Short communication

Ivermectin inhibits ovine ABCG2-mediated in vitro transport of meloxicam and reduces its secretion into milk in sheep

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Abstract

The ATP-binding cassette transporter G2 (ABCG2) is an efflux protein involved in the bioavailability and secretion into milk of several compounds including anti-inflammatory drugs. The aim of this work was to determine the effect in sheep of an ABCG2 inhibitor, such as the macrocyclic lactone ivermectin, on the secretion into milk of meloxicam, a non-steroidal anti-inflammatory drug widely used in veterinary medicine, and recently reported as an ABCG2 substrate. In vitro meloxicam transport assays in ovine ABCG2-transduced cells have shown that ivermectin is an efficient inhibitor of in vitro transport of meloxicam mediated by ovine ABCG2, with a 75% inhibition in the transport ratio (24.85 ± 4.62 in controls vs 6.31 ± 1.37 in presence of ivermectin). In addition, the role of ovine ABCG2 in secretion into milk of meloxicam was corroborated using Assaf lactating sheep coadministered with ivermectin. Animals were administered subcutaneously with meloxicam (0.5 mg/kg) with or without ivermectin (0.2 mg/kg). No difference in plasma pharmacokinetic parameters was found between treatments. In the case of milk, a significant reduction in the area under concentration-time curve (AUC) (3.92 ± 0.66 vs 2.26 ± 1.52 vs µg·h/mL) and the AUC milk-to-plasma ratio (0.17 ± 0.03 vs 0.09 ± 0.06) was reported for ivermectin-treated animals compared to controls.

Keywords: ABCG2; Ivermectin; Meloxicam; Milk; Sheep.

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for their analgesic, anti-inflammatory and antipyretic properties in human and veterinary medicine (Lees et al., 2004). Meloxicam is an NSAID with high therapeutic potential in ruminants for pain (Colditz et al., 2019), including lactation-related pathologies such as mastitis (McDougall et al., 2009; Fitzpatrick et al., 2013), which implies economic benefits for farmers (van Soest et al., 2018). However, its use in lactating cattle is reduced due to its high withdrawal period in milk (5 days) with a maximum residue limit of 15 μg/kg (European Medicines Agency, 2019).

The ATP-binding cassette transporter G2 (ABCG2) is one of the main factors involved in the active secretion of many compounds into milk, including veterinary drugs (Mealey, 2012; Mahnke et al., 2016; Garcia-Lino et al., 2019; Imperiale and Lanusse, 2021; Blanco-Paniagua et al., 2022) and also specifically anti-inflammatory drugs (García-Mateos et al., 2019), due to its induced expression during lactation in the mammary gland. Moreover, ABCG2 is expressed at the apical cellular surface in several tissues important for xenobiotic disposition and in association with a protective role (Yu et al., 2021). However, in the veterinary field, interest is focused on gaining information about potential mechanisms that may affect the appearance of drug residues in milk, including drug-drug interactions that lead to the inhibition of ABCG2 resulting in variation in drug secretion into milk (Real et al., 2011; Barrera et al., 2013).

Recently, ABCG2 has been identified as an important determinant of the secretion into milk of meloxicam using Abcg2-knockout mice (Garcia-Lino et al., 2020). However, whether this finding can be extrapolated to the secretion into milk of meloxicam in ruminants is unknown. In this study, therefore, the effect of a known ABCG2 inhibitor, such as the macrocyclic lactone ivermectin (Merino et al., 2009), on the secretion of meloxicam into milk was studied in sheep.

Beforehand, the role of ivermectin (10 mM) as an inhibitor of in vitro ovine ABCG2-mediated transport of meloxicam was assessed in vectorial transport assays using Transwell plates with MDCKII cells transduced with ovine variant of ABCG2, as previously described (González-Lobato et al., 2014). Madin-Darby Canine Kidney (MDCKII) cells transduced with ovine variant of ABCG2 were seeded on microporous polycarbonate membrane filters. To check the tightness of the monolayer, transepithelial resistance was measured in each well using a Millicell ERS ohmmeter (Millipore). The presence of meloxicam (Sigma-Aldrich) in the acceptor compartment was presented as the percentage of total meloxicam added to the donor compartment at the beginning of the experiment. Active transport across MDCKII monolayers mediated by the apically expressed efflux ABCG2 transporter was evaluated by the relative transport ratio, defined as the apically directed transport percentage divided by the basolaterally directed translocation percentage, after 4 h. Typically, ABCG2 substrates shows relative transport ratios higher in ABCG2-transduced cells compared to parental cells (without ABCG2). Samples were analyzed for meloxicam by HPLC-UV as described previously (Garcia-Lino et al., 2020). Standard samples in appropriate drug-free matrix were prepared at concentrations of 0.039–10 µg/mL for culture samples with correlation coefficients > 0.99. Precision coefficients of variation were < 10%, and relative standard deviations (accuracy) values were < 20%. The limit of quantification (LOQ) was 0.01 µg/mL. The Shapiro-Wilk normality test was used to test the normal distribution of the data. Statistical analysis for significant differences between the groups was then performed by either the Student's t-test or the Mann-Whitney U test. All analyses were carried out on the assumed significance level of $p \leq 0.05$ using SPSS Statistics software (v. 24.0; IBM, Armonk, New York, NY, USA).

