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In vitro interaction of the pesticides flupyradifurone, bupirimate and its metabolite ethirimol with the ATP-binding cassette transporter G2 (ABCG2)

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ABSTRACT

ABCG2 is an ATP-binding cassette efflux transporter that is expressed in absorptive and excretory organs such as liver, intestine, kidney, brain and testis where it plays a crucial physiological and toxicological role in protecting cells against xenobiotics, affecting pharmacokinetics of its substrates. In addition, the induction of ABCG2 expression in mammary gland during lactation is related to active secretion of many toxicants into milk. In this study, the in vitro interactions between ABCG2 and three pesticides flupyradifurone, bupirimate and its metabolite ethirimol were investigated to check whether these compounds are substrates and/or inhibitors of this transporter. Using in vitro transepithelial assays with cells transduced with murine, ovine and human ABCG2, we showed that ethirimol and flupyradifurone were transported efficiently by murine Abcg2 and ovine ABCG2 but not by human ABCG2. Bupirimate was not found to be an in vitro substrate of ABCG2 transporter. Accumulation assays using mitoxantrone in transduced MDCK-II cells suggest that none of the tested pesticides were efficient ABCG2 inhibitors, at least in our experimental conditions. Our studies disclose that ethirimol and flupyradifurone are in vitro substrates of murine and ovine ABCG2, opening the possibility of a potential relevance of ABCG2 in the toxicokinetics of these pesticides.

1. Introduction

ABCG2 is a described member of the ATP binding cassette superfamily (ABC), a group of proteins that play a major role in cell protection against xenotoxins (Mao and Unadkat, 2015; Khunweeraphong et al., 2019). Situated in the apical side of the cell membrane at anatomical sites relevant for xenobiotic disposition, such as the luminal membrane of enterocytes, the bile canalicular membrane of hepatocytes and the renal proximal tubule epithelia, it decreases the oral availability and systemic exposure of its substrates (Vlaming et al., 2009). Moreover, ABCG2 drug efflux transporter, is expressed on the placental syncytiotrophoblasts' membrane and the brain capillary endothelial cells (Jonker et al., 2000; van Hoppe et al., 2019), where it protects the fetus and brain from toxic compounds. ABCG2 is highly expressed in the alveolar cells from lactating mammary gland, and it is the only ABC transporter involved in active secretion of its substrates into milk and, therefore, in presence of toxic residues in milk (García-Lino et al., 2019). All these features contribute to the relevance of this transporter for toxicokinetics studies.

Previous studies have reported that some pesticides interact with drug transporters, including ABCG2 (Fardel et al., 2012; Bircsak et al., 2013; Halwachs et al., 2016; Herriage et al., 2022). However, potential interaction with ABCG2 remains to be studied for many pesticides.

In this context, bupirimate, a pyrimidine fungicide, and the insecticide flupyradifurone belonging to the class of butenolides, are two pesticides with a relevant spread in food chain and environment but their potential interaction with ABCG2 remains to be elucidated. Bupirimate is authorized for use on apple and sugar beet that might be fed to livestock (EFSA, 2019) and it has been detected in potato samples collected from local markets, exceeding the maximum residue level in

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EU by 1500% (Ahmed et al., 2014). Moreover, a potential dermal exposure to bupirimate residues, including its metabolite ethirimol, in tomato greenhouses during tying and pruning task, has been reported in a worker's re-entry exposure study (Kasiotis et al., 2017). Regarding flupyradifurone, it has become available since 2014 to be used on a wide range of crops in China (Zhong et al., 2021) and Europe (EFSA, 2016). Following a new economic assessment, it is currently one of the most commonly used imidacloprid substitutes in Californian agriculture (Huang et al., 2022). In-hive bees and foragers may be exposed to flupyradifurone during extended time periods. After field treatment, bees can ingest contaminated nectar which results in the detection of contaminated honey (Kammoun et al., 2019). Flupyradifurone has also been found in 33% of watersheds in Ontario (Canada) and relevant levels of residues were detected in the cloacal fluid of hummingbirds and in honeybee nectar (Metcalfe et al., 2019). Recently, after flupyradifurone intoxication, this pesticide was found in several human organs (Minji et al., 2022).

