ABSTRACT:
ATP-binding cassette transporter ABCG2 [breast cancer resistance protein (BCRP)] is a member of the ABC transporter superfamily that actively extrudes xenotoxins from cells and is a major determinant of the bioavailability of many compounds. ABCG2 expression is strongly induced during lactation in the mammary gland and is related to the active secretion of drugs into the milk. The presence of drug residues and environmental pollutants in milk is an outstanding problem for human milk consumption and milk industrial processes, involving important risks to public health and the dairy industry. In cows, a single nucleotide polymorphism (SNP) in this protein has been described previously (Tyr581) and is associated with higher fat and protein percentages and lower milk yield. However, whether this amino acid substitution affects ABCG2-mediated drug transport in cows, including milk secretion, required further exploration. We cloned the two variants of bovine ABCG2 and evaluated the effect of this SNP on mitoxantrone accumulation assays performed in ovine primary fibroblasts transiently expressing either of the variants. It is interesting to note that statistically significant differences in activity between both variants were observed, and the Ser581 variant was related with an increased efflux activity. In addition, we demonstrated that genistein is a very good inhibitor of bovine ABCG2 and identified new inhibitors of the transporter, such as the macrocyclic lactones, ivermectin, and selamectin. Moreover, the inhibitory effect of these compounds on human and murine ABCG2 homologs was confirmed using transduced Marbin-Dabin canine kidney II cells. These findings may have important implications regarding the presence of drug residues in milk and drug interactions affecting the pharmacological behavior of ABCG2 substrates.

Efflux transporters of the ATP-binding cassette (ABC) family play major physiological and pharmacological roles in mammals. These transmembrane proteins actively extrude a variety of drugs, carcinogens, and other xenotoxins across the cellular plasma membrane. This way, ABC transporters restrict the systemic exposure of many compounds by reducing their net intestinal uptake and by mediating their active excretion (van Herwaarden and Schinkel, 2006). One member of this family, ABCG2/breast cancer resistance protein (BCRP), has been detected in alveolar epithelial cells in the mammary gland (Jonker et al., 2005; Wu et al., 2008), where it is strongly expressed during lactation. It has been demonstrated that ABCG2 is the major factor involved in the active secretion of xenotoxins into milk (Jonker et al., 2005), although some physiological nutrients (such as riboflavin and vitamin K3) have also been identified as ABCG2 substrates (van Herwaarden et al., 2007; Shukla et al., 2007). However, in apparent contradiction with the protective role of this transporter in reducing drug bioavailability, the contamination of milk exposes suckling individuals as well as dairy consumers to xenotoxins.

The presence of drug residues and environmental pollutants in milk is an outstanding problem for human milk consumption and milk industrial processes, involving important risks to public health and the dairy industry. In addition, the health of the suckling calves could be impaired. Because ABCG2 transporter activity is one of the main determinants of xenotoxin presence in milk, polymorphisms in this protein become of great relevance and may contribute to differences in drug bioavailability and presence of drug residues in milk.

Human single nucleotide polymorphisms (SNPs) of ABCG2 have been extensively studied because of their relevance in drug pharmacokinetics. For instance, the significance of the Q141K SNP has been shown in vitro (Mizuurai et al., 2004) and in clinical studies (Sparrboom et al., 2004; Yamasaki et al., 2008). With regard to domestic animals, a single nucleotide change (A/C) in exon 14 of the bovine ABCG2 gene has been reported, encoding a substitution of tyrosine-581 to serine (Cohen-Zinder et al., 2005). This polymorphism has been proposed as a quantitative trait nucleotide on bovine chromosome 6 (Cohen-Zinder et al., 2005; Olsen et al., 2007), with the Tyr581 variant being associated with higher fat and protein percentages and lower milk yield. However, whether this SNP influences ABCG2-mediated drug transport in cattle and the presence of drug residues in milk remained to be addressed.

To further explore this matter, we cloned the two variants of the
full-length bovine ABCG2 cDNA and analyzed their functional activity in drug accumulation assays performed in ovine primary fibroblasts transiently expressing either of the variants. We also used this model to study the interactions of both variants with the macrocyclic lactones ivermectin and selamectin and the flavonoid genistein. Genistein is an isoflavone included in soy-based food and present in forage of ruminants. It has been shown to be a good inhibitor of the human transporter (Imai et al., 2004). Ivermectin and selamectin are potent anthelmintic drugs that interact with other ABC transporters like P-glycoprotein (Griffin et al., 2005; Brayden and Griffin, 2008). In this article, both antiparasitic drugs were identified as new inhibitors of the bovine, mouse, and human ABCG2.

