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INTERPRETATIVE SUMMARY

Effect of bovine ABCG2 Y581S polymorphism on concentrations in milk of enrofloxacin and its active metabolite ciprofloxacin by Otero et al. ABCG2 is a protein that contributes to the secretion of drugs into milk. This study demonstrates that the bovine ABCG2 Y581S genetic change modifies the transport of the fluoroquinolone antimicrobial enrofloxacin and its main active metabolite ciprofloxacin. Increased in vitro transport using cells over-expressing both variants of bovine ABCG2 indicates more efficient transport by the S581 variant. Lactating dairy cows with the Y581S polymorphism show significantly increased amounts of both enrofloxacin and ciprofloxacin in milk after subcutaneous administration of 2.5 mg/kg of enrofloxacin.

Y581S INCREASES AMOUNTS OF QUINOLONES IN MILK

Effect of bovine ABCG2 Y581S polymorphism on concentrations in milk of enrofloxacin and its active metabolite ciprofloxacin

J. A. Otero,*† D. García-Mateos,* A. de la Fuente*, J. G. Prieto,*‡ A. I. Álvarez,* and G. Merino*†[1]

*Department of Biomedical Sciences-Physiology, Veterinary Faculty, and

†Instituto de Desarrollo Ganadero y Sanidad Animal (INDEGSAL), and

‡Instituto de Biomedicina (IBIOMED), University of Leon, Campus de Vegazana 24071, Leon, Spain

[1]Corresponding author: Dr. Gracia Merino, Department of Biomedical Sciences-Physiology, Veterinary Faculty, University of Leon, Campus de Vegazana 24071, Leon, Spain. Phone.: +34-987291263; Fax +34-987291267; E-mail: gmerp@unileon.es

Abstract

The ATP-binding cassette transporter G2 (ABCG2) is involved in the secretion of several drugs into milk. The bovine Y581S ABCG2 polymorphism increases the secretion into milk of the fluoroquinolone danofloxacin in Holstein cows. Danofloxacin and enrofloxacin are the fluoroquinolones most widely used in veterinary medicine. Both enrofloxacin (ENRO) and its active metabolite ciprofloxacin (CIPRO) reach milk at relatively high concentrations. The aim of this work was to study the effect of the bovine Y581S ABCG2 polymorphism on in vitro transport as well as on concentrations in plasma and in milk of ENRO and CIPRO. Experiments using cells over-expressing bovine ABCG2 showed the effects of ABCG2 on the transport of CIPRO, demonstrating more efficient in vitro transport of this antimicrobial by the S581 variant as compared with the Y581 variant. Animal studies administering 2.5 mg/kg of ENRO subcutaneously to Y/Y 581 and Y/S 581 cows revealed that concentrations in plasma of ENRO and CIPRO were significantly lower in Y/S animals. Regardless of the genotype, the antimicrobial profile in milk after the administration of ENRO was predominantly of CIPRO. In respect of the genotype effects on the amounts of drugs present in milk, $AUC_{0\text{-}24}$ values were more than 1.2 times higher in Y/S cows for ENRO and 2.2 times for CIPRO, this indicating a greater capacity of Y581S to transfer these drugs into milk. These results emphasize the clinical relevance of this polymorphism as a factor affecting the concentrations in plasma and in milk of drugs of importance in veterinary medicine.

Keywords: Bovine ABCG2, polymorphism, enrofloxacin, ciprofloxacin.

INTRODUCTION

Enrofloxacin (ENRO) and its active metabolite ciprofloxacin (CIPRO) are broad spectrum antimicrobials belonging to the group of fluoroquinolones. Enrofloxacin was developed for veterinary use and is indicated for the treatment of many diseases in cattle, including respiratory and alimentary tract diseases of bacterial or mycoplasmal origin and mastitis (Lopez-Cadenas et al., 2013). It is widely distributed throughout the organism, with excellent tissue penetration and a long serum half-life. Both drugs, ENRO and CIPRO, reach high concentrations in milk (Kaartinen et al., 1995). Antimicrobial treatment of dairy cows creates residues in milk, and the avoidance of such residues is an important aspect of mastitis treatment (Pyörälä, 2009). At the present time, residues in milk of drugs administered to livestock pose growing concern, because of financial losses and the potential selection of antibiotic-resistant bacteria if dairy products that contain residues reach consumers (Virolainen and Karp, 2014). Although adherence to instructions on labelling relative to withdrawal periods is the principal step in controlling drug residues in milk, a knowledge of the factors involved in the secretion of drugs into milk may also be of assistance in overcoming this difficulty.

