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Research paper

Role of the Abcg2 transporter in plasma levels and tissue accumulation of the anti-inflammatory tolfenamic acid in mice

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ABSTRACT

The Breast Cancer Resistance Protein (BCRP/ABCG2) is an ATP-binding cassette efflux transporter that is expressed in the apical membrane of cells from relevant tissues involved in drug pharmacokinetics such as liver, intestine, kidney, testis, brain and mammary gland, among others. Tolfenamic acid is an anti-inflammatory drug used as an analgesic and antipyretic in humans and animals. Recently, tolfenamic acid has been repurposed as an antitumoral drug and for use in chronic human diseases such as Alzheimer. The aim of this work was to study whether tolfenamic acid is an in vitro Abcg2 substrate, and to investigate the potential role of Abcg2 in plasma exposure, secretion into milk and tissue accumulation of this drug. Using in vitro transepithelial assays with cells transduced with Abcg2, we showed that tolfenamic acid is an in vitro substrate of Abcg2. The in vivo effect of this transporter was tested using wild-type and Abcg2$^{-/-}$ mice, showing that after oral and intravenous administration of tolfenamic acid, its area under the plasma concentration-time curve in Abcg2$^{-/-}$ mice was between 1.7 and 1.8-fold higher compared to wild-type mice. Abcg2$^{-/-}$ mice also showed higher liver and testis accumulation of tolfenamic acid after intravenous administration. In this study, we demonstrate that tolfenamic acid is transported in vitro by Abcg2 and that its plasma levels as well as its tissue distribution are affected by Abcg2, with potential pharmacological and toxicological consequences.

1. Introduction

Tolfenamic acid is a non-steroidal anti-inflammatory drug (NSAID) included in the fenamates sub-group and used in human and veterinary medicine for postoperative pains, migraine and inflammatory processes due to its analgesic and antipyretic properties [1–3]. Moreover, this NSAID has shown antitumor effects by decrease of specific protein 1 (Sp1) and vascular endothelial growth factor [4–6] in different types of cancer such as ovarian cancer [7]. Degradation of Sp1 by tolfenamic acid is also related with improved therapeutic targets for Alzheimer diseases [8–10].

As with other NSAIDs, the mechanism of action of tolfenamic acid is based on cyclooxygenase (COX) inhibition [2,11]. Unfortunately, a long-term side effect of NSAIDs, principally COX-1 inhibitors, is severe gastrointestinal toxicity [12]. In particular, COX non-selective inhibitors like tolfenamic acid have been related to hepatotoxicity and nephrotoxicity [11,13]. Subsequently, the use of NSAIDs and their residues in food producing animals is strictly regulated, having been defined maximum residues levels of tolfenamic acid in milk, muscle, liver and kidney [13,14]. Moreover, the presence of residues of tolfenamic acid has been recently reported in human breastmilk as a result of continued environmental exposure [15,16]. A thorough knowledge of the factors that affect pharmacokinetics, milk secretion and tissue distribution of this drug is relevant for its therapeutic use and toxicity, and we hypothesize that the ATP-binding cassette (ABC) transporter ABCG2 could play a key role on this.

The drug efflux protein ABCG2 is one of the most important ABC transporters in terms of physiological, pharmacological and

Abbreviations: ABC, ATP-binding cassette efflux; ABCC1, ATP-binding cassette subfamily C1; A-B, apical to basal; AUC, area under the plasma concentration-time curve; B-A, basal to apical; BCRP/ABCG2, Breast Cancer Resistance Protein/ATP-binding cassette subfamily G2; COX, cyclooxygenase enzymes; DMEM, Dulbecco's modified Eagle's medium; LOD, limit of detection; LOQ, limit of quantification; MDCK-II, Madin-Darby Canine Kidney epithelial cells; NSAID, non-steroidal anti-inflammatory drug; Sp1, specific protein.

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toxicological processes [17]. ABCG2 is expressed in the apical membrane of cells from pharmacologically relevant tissues such as the canalicular membrane of hepatocytes, the luminal membrane of enterocytes, the kidney proximal tubule epithelia, the apical membrane of syncytiotrophoblasts, the blood-brain and blood-testis barriers and the alveolar cells from lactating mammary gland, among others [18–20]. Due to its localization, ABCG2 behaves like a pump that extrudes endogenous and exogenous substrates from the cells; so physiologically, ABCG2 has a detoxification role due to its effect in limiting the accumulation of xenobiotics in cells [21]. It is involved in the modulation of absorption, distribution and elimination of drugs, influencing pharmacokinetics and tissue distribution. By its localization in the apical membrane of cells from intestine, liver and kidney, Abcg2 extrudes substrates from the hepatocytes into the bile, from the renal tubule epithelia to the urinary lumen and from the intestinal epithelium into the intestinal lumen, reducing plasma levels of its substrates [18]. Furthermore, it is the only ABC transporter involved in active secretion of its substrates into milk [20].

