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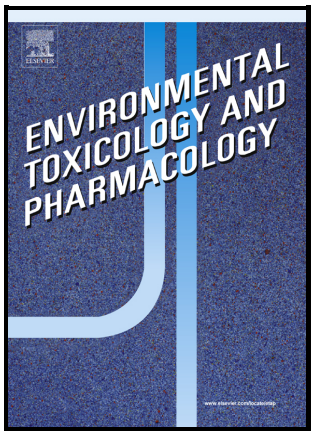

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Title

The ABCG2 protein in vitro transports the xenobiotic thiabendazole and increases the appearance of its residues in milk

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ABSTRACT

Thiabendazole (TBZ) is a broad–spectrum anthelmintic and fungicide used in humans, animals, and agricultural commodities. TBZ residues are present in crops and animal products, including milk, posing a risk to food safety and public health. ABCG2 is a membrane transporter which affects bioavailability and milk secretion of xenobiotics. Therefore, the aim of this work was to characterize the role of ABCG2 in the in vitro transport and secretion into milk of 5–hydroxythiabendazole (5OH–TBZ), the main TBZ metabolite. Using MDCK–II polarized cells

transduced with several species variants of ABCG2, we first demonstrated that 5OH–TBZ is efficiently in vitro transported by ABCG2. Subsequently, using Abcg2 knockout mice, we demonstrated that 5OH–TBZ secretion into milk was affected by Abcg2, with a more than 2–fold higher milk concentration and milk to plasma ratio in wild–type mice compared to their Abcg2$^{-/-}$ counterpart.

KEYWORDS: ABCG2, cell culture, MDCK–II, milk secretion, thiabendazole.

ABBREVIATIONS: AB, apical to basal; ABC, ATP–binding cassette efflux; ABCG2, ATP–binding cassette subfamily G2; BA, basal to apical; DMEM, Dulbecco's modified Eagle's medium; LOD, limit of detection; LOQ, limit of quantification; MDCK–II, Madin–Darby canine kidney epithelial cells; MRL, maximum residue limit; TBZ, thiabendazole.

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1. INTRODUCTION

Thiabendazole (2–(4–thiazolyl)benzimidazole, TBZ) is a broad–spectrum anthelmintic widely used in humans and cattle. It is also used as post–harvest agricultural fungicide, thus preserving crop quality and marketability (Hajikhani et al., 2024). Adverse effects after TBZ exposure including endocrine, nephrogenic, hepatogenic, teratogenic and neurological effects have been reported in mammals (Ekman et al., 2014). Etiological factors behind TBZ toxicity remain ambiguous; however, it has been hypothesized that it may be due to its bioactivation to 5OH–TBZ by the cytochrome P450 1A2 (CYP1A2) (Coulet et al., 1998a; Jamieson et al., 2011) (Fig. 1). Therefore, National and International Food Safety Authorities have established regulations for the usage of TBZ on food products setting maximum residue limits (MRLs) for the sum of TBZ and its metabolites in both agricultural and animal products ranging from 0.01 to 7 mg/kg depending on the product (European Food Safety Authority (EFSA) et al., 2021; U.S. Food and Drug Administration (FDA), 2021). Unfortunately, MRLs derived from pesticide use are equal to or higher than veterinary MRLs for all commodities, and the possible aggregated exposure from TBZ residues has not been studied, making it impossible to rule out an overexposure to TBZ through the food chain.

Chemical residues in milk for human nutrition arouses increasing concern as regards the unintended transfer of toxic xenobiotic metabolites, posing a health risk to consumers, especially children who are particularly vulnerable. Additionally, animal health and welfare as well as animal productiveness may be impaired by the transfer of xenobiotics during lactation (Caruso et al., 2009). Knowledge of the factors affecting the presence of residues of xenobiotics in milk is therefore relevant for animal and human health. The ATP–binding cassette (ABC) transporter ABCG2 is an important and widely described mechanism of transfer of compounds into milk and one of the most toxicologically relevant proteins in mammals (Yuan et al., 2014). It is expressed in a wide range of tissues and organs, including the mammary gland (Jonker et al., 2005), affecting the bioavailability (Malnoë et al., 2022) and the active milk secretion of its substrates (Schrickx and

Fink-Gremmels, 2008), including antibiotics (Otero et al., 2013, 2016a), anti–inflammatories (Garcia-Mateos et al., 2019; Blanco-Paniagua et al., 2022b), and antiparasitic drugs (Mahnke et al., 2016; Blanco-Paniagua et al., 2021, 2022a; Gunes et al., 2023).