Several fluoroquinolones such as CIPRO or danofloxacin have been reported to be secreted into milk by the ATP-binding cassette transporter G2 (**ABCG2**) in experiments using Abcg2 knockout mice, this contributing to the high concentrations of these antimicrobials in milk (Merino et al., 2006; Real et al., 2011a). The superfamily of ABC-transporters consists of a group of proteins that usually play a protective role in cells and tissues against toxic compounds and limit organism exposure to potentially harmful molecules (Petzinger and Geyer, 2006). Several in vivo studies have indicated that ABCG2 mediates intestinal, hepatobiliary and renal excretion of its substrates (reviewed by Jani et al., (2014)) affecting drug efficacy, drug–drug interactions and

adverse effects from drugs (Ballent et al., 2012; Mealey, 2013). Jonker et al., (2005) showed that expression of ABCG2 in the mammary gland is induced during lactation and plays an important role in the secretion of drugs into mouse milk. Although this induction is not exclusive to this ABC transporter in the lactating mammary gland (Mani et al., 2009; Ito et al., 2014), a major role for ABCG2 in the secretion of antimicrobials into the milk of ruminants has also been reported (Real et al., 2011a; Mealey, 2013).

Enrofloxacin was the first veterinary fluoroquinolone to be identified as an in vitro substrate of ABCG2 (Pulido et al., 2006; Wassermann et al., 2013a). Administration of ENRO in vivo jointly with ABCG2 inhibitors decreased ENRO concentrations in sheep milk (Pulido et al., 2006). In cattle, the genetic variant Y581S ABCG2 (rs43702337) has been described as an in vitro and in vivo gain-of-function polymorphism with a greater capacity to transport compounds in vitro, including ENRO and danofloxacin (Real et al., 2011b). Administration of danofloxacin to cows carrying the Y581S polymorphism resulted in higher concentrations in milk of this fluoroquinolone in comparison with the results in wild-type animals (Otero et al., 2013, 2015).

In the present study we assessed the differential in vitro transport of CIPRO by both variants of the Y581S polymorphism using cells over-expressing bovine ABCG2. The effect of this polymorphism on the concentrations in plasma and in milk of ENRO and of CIPRO was also investigated by administering ENRO to Y/Y 581 and Y/S 581 cows.

MATERIALS AND METHODS

Reagents and Drugs

The ciprofloxacin used in cell cultures was obtained from Sigma-Aldrich (St. Louis, MO). Baytril® (ENRO 10%) was purchased from Bayer (Barcelona, Spain) for use in the in vivo studies. The specific ABCG2 inhibitor Ko143 was obtained from Tocris (Bristol, UK). All the other chemicals were analytical grade and available from commercial sources.

Cell Cultures

Madin-Darby canine kidney epithelial cell (MDCK-II) parental cells were provided by Dr A.H. Schinkel (Netherlands Cancer Institute). MDCK-II cells stably transduced with both bovine variants (S581 and Y581) of ABCG2 had previously been generated and characterized by the research group (Real et al., 2011b). These transduced cells express bABCG2 protein at similar levels and the polymorphism had no effect on transporter trafficking to the cell surface (Real et al., 2011b). Cell culture conditions were the same as those described by González-Lobato et al. (2014); briefly, cells were cultured at 37ºC and pH 7.4 in an atmosphere with 5% CO2 in Dulbecco's modified Eagle medium (DMEM) with GlutaMAX (Life Technologies, Inc., Carlsbad, CA), supplemented with 10% foetal calf serum (Life Technologies), penicillin (50 U/mL) and streptomycin (50 μg/mL) (Life Technologies).

Transport Studies

Transepithelial transport assays using Transwell plates were carried out as described elsewhere (Perez et al., 2013) with minor modifications. Cells were seeded on

138 microporous polycarbonate membrane filters (3.0 μm pore size, 24 mm diameter;
139 Transwell 3414; Corning, NY) at a density of 1.0 x 10^6 cells per well. Cells were grown
140 for 3 days, the medium being replaced each day. To check the tightness of the
141 monolayer, transepithelial resistance was measured in each well using a Millicell ERS
142 ohmmeter (Millipore, Bedford, MA, USA). After corrections had been made for the
143 resistance obtained in blank control wells, those wells which registered a resistance of
144 200 ohms or greater were used in the transport experiments.

145 Before the start of the experiment, the medium on both sides of the monolayer
146 was replaced with 2 mL of Optimem medium (Life Technologies), free of serum, and
147 either containing or not the specific ABCG2 inhibitor 1 μM Ko143, for 2 h. After
148 incubation, the experiment was started (t = 0) by replacing the medium in either the
149 apical or basolateral compartment with fresh Optimem medium containing 1 μM
150 CIPRO, with or without the inhibitor 1 μM Ko143. Aliquots of 100 μL were taken from
151 the opposite compartment, after 2 and 4 h of incubation, and stored at -20ºC until high
152 performance liquid chromatography (HPLC) analysis could be undertaken. At least
153 three replicates of each setting were performed. The fraction of CIPRO transported to
154 the acceptor compartment was presented as a percentage of the total amount of CIPRO
155 added to the donor compartment at the beginning of the experiment. Active transport
156 across MDCK-II monolayers was expressed as the relative transport ratio, defined as the
157 apically directed transport percentage divided by the basolaterally directed translocation
158 percentage, after 4 h. The relative transport ratio at 4 h is a parameter which has been
159 previously used for comparison purposes in transport assays (Barrera et al., 2013;
160 Moreno-Sanz et al., 2014).

