SHORT COMMUNICATION

The bovine ATP-binding cassette transporter ABCG2 Y581S single nucleotide polymorphism increases milk secretion of the fluoroquinolone danofloxacin

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Running title: Bovine ABCG2 variants affect milk secretion of danofloxacin

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Abbreviations: ABC, ATP-binding cassette; AUC, area under the curve; BCRP, Breast Cancer Resistance Protein; MRT, mean residence time; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; T_{1/2 el}, elimination half-life
Abstract

The bovine ATP-binding cassette transporter ABCG2 [Breast Cancer Resistant Protein (BCRP)] polymorphism Y581S has recently been shown to increase in vitro transepithelial transport of antibiotics. Since this transporter has been extensively related to the active secretion of drugs into milk, the potential in vivo effect of this polymorphism on secretion of xenobiotics in livestock could have striking consequences for milk production, the dairy industry and Public Health. Our purpose was to study the in vivo effect of this polymorphism on the secretion into milk of danofloxacin, a widely used veterinary antibiotic. Danofloxacin (1.25 mg/kg) was administered to six Y/Y 581 homozygous and six Y/S 581 heterozygous lactating cows, and plasma and milk samples were collected and analysed by HPLC. No differences were found in the pharmacokinetic parameters of danofloxacin in plasma between the two groups of animals. In contrast, Y/S heterozygous cows showed a two-fold increase in danofloxacin levels in milk. In addition, the pharmacokinetic elimination parameters MRT and $T_{1/2}^{el}$ were significantly lower in the milk of the animals carrying the Y/S polymorphism. These in vivo results are in agreement with our previously published in vitro data, which showed a greater capacity of the S581 variant in accumulation assays, and demonstrate for the first time an important effect of the Y581S SNP on antibiotic secretion into cow milk. These findings could be extended to other ABCG2 substrates and may be relevant for the treatment of mastitis and for the design of accurate and novel strategies to handle milk residues.
Introduction

The ATP-binding cassette transporter ABCG2 [Breast cancer resistant protein (BCRP)] is expressed in a wide range of tissues and organs including intestine, liver, blood-brain barrier and the mammary gland (van Herwaarden and Schinkel, 2006). It affects the bioavailability of its substrates and mediates the active secretion of xenobiotics and several vitamins in milk (van Herwaarden et al., 2007). The occurrence of drug residues in milk could lead to the development of bacterial resistance, allergies, or hypersensitivity reactions in consumers (McManaman and Neville, 2003). On the other hand, effective treatments for mastitis may require a considerable transfer of drugs into milk (Escudero et al., 2007). All these issues are of outstanding concern for general Public Health, the dairy industry and veterinary therapeutics. Hence, the identification of relevant factors for the transfer of drugs into milk in livestock constitutes a priority.

Changes both in the expression and/or the function of ABCG2 can lead to dramatic variations in the pharmacokinetics and secretion of its substrates into milk (Jonker et al., 2007; Ni et al., 2010). Several single nucleotide polymorphisms (SNPs) have been studied in human ABCG2 and, of these, Q141K is one of the most important, causing impaired urate transport which may contribute to gout (Woodward et al., 2009). There is also evidence that this SNP results in increased plasma levels of diflomotecan, fluvastatin and simvastatin, among other chemotherapeutic agents (Morisaki et al., 2005; Keskitalo et al., 2009).

With regard to ABCG2 SNPs in the veterinary field, the discovery of a nucleotide missense A/C mutation encoding a replacement of tyrosine-581 with serine (Y581S) localized on the fifth extracellular region of the bovine ABCG2 transporter constitutes a crucial finding. Y581S SNP is widely spread in some bovine breeds, reaching a frequency of 20% in the Israeli Holstein population (Ron et al., 2006), and it has been suggested as the causative polymorphism of a quantitative trait locus affecting the production of milk and its protein and fat composition (Cohen-Zinder et al., 2005; Weikard et al., 2012). In addition, a negative correlation has been suggested between this SNP and fertility (Komisarek and Dorynek, 2009). With regard to the effect of this SNP on drug transport, our previous in vitro results described the Y581S SNP as a gain-of-function polymorphism, showing a lower mitoxantrone accumulation in ovine primary fibroblast transiently transfected with the S581
variant, when compared to the Y581 variant (Merino et al., 2009). Recently, the greater activity of the S581 allele was confirmed on transepithelial transport of antibiotics using stably transduced cell models (Real et al., 2011a). The present study compares plasma and milk pharmacokinetics in Y/S 581 heterozygous and Y/Y 581 homozygous lactating cows that received danofloxacin, a widely used antibiotic for the treatment of infections of the respiratory tract, the intestinal tract and the mammary glands (Poutrel et al., 2008; Schrickx and Fink-Gremmels, 2008). The aim of our study is to show for the first time the potential in vivo applicability of our previous in vitro results relative to the effect of Y581S SNP on drug secretion into milk in livestock.
Material and methods