Table 1 shows the results obtained in the meloxicam transport assay (30 μM in DMSO) in absence or presence of ivermectin at 10 μM (chosen concentration based on Gonzalez Lobato et al., 2014) as ABCG2 inhibitor using ovine ABCG2-transduced cells. When ivermectin at 10 μM was added, a reduction of 75% in the relative transport ratio of meloxicam was reported (24.85 ± 4.62 in absence of ivermectin vs 6.31 ± 1.37 in presence of ivermectin, $p \leq 0.05$). We showed recently that meloxicam is an in vitro substrate for ovine ABCG2 with a relative transport ratio higher in the ovine ABCG2-transduced cells compared with the MDCKII parental cell line (Garcia-Lino et al., 2021). We now report that this high relative transport ratio in the ovine ABCG2-transduced cells is inhibited by the addition of ivermectin, showing that ivermectin inhibits meloxicam transport mediated by ovine ABCG2, as shown previously for other substrates (Merino et al., 2009; Real et al., 2011).

Therefore, to check for possible in vivo interactions, studies with sheep were conducted according to institutional guidelines complying with European legislation (2010/63/EU), and approved by the Animal Care and Use Committee of the University of León ULE_008_2016 (09/06/2016). Eleven lactating Assaf sheep (3–4 months in lactation) weighing 70 to 85 kg were stratified according to milk production and number of days' post-partum, and then randomly distributed in 2 experimental groups. They received a subcutaneous injection of 0.5 mg/kg of meloxicam (Metacan®, 20 mg/mL, Boehringer, Germany) alone or together with another subcutaneous injection of ivermectin (Ivomec®, Merial, France) (0.2 mg/kg) in the contralateral side. The animals were parasite-free and drinking water was available ad libitum. Blood samples were collected from the jugular vein and milk samples were collected after completing milking of the gland before each treatment at 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, 48 and 72 h after meloxicam administration. Individual plasma and milk samples were analyzed by HPLC-

116 UV analysis as described previously (Garcia-Lino et al., 2020). Standard samples in
117 appropriate drug-free matrix were prepared at concentrations of 0.019-2.5 μg/mL for
118 plasma and of 0.019-1.25 μg/mL for milk with correlation coefficients > 0.99. Precision
119 coefficients of variation were < 15%, and relative standard deviations (accuracy) values
120 were < 20%. The extraction recovery levels for concentration in the standard curve were
121 88% for plasma and 90 % for milk samples. The LOQs were 0.017 μg/mL for plasma and
122 0.015 μg/mL for milk. Pharmacokinetic parameters were determined as reported
123 elsewhere (Otero et al., 2018) with a computer program (PK solution 2.0, Summit
124 Research Services, Ashland, OH).

125 No significant differences in plasma levels of meloxicam were found between
126 both groups of animals (Fig. 1A), which is reflected in the absence of significant
127 differences in the plasma pharmacokinetic parameters (Table 2). This lack of differences
128 in plasma concentrations has been reported before for other ABCG2 substrates such as
129 danofloxacin in presence of ivermectin (Real et al., 2011). However, a significantly lower
130 milk concentration of meloxicam was found in the animals coadministered with
131 ivermectin at 12 h (0.137 ± 0.040 μg/mL in controls vs 0.069 ± 0.057 μg/mL in
132 ivermectin-coadministered animals) (Fig. 1B). The values of the area under
133 concentration-time curve ($AUC_{(0-t)}$) for milk and the AUC milk-to-plasma ratio were
134 significantly reduced by more than 40% in ivermectin-coadministered animals compared
135 with control animals (Table 2). Although ivermectin interacts with other ABC
136 transporters, such as P-glycoprotein (Lespine et al., 2009), the effect of ivermectin on
137 meloxicam secretion into sheep milk can be attributed to ABCG2-mediated interaction
138 since no other ABC transporters are substantially expressed or induced in lactating
139 mammary gland (Van Herwaarden and Schinkel, 2006). This kind of drug-drug
140 interaction mediated by the ABCG2 transporter has been observed previously with the