ABCG2 can confer multidrug resistance to cancer cells, a major impediment to successful cancer chemotherapy [22,23]. In addition, a protective role of ABCG2 at the blood-brain barrier and its implication in Alzheimer disease have been reported. ABCG2 avoids beta-amyloid accumulation in the brain which is associated with Alzheimer pathogenesis. In patients with Alzheimer, ABCG2 levels have been reported to be improved as a compensatory mechanism and a protecting reaction against inflammation and oxidative stress condition [24].

Interactions between NSAIDs and ABCG2 have also been previously reported and some of them have been even described as ABCG2 substrates, such as diclofenac [25], flunixin [26] and meloxicam [27], or as ABCG2 inhibitors such as salicylic acid [28]. The interaction between ABCG2 and tolfenamic acid, however, has not yet been described. Our aim, therefore, was to study whether tolfenamic acid is an in vitro Abcg2 substrate and to characterize the potential effect of Abcg2 in plasma, milk and tissue distribution of this anti-inflammatory drug using wild-type and Abcg2$^{-/-}$ mice.

2. Materials and methods

2.1. Chemicals

Tolfenamic acid, flunixin, flufenamic acid and Lucifer Yellow were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ko143 was acquired from Tocris (Bristol, United Kingdom). For in vivo studies, tolfenamic acid (Tolfedine® 4%) was acquired from Vetoquinol, Madrid (Spain); isoflurane (Isovet®) was obtained from Braun VetCare, Barcelona (Spain) and oxytocin (Facilpart®) from Syva, León (Spain). All the other compounds used were reagent grade and were available from commercial sources.

2.2. Cell cultures

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

2.2.1. Transport assays

Transport assays were carried out as previously described by Merino et al. [29] with minor variations. Cells (passage 20–35) were seeded on microporous membrane filters (3.0 μm pore size, 24 mm diameter; Transwell 3414; Costar, Corning, NY) at a density of 1.0×10^6 cells per well. Cells were grown for 3 days and medium was replaced every day. Transepithelial resistance was measured to check the tightness of the monolayer using Millicell ERS (Millipore Burlington, MA). At the end of the experiment, confluence of the monolayer was also measured with Lucifer Yellow permeability assays [30]. Transport proficiency of these cells is constantly checked by testing a typical ABCG2 substrate like danofloxacin [31].

Two hours before the start of the experiment, medium in both compartments, apical and basal, was replaced with 2 ml of OptiMEM medium (Invitrogen, Carlsbad, CA), with or without 1 μM Ko143. The experiment began by replacing the medium on both sides with fresh OptiMEM medium, with or without 1 μM Ko143 and 20 μM tolfenamic acid. Cells were incubated at 37 °C in 5% CO_2 and 100 μl aliquots were taken at 2 and 4 h on the opposite side where tolfenamic acid was added and this volume was replaced with fresh medium; 600 μl aliquots were taken at 6 h on both sides of the well. Aliquots were stored at −20 °C until analysis. The appearance of tolfenamic acid in the opposite compartment was measured by HPLC as described below and was related to the total tolfenamic acid added at the beginning of the experiment. Finally, the relative efflux transport ratio was calculated as the apically directed transport percentage divided by the basolaterally directed transport percentage after 6 h.

2.3. 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_2016). Animals used were male and lactating female Abcg2$^{-/-}$ and wild–type mice, all of >99% FVB genetic background between 8 and 17 weeks of age. Animals, generated [32] and kindly provided by Dr. A. H. Schinkel (The Netherlands Cancer Institute), were kept in a controlled temperature environment with 12 h of light and 12 h of darkness, and received a standard diet and water ad libitum.