**Materials and Methods**

**Drugs and Chemicals.** Mitoxantrone (MXR), ivermectin, selamectin, and genistein were purchased from Sigma-Aldrich (St. Louis, MO). Ko143 was purchased from Sigma-Aldrich (St. Louis, MO). DNA polymerase (Invitrogen). Primers were designed according to the published sequence (GenBank accession no. AJ871176; 5′-GAAAGGCG- GAATATGCTAAAAATTAAAGAGGA-ATTTAAG-3′ and 3′-GACCAAATTAAAGAGGA- ATTTAAG-3′. The 2-kb polymerase chain reaction (PCR) product was cloned using the TOPO TA Cloning kit (Invitrogen). Different clones were obtained, some of which contained the Tyr581 ABCG2 allele, and others contained the Ser581 ABCG2 allele. Each allele was subcloned in pEF1α-IRES-GFP vector (S. Nicolas, M. F. Baro, and M. M. Marques, unpublished data).

**Construction of Expression Plasmids.** Total RNA was isolated from bovine liver using TRI Reagent (Ambion, Carlsbad, CA), and then ABCG2 cDNA was amplified with Platinum Pfx DNA polymerase (Invitrogen). Primers were designed according to the published bovine sequence (GenBank accession no. AJ871176; 5′-GAAAGGCG-GAAATGTCTAAAATGTCTCCC-3′ and 3′-GACCAAATTAAAGAGGA-ATTTAAG-3′. The 2-kb polymerase chain reaction (PCR) product was cloned using the TOPO TA Cloning kit (Invitrogen). Different clones were obtained, some of which contained the Tyr581 ABCG2 allele, and others contained the Ser581 ABCG2 allele. Each allele was subcloned in pEF1α-IRES-GFP vector (S. Nicolas, M. F. Baro, and M. M. Marques, unpublished data).

**Accumulation Assays.** In vitro accumulation assays were carried out as described previously (Pavek et al., 2005). MXR (5 μM) was used as the fluorescent substrate, and tested compounds were used as inhibitors. In brief, subconfluent cultures were used after 24 (SBFF1 cells) or 36 h (MDCKII cells).
from seeding. Medium was aspirated, and cells were incubated in prewarmed Optimem medium with or without Ko143 inhibitor (1 μM) or tested compound for 60 min before the addition of MXR. Accumulation of MXR was allowed for 1 h at 37°C. Then, cells were washed with ice-cold PBS and trypsinized. Cells were collected and resuspended in PBS with 2.5% fetal calf serum. Relative cellular accumulation of MXR was determined by flow cytometry using a FACSCalibur cytometer (BD Biosciences, San Jose, CA). Excitation and emission wavelengths for MXR were 488 and 650 nm, respectively. MXR fluorescence of at least 5000 GFP-gated positive cells was quantified from histogram plots using the median of fluorescence (MF). At least three independent experiments were performed. Flow cytometry data were processed and analyzed using WinMDI version 2.8 software (The Scripps Research Institute, La Jolla, CA). For kinetic experiments with different MXR concentrations, an indirect estimation of MXR relative efflux was calculated by subtracting the MF of ABCG2-expressing cells from the MF of vector control cells. The resulting data were used for semiquantitative analysis of relative kinetic constants by the fitting software SIMFIT (University of Manchester, Manchester, UK) (Bardsley, 2001), assuming Michaelis-Menten kinetics. Inhibitory potencies of compounds were calculated as previously described (Pavek et al., 2005) in MDCKII-ABCG2 or MDCKII-Abcg2 cells according to the following equation: Inhibitory potency = (MF with tested compound-MF without inhibitor)/(MF with Ko143-MF without inhibitor) × 100%.

Statistical Analysis. Statistical analysis for significant differences was performed using the two-tailed Student’s t test. A probability of <0.05 was considered to be statistically significant.