Therefore, the main goal of this study was to assess through in vitro assays the transport of these pesticides by ABCG2 using parental MDCK-II cells and their subclones transduced with murine Abcg2 and ovine and human ABCG2 cDNAs to test whether they are substrates of the different variants of the transporter. Murine Abcg2 was tested as mouse is a preclinical model and can be considered to be representative of general mammal exposure. Ovine ABCG2 was tested due to the relevance of sheep in food chain, mainly regarding dairy products. In addition, the potential role of these pesticides as in vitro inhibitors of ABCG2 was also evaluated with these cells using mitoxantrone accumulation assays.

2. Materials and methods

2.1. Chemicals

Bupirimate (CAS 41483–43–6, purity ≥ 98%), ethirimol (CAS 23947–60–6, purity ≥ 98%), flupyradifurone (CAS 951659–40–8, purity ≥ 98%), mitoxantrone (CAS 70476–82–3, purity ≥ 97%) and Lucifer Yellow (CAS 67769–47–5, purity ≥ 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ko143 (CAS 461054–93–3, purity ≥ 99%) was acquired from Tocris (Bristol, United Kingdom). Stock solutions (10 mM) were prepared in DMSO and stored at − 20 °C. Solvent concentration did not exceed 0.1% in all experiments. All the other chemicals used in this study were of analytical grade and were available from commercial sources.

2.2. Cell cultures

MDCK-II (Madin-Darby canine kidney epithelial cells) cell lines, parental and subclones transduced with murine Abcg2 and human ABCG2 were described elsewhere (Jonker et al., 2002; Pavek et al., 2005). All these cell types were kindly provided by Dr. A.H. Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands). Stably-transduced MDCK-II cells with ovine variant of ABCG2 were generated and characterized by our research group in previous studies (González-Lobato et al., 2014). Cells were cultured in DMEM (Dulbecco's modified Eagle's medium) with glutamax supplemented with FBS (10% v/v), penicillin (50 U/ml) and streptomycin (50 μg/ml) at 37 °C in an atmosphere with 5% CO_2 and were passaged every 3–4 days by trypsinization with trypsin-EDTA.

2.3. Cytotoxicity assays

Cytotoxicity assays were performed as previously described, with modifications (González-Lobato et al., 2014). Briefly, cells were plated at 6000–10,000/well in 96-well plates 24 h prior to the addition of pesticide. A concentration series of pesticide was applied along one plate axis and incubated for 24 h at 37 °C and 5% CO_2. Relative cell proliferation was quantified with MTT.

2.4. Accumulation assays

In vitro accumulation assays were carried out based on previous published methods (Pavek et al., 2005) using mitoxantrone (10 μM) as a fluorescent substrate. Briefly, subconfluent cultures were used after 36 h from seeding. Medium was aspirated and cells were incubated in Opti-MEM medium with or without Ko143 inhibitor (1 μM) or pesticides at different concentrations for 60 min before the addition of mitoxantrone. Accumulation of mitoxantrone was allowed for 1 h at 37 ºC. Cells were then washed with ice-cold PBS and trypsinized. Collected cells were sedimented and resuspended in PBS with 2.5% fetal calf serum. Relative cellular accumulation of mitoxantrone (excitation and emission wavelengths 635 and 650 nm, respectively) of at least 5000 was quantified from histogram plots using the median of fluorescence in a MACSQuant® flow cytometer (Miltenyi Biotech, Germany). Inhibitory potencies of compounds were calculated as previously described (Pavek et al., 2005) in ABCG2-transduced cells according to the following equation: inhibitory potency = (MF with tested compound – MF without inhibitor) / (MF with Ko143 – MF without inhibitor) x 100%.

2.5. Transport assays

Transepithelial transport assays, using 6-well Transwell plates (Transwell 3414, Corning Life Science, Corning, NY, USA), were carried out as previously described by Merino et al. (2005) with minor modifications. Cells (passage 20–35) were seeded at a density of 1.0×10^6 cells per well on microporous polycarbonate membrane filters (3.0 μm pore size, 24 mm diameter). Cells were grown for 3 days and medium was changed daily.

To check the integrity of the monolayer, transepithelial resistance was measured in each well using a Millicell ERS ohmmeter (Millipore, Burlington, MA). Confluence of the monolayer was also assessed with Lucifer Yellow permeability tests at the end of the experiment. Transport proficiency of cells was simultaneously tested using a typical ABCG2 substrate at the concentration used in previous in vitro studies, danofloxacin (10 μM) (Real et al., 2011) (Supplementary Table S1).

The media in both compartments, apical and basal, was replaced with 2 ml of OptiMEM medium (Invitrogen, Carlsbad, CA) and, either with or without the specific ABCG2 inhibitor Ko143 (1 μM), two hours before the assay began. The experiment started (t = 0) by replacing the medium on both sides with fresh OptiMEM medium, with or without 1 μM Ko143 and 10 μM of bupirimate, ethirimol or flupyradifurone.