2.3.1. Pharmacokinetic experiments

For oral administration of tolfenamic acid at 4 mg/kg, Tolfedine® 4% dissolved in saline was dosed at 300 μl per 30 g of body weight by gavage into the stomach after 3–4 h of fasting. For intravenous administration of tolfenamic acid at 4 mg/kg, Tolfedine® 4% dissolved in saline was dosed at 150 μl per 30 g of body weight by injection into the tail of mice lightly anesthetized with isoflurane. Blood was collected at different time points by cardiac puncture after anaesthesia with isoflurane, and heparinized blood samples were centrifuged immediately at 3000 *g* for 15 min to obtain plasma. Organs were collected after euthanasia by cervical dislocation and stored at −20 °C until analysis by HPLC. Four to nine animals were used at each time point. The area under the plasma concentration-time curve (AUC) was calculated using the linear trapezoidal rule.

2.3.2. Milk secretion experiments

For milk experiments, pups of approximately 10 days old were separated from their mothers 4 h before starting the experiment. To stimulate milk secretion, 200 μl of oxytocin (1 IU/ml) was administrated subcutaneously to lactating mice 10 min before sample collection. Tolfedine® 4% dissolved in saline (4 mg/kg) was injected into the tail vein 30 min before the milk was collected. Blood was collected from the retro-orbital sinus under anaesthesia with isoflurane, and then milk was collected from the mammary glands by pressing around the nipple using capillaries. Finally, animals were sacrificed by cervical dislocation. Milk and plasma were stored at −20 °C until the time of HPLC analysis. Four to six animals were used for each group of mice.

2.4. Sample extraction and high performance liquid chromatography (HPLC) analysis

The conditions for HPLC analysis of tolfenamic acid were based on previously described methods [3,33] with minor modifications. Tissue samples were homogenized with a mixture of 50% water: 50% methanol with 0.5% HCl; 1 ml of solution per 0.1 g of organ was used. To each 100 μl aliquots of milk, plasma and tissue homogenates, 10 μl of internal standard and 400 μl of acetonitrile were added in a 1.5 ml reaction tube. Flunixin (6.25 μg/ml and 5 μg/ml) was used as internal standard for pharmacokinetic and milk secretion experiments, respectively, and flufenamic acid (6.25 μg/ml) was used for tissue samples. The mixture was vortexed for 30 s and then the samples were centrifuged at 4000 *g* for 5 min at 4 °C. The supernatant was collected and evaporated to dryness under N_2 at 45–50 °C. Samples were resuspended in 100 μl of methanol (Merck, Darmstadt, Germany) and injected into the HPLC system. Samples from the transport assays were not processed and 100 μl of the culture media were directly injected into the HPLC system. The system consisted of a Waters 600 pump, a Waters 717 autosampler and a photodiode array detector. Separation was performed on a reversed-phase column (4 mm particle size, 250 × 4.6 mm, Max-RP 80 Å, Phenomenex®, Torrance, CA, USA). The mobile phase used was orthophosphoric acid 10 mM: acetonitrile (30:70). The flow rate of the mobile phase was set to 1.2 ml/min and UV absorbance was measured at 280 nm. The limit of quantification (LOQ) was 0.02 μg/ml and the limit of detection (LOD) 0.01 μg/ml for cell culture samples; LOQ 0.3 μg/ml and LOD 0.1 μg/ml for plasma samples; LOQ 0.1 μg/ml and LOD 0.05 μg/ml for milk samples and for tissues LOQ 0.04–0.10 μg/ml and LOD 0.01–0.04 μg/ml. LOD and LOQ were calculated as described by Tavernierset al. [34].

2.5. Statistical analysis

The SPSS Statistics software (v. 24.0; IBM, Armonk, New York, NY, USA) was used for the statistical analysis. The Shapiro-Wilk normality test was performed to check normal distribution. The two-tailed unpaired Student's *t*-test was applied to normal distribution data and the Mann-Whitney *U* test, a non-parametric test, was performed as an independent sample analysis for non-normal distribution data. *P* value $\leq$ 0.05 indicates that the differences were statistically significant.

