Role of the Abcg2 transporter in the secretion into milk of the anthelmintic clorsulon: interaction with ivermectin.

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Running head: Clorsulon and ABCG2 transporter

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ABSTRACT

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

KEY WORDS: ABCG2, clorsulon, substrate, milk, ivermectin.

ABBREVIATIONS: ABC, ATP-binding cassette; ABCG2, ATP-binding cassette transporter G2; HPLC, high performance liquid chromatography; i.p., intraperitoneal administration; i.v., intravenous administration; DMEM, Dulbecco's modified Eagle's medium; MDCK-II, Madin-Darby Canine Kidney epithelial cells; LOD, limit of detection; LOQ, limit of quantification.

INTRODUCTION

Helminthic infections such as fascioliasis, which affects a wide range of domestic and wild animals and is caused by food-borne trematodes, produce significant economic losses in 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 a serious problem of anthelmintic resistance (3, 4). The increase in the aforementioned phenomenon has prompted to study new strategies to slow down its development and the use of anthelmintic combination has been described as one of them (4–6).

Clorsulon is a benzenesulphonamide antiparasitic used for the 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 *in vitro* tested against *Echinococcus* spp that causes helminthic zoonosis in humans, albeit results are controversial (11, 12). Furthermore, and according to the principles of drug repurposing, it has been 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 effective, including against anthelmintic-resistant flukes, in sheep, rats and cattle (14–17) . Clorsulon is well absorbed and eliminated via urinary tract without being metabolized (12, 18, 19).

Despite the 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,

78 the presence of residues of clorsulon parent drug is used as a marker to
79 establish the maximum residue limit in milk (16 µg/ml) and a withdrawal period
80 in combination with ivermectin of 66 days has been established in bovine milk
81 (21–24). In spite of this disadvantage, at present there is a lack of authorised
82 products for the treatment of immature fluke in animals producing milk for
83 human consumption and the availability of an adequate range of products for
84 the treatment of fasciolasis, a highly debilitating disease, is essential in order to
85 avoid unnecessary suffering of the animals (21). In this context, the study of
86 potential mechanisms or factors that affect safety in anthelmintic therapeutics
87 including the appearance of residues in milk and drug-drug interactions is of the
88 outmost relevance in order to develop new treatment strategies. The activity of
89 the ATP-binding cassette (ABC) transporter Breast Cancer Resistance Protein
90 (BCRP/ABCG2) is one of the main factors that affects the excretion pattern of
91 several compounds at the mammary gland level determining the appearance of
92 drug residues in milk (25).

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

drugs that interact with the ABCG2 transporter affect drug pharmacokinetics and excretion pattern into milk (30–34).

Subsequently, the aim of this study was to determine if the antiparasitic clorsulon is an *in vitro* substrate for murine Abcg2 and human ABCG2 as well as to analyse the involvement of this transporter in the secretion into milk of clorsulon. 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 (MDCK-II) cells, 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 confluent polarized monolayer and efflux transport of clorsulon at 10 µM was determined.

For MDCK-II parental cells apically and basolaterally translocation of clorsulon were similar (Fig. 1A). However, an increase in apically directed translocation of clorsulon in murine Abcg2 (Fig. 1B) and human ABCG2 (Fig. 1C) were observed. Concretely, relative efflux transport ratio for clorsulon in murine Abcg2-transduced cells was significantly higher (2.20 ± 0.13) than in parental cells (1.05 ± 0.08; p = 0.0002, Table 1). Similarly, for human ABCG2-transduced cells (Fig. 1C), significant differences in relative efflux transport ratio were also shown between human ABCG2-transduced cells and parental cells (1.63 ± 0.17 vs. 1.05 ± 0.08, respectively; p = 0.005, Table 1). Selective participation of ABCG2 transport was confirmed using a specific inhibitor of ABCG2, Ko143 (Fig. 1D-F). In presence of Ko143, the efflux transport ratio in murine Abcg2 and human ABCG2 transduced cells was similar to parental MDCK-II cells (Table 1). These outcomes reported 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-I). For both murine (Fig. 1H) and human (Fig. 1I) ABCG2-transduced cells, apically directed translocation was inhibited in presence of ivermectin compared to clorsulon

