49, 50), anti-inflammatory (51, 52), antiparasitic (41), and natural (53, 54) compounds.

As the coadministration of drugs may affect the pattern of secretion into milk, drug residues may consequently be altered (38). Along these lines, in the present study, we also assessed *in vivo* the potential effect of the macrocyclic lactone ivermectin on the plasma levels and milk secretion of the substrate clorsulon at the recommended dose in veterinary practice (21) (1:0.1 ratio [5 mg/kg clorsulon–0.5 mg/kg ivermectin]). Our results showed that after treatment with ivermectin, the milk concentrations of clorsulon (Fig. 2B) and the milk-to-plasma ratios (Fig. 2C) in wild-type mice were approximately 2-fold lower than those with clorsulon alone. As a result, the complete inhibition of Abcg2-mediated milk secretion of clorsulon in wild-type mice was observed, as the levels were equal to those in Abcg2$^{-/-}$ mice.

The effect of ivermectin on clorsulon secretion into milk can be attributed to interactions mediated by Abcg2 because it is the main ABC transporter with induced expression in the mammary gland during lactation (29, 48), but also, no differences in milk concentrations or milk-to-plasma ratios were detected between wild-type and Abcg2$^{-/-}$ mice after ivermectin treatment, indicating that ivermectin's effect is Abcg2 specific. Similar results were previously reported using *in vivo* assays with mice in which ABCG2 inhibitors such as isoflavones and triclabendazole sulfoxide decreased the secretion of nitrofurantoin into milk (37, 55). This effect of ivermectin on the ABCG2-mediated secretion of clorsulon into milk may be

translated to the clinical situation, although this remains to be proven. In fact, reductions of the secretion of ABCG2 substrates like danofloxacin (31) or meloxicam (56) into milk after the coadministration of ivermectin were reported in sheep. Regarding antiparasitics, the milk concentrations of moxidectin were reduced after coadministration with triclabendazole (34).

Regarding plasma levels, no significant differences were noted between both types of mice at the doses and collection times tested when clorsulon was administered alone or with ivermectin (Fig. 2A). Although clorsulon is a substrate of Abcg2, and this transporter can affect the plasma disposition of its substrates (27), additional factors such as the potential *in vivo* involvement of other transporters could conceal an effect of Abcg2 on the systemic disposition of clorsulon. However, previous studies using cells overexpressing P-glycoprotein failed to show any interaction of clorsulon with this relevant ABC transporter that is modulated by ivermectin (3).

In conclusion, the role of ABCG2 in the *in vitro* transport of clorsulon by murine Abcg2 and human ABCG2 and its involvement in the active secretion of clorsulon into milk were revealed. Besides, drug-drug interactions mediated by ABCG2 in this process were shown using the macrocyclic lactone ivermectin as an ABCG2 inhibitor, contributing to the understanding of the potential factors that could influence the transfer of antiparasitic drugs into milk.

MATERIALS AND METHODS

Reagents and drugs. Clorsulon, oxfendazole, and Lucifer yellow were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ko143 was acquired from Tocris (Bristol, UK). For *in vivo* assays, ivermectin (Ivomec) was purchased from Boehringer Ingelheim (Barcelona, Spain), isoflurane (Isovet) was purchased from Braun VetCare (Barcelona, Spain), and oxytocin (Facilpart) was purchased from Syva (León, Spain). All the other compounds used were of reagent grade and were available from commercial sources.

Cell cultures. For transcellular transport assays, the polarized cell line MDCK-II was used. Murine Abcg2- and human ABCG2-transduced subclones were provided by A. H. Schinkel of The Netherlands Cancer Institute (Amsterdam, The Netherlands). The culture conditions were previously described (28). Briefly, cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with GlutaMAX (Life Technologies, Inc., Rockville, MD, USA), penicillin (50 U/mL), streptomycin (50 μg/mL), and 10% (vol/vol) fetal calf serum (MP Biomedicals, Solon, OH, USA) at 37°C in the presence of 5% CO_2. The cells were trypsinized every 3 to 4 days for subculturing.