Furthermore, genetic variants of ABCG2 may alter the bioavailability of its substrates (Mealey, 2013; Rocha et al., 2018). In cattle, the Y581S polymorphism was described in Holstein population (Cohen-Zinder et al., 2005) as a gain–of–function polymorphism directly involved in milk quality by affecting the presence of different compounds including xenobiotics in milk (Real et al., 2011a; Otero et al., 2015; Garcia-Lino et al., 2021).

This research was therefore intended to investigate the in vitro interaction of TBZ and its hydroxylated metabolite, 5OH–TBZ, with different species variants of ABCG2, including the bovine S581 polymorphism. In vivo studies with Abcg2$^{-/-}$ mice exogenous administered with 5OH–TBZ were used to correlate in vitro and in vivo results and to evaluate the role of ABCG2 on secretion of this TBZ metabolite into milk.

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2. MATERIAL AND METHODS

2.1. Chemicals

Reference standard for TBZ and its hydroxylated metabolite, 5OH–TBZ, were supplied by LGC Standards (Teddington, Middlesex, UK). Ko143 was purchased from Tocris (Bristol, United Kingdom) and Lucifer Yellow was obtained from Sigma–Aldrich (St. Louis, MO, USA). For in vivo studies, oxytocin (Facilpart®) was acquired from Syva (León, Spain) and isoflurane (Isovet®) from Braun VetCare (Barcelona, Spain). All the other chemicals used were of analytical grade and available from commercial sources.

2.2. Cell cultures

Polarized Madin–Darby canine kidney epithelial cells (MDCK–II) parental and the murine Abcg2 and human ABCG2–transduced subclones were gently provided by Dr. A.H. Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands). MDCK–II cells stably transduced with the ovine and the two polymorphic bovine variants (Y581 and S581) of ABCG2 were previously generated and characterized by our research group (Real et al., 2011b; González-Lobato et al., 2014). Cells were cultured at 37°C in an atmosphere with 5% CO2 in Dulbecco's modified Eagle's medium with GlutaMAX (Life Technologies Inc., Rockville, MD, USA), supplemented with 10% fetal calf serum (Life Technologies), and 1% mixture of antibiotics (penicillin (50 U/mL) and streptomycin (50 μg/mL), Life Technologies).

2.3. Transport studies

Transport assays were carried out as previously described by our group (Blanco-Paniagua et al., 2021) with minor modifications. Cells (passage 30–35) were grown for 3 days after seeding on microporous polycarbonate membrane filters (3.0 μm pore size, 24 mm diameter, Transwell® 3414, Costar, Corning, NY) at a density of 1.0 x 10^6 cells per well and medium was replaced every day. Two hours before beginning the experiment, transepithelial resistance was measured to check the tightness of the monolayer using a Millicell ERS ohmmeter (Millipore Burlington, MA).

Subsequently, medium in both apical and basal compartments was replaced with 2 mL of Optimem medium, free of serum, and either containing or not the specific ABCG2 inhibitor Ko143 (1 μM). The experiment started (t = 0) by replacing the medium in either the apical or basal compartment with fresh Optimem medium containing 10 μM TBZ or 5OH–TBZ, with or without the inhibitor Ko143 (1 μM). Aliquots of 100 μL were collected from the opposite compartment where the compounds were added after 1, 2 and 3 hours of incubation, and 600 μL aliquots were taken from both sides of the monolayer after 4 hours, and stored at −20°C until HPLC analysis could be undertaken. At the end of the experiment, confluence of the monolayer was measured with the previously described Lucifer Yellow permeability test (Mahnke et al., 2016), with minor modifications. The appearance of TBZ and 5OH–TBZ in the opposite side of the well was measured by HPLC as later described and active transport across MDCK–II monolayers was referred to the initial concentration. The apparent permeability (Papp) coefficient across cells monolayers in both apical to basal (Papp AB) and basal to apical (Papp BA) directions was calculated using the following equation:

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

being ΔQ/Δt the rate of xenobiotic appearing in the receiver compartment, obtained as the slope of the regression line on the transport–time profile of TBZ and 5OH–TBZ metabolite across the cell monolayers; C_0 the initial concentration of the studied compound; and A the cell monolayer surface area (4.67 cm^2). The efflux ratio was defined as the quotient between the Papp coefficients in BA and AB directions (Papp BA/ Papp AB), after 4 hours of incubation. Transport proficiency of cells was simultaneously checked by testing a well–known ABCG2 substrate; in this case, the fluoroquinolone danofloxacin (Real et al., 2011a). The experiments were performed at least in quadruplicate and at least in two different days.