161

162

163 ***Animal Studies***

164 Animals were handled in accordance with institutional guidelines that complied
165 with European legislation (2010⁄63⁄EU). The experiments were approved by the
166 Research Committee for Animal Use of the University of Leon (approval number 13-
167 2011, date of approval 7 November 2011).

168 Lactating Holstein-breed cows aged between 2 and 5 years and weighing 650 to
169 830 kg were used. Their daily milk yield was on average 45 ± 8 kg. There was no
170 difference in age, weight or milk yield between the two sets of cows. The normal
171 milking routine for all the animals involved milk being taken twice each day. The
172 experiments were performed at a private farm located at Santa María del Monte del
173 Condado, Leon (Spain). Y581S genotypes were determined in accordance with the
174 procedure described by Komisarek et al. (2009). During the genotyping procedure, S/S
175 animals could not be found. Animals were divided into two groups of 5 Y/S 581
176 heterozygous and 6 Y/Y 581 homozygous cows. Immediately before the administration
177 of the drug (time 0), the animals were milked, this coinciding with their routine morning
178 milking. Both groups received a single dose of 2.5 mg/kg of ENRO subcutaneously
179 (Baytril® 10%, Bayer). Blood samples were collected from the tail vein at 1, 5, 11, 24
180 and 48 h after treatment. Milk samples were collected manually at 1, 2, 5, 11 (the
181 routine afternoon milking), 24 and 48 h after treatment. A complete evacuation of the
182 udder was carried out at each sampling to avoid any dilution effect. Plasma was
183 separated by centrifugation at 1000 g for 15 min, and plasma and milk samples were
184 stored at –20°C until HPLC analysis.

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188 ***High Performance Liquid Chromatography (HPLC) Analysis***

189 The conditions for HPLC analysis of ENRO and CIPRO concentration from
190 transport assays and pharmacokinetic studies in cows were based on Perez et al. (2013)
191 and Barrera et al. (2013). Danofloxacin (0.01 μg⁄mL) for the plasma analysis and
192 difloxacin (0.01 μg⁄mL) for the milk analysis were used as internal standards. 600 μL of
193 chloroform were added to each 100 μL aliquot of sample, which was shaken for 30 min
194 and centrifuged at 5,000 x g for 6 min. The organic phase was separated and evaporated
195 until dry in a stream of nitrogen. The residue was reconstituted in 100 μL of methanol
196 and injected into the HPLC system. Samples from the transport assays were not
197 extracted with chloroform, with 50 μL of the culture media injected directly into the
198 HPLC system. The system consisted of a Waters 600 pump, a Waters 717 plus
199 autosampler, a Waters 486 fluorescence detector (Waters Corporation, Milford, MA)
200 and a C18 reversed-phase column (Mediterranea Sea 18 5μ 25x0.46cm; Teknokroma,
201 Barcelona, Spain). The mobile phase consisted of 25 mM orthophosphoric acid
202 supplemented with 0.1 % triethylamine (pH 3.0):acetonitrile (87:13). The flow rate was
203 set up at 1.25 mL⁄min. Fluorescence was detected at 280 nm (excitation) and 460 nm
204 (emission). Integration was performed using MILLENNIUM 32 software (Waters). The
205 limit of quantification (LOQ) values in plasma were 3.9 ng/mL for ENRO and 7.8
206 ng/mL for CIPRO, and in milk 7.8 ng/mL for both ENRO and CIPRO.

207

208 ***Pharmacokinetic Analysis***

209 Plasma and milk concentrations for each animal were analysed using a computer
210 program (PK solution 2.0, Summit Research Services, Ashland, OH). Peak
211 concentrations (Cmax) and time–peak concentrations (Tmax) were read from the plotted
212 concentration–time curve for each animal. The area under the plasma concentration–

time curves ($AUC_{0\text{-}\rightarrow\infty}$) was calculated using the linear trapezoidal rule from time zero with extrapolation to time infinity. The drug mean residence time (MRT) was calculated by the linear trapezoidal rule with extrapolation to time infinity, using the formula: MRT = AUMC/AUC, where AUMC is the mean area under the momentum curve.

Statistical Analysis

Statistical analysis for significant differences was performed using the two-tailed Student's T test. Results are reported as mean values ± S.D. A probability of $P < 0.05$ was considered to be statistically significant.