148 alone treatment. As a result, the efflux transport ratio significantly decreased
149 from 2.20 ± 0.13 (without ivermectin) to 1.13 ± 0.16 (with ivermectin) in murine
150 Abcg2 ($p = 0.00003$; Table 1) and from 1.63 ± 0.17 to 1.28 ± 0.17, without
151 ivermectin or with ivermectin, respectively, in human ABCG2 ($p = 0.033$; Table
152 1). Moreover, efflux transport ratios in presence of ivermectin of each cell line
153 did not show significant differences when compared to Ko143 treatment.
154 Concisely, clorsulon transport was inhibited in presence of ivermectin, leading
155 to an inhibition similar to which was attained with the specific ABCG2 inhibitor,
156 Ko143. These results reveal that ivermectin affects Abcg2/ABCG2 transport of
157 clorsulon, acting as an inhibitor.

158 **Secretion of clorsulon into milk in Abcg2$^{-/-}$ and wild-type mice: interaction**
159 **with ivermectin.**

160 To assess whether Abcg2 is involved in active secretion of clorsulon into milk as
161 well as whether ivermectin has any effect in the secretion of clorsulon into milk
162 mediated by Abcg2, clorsulon (5 mg/kg) was intravenously (i.v.) administrated
163 to lactating wild-type and Abcg2$^{-/-}$ female mice; with or without ivermectin
164 administration (0.5 mg/kg) intraperitoneally (i.p.), 10 min prior to i.v.
165 administration of clorsulon. After 30 min of clorsulon administration, milk and
166 plasma was collected.

167 No differences were reported in plasma concentrations of clorsulon between
168 wild-type and Abcg2 $^{-/-}$ mice in the treatment neither with clorsulon alone (3.09 ±
169 1.29 µg/ml vs. 3.81 ± 1.65 µg/ml; respectively) (Fig. 2A) and nor with the
170 combination of clorsulon and ivermectin (3.71 ± 1.24 µg/ml vs. 4.76 ± 1.32
171 µg/ml; respectively) (Fig. 2A). Additionally, no differences in plasma

concentrations were shown when treatments with or without ivermectin were compared in both types of mice.

After administration of clorsulon alone, milk concentrations (Fig. 2B) were 1.6-fold higher in wild-type compared to Abcg2$^{-/-}$ mice (2.56 ± 0.75 µg/ml vs. 1.61 ± 0.65 µg/ml; p = 0.01). In the same way, milk to plasma ratio of clorsulon (Fig. 2C) was significantly higher in wild-type than in Abcg2$^{-/-}$ mice (1.01 ± 0.61 µg/ml vs. 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 is administered with ivermectin, milk concentrations of clorsulon (Fig. 2B) were 1.7-fold lower (1.47 ± 0.45 µg/ml) compared to clorsulon alone in wild-type animals (2.56 ± 0.75 µg/ml; p = 0.016). Likewise, milk to plasma ratio in wild-type treated with ivermectin (Fig. 2C) was significantly lower compared to animals treated with clorsulon alone (0.43 ± 0.15 µg/ml vs. 1.01 ± 0.61 µg/ml, respectively; p = 0.028). No differences in milk concentrations (Fig. 2B) or milk to plasma ratio (Fig. 2C) of clorsulon were reported between treatment with or without ivermectin in Abcg2$^{-/-}$ mice.