Cells were then incubated at 37 °C in 5% CO_2 and 100 μl aliquots were collected at 2 and 4 h in the opposite compartment and this volume was replaced with new medium. At 6 h, 600 μl aliquots were taken from both sides of the well. Samples are stored at − 20 °C until HPLC analysis. At least three independent assays were performed with each drug. Results were represented as the percentage of pesticide concentration appeared in the opposite compartment related to the total pesticide added at the beginning of the assay.

At last, the relative efflux transport ratio was calculated as the apically directed transport percentage (B-A) divided by the basolaterally directed translocation percentage (A-B) after 6 h.

2.6. High performance liquid chromatography (HPLC) analysis

The chromatographic system consisted of a Waters 2695 separation module and a Waters 2998 UV photodiode array detector. Samples from the transport assays were not processed and 100 μl of the culture media were directly injected into the HPLC system in isocratic conditions. Representative chromatograms obtained for each compound are shown in Supplementary Fig. S1.

The conditions for HPLC analysis of bupirimate were based on previously described methods (Kasiotis et al., 2017) with slight modifications. Samples maintained at 4 °C were separated on a reversed-phase column (Mediterranea, 25 × 0.46 cm) at room temperature. The

mobile phase used was formic acid 0.1%: acetonitrile (25:75). The flow rate of the mobile phase was set to 0.8 ml/min and UV absorbance was measured at 239 nm. The limit of quantification (LOQ) was 0.02 µg/ml and the limit of detection (LOD) 0.01 µg/ml. LOD and LOQ were calculated as described by Taverniers et al. (2004).

For ethirimol, the separation of the samples was performed on a reverse-phase column (4 mm particle size, 250 × 4.6 mm, Max-RP 80 Å, Phenomenex®, Torrance, CA, USA). The mobile phase consisted of methanol formic acid (0.1%):water formic acid (0.1%) (50:50), the flow rate of the mobile phase was set to 0.9 ml/min and UV absorbance was measured at 268 nm. The temperature of the samples was 4 °C and the column was maintained at 40 °C. The limit of quantification (LOQ) was 0.07 µg/ml and the limit of detection (LOD) 0.03 µg/ml.

The conditions for HPLC analysis of flupyradifurone were modified according to previously published methods (Fang et al., 2020). Separation was performed on a reversed-phase column (Mediterranea, 25 ×