RESULTS

In Vitro Transport of CIPRO by Both Bovine ABCG2 Variants (S581 and Y581)

To determine whether the bovine ABCG2 variants could transport CIPRO differentially, transport assays were performed using polarized MDCK-II parental cells and their transduced subclones with both bovine ABCG2 variants (S581 and Y581). These cells with the protein located on the apical membrane preferentially transported ABCG2 substrates to the apical site.

Basal-to-apical transport of CIPRO was strongly increased in the bABCG2-transduced subclones, as compared to parental MDCK-II cells (Figure 1A-C), indicating a specific transport of CIPRO by bovine ABCG2, thanks to its apical location. Moreover, the relative transport ratio (i.e., the apical directed translocation divided by the basolateral directed translocation at t = 4 h) was higher for the S581 variant, as compared to the Y581 variant (1.70 ± 0.22 vs. 1.41 ± 0.11, $P < 0.05$), thus showing a greater capacity of the first variant to transport CIPRO. In all cases, bovine ABCG2-mediated transport was completely inhibited with the specific ABCG2 inhibitor Ko143 (Figure 1D-F). These data demonstrated that CIPRO is a substrate of bovine ABCG2 and that the S581 variant transports CIPRO more efficiently than the Y581 variant.

Effect of the Y581S Bovine Polymorphism on Concentrations in Plasma and in Milk of ENRO and CIPRO in Cattle

To study in more depth the in vivo effect of the Y581S polymorphism on the concentrations in plasma and secretion into milk of ENRO and its main active metabolite CIPRO, plasma and milk concentrations were analysed after the administration of ENRO at 2.5 mg/kg to Y/Y 581 and Y/S 581 cows (Figures 2 and 3).

Enrofloxacin plasma concentrations at 11 h after ENRO treatment were significantly lower for Y/S (0.045 ± 0.008 μg/mL) compared to Y/Y cows (0.063 ± 0.016 μg/mL). In the case of CIPRO, significantly smaller concentrations in Y/S relative to those obtained in Y/Y animals were observed at 5 h (0.074 ± 0.010 μg/mL vs. 0.097 ± 0.019 μg/mL) and 11 h (0.039 ± 0.007 μg/mL vs. 0.051 ± 0.008 μg/mL). These data indicate that the Y581S polymorphism decreases the concentrations in plasma of these antimicrobials.

With regard to secretion into milk, the area under the milk concentration curve (AUC_{0-48}) for ENRO increased significantly in Y/S cows and was around 1.2 times higher for Y/S (1.42 ± 0.24 μg·h/mL) than for Y/Y cows (1.11 ± 0.14 μg·h/mL), with no differences in C_{max} values (Table 1). For CIPRO, concentrations in milk were significantly higher in Y/S than in Y/Y cows at 5, 11, 24 and 48 h (Figure 3). The maximum concentration in milk (C_{max}) increased significantly for Y/S (1.03 ± 0.25 μg/mL), as compared with Y/Y animals (0.51 ± 0.24 μg/mL). Milk AUC_{0-48} for CIPRO was 2 times higher in the cows carrying the Y581S polymorphism (13.33 ± 2.26 μg·h/mL) in comparison with non-carriers (5.94 ± 2.41 μg·h/mL) (Table 1). These results indicate that the Y581S polymorphism increases the amounts of CIPRO and ENRO in milk after the administration of ENRO at 2.5 mg/kg.

DISCUSSION

It is widely known that genetic variants of human ABCG2 can lead to altered drug pharmacokinetics, affect responses to therapy and clinical outcomes, and be associated with diseases such as gout (Bruhn and Cascorbi, 2014). In the veterinary field, recent research has shown that ABCG2 plays a critical role in drug disposition and safety in animals (Schrickx and Fink-Gremmels, 2008; Mealey et al., 2012), including its involvement in the secretion of substrate antimicrobials into ovine milk (Pulido et al., 2006; Perez et al., 2013). ABCG2 over-expression has recently been associated with in vivo drug resistance in canine multi-centric lymphoma (Zandvliet et al., 2015). With regard to genetic variants in animals, previous studies have reported that defective feline ABCG2 is responsible for fluoroquinolone-induced retinal toxicity in cats (Ramirez et al., 2011), and that the bovine ABCG2 Y581S polymorphism alters the yield and composition of milk (Cohen-Zinder et al., 2005; Olsen et al., 2007), and affects the secretion into milk of danofloxacin in cows (Otero et al., 2013, 2015). Wassermann et al., (2013b) predicted that the Y581S polymorphism of the bovine ABCG2 is located in the extracellular area between transmembrane domains 5 and 6 of the transporter. The frequency of this bovine polymorphism can reach 20% in some Holstein populations but it may be lower in some of them (Ron et al., 2006). Therefore, heterozygosity would be more common than homozygosity for this polymorphism. In this case, the use of heterozygous animals would be the nearest scenario to the real situation (Otero et al., 2015). The present study demonstrated that lactating dairy cows with the Y581S polymorphism showed significantly increased amounts of both ENRO and CIPRO in their milk.