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

DISCUSSION

Unfortunately, necessary use of anthelmintic drugs has led to a serious problem of anthelmintic resistance which consequently affects animal health and production. Combination of anthelmintics has been proposed as a strategy to slow down the development of resistance even when strong resistance to one component of the combination is reported (4). The rationale behind using drug coadministration is based on a lower degree of resistance to multiple drugs compared to single treatments (6); for example, it has been described that the combination of nematicides like ivermectin and flukicides such as clorsulon improves efficacy and broaden the spectrum of anthelmintic activity, as well as limits resistance emergence (3). Several other combinations of antiparasitic have been tested against soil-transmitted helminth infections to increase drug efficiency and avoid resistances, 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 reversal 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 has been recognized as an important mechanism for clinically relevant drug-drug interactions (38). In this study, *in vitro* and *in vivo* interactions of clorsulon with ABCG2 and the effect of ivermectin in 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 an antiparasitic drug from the benzenesulphonamide group has ever been reported as an ABCG2 substrate. Formerly, it has been described that other antiparasitics show *in vitro* interactions with ABCG2, mainly benzimidazole drugs like albendazole sulfoxide, oxfendazole (39) and pantoprazole (40) that were reported to be *in vitro* ABCG2 substrates with transport ratios equal or higher than 6. Recently, albendazole metabolites, albendazole sulphone and albendazole amino-sulphone, were also described as *in vitro* substrates of murine Abcg2 and human ABCG2 (41) with transport ratios around 4 and 2 respectively, similar to our transport ratios with clorsulon (Fig. 1B,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 around 36% in murine Abcg2 and 95% in human ABGC2 at 50 μM (35). In addition, IC_{50} values in the 1-1.5 μM range for human ABCG2 were described (42). As it was mentioned above, clorsulon and ivermectin combination is commonly used and marketed (3, 43) so we also evaluated *in vitro* drug-drug interactions between clorsulon and ivermectin conducting transcellular transport assay in presence of ivermectin (10 μM) in MDCK-II cells transduced with murine Abcg2 and human ABCG2. We showed that ivermectin at 10 μM inhibits clorsulon ABCG2-mediated transport in murine Abcg2 (Fig. 1H) and human ABCG2 (Fig. 1I) to the same extent resulting in a similar relative efflux ratio compared to parental cells (Table 1). Previous *in vitro*

244 studies have reported that ivermectin can block ABCG2-mediated transcellular
245 transport using albendazole sulphoxide (44) or danofloxacin (31) as substrates.

246 After treatments with antiparasitic drugs, presence of residues in edible
247 products such as milk constitutes one of the main hazards for public health (38,
248 45). In such manner, interactions with ABCG2 are gaining clinical importance.
249 ABCG2 is the main factor involved in the active secretion of numerous
250 compounds into milk leading to important clinical and toxicological
251 consequences (25). Therefore, influence of Abcg2 in clorsulon secretion into
252 milk was also evaluated in our study. Doses used (5 mg/kg) are chosen from
253 previous studies with clorsulon and rodents (46, 47). We clearly showed that
254 clorsulon is actively secreted into milk by Abcg2, as indicated by higher milk
255 concentrations (Fig. 2B) and milk to plasma ratio of clorsulon (Fig. 2C) in wild-
256 type compared to Abcg2$^{-/-}$ mice. Secretion into milk mediated by Abcg2 have
257 been previously reported for others drugs of different classes such as
258 antitumoral (48), antibiotics (31, 49, 50), anti-inflammatory (51, 52), antiparasitic
259 (41) and natural compounds (53, 54).

260 As coadministration of drugs may affect the secretion pattern into milk, drug
261 residues may consequently be altered (38). Along these lines, in the present
262 study, we also assess *in vivo* the potential effect of the macrocyclic lactone
263 ivermectin in plasma levels and milk secretion of the substrate clorsulon with the
264 recommended dose rate in veterinary practice (21) (1:0.1, 5 mg/kg clorsulon:
265 0.5 mg/kg ivermectin). Our results showed that after treatment with ivermectin,
266 milk concentrations of clorsulon (Fig. 2B) and milk to plasma ratio (Fig. 2C) in
267 wild-type mice were approximately 2-fold lower compared to clorsulon alone. As

a result, a complete inhibition of Abcg2-mediated milk secretion of clorsulon in wild-type mice were reported, as levels were equal to those in Abcg2$^{-/-}$ mice.