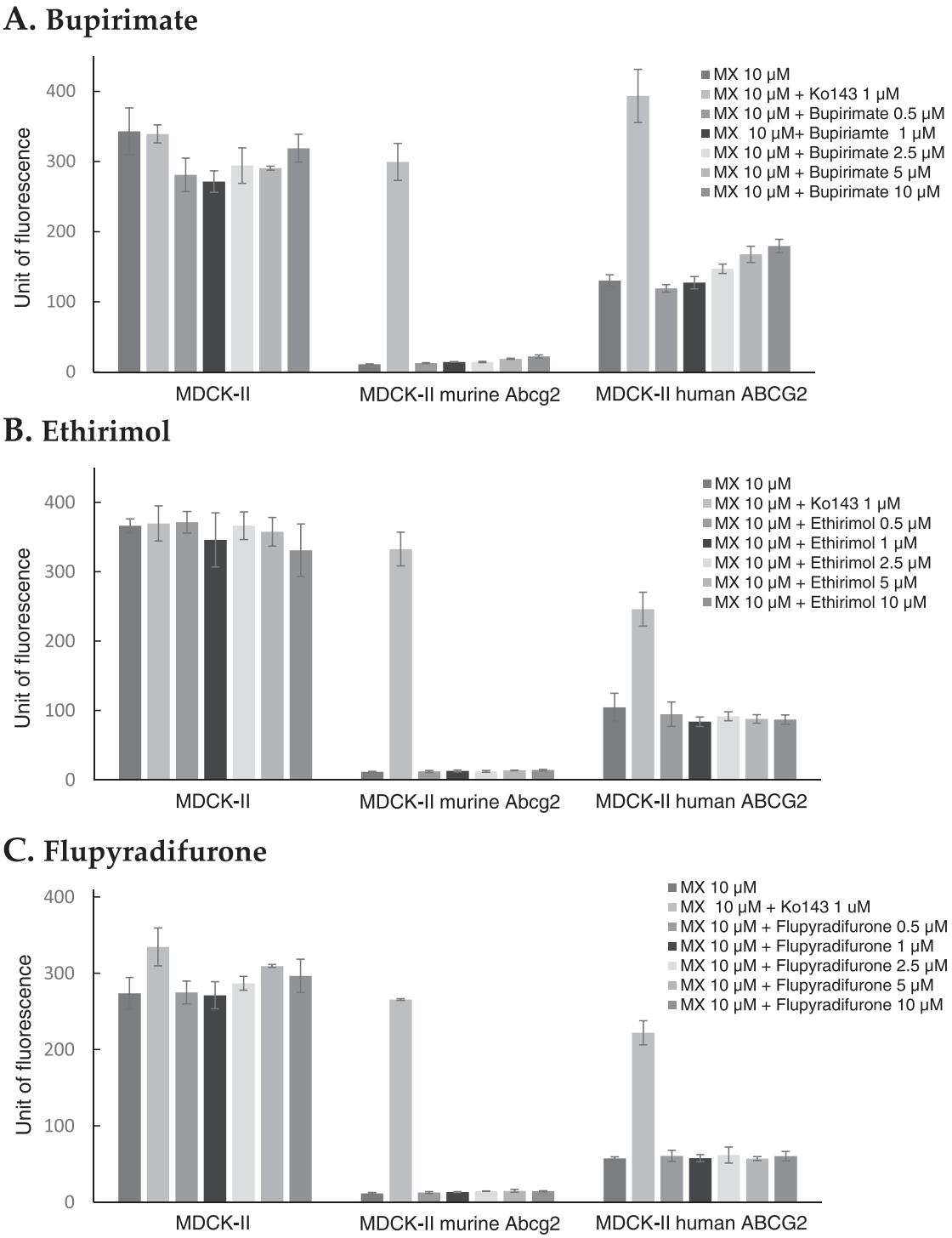

Fig. 1. Effect of bupirimate, ethirimol and flupyradifurone on accumulation of mitoxantrone (10 µM) at different concentrations in parent MDCK-II cells and in their murine Abcg2 and human ABCG2-transduced derivatives. Results (units of fluorescence, median) are expressed as the means of at least three experiments; error bars indicate SDs.