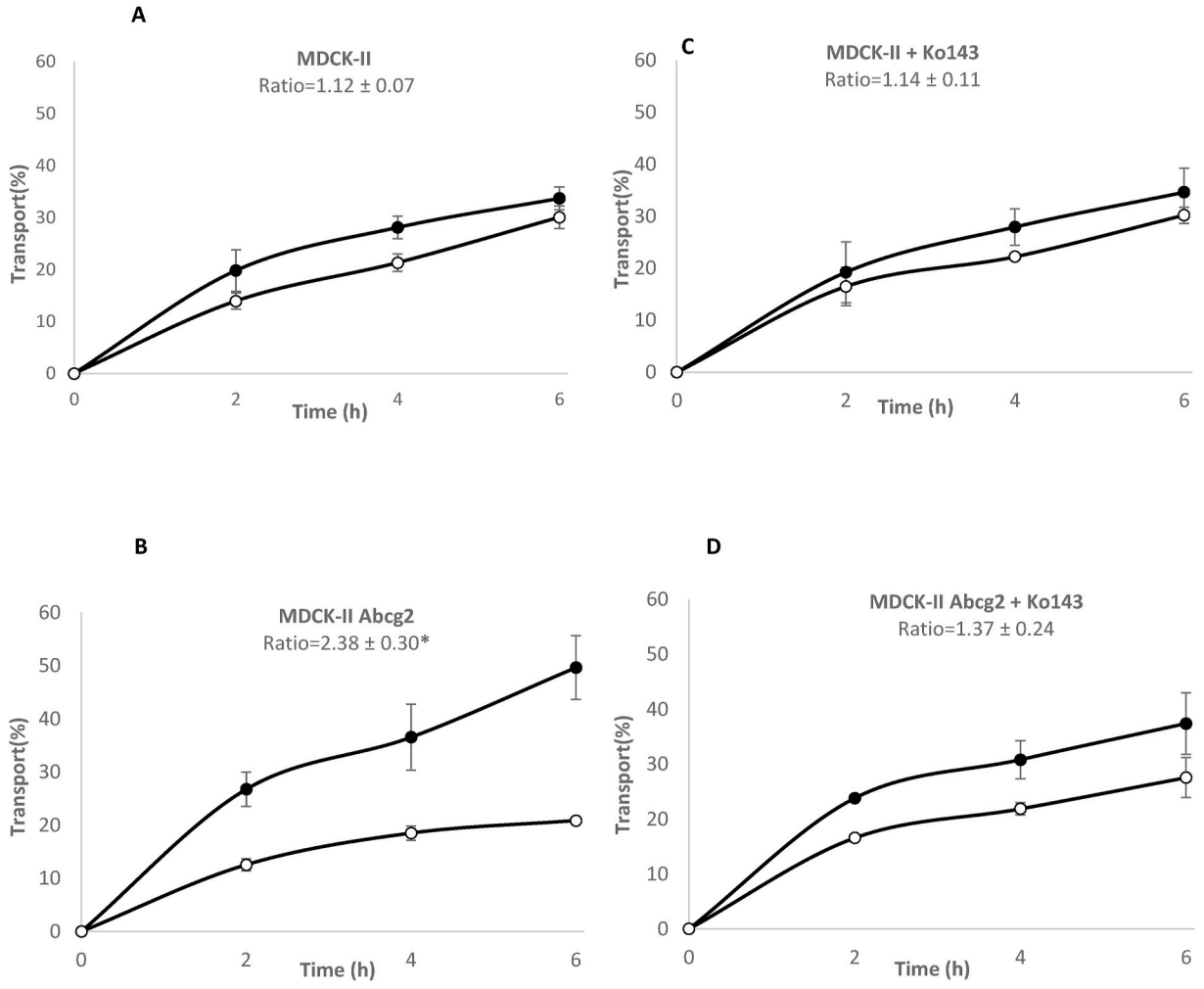

Fig. 1. Transcellular transport of tolfenamic acid (20 μM) with or without Ko143 (ABCG2 inhibitor) in parental MDCK-II cells (A and C, respectively) and MDCK-II cells transduced with murine Abcg2 (B and D, respectively). The assay was started by changing the medium in apical or basolateral compartment with fresh culture medium with or without Ko143 at 1 μM and 20 μM of tolfenamic acid. Aliquots of 100 μl were taken at 2 and 4 h on the opposite side from where tolfenamic acid was added and aliquots of 600 μl were taken at 6 h on both sides of the well; all of these were measured by HPLC. The appearance of tolfenamic acid in the opposite comportment was related to the total drug added at the beginning of the experiment. Results represented the mean and error bars indicate S.D. The relative efflux transport ratio, apical directed translocation divided by basolateral directed translocation at 6 h is indicated. (●) transport from basal to the apical compartment; (○) transport from apical to the basal compartment. (n = 3–6). (*) significant differences in transport ratio compared to parental MDCK-II cells ($p \leq 0.05$).