MDCK-II cells are one of the most commonly used models for studying ABCG2-mediated transport of drugs (Xia et al., 2007). MDCK-II cells over-expressing

307 ABCG2 have shown strong predictive ability for the effect of ABCG2 on the secretion
308 of drugs into milk (Barrera et al., 2013; Perez et al., 2013; Gonzalez-Lobato, 2014). The
309 present in vitro results using MDCK-II cells over-expressing bovine ABCG2
310 demonstrated for the first time that bovine ABCG2 extrudes and therefore interacts with
311 CIPRO in a specific way (Figure 1). As previously shown for ENRO (Real et al.,
312 2011b), cells over-expressing the S581 variant transport CIPRO in the basolateral to
313 apical direction more efficiently than cells with the Y581 variant, indicating a greater in
314 vitro transport capacity for this variant, which is in agreement with the results obtained
315 previously for other drugs such as difloxacin, danofloxacin, marbofloxacin and
316 nitrofurantoin (Real et al., 2011b, González-Lobato et al., 2014). The results reported
317 here support our in vitro model as a valuable tool for the assessment of bovine ABCG2-
318 mediated transport, but it is not possible to rule out other outcomes if other cell models,
319 such as mammary cell lines, were to be used. In fact, bovine mammary epithelial cells
320 (BME) and bovine mammary alveolar cells (MAC-T) have previously been used for
321 transport studies (Cavret et al., 2005; Halwachs et al., 2013).

322 Because of the widespread use of ENRO, it appeared necessary to assess the
323 effect of the bovine Y581S polymorphism on the concentrations in plasma and in milk
324 of ENRO and of CIPRO. Hence, ENRO (Baytril® 10%) was administered to 5 Y/S 581
325 and 6 Y/Y 581 cows to provide an in vivo setting. The concentrations in plasma
326 recorded for ENRO and CIPRO (Figure 2) were in the same range as those obtained by
327 McKellar et al. (1999) and Fu et al. (2008). With regard to the genotype-driven effect,
328 concentrations in plasma for both compounds were lower for Y/S than for Y/Y cows.
329 ABCG2 was first described as a transporter involved in the absorption and excretion of
330 drugs (Jonker et al., 2000). Its expression in the liver, kidney and intestine of cattle
331 (Zancanella et al., 2013; Lindner et al., 2013; Haslam and Simmons, 2014), together

with its induced expression during lactation in the bovine mammary gland (Jonker et al., 2005), have all been confirmed. Thus, the decrease noted in the concentrations in plasma of ENRO in Y/S cows is probably due to the enhanced function of the Y581S polymorphism which may promote a higher clearance of the drug into urine, the primary route for excretion of ENRO (Martinez et al., 2006), into bile, into the intestinal lumen and into milk. However, in the case of CIPRO, the differences between the two sets of cows might be attributed not only to an effect of the Y581S polymorphism, but also in part to the metabolism of ENRO, which is converted to CIPRO mainly in the liver (Lopez-Cadenas et al., 2013). Different levels of ENRO were available for metabolizing to CIPRO in the different sets of cows and the lower concentrations in plasma of CIPRO in Y/S cows might be a consequence of lower concentrations in plasma of ENRO in these animals.

In respect of genotype-driven differences in milk parameters, an effect due to the gretaer transport capacity of the Y581S polymorphism was also observed. Despite lower concentrations in plasma, the higher milk $AUC_{0\text{-}48}$ values in Y/S, as compared with Y/Y animals (Table 1) indicate a higher secretion into milk of ENRO and CIPRO by Y/S cows, similar to the outcome previously reported for danofloxacin by Otero et al. (2013, 2015) after its administration at two different dose levels. Although ENRO is mainly metabolized in the liver (Lopez-Cadenas et al., 2013), it is not possible to rule out the possibility that part of the difference observed between the two genotypes in the secretion into milk of CIPRO might be due to a partial metabolization of ENRO to CIPRO in the udder (Malbe et al., 1996). However, the much greater differences between Y/S and Y/Y cows in $AUC_{0\text{-}48}$ for CIPRO (2.2 times higher), as compared with ENRO (1.2 times higher) (Table 1) indicate a striking effect of the Y581S

356 polymorphism on the secretion of CIPRO into milk, regardless of any potential ENRO
357 metabolism in the udder.