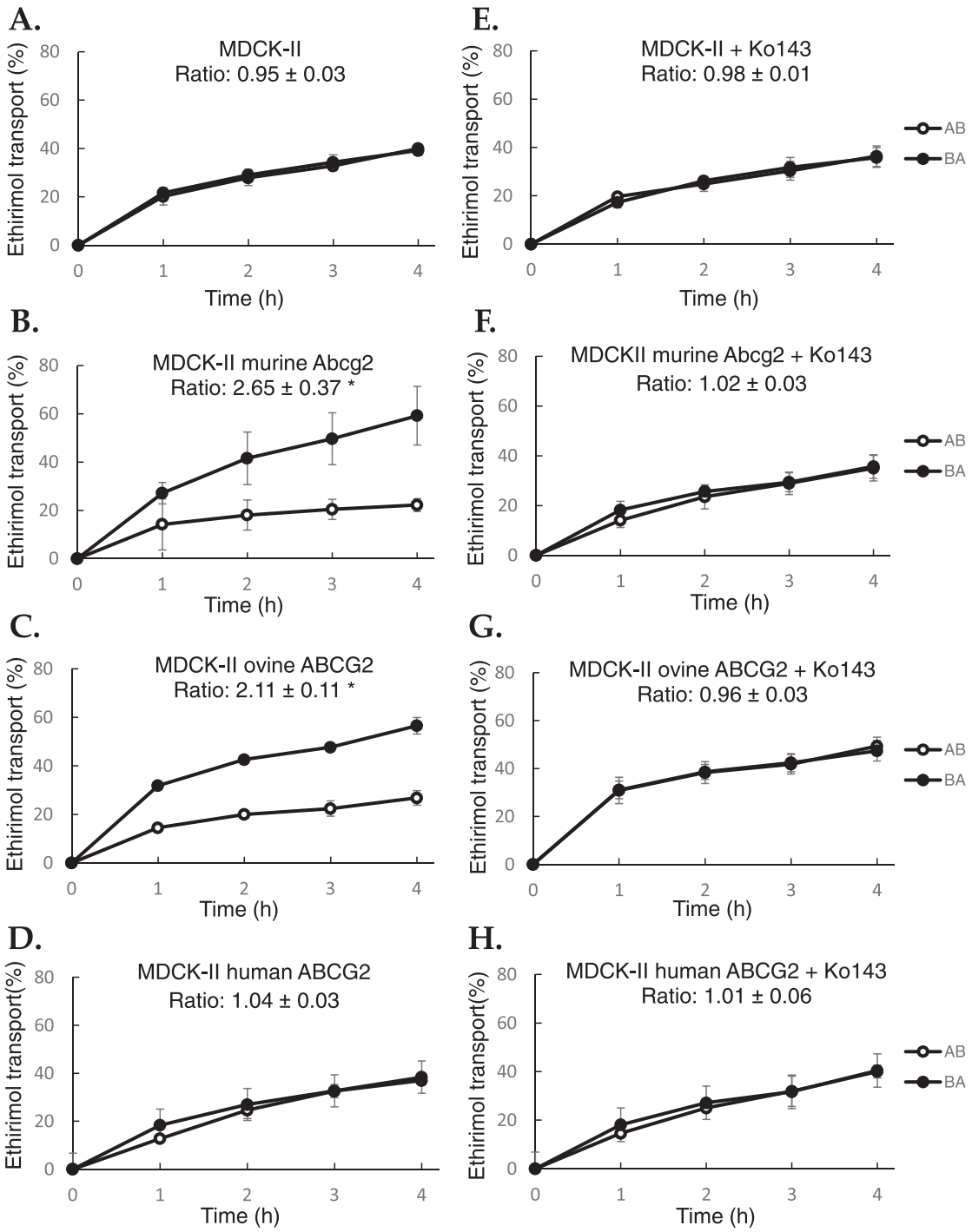

Fig. 2. Transepithelial transport of ethirimol (10 μM) with or without 1 μM Ko143 (ABCG2 inhibitor) in parental MDCK-II cells (A and E respectively) and in their murine Abcg2 (B and F respectively), ovine (C and G respectively) and human (D and H, respectively) ABCG2-transduced derivatives. The experiment was started with the addition of ethirimol to one compartment (basolateral or apical). After 2, 4 and 6 h, the percentage of pesticide appearing in the opposite compartment was measured by HPLC and plotted. Results are means, and error bars (sometimes smaller than the symbols) indicate SDs (n = 3). (•) translocation from the basolateral to the apical compartment; (O) translocation from the apical to the basolateral compartment. The relative transport ratio (the apically directed translocation divided by the basolaterally directed translocation) is indicated at 6 h. (*) significant differences in transport ratio compared to parental MDCK-II cells ($p \leq 0.05$).

0.46 cm). The composition of the mobile phase was orthophosphoric acid 10 mM: acetonitrile at a proportion of 60:40. The flow rate of the mobile phase was set to 1 ml/min and UV absorbance was measured at 261 nm. The temperature of the samples was 4 °C and the temperature of the column was 40 °C. The limit of quantification (LOQ) was 0.008 μg/ml and the limit of detection (LOD) 0.004 μg/ml.

2.7. 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. Comparisons between groups were performed by the Student's t-test (normal variables) and the Mann-Whitney U test, a non-parametric test, was applied as an