3. Results

3.1. In vitro transport of tolfenamic acid

To evaluate whether Abcg2 is involved in the in vitro transport of tolfenamic acid, MDCK-II cells and its subclon transduced with murine Abcg2 cDNAs were used. The parental and subclon cell lines were grown to confluent polarized monolayers and vectorial transport of 20 μM tolfenamic acid across the monolayers was determined. The results obtained in the MDCK-II parental cells for the apically and basolaterally translocation were similar (Fig. 1A). However, the basal to apical (B-A) transport of tolfenamic acid in Abcg2-transduced cells (Fig. 1B) was higher than the apical to basal (A-B) transport, with a relative transport ratio (B-A/A-B at 6 h) significantly higher (2.38 ± 0.30) than in the parental cells (1.12 ± 0.07; $p \leq 0.05$). To confirm that this effect is specifically caused by Abcg2, the specific Abcg2 inhibitor Ko143 was used [35] (Fig. 1C and D) resulting in a similar efflux ratio to that of the MDCK-II parental cell line (Fig. 1A). These data demonstrate that tolfenamic acid is an in vitro substrate of Abcg2.

3.2. Plasma levels of tolfenamic acid in Abcg2$^{-/-}$ and wild-type mice

We measured plasma levels of tolfenamic acid in Abcg2$^{-/-}$ and wild-type male mice to determine whether in vitro Abcg2-mediated transport of tolfenamic acid was also reflected in vivo. Plasma concentrations of tolfenamic acid after its oral and intravenous (Figs. 2 and 3) administration, two of the most common routes of administration of the drug, were determined in Abcg2$^{-/-}$ and wild-type mice at the dose of 4 mg/kg, based on previous studies in rats [3]. After oral administration, a rapid absorption of the drug was reported with the observed C_{max} at the first sampling point (15 min) (Fig. 2). Plasma AUC of Abcg2$^{-/-}$ mice was 1.8-fold higher compared to wild-type mice (47.49 ± 4.6 versus 26.11 ± 2.03 μg*h/ml; $p \leq 0.05$). There were significant differences in plasma concentrations between Abcg2$^{-/-}$ and wild-type mice at 45 min, 2 and 3 h ($p \leq 0.05$) (Fig. 2). For intravenous administration, plasma AUC of Abcg2$^{-/-}$ mice increased 1.7-fold compared to wild-type mice (89.55 ± 5.8 versus 51.51 ± 5.33 μg*h/ml; $p \leq 0.05$). Significant differences in plasma concentrations at 30 min, 1 and 2 h ($p \leq 0.05$) were also found (Fig. 3). These results demonstrate that Abcg2 activity affects plasma levels of tolfenamic acid after oral and intravenous administration.

3.3. Tissue distribution of tolfenamic acid in Abcg2$^{-/-}$ and wild-type mice

Concentrations of tolfenamic acid were analysed in different tissues: liver, kidney, intestine, intestinal content, brain and testis of wild-type and Abcg2$^{-/-}$ male mice 2 and 4 h after intravenous administration of tolfenamic acid at 4 mg/kg (Table 1). Regardless of Abcg2 expression, organ concentrations were higher at 2 h than at 4 h except for testis. In fact, at 4 h many samples were below LOQ, including liver samples from wild-type mice but not from Abcg2$^{-/-}$ mice. These higher levels of tolfenamic acid in liver samples from Abcg2$^{-/-}$ animals compared to wild-type mice were also reported at 2 h. In testis, Abcg2$^{-/-}$ mice showed a higher accumulation of tolfenamic acid compared to wild-type at both time points, with a difference of almost 3-fold at 4 h. No significant differences were found in other organs between wild-type and Abcg2$^{-/-}$ mice. Brain levels were below LOQ in almost all samples and were not included in Table 1.

3.4. Secretion of tolfenamic acid into milk in Abcg2$^{-/-}$ and wild-type mice

To assess whether Abcg2 is involved in the secretion of tolfenamic acid into milk, we administrated this drug intravenously (4 mg/kg) to lactating Abcg2$^{-/-}$ and wild-type female mice, and milk and blood samples were collected 30 min after administration. There were no differences between wild-type and Abcg2$^{-/-}$ in plasma concentration (43.81 ± 23.24 versus 38.56 ± 11.25 μg/ml, respectively), milk concentration (8.92 ± 6.07 versus 14.23 ± 3.74 μg/ml, respectively) or in milk to plasma ratio (0.24 ± 0.1 versus 0.39 ± 0.19 μg/ml, respectively). Based on these data, we determine that Abcg2 does not show any effect in secretion of tolfenamic acid into milk in our experimental conditions.

4. Discussion

Therapeutic success and drug toxicity events are closely related with drug bioavailability. Studies of factors that could affect this systemic exposure are crucial and the efflux transporter activity of ABCG2 could be one of such factors. This study shows for the first time the in vitro and in vivo interaction between Abcg2 and tolfenamic acid, an anti-inflammatory drug.