Transcellular transport assays. Transcellular transport assays using transduced MDCK-II cells were carried out as previously described (50), with minor modifications. Cells were seeded onto microporous membrane filters (3.0-μm pore size and 24-mm diameter) (Transwell 3414; Costar, Corning, NY) at a density of 1.0×10^6 cells per well. Cells were grown for 3 days, and the medium was replaced every day. At the beginning and the end of the assay, transcellular resistance was measured to check the tightness of the monolayer using Millicell Electrical Resistance System (ERS) (Millipore, Burlington, MA); wells registering a resistance of 150 Ω or higher were used in the transport experiments. Moreover, a Lucifer yellow permeability assay was used to measure the confluence of the monolayer at the end of the experiment. Results from monolayers with Lucifer yellow transport rates of >3% were discarded. The transport proficiency of these cells was recurrently checked by testing a typical ABCG2 substrate, danofloxacin (31).

Two hours before the start of the experiment, the medium in both the apical and basal compartments was replaced with 2 mL of transport medium with or without the inhibitors (Ko143 [1 μM] and ivermectin [10 μM]) (35, 57). The transport medium consisted of Hanks' balanced salt solution (Sigma-Aldrich) supplemented with HEPES (25 mM). The assay was started by replacing the medium in both compartments with fresh transport medium with or without inhibitors (Ko143 [1 μM] and ivermectin [10 μM]) and clorsulon (10 μM). Cells were incubated at 37°C with 5% CO_2, and 100-μL aliquots were taken at 1, 2, and 3 h on the opposite side from where clorsulon was added; this volume was replaced with fresh medium. Finally, 600-μL aliquots were taken from both sides of the well at 4 h. Aliquots were stored at −20°C until analysis by high-performance liquid chromatography (HPLC), as described below. The concentration of clorsulon in the acceptor compartment was recorded as a percentage of the total drug added to the donor compartment at the beginning of the experiment. The relative efflux transport ratio was calculated as the basal-to-apical-direction transport percentage divided by the apical-to-basal-direction transport percentage at 4 h.

Animals. Mice were housed and handled according to institutional and ARRIVE guidelines in compliance 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). Lactating female Abcg2$^{-/-}$ and wild-type mice were used, all of a >99% FVB genetic background and between 8 and 17 weeks of age. Animals were generated (58) and kindly provided by A. H. Schinkel (The Netherlands Cancer Institute). Animals 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*.

Milk secretion experiments. Four hours before the start of the experiment, pups of approximately 10 days of age were separated from their mothers. Clorsulon (5 mg/kg) was administered in the tail vein to wild-type and Abcg2$^{-/-}$ lactating female mice as a solution of 10% ethanol, 40% polyethylene glycol

400 (PEG 400), and 50% saline. Intravenous (i.v.) administration consisted of 150 μL of solution per 30 g of body weight. Ivermectin (Ivomec) at 0.5 mg/kg or the vehicle (saline) was administered intraperitoneally (i.p.) (200 μL of solution per 30 g of body weight) 10 min before the intravenous administration of clorsulon. To stimulate milk secretion, oxytocin (200 μL of a 1-IU/mL solution) was administered subcutaneously to lactating mice 10 min before sample collection. Blood and milk samples were collected 30 min after clorsulon administration under anesthesia by isoflurane. First, blood samples were collected by orbital bleeding, and heparinized blood samples were centrifuged at 3,000 $\times$ *g* for 15 min to obtain plasma. Next, milk was collected from the mammary gland by gentle pinching around the nipple using capillaries. Animals were killed by cervical dislocation at the end of the experiment. Plasma and milk samples were stored at −20°C until HPLC analysis.

High-performance liquid chromatography analysis. The conditions for HPLC analysis of clorsulon were based on a previously described method (18), with modifications.