2.4. Milk secretion experiment

Milk secretion experiment was carried out using lactating female Abcg2$^{-/-}$ and wild–type mice, all of > 99% FVB genetic background, and aged between 11–17 weeks. Animals, generated and kindly supplied by Dr. A. H. Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands), were kept in a controlled temperature environment with a 12–hour light/12–hour dark cycle with *ad libitum* access to a standard diet and water, and housed and handled complying with institutional and ARRIVE guidelines and European legislation (EU Directive 2010/63/EU for animal experiments). 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).

For the experiment, pups of approximately 10 days old were separated from their mothers (wild–type (n = 6) and Abcg2$^{-/-}$ (n = 8) female mice) 3 hours before starting the experiment. 5OH–TBZ (10 mg/kg) was intravenously injected as a solution of 10% ethanol, 40% polyethylene glycol 400 and 50% sodium chloride (0.9%) at 150 μL per 30 g of body weight into the tail vein of mice lightly anaesthetized with isoflurane. Ten minutes before milk and blood collection, 200 μL oxytocin (1 IU/mL) was administrated subcutaneously to lactating females to stimulate milk secretion. Blood was collected 30 min after 5OH–TBZ administration from retro–orbital sinus under anaesthesia with isoflurane and, subsequently, milk was obtained from the mammary glands by pressing the nipple. At the end of the experiment, mice were sacrificed by cervical dislocation. Heparinized blood samples were centrifugated at 3000g for 15 min to obtain the plasma, and milk and plasma samples were stored at –20 ºC until extraction and HPLC analysis.

2.5. HPLC analysis

HPLC analysis was used to determine the concentrations of the studied compounds in transepithelial transport assays and milk secretion analysis. The chromatographic system consisted of a Waters 2695 separation module and a Waters 2998 UV photodiode array detector set at 319 nm. Chromatographic separation was achieved on a reverse–phase column (Synergy 4μm Hydro–RP 80 Å, 250 x 4.60 mm; Phenomenex®, Torrance, CA, USA) using an isocratic binary mobile

phase consisting of methanol:water enriched with 0.1% formic acid (70:30 for culture samples and 80:20 for animal samples) at a flow rate of 1.1 mL/min. Autosampler and column temperature were 4 ºC and 40 ºC, respectively.

Plasma and milk samples from each animal were individually processed and analysed without pooling. The entire amount of collected milk (63.4–168.7 mg) and 100 µL plasma were processed. To each sample, 10 µL of albendazole sulfoxide (ABZSO) (2 µg/mL) was added as an internal standard and 1 mL of ethyl acetate was used to precipitate the proteins. The mixture was vortexed horizontally for 15 min and centrifugated at 6,000 g for 6 min at 4 ºC. The supernatant was transferred to a clean tube and evaporated to dryness under a nitrogen stream at 40 ºC. After reconstitution with 100 µL of mobile phase, 50 µL was injected into the HPLC system. Samples from transport assays were not processed, and 50 µL of culture media was directly injected into the HPLC system. Standards used for calibration curves were subjected to the same procedures as samples. Standard samples of TBZ and 5OH–TBZ for calibration curves were prepared at concentrations of 0.039–10 µg/mL, with coefficients of correlation higher than 0.99. Limit of detection (LOD) and limit of quantification (LOQ) were calculated as previously described by Taverniers *et al*. (2004). LOD was 0.009 µg/mL and LOQ 0.019 µg/mL for cell culture samples, LOD 0.021 µg/mL and LOQ 0.067 µg/mL for plasma samples; LOD 0.014 µg/mL and LOQ 0.054 µg/mL for milk samples.

2.6. Statistical analysis

Comparisons between groups were performed using the two–tailed unpaired Student's t–test (normally distributed variables) and the non–parametric Mann–Whitney U test (not–normally distributed variables). Normal distribution of the variables was checked applying the Shapiro–Wilk normality test. All analysis were carried out on the assumed significance level of $p \leq 0.05$ using SPSS Statistics software v. 26 (IBM, Armonk, New York, USA). The results are shown as mean ± standard deviation (SD).

3. RESULTS

3.1. In vitro transport of TBZ and 5OH–TBZ

To assess the in vitro transport of TBZ and its hydroxylated metabolite, 5OH–TBZ, by the ABCG2 membrane transporter, MDCK–II polarized cells and its subclones transduced with murine Abcg2 and human, ovine and bovine ABCG2 were used. Parental cell line and transduced subclones were grown to confluent polarized monolayers and vectorial transport of 10 μM TBZ and 5OH–TBZ across monolayers was evaluated.