The effect of ivermectin on clorsulon secretion into milk can be attributed to Abcg2-mediated interactions because it is the main ABC transporter with an induced expression in mammary gland during lactation (29, 48), but also no differences in milk concentrations or milk to plasma ratio were disclosed between wild-type and Abcg2$^{-/-}$ mice after ivermectin treatment, indicating that ivermectin 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 secretion of nitrofurantoin into milk (37, 55). This ivermectin effect on ABCG2 mediated secretion into milk of clorsulon may be translated to the clinical situation, although this remains to be proved. In fact, reduction of secretion into milk of ABCG2 substrates like danofloxacin (31) or meloxicam (56) after coadministration of ivermectin were reported in sheep. Related to antiparasitics, 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 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 clorsulon systemic disposition. 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 active secretion into milk of clorsulon were disclosed. Besides, drug-drug interactions mediated by ABCG2 in this process were showed using the macrocyclic lactone ivermectin as an ABCG2 inhibitor, contributing to the understanding of the potential factors that could influence in the transfer of antiparasitic drugs into the milk.

MATERIALS AND METHODS

Reagents and drugs

Clorsulon, oxfendazol 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®) from Boehringer Ingelheim (Barcelona, Spain), isoflurane (Isovet®) from Braun VetCare, Barcelona (Spain) and oxytocin (Facilpart®) from SYVA, León (Spain) were purchased. All the other compounds used were reagent grade and were available from commercial sources.

Cell Cultures

For transcellular transport assays, the polarized cell line MDCK-II was used. Concretely, 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 described (28). Briefly, cells were cultured in DMEM (Dulbecco's modified

316 Eagle's medium) supplied with glutamax (Life Technologies, Inc., Rockville, MD,
317 USA) and supplemented with penicillin (50 units/mL), streptomycin (50 μg/mL),
318 and 10% (v/v) fetal calf serum (MP Biomedicals, Solon, OH, USA) at 37ºC in
319 the presence of 5% CO_2. The cells were trypsinized every 3 to 4 days for
320 subculturing.

321

322 **Transcellular Transport Assays**

323 Transcellular transport assays using MDCK-II-transduced cells were carried out
324 as previously described (50) with minor modifications. Cells were seeded on
325 microporous membrane filters (3.0 μm pore size, 24 mm diameter; Transwell
326 3414; Costar, Corning, NY) at a density of 1.0×10^6 cells per well. Cells were
327 grown for 3 days and the medium was replaced every day. At the beginning and
328 the end of the assay, transcellular resistance was measured to check the
329 tightness of the monolayer using Millicell ERS (Millipore Burlington, MA); wells
330 registering a resistance of 150 Ω or greater were used in the transport
331 experiments. Moreover, Lucifer Yellow permeability assay was used to measure
332 the confluence of the monolayer at the end of the experiment. Results from
333 monolayers with Lucifer Yellow transport higher than 3% were discarded.
334 Transport proficiency of these cells is recurrently checked by testing a typical
335 ABCG2 substrate, danofloxacin (31).

336 Two hours before the start of the experiment, medium in both compartments,
337 apical and basal, was replaced with 2 ml of transport medium with or without the
338 inhibitors (Ko143 (1 μM) and ivermectin (10 μM)) (35, 57). The transport
339 medium consisted of Hanks' balanced salt solution (Sigma-Aldrich)

supplemented with HEPES (25 mM). The assay started by replacing the medium on 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 ºC in 5% CO_2 and 100 μl aliquots were taken at 1, 2 and 3 h on the opposite side where clorsulon was 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 -20ºC until analysis by high-performance liquid chromatography (HPLC) as described below. The amount 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 directed transport percentage divided by the apical to basal directed transport percentage at 4 hours.

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). Lactating females Abcg2$^{-/-}$ and wild-type mice were used, all of > 99% FVB genetic background between 8 and 17 weeks of age. Animals were generated (58) and kindly provided by Dr. 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 starting the experiment, pups of approximately 10 days old were separated from their mothers. Clorsulon (5 mg/kg) was administrated in the tail vein to wild-type and Abcg2$^{-/-}$ lactating female mice as a solution of 10% ethanol, 40% PEG400 and 50% saline. The 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) were administrated intraperitoneally (i.p.) (200 µl of solution per 30 g of body weight) 10 min before intravenous administration of clorsulon. To stimulate milk secretion, oxytocin (200 µl of a 1 IU/ml solution) was administrated subcutaneously to lactating mice 10 min before sample collection. Blood and milk samples were collected 30 min after clorsulon administration under anesthesia by isoflurane. Firstly, blood samples were collected by orbital bleeding and heparinised blood samples were centrifuged at 3000g for 15 min to obtain plasma. Then, 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 -20ºC until HPLC analysis.