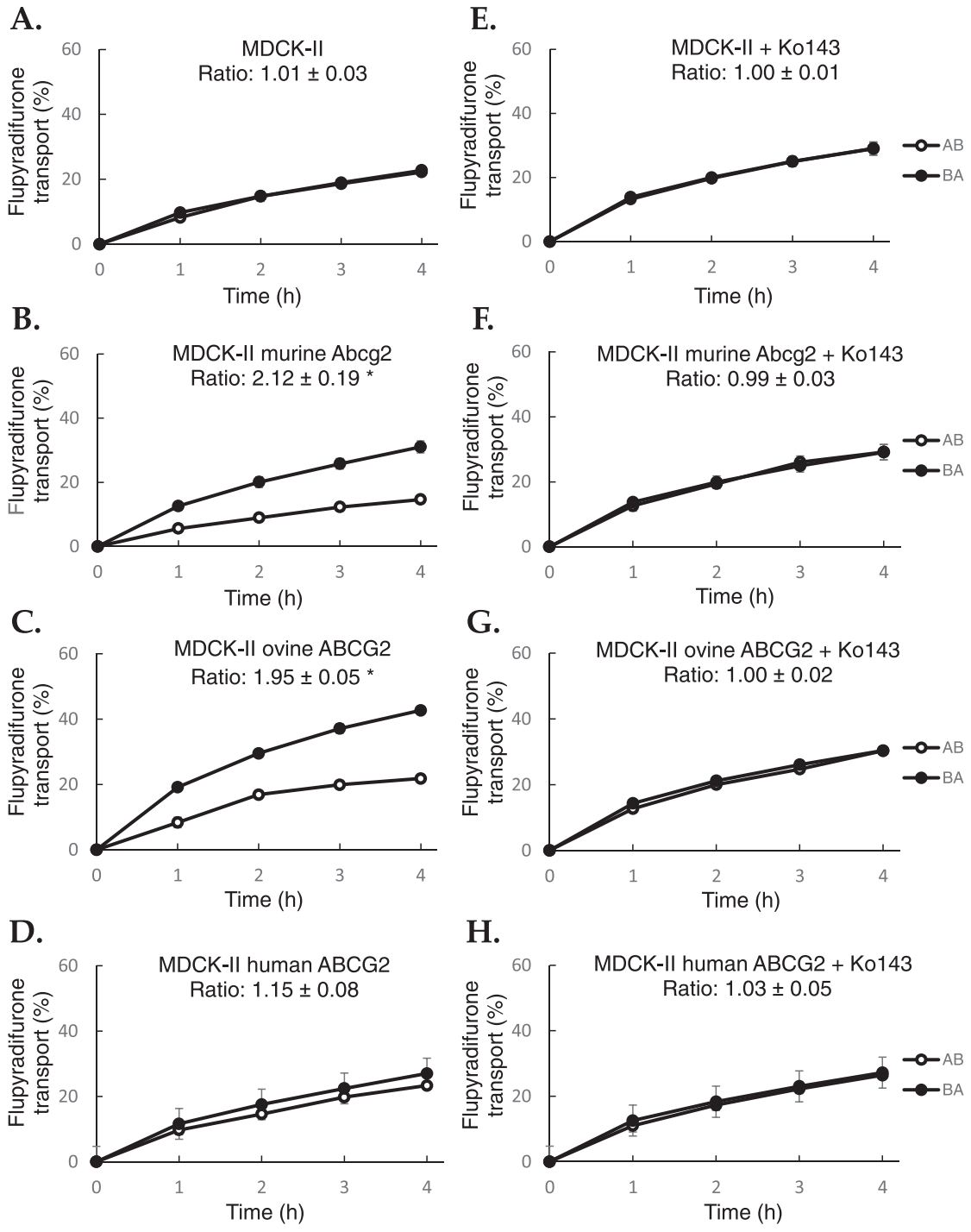

Fig. 3. Transepithelial transport of flupyradifurone (10 μM) with or without 1 μM Ko143 (ABCG2 inhibitor) in parental MDCK-II cells (A and E respectively) and in their murine Abcg2 (B and F respectively), ovine (C and G respectively) and human (D and H, respectively) ABCG2-transduced derivatives. The experiment was started with the addition of flupyradifurone to one compartment (basolateral or apical). After 2, 4 and 6 h, the percentage of pesticide appearing in the opposite compartment was measured by HPLC and plotted. Results are means, and error bars (sometimes smaller than the symbols) indicate SDs (n = 3). (●) translocation from the basolateral to the apical compartment; (O) translocation from the apical to the basolateral compartment. The relative transport ratio (the apically directed translocation divided by the basolaterally directed translocation) is indicated at 6 h. (*) significant differences in transport ratio compared to parental MDCK-II cells ($p \leq 0.05$).

independent sample analysis for non-normal distribution data. Results are presented as mean ± standard deviation (SD). Differences were considered to be statistically significant when P value was ≤ 0.05.

3. Results

3.1. Citotoxicity assays

To determine the appropriate concentration for accumulation and transport assays, the cytotoxicity of bupirimate, ethirimol and

flupyradifurone (1, 5, 10, 25, 50 and 100 μM) was assessed using the MTT assay. As shown in Supplementary Fig. S2, the results showed that the cell viability was above 80% until 10 μM and this maximum concentration could be used for the rest of the experiments.