In vitro assays show that tolfenamic acid is transported by the Abcg2 murine variant (Fig. 1). Our reported transport ratio of tolfenamic acid in Abcg2-transduced cells (2.38 ± 0.30) (Fig. 1B) was lower than those

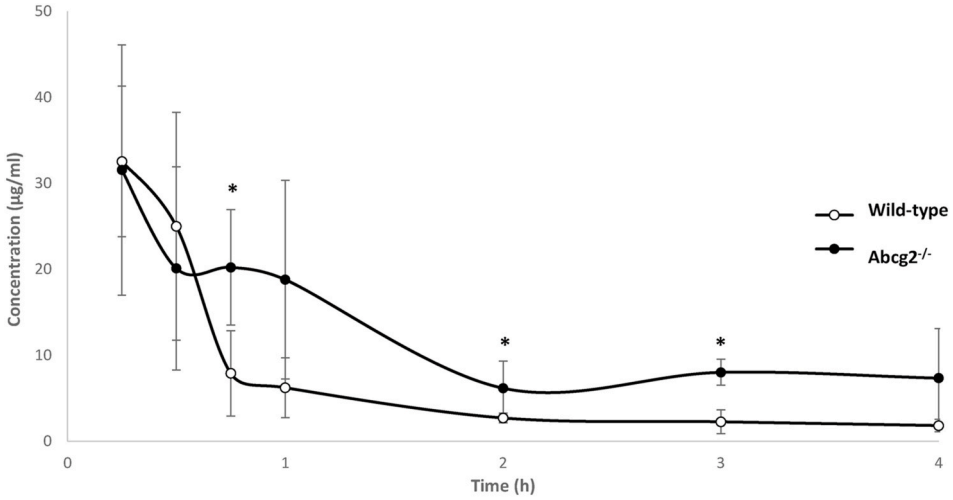

Fig. 2. Plasma concentrations after oral administration of Tolfedine® (4 mg/kg) to wild-type and Abcg2$^{-/-}$ male mice. Plasma samples were collected at 15, 30, 45 min, 1, 2, 3 and 4 h. Plasma levels of tolfenamic acid were determined by HPLC. Results are means and error bars indicate S.D. (*) $p \leq 0.05$ significant differences between both groups of mice (n = 4–8).

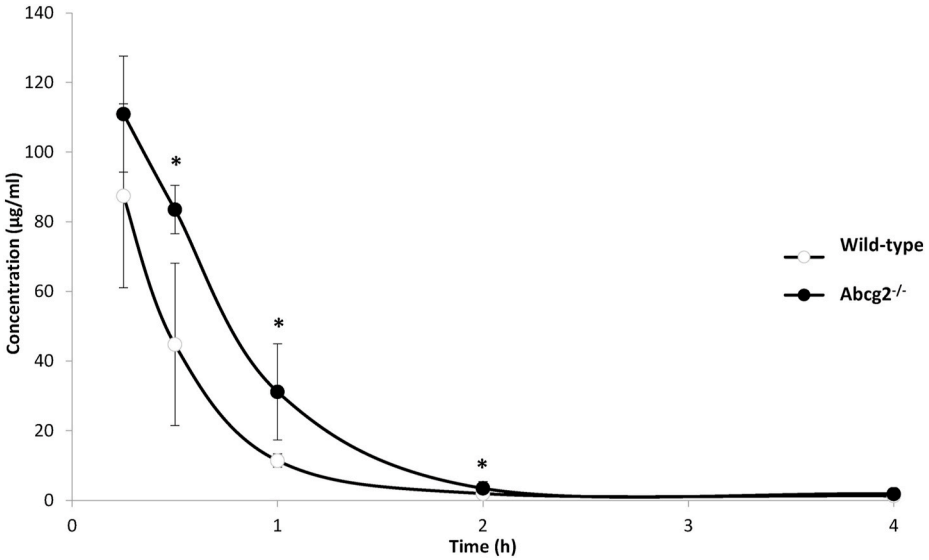

Fig. 3. Plasma concentrations after intravenous administration of Tolfedine® (4 mg/kg) to wild-type and Abcg2$^{-/-}$ male mice. Plasma samples were collected at 15, 30 min, 1, 2 and 4 h. Plasma levels of tolfenamic acid were determined by HPLC. Results are means and error bars indicate S.D. (*) $p \leq 0.05$ significant differences between both groups of mice (n = 4–9).