Ten microliters of the internal standard (oxfendazole at 10 μg/mL) and 200 μL of ethyl acetate were added to each 100-μL aliquot of milk and plasma. The mix was vortexed horizontally for 1 min and then centrifuged at 1,200 $\times$ *g* for 10 min at 4°C. The supernatant was collected and evaporated to dryness under N_2 at 30°C. Five hundred microliters of hexane and 300 μL of acetonitrile were added to evaporated samples, and the mix was vortexed horizontally for 1 min and then centrifuged at 1,200 $\times$ *g* for 10 min at 4°C. Hexane was eliminated, and the rest was evaporated to dryness under N_2 at 30°C. Samples were resuspended in 100 μL of cold methanol and injected into the HPLC system. Samples from *in vitro* assays were injected directly into the HPLC system. For the sample analysis, a Waters 2695 separation module and a Waters 2998 UV photodiode array detector were used as the chromatographic system. 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 potassium phosphate (pH 7)-acetonitrile (75:25) with a flow rate of 1.20 mL/min and a UV absorbance of 225 nm.

Standard samples of clorsulon for calibration curves were prepared at concentrations of 0.078 to 10 μg/mL for culture samples, 0.078 to 5 μg/mL for milk samples, and 0.156 to 5 μg/mL for plasma samples. The coefficients of correlation for clorsulon ranged between 0.986 and 0.999 for the analyzed samples. Precision coefficients of variation were <15%, and relative standard deviation (SD) (accuracy) values were <20%. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as described previously by Taverniers et al. (59). The LOQ was 0.02 μg/mL and the LOD was 0.01 μg/mL for cell culture samples. For milk samples, the LOQ was 0.09 μg/mL, and the LOD was 0.03 μg/mL. Finally, for plasma samples, the LOQ was 0.07 μg/mL, and the LOD was 0.03 μg/mL.

Statistical analysis. SPSS Statistics software (v.26.0; IBM, Armonk, NY, USA) was used for the statistical analysis. Comparisons between groups were made using Student's *t* test and a Mann-Whitney U test for normally and not normally distributed variables, respectively. A *P* value of ≤0.05 indicates that the differences were statistically significant.

ACKNOWLEDGMENTS

We thank A. H. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands), who provided the parental MDCK-II cell line, its murine Abcg2- and human ABCG2-transduced subclones, and Abcg2 knockout mice.

This work was supported by research projects RTI2018-100903-B-I00 and PID2021-125660OB-I00 (MCIN/AEI/10.13039/501100011033/FEDER Una Manera de Hacer Europa) and by predoctoral grants (grant FPU18/01559 to E.B.-P. and grant FPU19/04169 to L.A.-F.) from the Spanish Ministry of Education, Culture, and Sport.

E.B.-P. Conceptualization, Methodology, Data Curation, Formal Analysis, Investigation, and Writing – Original Draft. L.Á.-F., A.R.-A., and A.M.-G. Methodology, Data Curation, Formal Analysis, and Investigation. A.I.Á. Conceptualization, Methodology, Funding Acquisition, Validation, Supervision, and Writing – Review and Editing. G.M. Conceptualization, Methodology, Funding Acquisition, Validation, Project Administration, Supervision, and Writing – Review and Editing.

REFERENCES

1. Duthaler U, Smith TA, Keiser J. 2010. In vivo and in vitro sensitivity of Fasciola hepatica to triclabendazole combined with artesunate, artemether, or OZ78. Antimicrob Agents Chemother 54:4596–4604. https://doi.org/10.1128/AAC.00828-10.
2. Keiser J, Utzinger J. 2009. Food-borne trematodiases. Clin Microbiol Rev 22:466–483. https://doi.org/10.1128/CMR.00012-09.
3. Dupuy J, Alvinerie M, Ménez C, Lespine A. 2010. Interaction of anthelmintic drugs with P-glycoprotein in recombinant LLC-PK1-mdr1a cells. Chem Biol Interact 186:280–286. https://doi.org/10.1016/j.cbi.2010.05.013.
4. Fissiha W, Kinde MZ. 2021. Anthelmintic resistance and its mechanism: a review. Infect Drug Resist 14:5403–5410. https://doi.org/10.2147/IDR.S332378.
5. Bartram DJ, Leathwick DM, Taylor MA, Geurden T, Maeder SJ. 2012. The role of combination anthelmintic formulations in the sustainable control of sheep nematodes. Vet Parasitol 186:151–158. https://doi.org/10.1016/j.vetpar.2011.11.030.
6. Lanusse C, Canton C, Virkel G, Alvarez L, Costa-Junior L, Lifschitz A. 2018. Strategies to optimize the efficacy of anthelmintic drugs in ruminants. Trends Parasitol 34:664–682. https://doi.org/10.1016/j.pt.2018.05.005.
7. Elitok B, Elitok ÖM, Kabu M. 2006. Field trial on comparative efficacy of four fasciolicides against natural liver fluke infection in cattle. Vet Parasitol 135:279–285. https://doi.org/10.1016/j.vetpar.2005.10.008.
8. Committee for Medicinal Products for Veterinary Use, European Medicines Agency. 2008. European public MRL assessment report (EPMAR). Clorsulon. Cattle. EMEA/CVMP/358525/2008. European Medicines Agency, Amsterdam, The Netherlands.
9. Fairweather I, Boray JC. 1999. Fasciolicides: efficacy, actions, resistance and its management. Vet J 158:81–112. https://doi.org/10.1053/tvjl.1999.0377.