358 Independently of the genotype, the antimicrobial profile in milk after the
359 administration of ENRO was mainly dominated by CIPRO (Figure 3). Milk AUC_{0-48}
360 values for CIPRO were higher than for ENRO (5 times higher for Y/Y animals and 9
361 times higher for Y/S animals, Table 1), showing that CIPRO was more efficiently
362 accumulated in cow milk than was ENRO. Such higher amounts in milk of CIPRO
363 relative to ENRO have been reported previously (Rantala et al., 2002; Kaartinen et al.,
364 1995; Idowu et al., 2010). Thus, in the case of mastitis, CIPRO would be the key
365 molecule acting against the infectious agent. Moreover, the Cmax values obtained for
366 milk (Table 1, Figure 3) were higher than the in vitro minimal inhibitory concentrations
367 (MIC) for isolates from mastitis (Grobbel et al., 2007; Thomas et al., 2015). Bearing in
368 mind that the MIC values of ENRO and CIPRO for many pathogens have been reported
369 to be < 0.1 μg/mL (Prescott and Yielding, 1990), if this concentration (0.1 μg/mL) is
370 taken as the MIC of these compounds, the therapeutic Cmax/MIC ratios can be
371 estimated using the milk parameters for the active metabolite CIPRO (Table 1), which
372 highlight an increase from 5.1 in Y/Y to 10.3 in Y/S animals, respectively. Cmax/MIC
373 is important in determining successful outcomes and, in particular, killing the more
374 resistant subpopulations of bacteria (Escudero et al., 2007). Previous research has
375 shown that for fluoroquinolones a Cmax/MIC > 3 produced a 99% reduction in bacterial
376 counts and a Cmax/MIC of 8 or greater prevented the emergence of resistant organisms
377 (Craig, 1998). The data reported here may be relevant in the therapeutic use of these
378 compounds, since prevention of the development of resistance is correlated with the
379 Cmax/MIC ratio (Aliabadi and Lees, 2000).

380

CONCLUSIONS

This work demonstrates that lactating dairy cows carrying the Y581S polymorphism produced milk with higher amounts of both ENRO and CIPRO. However, the effect of this polymorphism may not be restricted only to fluoroquinolones, since other ABCG2 substrate drugs might also be affected. The findings of this study provide evidence that genetic factors such as this polymorphism must be taken into account when designing appropriate veterinary therapies, since they can alter the expected drug profile in plasma and in milk and may lead to the failure of treatments or to variable amounts of drugs in milk.

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578 **Table 1.** Pharmacokinetic parameters (means ± SD) for ENRO and CIPRO in milk after
579 subcutaneous administration of ENRO at a dosage of 2.5 mg/kg in Y/Y (n = 6) and Y/S
580 cows (n = 5).

Parameter[1]	Y/Y 581	Y/S 581
ENRO		
AUC $_{0\text{-}48}$ (μg·h/mL)	1.11 ± 0.14[a]	1.42 ± 0.24[b]
AUC $_{0\text{-}\infty}$ (μg·h/mL)	1.23 ± 0.08[a]	1.55 ± 0.18[b]
C_{max} (μg/mL)	0.11 ± 0.03	0.13 ± 0.01
T_{max} (h)	3.00 ± 1.41	3.20 ± 1.45
MRT (h)	10.08 ± 4.38	9.81 ± 2.21
CIPRO		
AUC $_{0\text{-}48}$ (μg·h/mL)	5.94 ± 2.41[a]	13.33 ± 2.26[b]
AUC $_{0\text{-}\infty}$ (μg·h/mL)	6.04 ± 2.38[a]	13.46 ± 2.28[b]
C_{max} (μg/mL)	0.51 ± 0.24[a]	1.03 ± 0.25[b]
T_{max} (h)	5.00 ± 0.00	6.20 ± 2.40
MRT (h)	10.68 ± 2.35	10.88 ± 0.84

581 [a-b] Means within a row with different superscripts differ ($P < 0.05$).

582 [1]AUC, area under the curve; C_{max}, maximum concentration; MRT, mean residence time;
583 $T_{1/2}$, elimination half-life; T_{max}, time to peak concentration.