3.2. Mitoxantrone accumulation assays

To characterize the interactions of bupirimate, ethirimol and flupyradifurone with the different variants of ABCG2, we started testing the ability of these compounds to inhibit ABCG2 mediated-transport by modulating mitoxantrone accumulation in MDCK-II cells and their subclones transduced with murine and human ABCG2 in flow cytometry experiments (Fig. 1). Accumulation of mitoxantrone in ABCG2-transduced cells increased by ABCG2 inhibition with Ko143, a model ABCG2 inhibitor, and consequently increased the fluorescence to similar levels to those in the parental MDCK-II cells. The results obtained in the different ABCG2-transduced subclones showed that there was no observed effect on the accumulation of mitoxantrone (10 μM) by the addition of the tested pesticides, suggesting that they were not probably effective ABCG2 inhibitors at concentrations ranging between 0.5 and 10 μM. Particularly, bupirimate only reached an inhibitory potency of 18.7 ± 3.6% in MDCKII-human ABCG2 at a concentration of 10 μM (Fig. 1).

3.3. Transport assays

Afterwards, to investigate whether ABCG2 is involved in the in vitro transport of bupirimate, ethirimol and flupyradifurone, vectorial transepithelial transport of these compounds (10 μM) across the monolayers was studied. For ethirimol and flupyradifurone, the results obtained for the apically (B-A) and basolaterally (A-B) translocation in the MDCK-II parental cells were similar (Fig. 2A and Fig. 3A). However, the basal to apical (B-A) transport was higher than the apical to basal (A-B) transport in murine Abcg2 and ovine ABCG2-transduced cells (Figs. 2B, C, 3B and C), with relative transport ratios (B-A/A-B) at 6 h significantly higher than in the parental cells. In fact, the relative transport ratios for ethirimol in murine Abcg2 and ovine ABCG2-transduced cells were 2.65 ± 0.37 and 2.11 ± 0.11, respectively, versus 0.95 ± 0.03 in MDCK-II parental cells ($p \leq 0.05$) (Fig. 2A-C); whereas, for flupyradifurone, the efflux ratios were 2.12 ± 0.19 and 1.95 ± 0.05 in murine Abcg2 and ovine ABCG2-transduced cells, respectively, compared with parental cells (1.01 ± 0.03) ($p \leq 0.05$) (Fig. 3A-C). In order to confirm that this effect is specifically caused by ABCG2, the selective ABCG2 inhibitor Ko143 (Allen et al., 2002) was used which resulted in efflux ratios equivalent to those of the MDCK-II parental cells (Fig. 2E-G and Fig. 3E-G). These results show that ethirimol and flupyradifurone are in vitro substrates of murine and ovine ABCG2.

Conversely, when the human ABCG2-transduced cells were used, a low transport ratio similar to that of the parental cells was obtained for both ethirimol and flupyradifurone (Figs. 2D, H, 3D and H), indicating that they are not substrates of human ABCG2.

For bupirimate, in the MDCK-II parental cell line, the translocation in the basolateral and apical directions was similar and no significant vectorial transport was observed for bupirimate in subclones transduced with murine Abcg2 and human ABCG2 (Fig. 4).