10. Álvarez-Sánchez MA, Mainar-Jaime RC, Pérez-García J, Rojo-Vázquez FA. 2006. Resistance of Fasciola hepatica to triclabendazole and albendazole in sheep in Spain. Vet Rec 159:424–425. https://doi.org/10.1136/vr.159.13.424.
11. Xin Q, Yuan M, Li H, Song X, Lu J, Jing T. 2019. In vitro and in vivo effects of 3-bromopyruvate against Echinococcus metacestodes. Vet Res 50:96. https://doi.org/10.1186/s13567-019-0710-7.
12. Richter D, Richter J, Grüner B, Kranz K, Franz J, Kern P. 2013. In vitro efficacy of triclabendazole and clorsulon against the larval stage of Echinococcus multilocularis. Parasitol Res 112:1655–1660. https://doi.org/10.1007/s00436-013-3321-7.
13. Ferraroni M, Angeli A, Carradori S, Supuran CT. 2022. Inhibition of Schistosoma mansoni carbonic anhydrase by the antiparasitic drug clorsulon: X-ray crystallographic and in vitro studies. Acta Crystallogr D Struct Biol 78:321–327. https://doi.org/10.1107/S2059798322000079.
14. Sibille P, Calléja C, Carreras F, Bigot K, Galtier P, Boulard C. 2000. Fasciola hepatica: influence of gender and liver biotransformations on flukicide treatment efficacy of rats infested and cured with either clorsulon/ivermectin or triclabendazole. Exp Parasitol 94:227–237. https://doi.org/10.1006/expr.2000.4501.
15. Heredia R, Aguilar E, Romero C, Bautista L, Mendoza G. 2016. Evaluation of five treatments to control intestinal parasites in sheep in Ayapango, State of Mexico. Vet World 9:1233–1237. https://doi.org/10.14202/vetworld.2016.1233-1237.
16. Ibarra-Velarde F, Vera-Montenegro Y, Nájera-Fuentes R, Sánchez-Albarran A. 2001. Efficacy of combined chemotherapy against gastrointestinal nematodes and Fasciola hepatica in cattle. Vet Parasitol 99:199–204. https://doi.org/10.1016/s0304-4017(01)00460-5.
17. Loyacano AF, Skogerboe TL, Williams JC, DeRosa AA, Gurie JA, Shostrom VK. 2001. Effects of parenteral administration of doramectin or a combination of ivermectin and clorsulon on control of gastrointestinal, nematode and liver fluke infections and on growth performance in cattle. J Am Vet Med Assoc 218:1465–1468. https://doi.org/10.2460/javma.2001.218.1465.
18. Sundlof SF, Whitlock TW. 1992. Clorsulon pharmacokinetics in sheep and goats following oral and intravenous administration. J Vet Pharmacol Ther 15:282–291. https://doi.org/10.1111/j.1365-2885.1992.tb01018.x.
19. Committee for Veterinary Medicinal Products, European Agency for the Evaluation of Medicinal Products. 1995. Clorsulon. EMEA/MRL/037/95-FINAL. European Agency for the Evaluation of Medicinal Products, Amsterdam, The Netherlands.
20. Statham JME. 2015. Control of liver fluke: an emerging issue in terms of veterinary residues. Vet Rec 177:519–521. https://doi.org/10.1136/vr.h6287.
21. Committee for Medicinal Products for Veterinary Use, European Medicines Agency. 2014. European public MRL assessment report (EPMAR). Clorsulon (bovine milk)—after the provisional maximum residue limit (MRL). EMA/CVMP/752339/2013. European Medicines Agency, Amsterdam, The Netherlands.
22. European Medicines Agency. 2014. Opinion of the Committee for Medicinal Products for Veterinary Use on the establishment of maximum residue limits. EMA/CVMP/741367/2013-Rev1. European Medicines Agency, Amsterdam, The Netherlands.