Table 1
Concentrations of tolfenamic acid (µg/ml) in wild-type and Abcg2$^{-/-}$ male mice tissues 2 and 4 h after intravenous administration of Tolfedine® at 4 mg/kg (n = 4–9).

	Wild-type	Abcg2$^{-/-}$	Wild-type	Abcg2$^{-/-}$
	2 h		4 h	
Liver	0.22 ± 0.07	0.40 ± 0.13*	<LOQ	0.11 ± 0.03
Kidney	0.09 ± 0.05	0.13 ± 0.02	<LOQ	<LOQ
Small Intestine (Tissue)	0.32 ± 0.33	0.33 ± 0.22	<LOQ	<LOQ
Small Intestinal content	0.76 ± 0.57	1.55 ± 1.10	0.35 ± 0.19	0.38 ± 0.18
Testis	0.13 ± 0.01	0.15 ± 0.01*	0.54 ± 0.38	1.42 ± 0.57*

Results are presented as means ± S.D.
*: $p \leq 0.05$ significant differences versus wild-type.
< LOQ: sample concentration below limit of quantification.

obtained for other NSAIDs. For example, flunixin and meloxicam have been identified as efficient substrates for Abcg2 with transport ratios of around 30 [26,27]. Diclofenac and 5-hidroxyflunixin have also been identified as substrates for murine Abcg2 with estimated ratios between 2 and 4 [25,26]. These differences in Abcg2 transport efficiency between NSAIDs could be due to differences in physicochemical properties of the molecules involved in the efflux transport; for instance, lipid-water partition coefficient, hydrophobicity, polarity, solubility or molecular weight [36–39]. In relation with the latter, ABCG2 substrates typically have a molecular weight around 400 g/mol [36]. Tolfenamic acid, compared with these other NSAIDs, has the lowest molecular weight, 261.7 g/mol, since the molecular weight of flunixin, meloxicam, diclofenac and 5-hidroxyflunixin is above 290.

Although tolfenamic acid is not a NSAID with a high in vitro transport ratio mediated by Abcg2, our in vivo assays reveal that Abcg2 is an important determinant of the exposure of this drug since Abcg2 mediates plasma levels and tissue accumulation of tolfenamic acid. Plasma concentrations of tolfenamic acid in both administrations, oral (Fig. 2) and intravenous (Fig. 3), exhibited clear differences between wild-type and Abcg2$^{-/-}$ mice and higher plasma levels of tolfenamic acid in the absence of the transporter were reported. Tolfenamic acid was quickly absorbed in both mouse strains after oral administration, while plasma levels stayed higher in Abcg2$^{-/-}$ mice than in wild-type between 30 min and 2 h (Fig. 2) resulting in a higher systemic exposure of tolfenamic acid in Abcg2$^{-/-}$ mice. Unfortunately, there is no data available on whether metabolites of tolfenamic acid are substrates of Abcg2 which may partially explain the higher plasma levels of tolfenamic acid in Abcg2$^{-/-}$ mice due to product inhibition effect. Nevertheless, the higher levels of tolfenamic acid in liver from Abcg2$^{-/-}$ mice than in wild-type animals at 2 and 4 h after intravenous administration (Table 1) and the lack of significant differences in intestine and kidney accumulation between wild-type and Abcg2$^{-/-}$ mice indicate that this effect in plasma is probably due to Abcg2 present in liver and more specifically Abcg2-mediated biliary secretion. These differences in liver accumulation were also observed for other NSAIDs such as meloxicam [27] and they may be partially related to the differences in plasma concentrations caused by Abcg2 (Fig. 3). In any case, differences in tissue levels are relevant since they can cause toxicity problems. It will be important to assess whether this also applies to humans. NSAIDs are generally used in long-term treatments and are related to idiosyncratic hepatotoxicity and gastrointestinal injury [40]. The mechanisms involved in gastrointestinal and liver damage are not well described but for example, in the gastrointestinal tract, the accumulation of NSAID has been proposed to cause the damage [41]. Li et al. [42] attribute the liver cytotoxicity of tolfenamic acid to the diphenylamine that contained in its structure and which could be connected to mitocondrial permeability transition. Hepatotoxicity is also associated with N-phenylanthranilic acid or fenamic acid from which tolfenamic acid is derived [43]. In humans, a decrease in ABCG2 protein liver levels was reported in the elderly population compared to adult or children [17], which could result in high exposure to the drug and consequently in toxic effects.