Concerning 5OH–TBZ, a similar transport with equal apically (basal to apical, BA) and basolaterally (apical to basal, AB) vectorial translocation was observed in the parental MCDK–II cell line (Fig. 2A, Table 1). In the murine Abcg2 and ovine ABCG2–transduced cells and, to a lesser extent, in the human and both bovine variants of the transporter, an increased translocation from the basal to the apical (BA) compartment and a reduced translocation from the apical to the basal (AB) compartment were observed compared with the non–transduced (parental) MDCK–II cells (Fig. 2B–F, Table 1). Additionally, efflux ratios (Papp BA/Papp AB) at 4 hours were significantly higher in transduced subclones related to parental cells (Table 1). To evaluate the role of the bovine Y581S polymorphism in the transport of 5OH–TBZ, MDCK–II cells overexpressing the wild–type Y581 and the polymorphic S581 bovine variant of ABCG2 were used (Fig. 2E–F). A ratio of 5OH–TBZ significantly higher in the S581 variant compared to the wild–type one was found (Table 1), therefore confirming that the Y581S polymorphism affects the in vitro transport of 5OH–TBZ, with a higher in vitro transport capacity for the S581 variant. In all cases, the specificity of ABCG2–mediated transport was checked using the selective inhibitor, Ko143. ABCG2–mediated transport was inhibited by Ko143 for all types of transduced cells (Fig. G–L). These results demonstrate that 5OH–TBZ is an efficient in vitro substrate of ABCG2. This is in contrast to the parent compound, which was not identified as an in vitro ABCG2 substrate. The outcome obtained for TBZ in the MDCK–II parental cells and cells transduced with different species variants

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of the ABCG2 transporter for apically and basolaterally translocation was similar, and no significant differences between the efflux ratios were observed (Table 1).

3.2. Milk secretion of 5OH–TBZ in wild–type and Abcg2$^{-/-}$ mice

To determine the role of ABCG2 in the active secretion of 5OH–TBZ into milk, 10 mg/kg of the 5–hydroxylated TBZ metabolite was intravenously administered to lactating Abcg2$^{-/-}$ and wild–type female mice (Fig. 3). Blood and milk samples were collected 30 min after administration and HPLC analysis was performed to quantify the amount of compound present in biological samples. Similar concentration of 5OH–TBZ was obtained in plasma from both types of mice (0.46 ± 0.22 μg/mL in wild–type vs. 0.44 ± 0.16 μg/mL in Abcg2$^{-/-}$ mice). In contrast to plasma, milk concentration of 5OH–TBZ was 2.1–fold higher (0.75 ± 0.43 μg/mL in wild–type vs. 0.36 ± 0.11 μg/mL in Abcg2$^{-/-}$ mice; $p = 0.03$). Moreover, milk to plasma ratio was also 2.1-fold higher in wild–type mice compared with Abcg2$^{-/-}$ lactating females (1.79 ± 0.71 vs. 0.85 ± 0.17, respectively; $p = 0.01$). These results clearly show that ABCG2 is involved in the active secretion of 5OH–TBZ into milk.

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4. DISCUSSION

Food is the main potential source of human exposure to TBZ residues, both plant products and animal commodities, considering dietary exposure to pesticide residues and chemotherapy for fungal and parasite control (Moldovan et al., 2023). In fact, it was listed in the top 10 most frequently found xenobiotics in human food by the American Food and Drug Administration (FDA) (2021). In this research, we comprehensively demonstrate, for the first time, the in vitro and in vivo interaction between TBZ and its main metabolite, 5OH–TBZ, with the ABCG2 carrier efflux protein, affecting the presence of milk residues of 5OH–TBZ.

In vitro experiments using MDCK–II cells overexpressing murine Abcg2 and human, ovine, and bovine ABCG2 demonstrated that 5OH–TBZ is efficiently transported by all variants tested (Fig. 2, Table 1). Our outcomes should be considered only in qualitative terms. Comparison between species variants should not be established since although apparent differences between species subclones may be due to differences in the affinity/selectivity of ABCG2 species variants for their substrates (Mizuno et al., 2004), potential differences in efficiency of ABCG2 transduction cannot be excluded.