23. Chiu SHL, Baylis FP, Taub R, Green M, Halley BA, Bodden RM. 1989. Depletion of [14C]clorsulon in cows' milk. J Agric Food Chem 37:819–823. https://doi.org/10.1021/jf00087a054.
24. Escribano M, San Andres MI, de Lucas JJ, Gonzalez-Canga A. 2012. Ivermectin residue depletion in food producing species and its presence in animal foodstuffs with a view to human safety. Curr Pharm Biotechnol 13:987–998. https://doi.org/10.2174/138920112800399121.
25. Garcia-Lino AM, Álvarez-Fernández I, Blanco-Paniagua E, Merino G, Álvarez AI. 2019. Transporters in the mammary gland—contribution to presence of nutrients and drugs into milk. Nutrients 11:2372. https://doi.org/10.3390/nu11102372.
26. Khunweeraphong N, Szöllősi D, Stockner T, Kuchler K. 2019. The ABCG2 multidrug transporter is a pump gated by a valve and an extracellular lid. Nat Commun 10:5433. https://doi.org/10.1038/s41467-019-13302-2.
27. Vlaming MLH, Lagas JS, Schinkel AH. 2009. Physiological and pharmacological roles of ABCG2 (BCRP): recent findings in Abcg2 knockout mice. Adv Drug Deliv Rev 61:14–25. https://doi.org/10.1016/j.addr.2008.08.007.
28. González-Lobato L, Real R, Herrero D, de la Fuente A, Prieto JG, Marqués MM, Álvarez AI, Merino G. 2014. Novel in vitro systems for prediction of veterinary drug residues in ovine milk and dairy products. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 31:1026–1037. https://doi.org/10.1080/19440049.2014.908261.
29. van Herwaarden AE, Schinkel AH. 2006. The function of breast cancer resistance protein in epithelial barriers, stem cells and milk secretion of drugs and xenotoxins. Trends Pharmacol Sci 27:10–16. https://doi.org/10.1016/j.tips.2005.11.007.
30. Pulido MM, Molina AJ, Merino G, Mendoza G, Prieto JG, Alvarez AI. 2006. Interaction of enrofloxacin with breast cancer resistance protein (BCRP/ABCG2): influence of flavonoids and role in milk secretion in sheep. J Vet Pharmacol Ther 29:279–287. https://doi.org/10.1111/j.1365-2885.2006.00744.x.
31. Real R, Egido E, Pérez M, González-Lobato L, Barrera B, Prieto JG, Álvarez AI, Merino G. 2011. Involvement of breast cancer resistance protein (BCRP/ABCG2) in the secretion of danofloxacin into milk: interaction with ivermectin. J Vet Pharmacol Ther 34:313–321. https://doi.org/10.1111/j.1365-2885.2010.01241.x.
32. Perez M, Otero JA, Barrera B, Prieto JG, Merino G, Alvarez AI. 2013. Inhibition of ABCG2/BCRP transporter by soy isoflavones genistein and daidzein: effect on plasma and milk levels of danofloxacin in sheep. Vet J 196:203–208. https://doi.org/10.1016/j.tvjl.2012.09.012.
33. Otero JA, García-Mateos D, Alvarez-Fernández I, García-Villalba R, Espín JC, Álvarez AI, Merino G. 2018. Flaxseed-enriched diets change milk concentration of the antimicrobial danofloxacin in sheep. BMC Vet Res 14:14. https://doi.org/10.1186/s12917-018-1341-3.
34. Barrera B, González-Lobato L, Otero JA, Real R, Prieto JG, Álvarez AI, Merino G. 2013. Effects of triclabendazole on secretion of danofloxacin and moxidectin into the milk of sheep: role of triclabendazole metabolites as inhibitors of the ruminant ABCG2 transporter. Vet J 198:429–436. https://doi.org/10.1016/j.tvjl.2013.07.033.