The testis is another organ with higher levels of tolfenamic acid in

Abcg2$^{-/-}$ mice (Table 1), similarly to meloxicam [27]. In the absence of Abcg2, higher levels of tolfenamic acid are accumulated in testis with the highest difference at 4 h as compared to 2 h (Table 1). If also true in humans, this outcome is relevant since damaged testis function has been reported for some NSAIDs. For example, in vitro exposure to analgesic drugs such as paracetamol, aspirin and indomethacin can induce multiple endocrine disturbances in the human adult and fetal testis [44,45]. Thus, in ex vivo cultures of human fetal testis, exposure to ibuprofen can affect testicular somatic cell populations and germ cells during particular periods of human fetal testis development [46]. In addition, some epidemiology studies have reported that exposure to some NSAIDs, mainly paracetamol, during the first and second trimesters of gestation may increase the risk of cryptorchidism, the most frequent abnormality in new born boys [47–49].

Our findings show the protective role of ABCG2 in the liver and the testis-blood barrier, as it avoids accumulation of high levels of tolfenamic acid in these organs. Tissue levels are also very relevant in relation to drug residues in edible tissues in livestock and in the case of tolfenamic acid, liver residues may be also affected by Abcg2, with potential consequences in the established withdrawal periods.

Regarding other relevant organs in tolfenamic therapeutics such as the brain, in the case of Alzheimer disease, this NSAID was not detected in significant amounts in this organ. Therefore, no relevant conclusions can be obtained in this regard.

Compensatory changes in the expression of genes coding for proteins involved in transport in the Abcg2$^{-/-}$ mice that could affect plasma levels and tissue distribution of tolfenamic acid cannot be completely excluded. However, indications for such disturbing effects in previous studies have never been shown [50–52].

In contrast to male pharmacokinetic experiments, assays of secretion into milk indicate that Abcg2 is not involved in the transfer of tolfenamic acid into milk since there were no differences in milk concentrations and milk/plasma ratios between both types of mice. This lack of Abcg2-mediated effect in secretion into milk may be related with the low in vitro transport ratio of tolfenamic acid in Abcg2-transduced cells (2.38 ± 0.30) (Fig. 1B) since, in the case of other NSAIDs with a higher transport ratio such as meloxicam, flunixin and 5-hidroxyflunixin flunixin (as indicated above), a clear in vivo effect of Abcg2 on their secretion into milk has been reported [26,27]. Nevertheless, other factors apart from ABCG2 may be contributing to the secretion into milk of tolfenamic acid. Drug transfer into milk could be influenced by physicochemical properties such as drug binding to proteins and partitioning of drug into the lipid fraction [53,54]. Although the possibility that an uptake transporter may be involved in the transfer of this drug into milk cannot be excluded [20], the low milk/plasma ratio reported makes this hypothesis highly unlikely.

No differences between wild-type and Abcg2$^{-/-}$ mice were reported in plasma concentration at 4 mg/kg in the lactating females in contrast to the male experiments (Fig. 3). Differences in the effect of Abcg2 on plasma levels of several substrates have been previously reported between both sexes, with a more pronounced effect of the transporter in males due to a higher Abcg2 expression in male liver [55,56].

Finally, factors affecting Abcg2 activity may influence tolfenamic acid bioavailability and tissue accumulation and, in consequence, its therapeutic and toxicity characteristics may be altered. Different expression of ABCG2 may affect drug pharmacokinetics [57,58]. Interactions and co-administration of drugs which are substrates and/or inhibitors of the transporter may also affect the pharmacokinetic parameters of tolfenamic acid with potential therapeutic and toxicology consequences. Previous studies with different families of ABCG2 substrates have reported these interactions and their consequences [59–61].

5. Conclusions

In this study we identify tolfenamic acid as an in vitro Abcg2 substrate and defined the role of the Abcg2 transporter as a factor involved in the systemic exposure of tolfenamic acid and its tissue distribution in organs such as liver or testis.

CRediT author statement

Esther Blanco Paniagua: Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Investigation, Writing-Original Draft. Alba M. Garcia-Lino: Methodology, Data curation, Formal analysis, Visualization, Investigation. Dafne Garcia-Mateos: Methodology, Investigation. Ana I. Alvarez: Conceptualization, Methodology, Funding acquisition, Validation, Supervision, Writing-Review & Editing. Gracia Merino: Conceptualization, Methodology, Funding acquisition, Project administration, Resources, Supervision, Writing-Review & Editing.

Declaration of competing interest

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.

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