In the case of assays with both bovine polymorphic variants, previous studies reported that the Y581S SNP is an in vitro and in vivo gain-of-function polymorphism, confirming that the higher activity of this polymorphism was not linked to higher ABCG2 mRNA or protein levels (Real et al., 2011b; Otero et al., 2015). In fact, Western blot analysis of our MDCK–II cells transduced with both bovine variants detect similar amount of ABCG2 protein (Real et al., 2011b). Therefore, in this case, we can confirm that our data report a more efficient in vitro transport for 5OH–TBZ by the bovine S581 polymorphic variant as compared with the Y581 variant (Fig. 2, Table 1), which is also in agreement with our previous results obtained for danofloxacin, marbofloxacin, and nitrofurantoin (Real et al., 2011b; González-Lobato et al., 2014).

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These results are in contrast with to the ones obtained for the parent compound (TBZ), which has not been identified as an in vitro ABCG2 substrate (Table 1). Interactions with ABCG2 are closely linked to physicochemical properties of molecules, particularly hydrophobicity. In our case, TBZ is metabolized into a more hydrophilic derivative (5OH–TBZ) (Coulet et al., 1998a), which is efficiently transported by ABCG2. This physicochemical phenomenon has also been described for other benzimidazole compounds such as albendazole. Previous studies carried out by our research group demonstrated that only its more hydrophilic metabolites, albendazole sulfoxide, albendazole sulfone and 2–aminosulphone were effectively transported by ABCG2 (Merino et al., 2005; Blanco-Paniagua et al., 2022a).

Regardless of parent compound, our in vitro results for 5OH–TBZ should be considered to be of interest as metabolic studies on ruminants have shown that 5OH–TBZ is the main metabolite that contributes to TBZ residues in animal tissues. Moreover, TBZ–induced toxicity observed in animals and humans has suggested to be exerted via 5OH–TBZ (Jamieson et al., 2011), based on the relationship between systemic peak concentrations of the hydroxylated metabolite and the onset of signs and symptoms attributable to its toxicity (Coulet et al., 1998b). On the other hand, 5OH–TBZ has been described to possess the same teratogenic potential as the parent drug TBZ, and that high exposure to this metabolite may be associated with a high asthma, odds of eczema and itchy rash in humans (Alhanti et al., 2022).

Attending the in vivo situation, several pharmacokinetics and residue studies have been previously undertaken with TBZ and 5OH–TBZ on different livestock species and food products (Galtier et al., 1994; Zheng et al., 2024). However, this is the first study to explore the in vivo role of the ABCG2 transporter in the active secretion of TBZ residues into milk. Milk residues are of concern for dairy industry due to financial losses and the development of resistance. They also pose a threat to health, as milk is essential for optimum growth and development of newborn mammals and an important component of human diet worldwide. For these reasons, research focus on the

better understanding of factors affecting xenobiotics secretion into milk are essential. Jonker et al. (2005) showed that the expression of ABCG2 in the mammary gland is induced during lactation and plays an important role in the secretion of xenobiotics into milk. Taking this into consideration and the positive outcome from the in vitro setting for 5OH–TBZ, the role of ABCG2 in 5OH–TBZ secretion into milk was studied by analysing plasma and milk samples from lactating wild–type and Abcg2$^{-/-}$ mice administered with 5OH–TBZ. The use of knockout mice as a preclinical set up in the study of in vivo interactions of different molecules with membrane transporters has been validated (The International Transporter Consortium et al., 2010) and it is a widely used model to test the in vivo role of some transporters such as P–glycoprotein or ABCG2 (Vlaming et al., 2009). We can therefore assure that our results with knockout mice reveal that Abcg2 is implicated in the secretion of 5OH–TBZ into milk as indicated by the 2.1–fold higher milk concentration and milk–to–plasma ratio in wild–type compared with the Abcg2$^{-/-}$ female mice after intravenous administration of the 5–hydroxylated metabolite (Fig. 3). While most molecules are transferred from the bloodstream to milk through passive diffusion, compounds actively transported into milk by Abcg2 exhibit elevated milk–to–plasma ratios, typically exceeding the unit (Alvarez et al., 2006). In our experiments, elevated milk–to–plasma ratios consistently show a specific role for Abcg2 in the milk secretion of 5OH–TBZ, as the mean ratio was higher than 1 in the presence of the membrane transporter (Fig. 3). These results are in accordance with our in vitro findings, which report that 5OH–TBZ was effectively transported by murine Abcg2 (Fig 2B). Conversely, no notable differences in plasma levels were observed at the tested doses and collection times between wild–type and Abcg2$^{-/-}$ female mice (Fig. 3). Similar findings have been reported for other ABCG2 substrates, such as flunixin and its metabolite (Garcia-Mateos et al., 2019), meloxicam (Garcia-Lino et al., 2020), as well as albendazole (Blanco-Paniagua et al., 2022a) in lactating female mice with wild–type and Abcg2–deficient backgrounds.