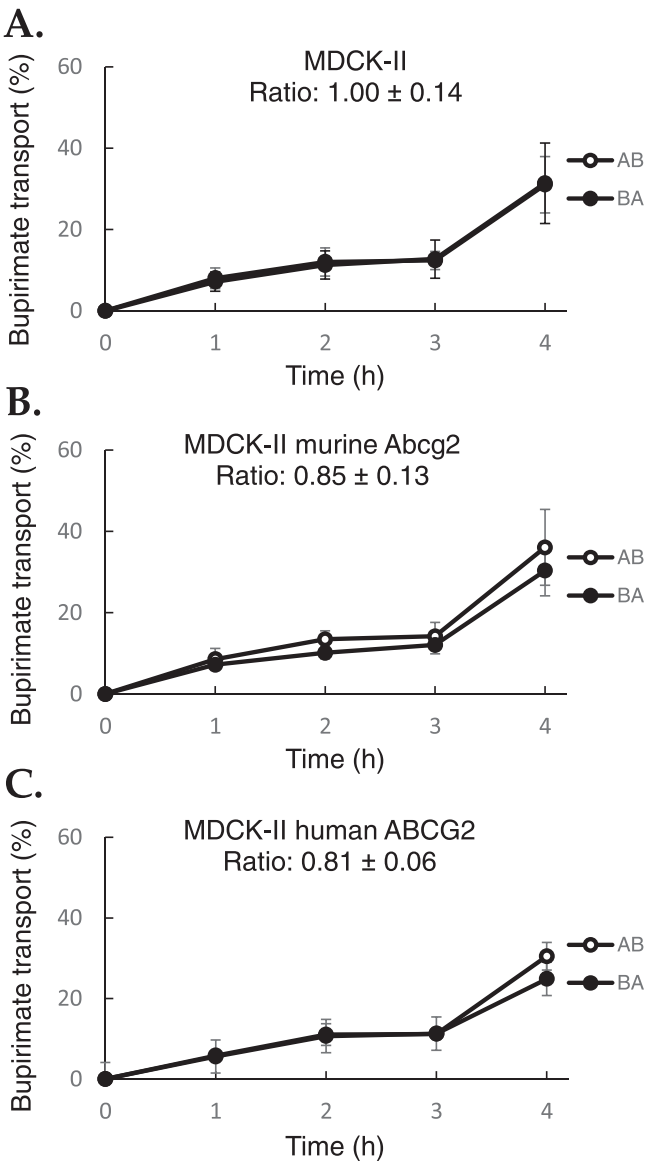

Fig. 4. Transepithelial transport of bupirimate (10 μM) in parental MDCK-II cells (A) and in their murine Abcg2 (B) and human (C) ABCG2-transduced derivatives. The experiment was started with the addition of bupirimate to one compartment (basolateral or apical). After 2, 4 and 6 h, the percentage of pesticide appearing in the opposite compartment was measured by HPLC and plotted. Results are means, and error bars (sometimes smaller than the symbols) indicate SDs (n = 3). (•) translocation from the basolateral to the apical compartment; (O) translocation from the apical to the basolateral compartment. The relative transport ratio (the apically directed translocation divided by the basolaterally directed translocation) is indicated at 6 h.

4. Discussion

In the present study, we investigated the interactions of the pesticides, bupirimate, ethirimol and flupyradifurone with ABCG2. Firstly, no relevant effect of these compounds as inhibitors of this transporter using mitoxantrone as a model substrate was reported (Fig. 1). However, due to the existence of multiple binding sites in this transporter (Yu et al., 2021), a different scenario when using substrates other than mitoxantrone cannot be completely ruled out. Furthermore, potential altered expression of this transporter by these compounds as has been reported for other pesticides (Halwachs et al., 2016; Herriage et al., 2022) deserves further study.

Secondly, ethirimol and flupyradifurone are efficiently transported by murine and ovine ABCG2, although they are not substrates of the human variant (Figs. 2 and 3). These differences in transport between MDCK-II murine Abcg2, ovine and human ABCG2 cell lines could be attributable to differences in the affinity/selectivity for substrates/inhibitors between different subclones and have been previously shown for other tested drugs (Blanco-Paniagua et al., 2021).

The fact that ethirimol and flupyradifurone are both substrates of murine and ovine ABCG2 may have important toxicological consequences. It should be noted that functional modulation (chemicals, other natural substrates) or changes in the expression of ABCG2 caused by

polymorphisms (Hira and Terada, 2018) might contribute to variations of the bioavailability (and therefore toxicology) of ABCG2 substrates by altering their intestinal absorption and their hepatobiliary and renal excretion, since ABCG2 is expressed in the enterocytes and biliary canalicular membrane of hepatocytes and in the luminal epithelium of kidney (van Herwaarden et al., 2003; Horsey et al., 2016). These potential in vivo effects are more probable in wild and domestic animals exposed to these compounds, especially, flupyradifurone, which is a food-borne pesticide (Haviland et al., 2019). This is specifically addressed by the positive results of these pesticides as substrates of murine and ovine ABCG2. Resulting from the lack of positive results in human ABCG2-transduced cells, such effects in humans are less probable.

Furthermore, pesticide secretion into milk can also be affected by modifications in the ABCG2 expression and/or functionality. Thus, intake of food contaminated with pesticides may result in ABCG2-mediated secretion of potential harmful compounds into milk leading to negative consequences for the health of suckling and dairy consumers. Notwithstanding, all these potential in vivo consequences remain to be elucidated.

In this study, we identify both ethirimol and flupyradifurone as in vitro murine Abcg2 and ovine ABCG2 substrates. These new findings will contribute to the understanding of the influence of this transporter in the pharmacokinetics of these pesticides and/or their secretion into milk, potentially altering their toxicity in mammals.

CRediT authorship contribution statement

Nada Ben Halima: Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Laura Álvarez-Fernández:** Investigation, Formal analysis, Visualization, Writing – review & editing. **Esther Blanco-Paniagua:** Formal analysis, Validation. **Salwa Abid-Essefi:** Conceptualization, Supervision. **Yosra Guedri:** Conceptualization, Supervision. **Gracia Merino:** 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.

Data Availability

Data will be made available upon request.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxlet.2023.03.012.

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