High performance liquid chromatography (HPLC) analysis

The conditions for HPLC analysis of clorsulon were based on a formerly described method (18) with modification.

To each 100 µl aliquots of milk and plasma, 10 µl of internal standard (oxfendazol 10 ug/ml) and 200 µl of ethyl acetate were added. The mix was vortexed horizontally for 1 min and then was centrifuged at 1200 g for 10 min at 4 ºC. The supernatant was collected and evaporated to dryness under N_2 at 30 ºC. 500 µl of hexane and 300 µl of acetonitrile were added to evaporated samples, the mix was vortexed horizontally for 1 min and then centrifuged at

1200g for 10 min at 4ºC. Hexane was eliminated and the rest was evaporated to dryness under N_2 at 30 ºC. Samples were resuspended in 100 µl of cold methanol and injected into HPLC system. Samples from *in vitro* assays were directly injected into HPLC system. For the samples analysis, Waters 2695 separation module and Waters 2998 UV photodiode array detector were used as chromatographic system. Separation was performed on a reversed-phase column (4 mm particle size, 250 x 341 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 UV absorbance of 225 nm.

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

Statistical analysis

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

413 variables, respectively. P value ≤ 0.05 indicates that differences were
414 statistically significant.

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TABLES

Table 1. Percentage of transport towards apical (BL-AP transport) or basal (AP-BL transport) compartments and relative transport efflux ratios (BL-AP/AP-BL) at 4 h for MDCK-II parental cells, murine Abcg2 and human ABCG2 transduced cells lines in presence of clorsulon (10 μM), clorsulon (10 μM) with ABCG2 inhibitors Ko143 (1 μM) or ivermectin (10 μM).

		BL-AP (%transport)	AP-BL (%transport)	Ratio 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]

Results are represented as mean ± SD (n ≥ 3).
[a]: significant differences in transport ratio compared to parental MDCK-II cells ($p \leq 0.05$).
[b]: significant differences in transport ratio of each cell line compared to clorsulon alone treatment ($p \leq 0.05$).

FIGURE LEGENDS

Figure 1. Transepithelial transport assays of clorsulon at 10 μM in parental MDCK-II cells and its subclones transduced with murine Abcg2 and human ABCG2 in the absence of inhibitors (A, B, C), in presence of ABCG2 inhibitor Ko143 1 μM (D, E, F) and ivermectin 10 μM (G, H, I). The assay was started by replacing the medium in either the apical or basolateral compartment with fresh transport medium containing 10 μM of clorsulon with or without Ko143 at 1 μM or ivermectin 10 μM. Aliquots were taken from de 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 represented as mean ± SD. (●) basolateral to apical transport; (○) apical to basolateral transport (n ≥ 3).

Figure 2. *In vivo* effect of Abcg2 and ivermectin in secretion of clorsulon into milk. (A) Plasma concentration 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 ratio of clorsulon in wild-type and Abcg2$^{-/-}$ lactating females. White columns represent i.v. administration of clorsulon (5 mg/kg). Black columns represent i.p. administration of ivermectin (0.5 mg/kg) 10 min prior to 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 means ± SD (n= 5-12). #, $p \leq 0.05$ significant differences between wild-type and Abcg2$^{-/-}$ mice. *, $p \leq 0.05$ significant differences between clorsulon alone treatment and clorsulon with ivermectin treatment.