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To extrapolate results from experiments carried out in mice to veterinary and human species, future experiments on target species are needed. Alterations in the administration route, dose rate, and sampling points cannot be ruled out as factors that may have an impact on the final outcome. In addition, the in vivo role of ABCG2 in the milk secretion of 5OH–TBZ must be considered together with our in vitro data. Transepithelial transport assays with MDCK–II cells overexpressing ABCG2 have shown a strong predictive ability for the effect of ABCG2 on the secretion of several xenobiotics into ruminant milk, both from sheep and cow (Barrera et al., 2013; Perez et al., 2013). In this sense, our results from cells overexpressing both bovine variants are of great importance because they disclose that the S581 variant shows higher in vitro transport of 5OH–TBZ compared to Y581 variant (Fig. 2 E-F, Table 1), and therefore they predict that milk levels of 5OH–TBZ might be higher in animals expressing the Y581S polymorphism. This in vitro and in vivo correlation has been confirmed in previous studies carried out by our group (Otero et al., 2013, 2015, 2016a), providing evidence that ABCG2 polymorphisms must be taken into account because they can alter the expected drug concentration in milk and may lead to variable amounts of drug residues in milk with an important health risk for human consumption. Apart from the genetic Y581S polymorphism, several factors could influence the activity of ABCG2, including gender, natural feed compounds and coadministration with drugs that interact with the transporter. In fact, ABCG2–mediated secretion of drugs into milk can be greatly diminished by administering ABCG2 inhibitors present in the diet such as flavonoids or lignans (Otero et al., 2016b; Gunes et al., 2023). Nevertheless, further in vivo studies are needed to confirm this hypothesis.

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5. CONCLUSIONS

Our findings point out the involvement of ABCG2 in the active in vitro transport of 5OH–TBZ in murine, human, ovine, and bovine variants of the transporter. Furthermore, our study highlights the crucial role of murine Abcg2 in the secretion of 5OH–TBZ into milk, as demonstrated through our in vivo experiments carried out in Abcg2$^{-/-}$ mice.

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CRediT author statement

Laura Álvarez-Fernández: Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Investigation, Writing-Original Draft. **Esther Blanco-Paniagua**: Methodology, Formal analysis, Visualization, Investigation. **Alicia Millán-García**: Writing-Review & Editing. **Miriam Velasco-Díez:** Data curation, Formal analysis, Visualization, Investigation. **Ana I. Álvarez**: Conceptualization, Funding acquisition, Supervision. **Gracia Merino**: Conceptualization, Methodology, Funding acquisition, Project administration, Resources, Validation, Supervision, Writing-Review & Editing.

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TABLES

Table 1. Apparent permeability (Papp) across cells monolayers in both basolateral (Papp AB) and apical (Papp BA) directions, and relative efflux ratio (Papp BA/Papp AB), at 4 hours, for transepithelial transport of TBZ (10 µM) and 5OH–TBZ (10 µM) in MDCK–II non–transduced cells (parental) and their subclones transduced with the murine (mAbcg2), human (hABCG2), ovine (oABCG2), and both variants of bovine (Y581 and S581 bABCG2) and variants of ABCG2 (n = 4–6).

Cell subclones	**Papp BA, x10^{-5} cm/s**	**Papp AB, x10^{-5} cm/s**	**Efflux ratio Papp BA/Papp AB**
TBZ			
Parental	0.90 ± 0.02	0.89 ± 0.04	1.02 ± 0.04
mAbcg2	0.95 ± 0.02	0.94 ± 0.03	1.01 ± 0.03
hABCG2	1.01 ± 0.04	0.97 ± 0.03	1.04 ± 0.01
oABCG2	1.03 ± 0.02	1.02 ± 0.03	1.01 ± 0.03
Y581 bABCG2	0.96 ± 0.05	0.93 ± 0.05	1.03 ± 0.05
S581 bABCG2	0.98 ± 0.06	1.01 ± 0.05	0.97 ± 0.11
5OH–TBZ			
Parental	1.11 ± 0.02	1.17 ± 0.14	0.95 ± 0.11
mAbcg2	1.59 ± 0.03	0.33 ± 0.02	4.79 ± 0.05*
hABCG2	1.42 ± 0.07	0.89 ± 0.04	1.60 ± 0.11*
oABCG2	1.72 ± 0.11	0.40 ± 0.05	4.35 ± 0.80*
Y581 bABCG2	1.29 ± 0.03	0.83 ± 0.10	1.57 ± 0.14*
S581 bABCG2	1.46 ± 0.03	0.64 ± 0.02	2.27 ± 0.12*$^{\#}$

Results are presented as means ± SD.