co-administration of ivermectin and other ABCG2 substrates, such as the antimicrobial danofloxacin, in sheep (Real et al., 2011). Although it cannot be excluded that other excretory or metabolic pathways of meloxicam may be affected by ivermectin, such as intestinal elimination, the lack of changes in plasma levels makes this hypothesis highly unlikely.

The present data show that secretion into milk of meloxicam can be modulated by ivermectin, producing drug-drug interaction, but also probably by other compounds that interact with the ABCG2 transporter, as other drugs or molecules present in the diet such as flavonoids (Pulido et al., 2006; Otero et al., 2018), with consequences regarding the amount of milk residues.

In conclusion, the major role of ABCG2 in the secretion of meloxicam into ovine milk and the effect of drug-drug interactions in this process using the macrocyclic lactone ivermectin as inhibitor of the transporter are demonstrated. These results will contribute to the understanding of the factors that influence the transfer of anti-inflammatory drugs into ruminant milk.

Declaration of interest: none

Acknowledgements

This work was supported by the research projects AGL2015-65626-R (MCIN/AEI/10.13039/501100011033/FEDER" Una manera de hacer Europa") and RTI2018-100903-B-I00 (MCIN/AEI/10.13039/501100011033/FEDER" Una manera de hacer Europa"); and by the predoctoral grants (FPU18/01559 grant to EBP, FPU19/04169

grant to LAF) from the Spanish Ministry of Education, Culture and Sport and BES-2016-077235 grant to AMGL (MCIN/AEI/10.13039/501100011033 y FSE "El FSE invierte en tu futuro"). We are grateful to Prof. James McCue for assistance with language editing.

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258 **Figure legends**

259 Figure 1. Concentration in plasma (A) and milk (B) vs. time curves for meloxicam
260 obtained from lactating Assaf sheep treated with a single dose of meloxicam (Metacam®)
261 at 0.5 mg/kg (s.c.) and co-administered with ivermectin (Ivomec®) at 0.2 mg/kg (s.c.).
262 The insets show semilogs plot of the data. Each point represents a mean; bars indicate the
263 standard deviation (n=5-6). (*) $p \leq 0.05$ significant differences (Student's t-test).