Results and Discussion

This manuscript presents the cloning and functional analysis of the two alleles described for the bovine ABCG2 gene. Both variants were successfully amplified from a heterozygous animal and subcloned in the bicistronic expression vector pEF1α-IRES-GFP. The A→C mutation was confirmed by sequence analysis as shown in Fig. 1A. Both alleles were transiently expressed in SBFF1 ovine fetal fibroblasts, which have undetectable background transporter expression (Fig. 1, B–D). This cell type was chosen to mimic the environment of a ruminant cell. No obvious differences were observed in the amount of protein between cells transfected with either variant. To evaluate drug transport activity of both variants, accumulation analyses were performed with a typical ABCG2 substrate as mitoxantrone (5 μM). GFP expression was monitored by fluorescence microscopy, and, according to that, cells were used 24 h post-transfection for flow cytometry assays. Transfection efficiency (ranging from 24.1 to 30.6%) and GFP fluorescence means were equivalent for both variants in each experiment. The GFP-positive cell population was chosen for the subsequent determinations of MXR fluorescence. As shown in Fig. 1E, MXR accumulation was 65 to 80% lower in ABCG2-expressing cells than in the vector control, demonstrating that bovine ABCG2 is a highly efficient transporter of MXR like its murine and human analogs. The accumulation effect was largely reversed by the potent and specific ABCG2 inhibitor Ko143, as shown by the increase in MXR levels in transfected cells. In addition, significant differences in activity between both variants were observed, with the Ser581 allele showing a 5-fold reduced MXR accumulation compared with the vector-transfected cells (14.48 ± 2.41 versus 72.41 ± 23.93 units of fluorescence), whereas a 3-fold reduction was observed for the Tyr581 ABCG2 allele (24.52 ± 0.90 versus 72.41 ± 23.93).

To further compare the transport kinetics of both variants, MXR accumulation assays using a range of substrate concentrations (1–500 μM) were conducted (Fig. 2). An indirect estimation of MXR relative efflux was calculated by subtracting the MF of BCRP-expressing cells from the MF of vector control cells, and the resulting data were used for semiquantitative analysis of relative kinetic constants. Those experiments confirmed the significant differences in activity of both variants for all of the tested concentrations. Semi-quantitative analysis showed a 1.4 fold difference (p < 0.01) for relative V_{max} values (2.43 ± 0.042 units of fluorescence/min for Ser581 versus 1.74 ± 0.042 units of fluorescence/min for Tyr581). However, estimation of K_M values showed no significant differences. These data suggest that the studied SNP could have an effect on the capacity of the ABCG2 bovine transporter, with the Ser581 variant being related with an increased efflux activity. In line with previous studies using human ABCG2-expressing cells, these differences from drug accumulation studies could result in larger changes in drug resistance profiles from transport studies (Mizuarai et al., 2004). On the other hand, due to the existence of multiple binding sites in this transporter (Clark et al., 2006), a different scenario when using substrates other than MXR can not be completely ruled out. This is the first time that a functional difference has been correlated with an ABC transporter polymorphism in ruminant species. However, reports of increased activity associated to human ABCG2 SNPs exist. For example, the I206L variant exhibits 2 to 3 times higher efflux activities and drug resistance capabilities than that of wild-type protein (Vethanayagam et al., 2005).

The presence of this polymorphism in bovine populations may strongly modify the pharmacokinetic properties of different drugs, including their active secretion to milk. Furthermore, it could imply important economic and health consequences regarding modifications in withdrawal time of the drug milk residues depending on the individual genotypes. Some of the ABCG2 substrates identified so far are anthelmintic drugs and antibiotics with established withdrawal periods in milk (Merino et al., 2005, 2006). In this context, we addressed whether our model could be useful to analyze the interaction of bovine ABCG2 with some macrocyclic lactones and the flavonoid genistein, by measuring their ability to reverse the reduced MXR accumulation in SBFF1 cells expressing either variant (Fig. 3A). A concentration of 30 μM was selected for the tested compounds because higher concentrations were cytotoxic. No inhibitory effect of the tested compounds was observed on the vector-transfected cells. It is remarkable that genistein showed a very...
strong inhibitory potency, with values close to 100%. Both ivermectin and selamectin had a more moderate effect at the tested concentration, with inhibitory potencies close to 50%. It is interesting to note that selamectin at the tested concentration was not as remarkable on the murine transporter, with inhibitory potencies of 36.75 ± 5.82 and 29.37 ± 0.79%, respectively. These data indicate that both macrocyclic lactones are not only inhibitors of bovine ABCG2 but also of murine and human transporters.

This study shows for the first time a functional difference linked to an ABC transporter polymorphism in ruminant species. In addition, it illustrates the usefulness of generating in vitro cell models to address drug interactions mediated by ABCG2, such as the ones described here for macrocyclic lactones and flavonoids. Further studies should resolve other issues such as whether differences in transport activity are related to fat and protein percentages in milk.

Acknowledgments. We thank Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands) for critical reading of the manuscript and for kindly supplying Ko143 and the MDCKII cells and its transduced subclones. We also thank Dr. Alvaro de la Fuente for technical help and assistance in experimental design.

Instituto de Desarrollo Ganadero y Sanidad Animal (G.M., M.F.B., M.M.M.), Area de Fisiología, Departamento de Ciencias Biomédicas (R.R., L.G.-L., J.G.P., A.I.A.), Universidad de León, Campus de Vegazana, León


References