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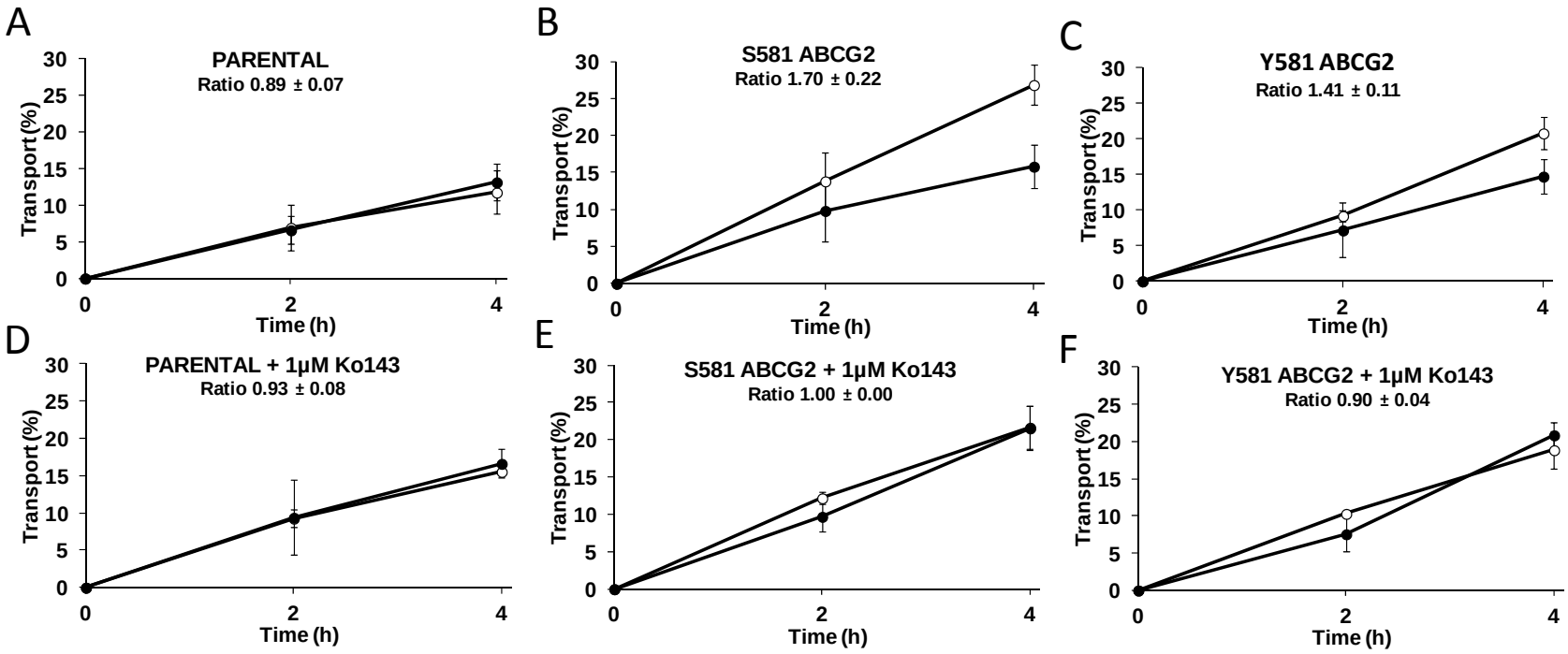
586 **Figure captions**

587 Figure 1. Transepithelial transport of CIPRO (10 μM) in polarized MDCK-II parental
588 (non-transduced), MDCK-II-S581-ABCG2 and MDCK-II-Y581-ABCG2 monolayers in
589 the absence (A, B, C) or presence (D, E, F) of the specific ABCG2 inhibitor Ko143 (1
590 μM). The experiment was started (t = 0 h) by replacing the medium in either the apical
591 or basolateral compartment with fresh Optimem medium containing 10 μM CIPRO,
592 with or without the inhibitor 1 μM Ko143. Aliquots of 100 μL were taken from the
593 opposite compartment at t = 2 and 4 h and measured by HPLC. The fraction of CIPRO
594 transported to the acceptor compartment was presented as a percentage of the total
595 amount of CIPRO added to the donor compartment at the beginning of the experiment.
596 Results are means and error bars indicate SD (n = 3). "Ratio" represents the relative
597 transport ratio (i.e., the apical directed translocation divided by the basolateral directed
598 translocation) at t = 4 h. ○ Translocation from the basolateral to the apical compartment.
599 ● Translocation from the apical to the basolateral compartment.