Reagents and Chemicals. For the pharmacokinetic studies, danofloxacin (Advocin 2.5%) was obtained from Pfizer (Cedex, France). All other chemicals were analytical grade and available from commercial sources.

Animals. Animals were housed and handled according to institutional guidelines, in compliance with European legislation (86/609/EEC). Lactating Holstein cows aged 2 to 5 years (600–800 kg) were used. Routine milking of the cows was undertaken twice daily. The animals were parasite free. Drinking water was available ad libitum for all of them. The experiments were performed on the private Garfi SAT farm located at Santa María del Monte del Condado, in the Province of Leon (Spain).

Cow Genotyping. DNA for animal genotyping was isolated from hair follicle using Chellex 100® (Walsh et al., 1991) or from peripheral blood by using the standard phenol-chloroform procedure. Genotypes were determined with the PCR-RFLP method described by Komisarek and Dorynek (2009).

Pharmacokinetic Experiments with Lactating Cows. Animals were divided into two groups of six individuals each, Y/S 581 heterozygous genotype carriers and Y/Y 581 homozygous animals. Both groups received a single dose of 1.25 mg/kg (i.m.) of danofloxacin (Advocin 2.5%). Blood samples were collected from the tail vein at 1, 2, 5, 11, 24 and 48 h after treatment. Milk samples were collected after complete milking of the gland before the treatment and at 2, 5, 11, 24, 35 and 48 h after treatment. Plasma was separated by centrifugation at 3000 rpm for 15 min, and plasma and milk samples were stored at -20°C until HPLC analysis.

HPLC Analysis. The conditions for HPLC analysis of danofloxacin were modified according to previously published methods (Garcia, et al. 2000). Difloxacin (2.5 µg/mL) for plasma samples and ciprofloxacin (5 µg/mL) for milk samples were used as internal standards and 600 µl of chloroform was added to each 100 µl aliquot of sample. Samples were shaken for 10 min and the organic phase was separated by centrifugation at 5000 g for 6 min and then evaporated to dryness under a nitrogen stream. The samples were resuspended in 100 µl of methanol and injected into the HPLC system, consisting of a Waters 600 pump, a Waters 717 plus autosampler and a Waters 486 fluorescence.
detector (Waters Corporation, Milford, MA). Sample separation was performed on a reversed-phase column (Phenomenex Synergi 4 µm Hydro-RP 80A). Mobile phase consisted of 25 mM orthophosphoric acid (pH 3.0) and acetonitrile (80:20), and the flow rate was set to 1.6 mL/min. Sample detection was performed by fluorescence detection at 338 nm (excitation) and 425 nm (emission). Integration was performed using Millenium32 software (Waters).

Standard samples were prepared in the appropriate drug-free matrix. Inter-assay precision coefficients of variation were < 15% and relative error (accuracy) values were < 20%. Limits of detection and limits of quantification were calculated as 3.3 and 10 times, respectively, the standard deviation of the signal corresponding to 10 blank solutions divided by the slope of the calibration curve. The limits of detection obtained were 0.8 ng/ml for plasma and 0.6 ng/ml for milk. The limits of quantification obtained were 2.3 ng/ml for plasma and 1.7 ng/ml for milk.

**Pharmacokinetic Calculations and Statistical Analyses.** Milk and plasma concentration versus time curves after treatment for each individual were analysed with the PK Solution 2.0 computer program (Ashland, OH, USA) to obtain the estimated kinetic parameters. Results are reported as the mean ± standard deviation (SD). Statistical analysis for significant differences was performed using the two-tailed Student’s t-test. A probability of $p < 0.05$ was considered to be statistically significant.
Results and Discussion

ABCG2 polymorphism research is pharmacologically and financially of relevance in the case of ruminants because of the involvement of this protein in the transport of xenobiotics and vitamins into milk. In this paper we studied the effect of the Y581S SNP of bovine ABCG2 on plasma bioavailability and secretion into milk of the widely used fluoroquinolone danofloxacin.