35. Merino G, Real R, Baro MF, Gonzalez-Lobato L, Prieto JG, Alvarez AI, Marques MM. 2009. Natural allelic variants of bovine ATP-binding cassette transporter ABCG2: increased activity of the Ser581 variant and development of tools for the discovery of new ABCG2 inhibitors. Drug Metab Dispos 37:5–9. https://doi.org/10.1124/dmd.108.022715.
36. Moser W, Schindler C, Keiser J. 2019. Drug combinations against soil-transmitted helminth infections. Adv Parasitol 103:91–115. https://doi.org/10.1016/bs.apar.2018.08.002.
37. Barrera B, Otero JA, Egido E, Prieto JG, Seelig A, Álvarez AI, Merino G. 2012. The anthelmintic triclabendazole and its metabolites inhibit the membrane transporter ABCG2/BCRP. Antimicrob Agents Chemother 56:3535–3543. https://doi.org/10.1128/AAC.06345-11.
38. Virkel G, Ballent M, Lanusse C, Lifschitz A. 2019. Role of ABC transporters in veterinary medicine: pharmaco-toxicological implications. Curr Med Chem 26:1251–1269. https://doi.org/10.2174/0929867325666180201094730.
39. Merino G, Jonker JW, Wagenaar E, Pulido M, Molina A, Alvarez AI, Schinkel A. 2005. Transport of anthelmintic benzimidazole drugs by breast cancer resistance protein (BCRP/ABCG2). Drug Metab Dispos 33:614–618. https://doi.org/10.1124/dmd.104.003319.
40. Breedveld P, Zelcer N, Pluim D, Sönmezer O, Tibben MM, Beijnen JH, Schinkel AH, van Tellingen O, Borst P, Schellens JHM. 2004. Mechanism of the pharmacokinetic interaction between methotrexate and benzimidazoles. Cancer Res 64:5804–5811. https://doi.org/10.1158/0008-5472.CAN-03-4062.
41. Blanco-Paniagua E, Álvarez-Fernández L, Garcia-Lino AM, Álvarez AI, Merino G. 2022. Secretion into milk of the main metabolites of the anthelmintic albendazole is mediated by the ABCG2/BCRP transporter. Antimicrob Agents Chemother 66:e00062-22. https://doi.org/10.1128/aac.00062-22.
42. Jani M, Makai I, Kis E, Szabó P, Nagy T, Krajcsi P, Lespine A. 2011. Ivermectin interacts with human ABCG2. J Pharm Sci 100:94–97. https://doi.org/10.1002/jps.22262.
43. Meaney M, Fairweather I, Brennan GP, McDowell LSL, Forbes AB. 2003. Fasciola hepatica: effects of the fasciolicide clorsulon in vitro and in vivo on the tegumental surface, and a comparison of the effects on young- and old-mature flukes. Parasitol Res 91:238–250. https://doi.org/10.1007/s00436-003-0863-0.
44. Muenster U, Grieshop B, Ickenroth K, Gnoth MJ. 2008. Characterization of substrates and inhibitors for the in vitro assessment of Bcrp mediated drug-drug interactions. Pharm Res 25:2320–2326. https://doi.org/10.1007/s11095-008-9632-1.
45. Imperiale F, Lanusse C. 2021. The pattern of blood-milk exchange for antiparasitic drugs in dairy ruminants. Animals (Basel) 11:2758. https://doi.org/10.3390/ani11102758.
46. Schulman MD, Valentino D, Cifelli S, Ostlind DA. 1982. Dose-dependent pharmacokinetics and efficacy of MK-401 against old, and young-mature infections of Fasciola hepatica in the rat. J Parasitol 68:603–608. https://doi.org/10.2307/3280917.
47. Schulman MD, Valentino D, Cifelli S, Lang R, Ostlind DA. 1979. A pharmacokinetic basis for the efficacy of 4-amino-6-trichloroethenyl-1,3-benzenedisulfonamide against Fasciola hepatica in the rat. J Parasitol 65:555–561. https://doi.org/10.2307/3280320.