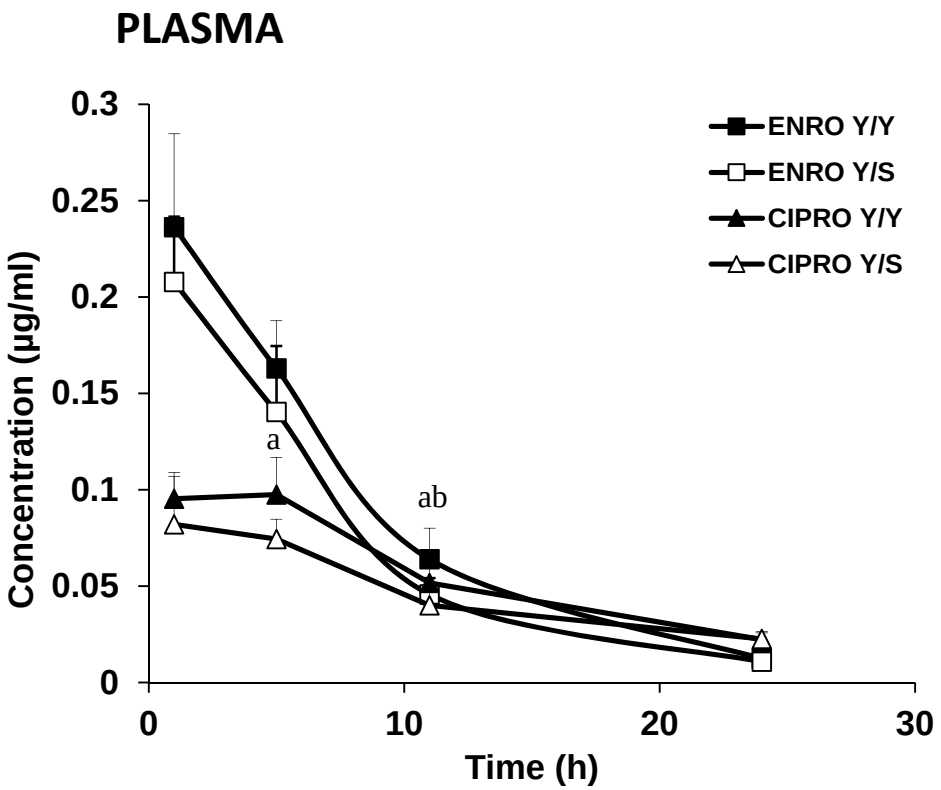
600 Figure 2. Mean plasma concentrations of ENRO and CIPRO after SC administration of
601 ENRO at a dosage of 2.5 mg/kg to Y/S 581 and Y/Y 581 lactating cows. Plasma
602 samples were collected at several points over 48 h. Concentrations were undetectable
603 after 48 h. Lowercase letter (a) represents significant differences ($P < 0.05$) between the
604 two genotypes for CIPRO. Lowercase letter (b) represents significant differences ($P <$
605 0.05) between the two genotypes for ENRO. Y/Y cows n = 6; Y/S cows n = 5.

606 Figure 3. Mean milk concentrations of ENRO and CIPRO after SC administration of
607 ENRO at a dosage of 2.5 mg/kg to Y/S 581 and Y/Y 581 lactating cows. The inset
608 shows ENRO data alone. Milk samples were collected at several points over 48 h.
609 Enrofloxacin concentrations were undetectable after 48 h. Lowercase letter (a)

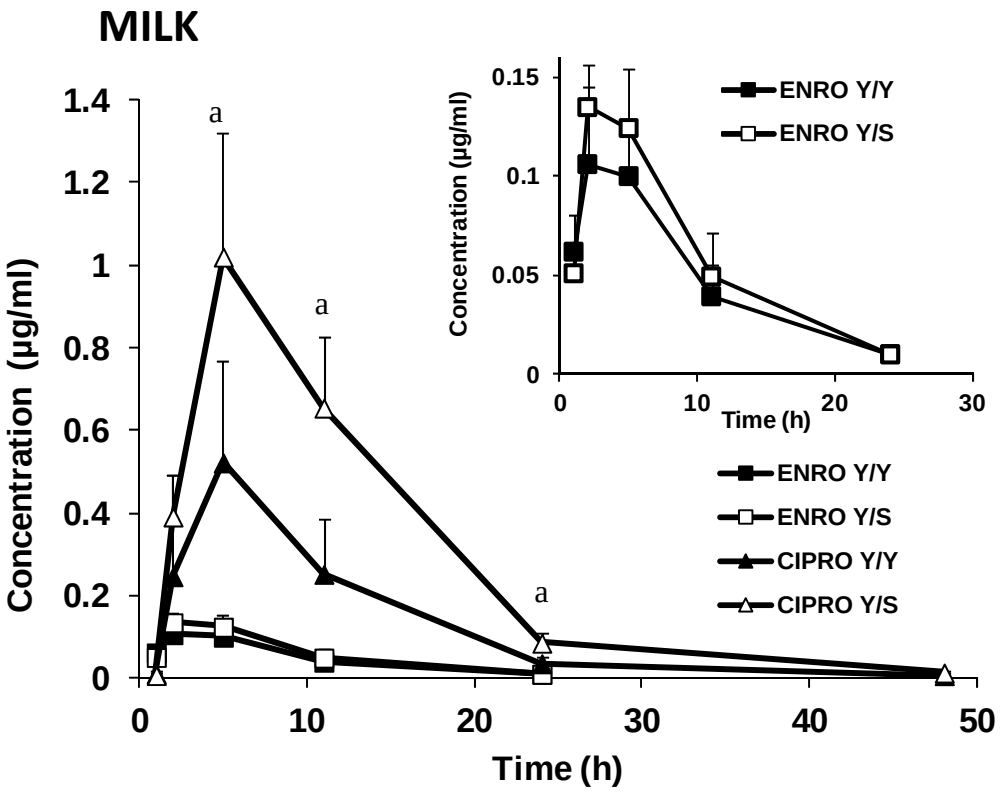
represents significant differences ($P < 0.05$) between the two genotypes for CIPRO. Y/Y cows n = 6; Y/S cows n = 5.



Otero et al. Figure 1



Otero et al. Figure 2



Otero et al. Figure 3