Figure 1

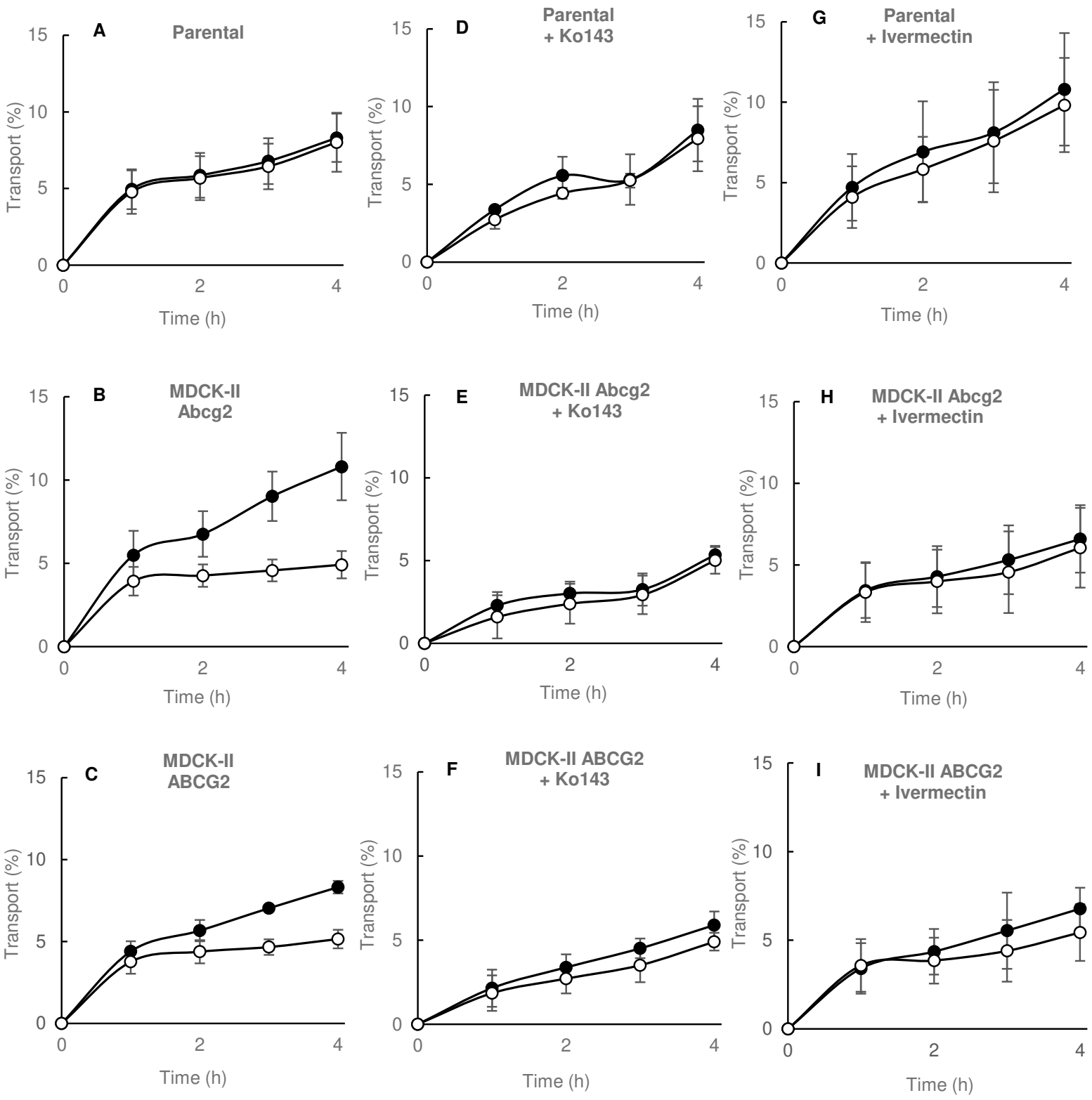
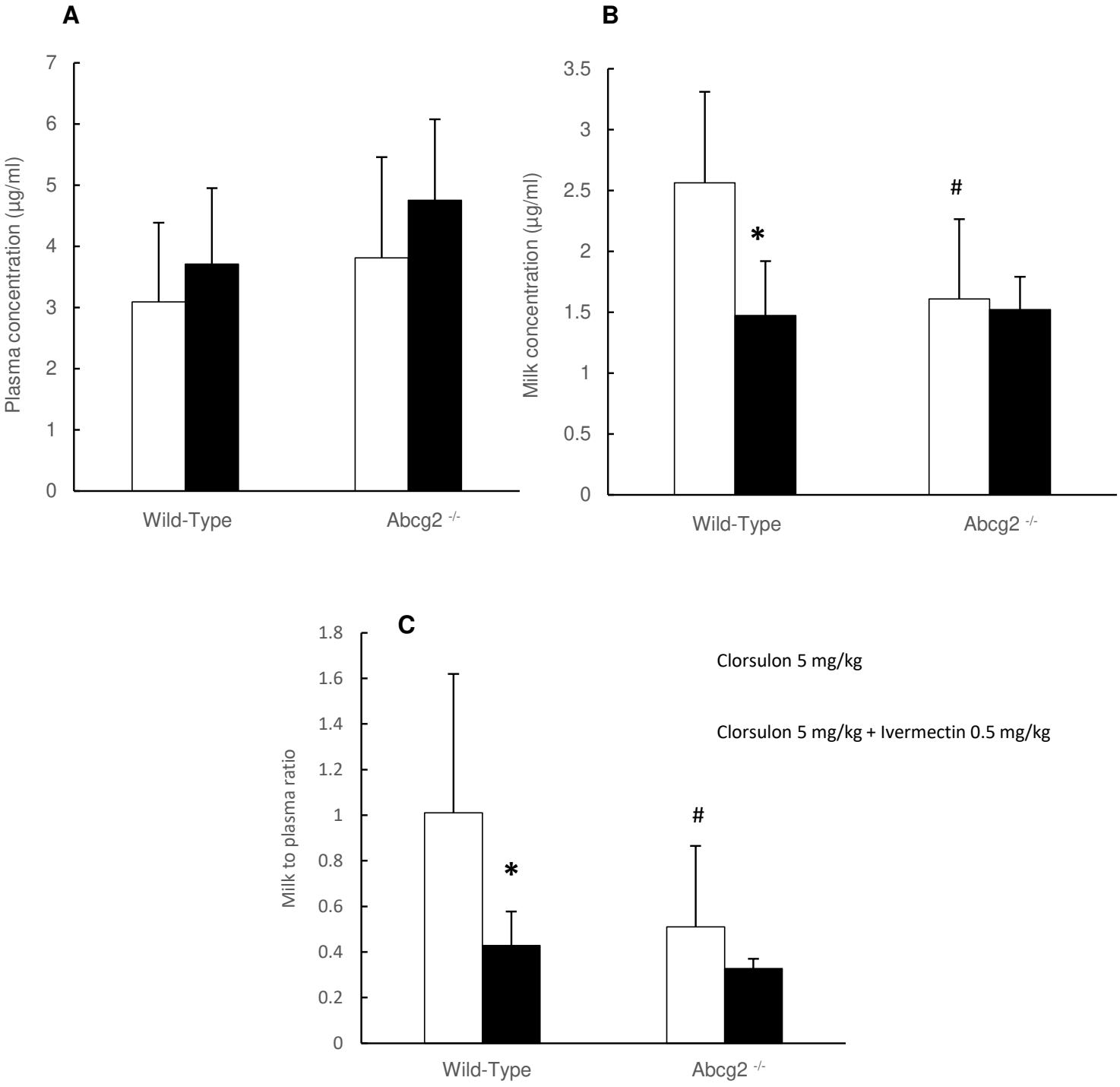

A
Parental
D
Parental
+ Ko143
G
Parental
+ Ivermectin
B
MDCK-II
Abcg2
E
MDCK-II Abcg2
+ Ko143
H
MDCK-II Abcg2
+ Ivermectin
C
MDCK-II
ABCG2
F
MDCK-II ABCG2
+ Ko143
I
MDCK-II ABCG2
+ Ivermectin
Transport (%)
Time (h)
0
5
10
15
0
2
4

Figure 2


A
Plasma concentration (µg/ml)
0
1
2
3
4
5
6
7
Wild-Type
Abcg2 -/-
B
Milk concentration (µg/ml)
0
0.5
1
1.5
2
2.5
3
3.5
*
#
Wild-Type
Abcg2 -/-
C
Milk to plasma ratio
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
*
#
Wild-Type
Abcg2 -/-
Clorsulon 5 mg/kg
Clorsulon 5 mg/kg + Ivermectin 0.5 mg/kg