Danofloxacin (1.25 mg/kg) was administered to 6 Y/Y 581 homozygous and 6 Y/S 581 heterozygous lactating cows and plasma and milk concentrations of the antibiotic after its administration were analysed. Plasma levels (Fig. 1) and plasma pharmacokinetic parameters (Table 1) obtained for both groups of animals were very similar to those obtained by Shem-Tov et al. (1998) and Shojaee Aliabadi and Lees (2003). Our results showed no significant differences according to the genotype. Thus, Y581S SNP does not affect systemic exposure (plasma AUC) of danofloxacin at the administered dose. A lack of any effect from Y581S SNP on the plasma disposition of danofloxacin is a positive outcome for the therapeutics of systemic infections, since a change in plasma levels of the antibiotic would affect treatment efficacy, as a result of the concentration-dependent effect of fluoroquinolones. Although, in general, ABCG2 does affect the plasma disposition of some of its substrates (Vlaming et al., 2009), no ABCG2-mediated effect in the systemic plasma profile of some ABCG2 substrates has been reported (Jonker et al., 2005; Zhou et al., 2008). Even human Q141K SNP does not affect the plasma disposition of all ABCG2 substrates (Keskitalo et al., 2009; Kim et al., 2007; Adkison et al., 2008). Furthermore, our previous studies using Abcg2(-/-) mouse models and sheep have shown that plasma concentrations of danofloxacin are not affected by this transporter in these species (Real et al., 2011b).

In the case of levels and milk pharmacokinetic parameters in milk (Fig. 2, Table 1), our data are in agreement with those previously published by Shem-Tov et al. (1998) but only for Y/Y homozygous cows. Levels in the milk of Y/S heterozygous animals were significantly higher when compared with Y/Y homozygous animals at 5 and 11 h (Fig. 2) and the milk C_max (0.88 ± 0.32 versus 1.72 ± 0.79 μg/ml, p < 0.05), AUC_{(0-∞)} values (6.53 ± 1.41 versus 12.59 ± 5.27 μg.h/ml, p < 0.05) and AUC milk/plasma ratio (4.09 ± 1.07 versus 8.81 ± 3.69, p < 0.05) were two-fold higher in the animals.
carrying the polymorphism. In addition, the elimination parameters MRT (6.30 ± 0.32 versus 5.72 ± 0.52 h, \( p < 0.05 \)) and \( T_{1/2 \text{el}} \) (4.36 ± 0.22 versus 3.96 ± 0.36 h, \( p < 0.05 \)) were significantly lower in the Y/S heterozygous, as compared to the Y/Y homozygous animals. The greater capacity to transfer danofloxacin into milk in the Y/S heterozygous animals indicates that Y581S SNP significantly affects the secretion into milk of this antibiotic. Our findings are in agreement with our previously published \textit{in vitro} data showing the greater \textit{in vitro} capacity of the S581 variant compared to the Y581 variant in terms of higher relative \( V_{\text{max}} \) values (Merino et al., 2009). Moreover, we recently reported a higher danofloxacin transport ratio in cells transduced with the S581 variant in comparison with Y581 cells (Real et al., 2011a). Withdrawal time for danofloxacin is set at 48 h. Experiments with other veterinary ABCG2 substrates with longer withdrawal periods and greater persistence in milk need to be performed in order to elucidate the therapeutic and financial impact of the effect of the S581 variant on the persistence of antibiotics in milk as a result of its greater capacity to extrude ABCG2 substrates. Our \textit{in vivo} data might be extended to other ABCG2 substrates whose secretion into milk may be affected by this SNP, since differences between both variants in the \textit{in vitro} transport of veterinary fluoroquinolones, such as enrofloxacin and difloxacin, have recently been reported (Real et al., 2011a). Milk residues of fluoroquinolones are particularly relevant, since these antibiotics are drugs with considerable stability when subjected to thermal treatments and cooking procedures. Consequently, they can remain in milk after dairy processing, and can thus reach consumers (Roca et al., 2010) if the withdrawal times are not fulfilled. Likewise, intermittent exposure of consumers of dairy products to low levels of fluoroquinolones levels of dairy-product-consumers can produce hypersensitivity reactions or affect the intestinal microflora via food intake (Normanno et al., 2005).