*: $p \leq 0.05$, significant differences in efflux ratio compared to parental MDCK–II cells.

#: $p \leq 0.05$, significant differences in efflux ratio between MDCK–II cells transduced with both bovine variants of the ABCG2 transporter.

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FIGURE CAPTIONS

Figure 1

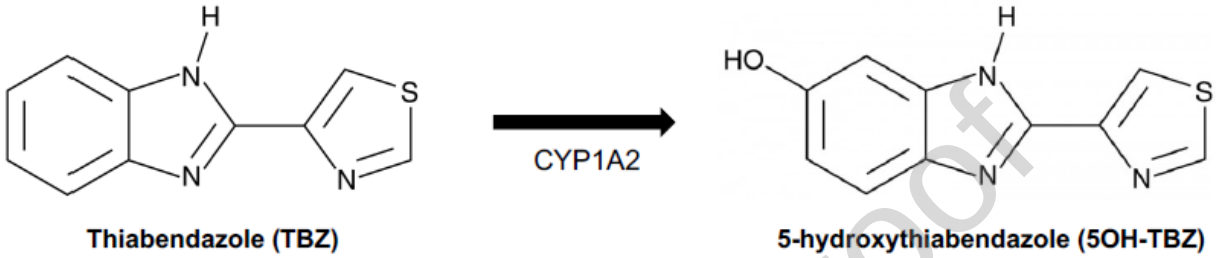

Fig. 1. Chemical structures of thiabendazole (TBZ) and its hydroxylated metabolite, 5–hydroxythiabendazole (5OH–TBZ). Hydroxylation reaction is mediated by the cytochrome 1A2 (CYP1A2).