48. Jonker JW, Merino G, Musters S, van Herwaarden AE, Bolscher E, Wagenaar E, Mesman E, Dale TC, Schinkel AH. 2005. The breast cancer resistance protein BCRP (ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. Nat Med 11:127–129. https://doi.org/10.1038/nm1186.
49. Merino G, Alvarez AI, Pulido MM, Molina AJ, Schinkel AH, Prieto JG. 2006. Breast cancer resistance protein (Bcrp/Abcg2) transports fluoroquinolone antibiotics and affects their oral availability, pharmacokinetics, and milk secretion. Drug Metab Dispos 34:690–695. https://doi.org/10.1124/dmd.105.008219.
50. Merino G, Jonker JW, Wagenaar E, van Herwaarden AE, Schinkel AH. 2005. The breast cancer resistance protein (BCRP/ABCG2) affects pharmacokinetics, hepatobiliary excretion, and milk secretion of the antibiotic nitrofurantoin. Mol Pharmacol 67:1758–1764. https://doi.org/10.1124/mol.104.010439.
51. Garcia-Mateos D, Garcia-Lino AM, Alvarez-Fernandez I, Blanco-Paniagua E, de la Fuente A, Alvarez AI, Merino G. 2019. Role of ABCG2 in secretion into milk of the anti-inflammatory flunixin and its main metabolite: in vitro-in vivo correlation in mice and cows. Drug Metab Dispos 47:516–524. https://doi.org/10.1124/dmd.118.085506.
52. Garcia-Lino AM, Blanco-Paniagua E, Astorga-Simon EN, Alvarez-Fernández L, Garcia-Mateos D, Alvarez-Fernandez I, Alvarez AI, Merino G. 2020. Abcg2 transporter affects plasma, milk and tissue levels of meloxicam. Biochem Pharmacol 175:113924. https://doi.org/10.1016/j.bcp.2020.113924.
53. García-Mateos D, García-Villalba R, Marañón JA, Espín JC, Merino G, Álvarez AI. 2017. The breast cancer resistance protein (BCRP/ABCG2) influences the levels of enterolignans and their metabolites in plasma, milk and mammary gland. J Funct Foods 35:648–654. https://doi.org/10.1016/j.jff.2017.06.038.
54. Miguel V, Otero JA, García-Villalba R, Tomás-Barberán F, Espín JC, Merino G, Álvarez AI. 2014. Role of ABCG2 in transport of the mammalian lignan enterolactone and its secretion into milk in abcg2 knockout mice. Drug Metab Dispos 42:943–946. https://doi.org/10.1124/dmd.113.055970.
55. Merino G, Perez M, Real R, Egido E, Prieto JG, Alvarez AI. 2010. In vivo inhibition of BCRP/ABCG2 mediated transport of nitrofurantoin by the isoflavones genistein and daidzein: a comparative study in Bcrp1−/− mice. Pharm Res 27:2098–2105. https://doi.org/10.1007/s11095-010-0208-5.
56. Blanco-Paniagua E, Garcia-Lino AM, Alvarez-Fernández L, Alvarez AI, Merino G. 2022. Ivermectin inhibits ovine ABCG2-mediated in vitro transport of meloxicam and reduces its secretion into milk in sheep. Res Vet Sci 153:88–91. https://doi.org/10.1016/j.rvsc.2022.10.019.
57. Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, Schellens JHM, Koomen G-J, Schinkel AH. 2002. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. Mol Cancer Ther 1:417–425.
58. Jonker JW, Buitelaar M, Wagenaar E, Van Der Valk MA, Scheffer GL, Scheper RJ, Plösch T, Kuipers F, Elferink RPJO, Rosing H, Beijnen JH, Schinkel AH. 2002. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. Proc Natl Acad Sci U S A 99:15649–15654. https://doi.org/10.1073/pnas.202607599.
59. Taverniers I, De Loose M, Van Bockstaele E. 2004. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. Trends Anal Chem 23:535–552. https://doi.org/10.1016/j.trac.2004.04.001.