Our findings could have considerable relevance to other major fields such as the quality of milk in respect to its vitamin composition, since some vitamins, such as riboflavin (van Herwaarden et al., 2007) are ABCG2 substrates and are secreted into milk. In addition, ABCG2-mediated transport of vitamins into milk might potentially affect the health of suckling calves. However, this effect of Y581S SNP on the concentrations of vitamins in milk remains to be addressed.

This study shows for the first time the \textit{in vivo} effect of a bovine SNP on the secretion of a drug into milk and confirms the relevance of ABCG2 polymorphisms, which might be used to predict milk
pollutant kinetics more accurately. Despite being expressed as a heterozygous allele, previous in vitro results have been corroborated, showing a two-fold greater capacity of Y/S animals to transfer the antibiotic danofloxacin into milk than Y/Y homozygous animals, with noteworthy consequences for veterinary therapeutics, milk residues and Public Health.

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Authorship Contributions

Participated in research design: Merino, Marqués, Álvarez and Prieto.

Conducted experiments: Otero, Real and de la Fuente.

Performed data analysis: Otero and Merino.

Wrote or contributed to the writing of the manuscript: Otero, Álvarez, Marqués and Merino.
References


Footnotes

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Figure legends
Figure 1. Plasma concentrations of danofloxacin after its intramuscular administration at a dosage of 1.25 mg/kg to Y/S 581 heterozygous and Y/Y 581 homozygous lactating cows. The insert shows a semi-log plot of the data. Plasma samples were collected at several points over 48 h. Plasma levels of danofloxacin were determined by HPLC. Concentrations at 48 h were undetectable. The results are means; error bars indicate standard deviation (n=6).

Figure 2. Milk concentrations of danofloxacin after its intramuscular administration at a dosage of 1.25 mg/kg to Y/S 581 heterozygous and Y/Y 581 homozygous lactating cows. Milk samples were collected at several points over 48 h. Milk levels of danofloxacin were determined by HPLC. Concentrations at 48 h were undetectable. The results are means; error bars indicate standard deviation (n=6).
Table 1. Mean (±SD) pharmacokinetic parameters of danofloxacin in plasma and milk after intramuscular administration at a dosage of 1.25 mg/kg in Y/Y and Y/S cows (n=6).

<table>
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<tr>
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<th>Y/Y 581</th>
<th>Y/S 581</th>
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<tbody>
<tr>
<td><strong>Plasma</strong></td>
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<tr>
<td>AUCₜₗₗ (µg·h/mL)</td>
<td>1.59 ± 0.29</td>
<td>1.44 ± 0.12</td>
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<tr>
<td>Cₘₚₜ (µg/mL)</td>
<td>0.19 ± 0.02</td>
<td>0.21 ± 0.03</td>
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<tr>
<td>MRT (h)</td>
<td>5.95 ± 1.29</td>
<td>5.42 ± 0.33</td>
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<tr>
<td>Tₘₚₜ (h)</td>
<td>1.33 ± 0.47</td>
<td>1.50 ± 0.50</td>
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<tr>
<td>T₁/₂ el (h)</td>
<td>4.12 ± 0.89</td>
<td>3.75 ± 0.23</td>
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<tr>
<td><strong>Milk</strong></td>
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<tr>
<td>AUCₜₗₗ (µg·h/mL)</td>
<td>6.53 ± 1.41</td>
<td>12.73 ± 5.16*</td>
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<tr>
<td>Cₘₚₜ (µg/mL)</td>
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<td>1.76 ± 0.74*</td>
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<td>MRT (h)</td>
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<td>Tₘₚₜ (h)</td>
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<td>T₁/₂ el (h)</td>
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<tr>
<td>AUC milk/plasma</td>
<td>4.09 ± 1.07</td>
<td>8.81 ± 3.69*</td>
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* p < 0.05, significantly different between the two groups of animals
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Fig. 2

- Y/Y 581
- Y/S 581

Concentration (µg/mL) vs Time (h)