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Figure 2

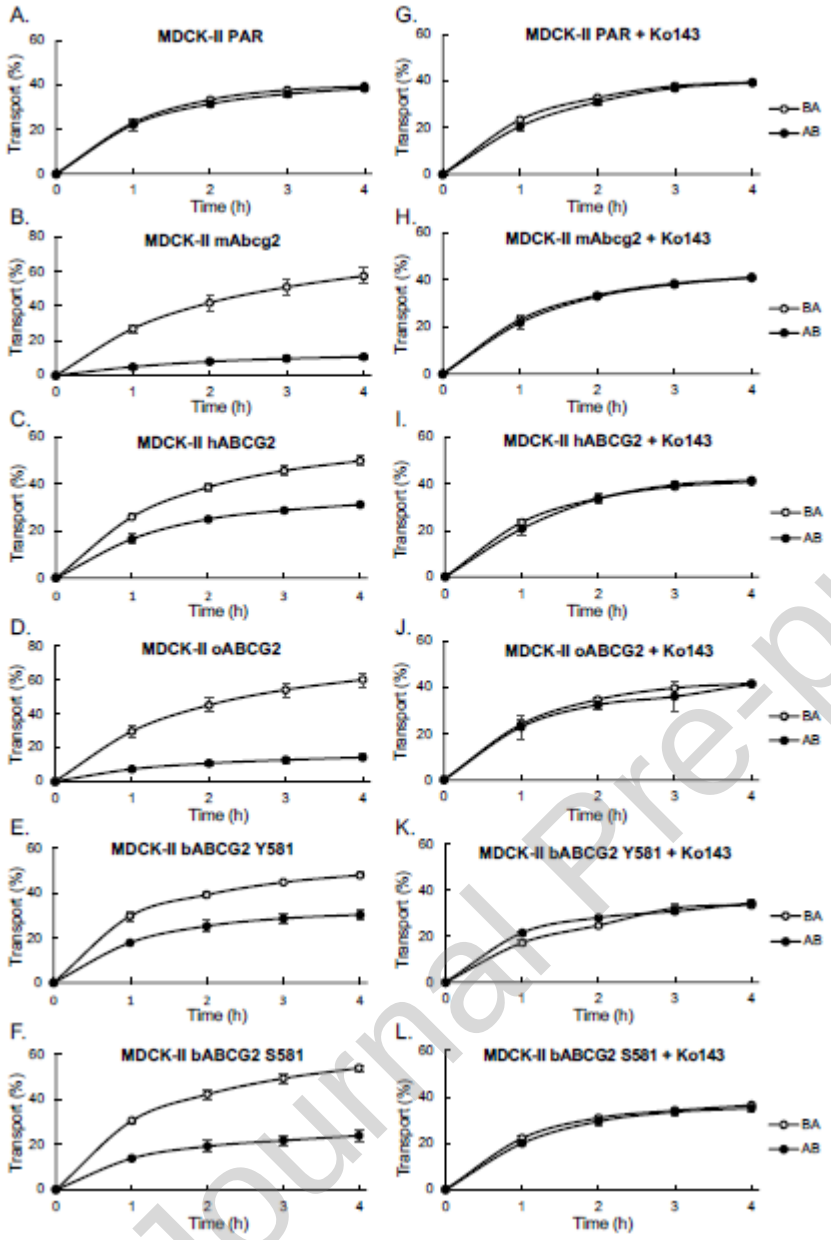

Fig. 2. Transepithelial transport of 5OH–TBZ at 10 µM in the absence (A–F) or presence (G–L) of the specific inhibitor of ABCG2, Ko143 (1 µM), in the parental MDCK–II cells (A, G) and its subclones transduced with murine Abcg2 (mAbcg2) (B, H), human (hABCG2) (C, I), ovine (oABCG2) (D, J), and bovine (Y581 and S581 bABCG2) (E, K and F, L, respectively) ABCG2 transporter. The experiment started when the medium in both sides was replaced with fresh transport solution, with or without Ko143, and containing the potential substrate. Aliquots were collected from the opposite compartment where the metabolite was added at 1, 2, 3 and 4 hours and

quantified by HPLC. Active transport across MDCK–II monolayers was presented as a percentage of total concentration added to the donor compartment at the beginning of the experiment. The appearance of 5OH–TBZ in the opposite compartment was related to the total compound added at the beginning of the experiment. The experiments were performed at least in quadruplicate and at least in two different days. Results are presented as means and error bars indicate SD.

Figure 3

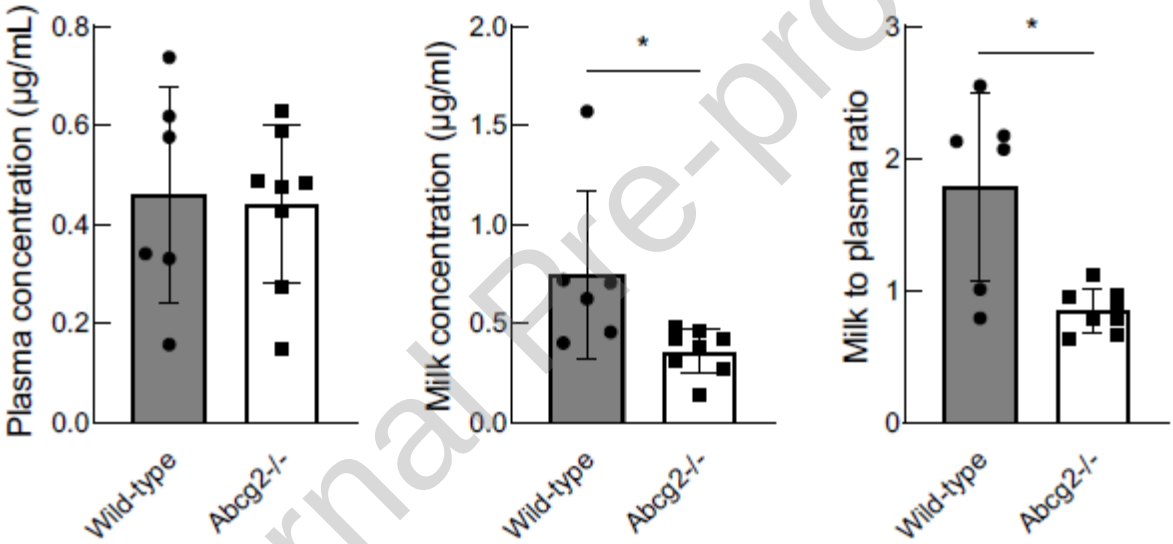

Fig. 3. Plasma and milk concentration and milk–to–plasma ratio of 5OH–TBZ in wild–type and Abcg2$^{-/-}$ female mice after intravenous administration at a dose of 10 mg/kg body weight. Plasma and milk samples were collected 30 min after administration and metabolite levels were determined by HPLC. Results are presented as individual data (dots) and means (columns), and error bars indicate SD. (*) $p \leq 0.05$, significant differences compared to wild–type mice (Student's t–test, normally distributed data) (n = 6–8).

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

HIGHLIGHTS

- 5OH-thiabendazole is in vitro transported by murine, human, ovine, and bovine ABCG2.
- In vitro transport of 5OH-thiabendazole is affected by the bovine Y581S SNP.
- ABCG2 transporter increases milk secretion of 5OH-thiabendazole.

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