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Figure 1

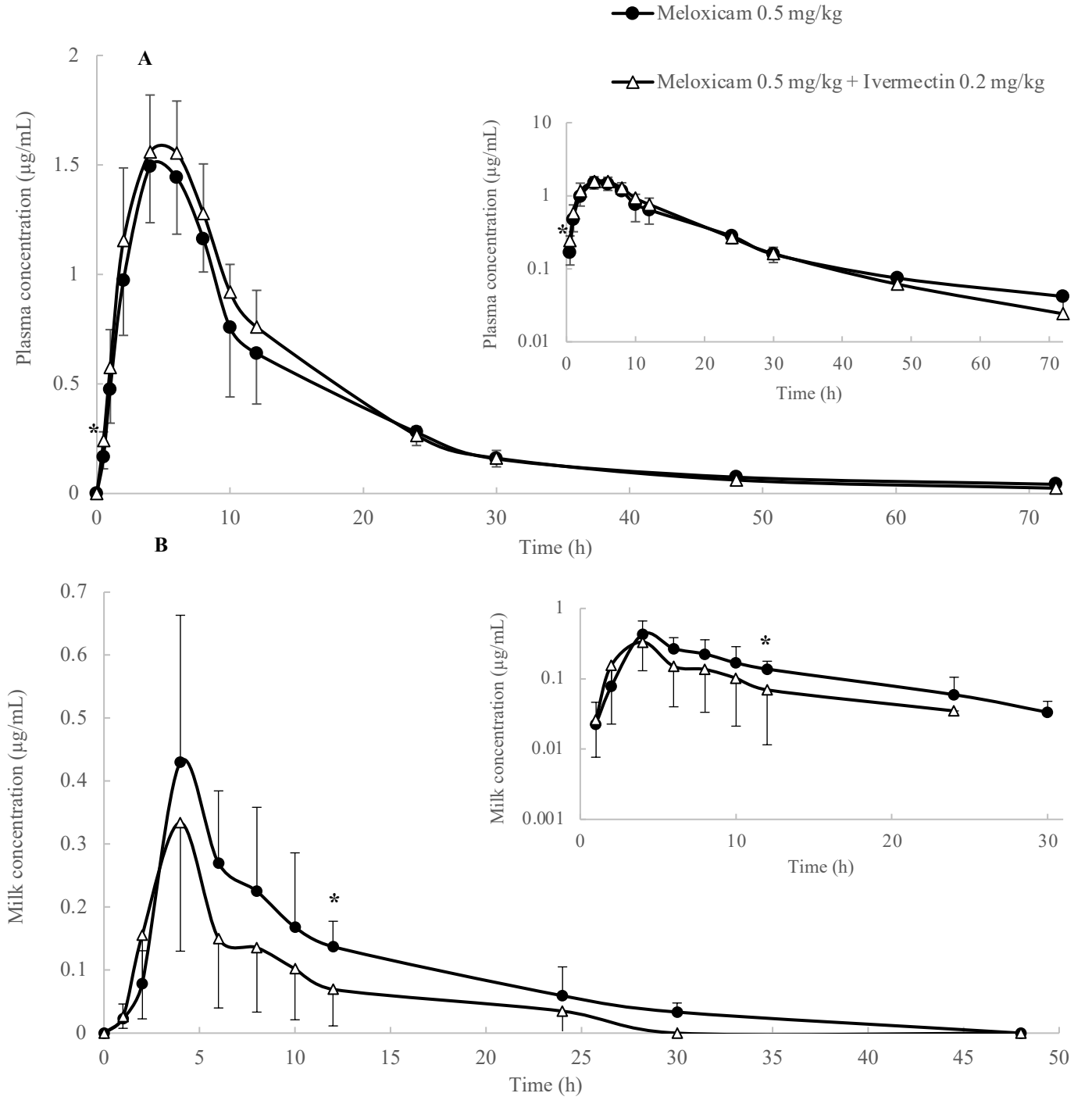

Table 1. Transport of meloxicam (30 µM) towards apical (BL-AP transport) or basal (AP-BL transport) compartments in MDCKII parental cells and the ovine-ABCG2 transduced cells in presence of ivermectin after 4 h (n= 3-7).

	Treatment	BL-AP (% transport)	AP-BL (% transport)	Relative transport ratio BL-AP/AP-BL	Transport inhibition
MDCKII ovine ABCG2	Control	62.87 ± 4.72	2.77 ± 0.75	24.85 ± 4.62	-
	+ Ivermectin	63.52 ± 3.38	10.36 ± 1.85 [a]	6.31 ± 1.37 [a]	75 %

Results are means ± SDs.
[a] $p \leq 0.05$, significant differences from MDCKII ovine ABCG2 cells without ivermectin (Student's t-test)

Table 2. Mean (±SD) pharmacokinetic parameters of meloxicam in plasma and milk after subcutaneous administration at a dosage of 0.5 mg/kg in sheep co-administered with ivermectin (0.2 mg/kg s.c.) (n=5-6).

		Meloxicam 0.5 mg/kg	Meloxicam 0.5 mg/kg + Ivermectin 0.2 mg/kg
Plasma	$AUC_{(0-t)}$ (µg·h/mL)	23.77 ± 3.94	23.42 ± 2.83
	C_{max} (µg/mL)	1.53 ± 0.29	1.68 ± 0.15
	T_{max} (h)	4.33 ± 0.82	4.00 ± 0.00
	$T_{1/2}$ (h)	8.93 ± 1.38	8.90 ± 0.42
	MRT (h)	16.85 ± 0.85	14.60 ± 2.09
Milk	$AUC_{(0-t)}$ (µg·h/mL)	3.92 ± 0.66	2.26 ± 1.52*
	C_{max} (µg/mL)	0.48 ± 0.23	0.30 ± 0.21
	T_{max} (h)	4.33 ± 0.82	3.60 ± 1.67
	$T_{1/2}$ (h)	8.50 ± 3.35	6.45 ± 2.77
	MRT (h)	13.55 ± 4.60	11.28 ± 4.73
Milk/plasma	AUC	0.17 ± 0.03	0.09 ± 0.06*

* $p \leq 0.05$, significant differences from meloxicam 0.5 mg/kg (Student's t-test)