Short Communication

The Bovine ATP-Binding Cassette Transporter ABCG2 Tyr581Ser Single-Nucleotide Polymorphism Increases Milk Secretion of the Fluoroquinolone Danofloxacin

Received September 13, 2012; accepted December 10, 2012

ABSTRACT

The bovine adenosine triphosphate-binding cassette transporter G2 (ABCG2/breast cancer resistance protein) polymorphism Tyr581Ser (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 secretion of danofloxacin, a widely used veterinary antibiotic, into milk. 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 analyzed by high-performance liquid chromatography. 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 2-fold increase in danofloxacin levels in milk. In addition, the pharmacokinetic elimination parameters, mean residence time and elimination half-life, 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 SS81 variant in accumulation assays, and demonstrate, for the first time, an important effect of the Y581S single-nucleotide polymorphism 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 (ABC) transporter ABCG2 (breast cancer resistance protein) is expressed in a wide range of tissues and organs, including the intestine, liver, blood-brain barrier, and 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 of these issues are of great concern for general public health, the dairy industry, and veterinary therapeutics. Hence, identification of relevant factors for the transfer of drugs into milk in livestock constitutes a priority.

Changes 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, Glu141Lys (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 (Murisaki et al., 2005; Keski-tilo et al., 2009).

With regard to ABCG2 SNPs in the veterinary field, the discovery of a nucleotide missense adenosine/cytosine 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 correlation has been suggested between this SNP and fertility (Komisarek and Dorynek, 2009).

This study was partially supported by the Spanish Ministry of Science and Technology; the European Regional Development Fund [Research Project AGL2009-11730 and Ramon y Cajal fellowship]; and the Basque Government in Spain [predoctoral fellowship].

dx.doi.org/10.1124/dmd.112.049056.

ABBREVIATIONS: ABC, ATP-binding cassette; ABCG2, adenosine triphosphate-binding cassette transporter G2; AUC, area under the curve; HPLC, high-performance liquid chromatography; Q141K, Glu141Lys; SNP, single-nucleotide polymorphism; Y581S, Tyr581Ser.
plasma and 1.7 ng/ml for milk. The limits of quantification obtained were 2.3 ng/ml for calibration curve. The limits of detection obtained were 0.8 ng/ml for plasma were calculated as 3.3 and 10 times, respectively, the standard deviation of the

Materials 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 (European Commission, 1986). 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 animals. The experiments were performed on the private Garfi SAT farm located at Santa María del Monte del Condao, in the Province of Leon (Spain).

Cow Genotyping. DNA for animal genotyping was isolated from hair follicles using Chellflex 100 (Sigma-Aldrich, St. Louis, MO) (Walsh et al., 1991) or from peripheral blood using the standard phenol-chloroform procedure. Genotypes were determined with the polymerase chain reaction–restriction fragment length polymorphism 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 hours after treatment. Milk samples were collected after complete milking of the gland before the treatment and at 2, 5, 11, 24, 35, and 48 hours after treatment. Plasma was separated by centrifugation at 3000 rpm for 15 minutes, and plasma and milk samples were stored at −20°C until high-performance liquid chromatography (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 minutes, and the organic phase was separated by centrifugation at 5000 g for 6 minutes and then evaporated to dryness under a nitrogen stream. The samples were reconstituted 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 (Synergi 4 µm Hydro RP 80A; Phenomenex, Torrance, CA). The 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 423 nm (emission). Integration was performed using Millennium32 software (Waters Corporation).

Standard samples were prepared in the appropriate drug-free matrix. Interassay 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 concentrations versus time curves after treatment of each individual were analyzed with the PK Solution 2.0 computer program (Summit Research Services, Ashland, OH) to obtain the estimated kinetic parameters. Results are reported as the mean ± S.D. 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 six Y/Y 581 homozygous and six Y/S 581 heterozygous lactating cows, and plasma and milk concentrations of the antibiotic after its administration were analyzed. 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 area under the curve (AUC)] of danofloxacin at the administered dose. A lack of any effect from Y581S SNP on the plasma disposition of danofloxacin is a positive result for the therapeutic use 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 (Kim et al., 2007; Adkison et al., 2008; Keskitalo et al., 2009). 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 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 hours (Fig. 2), and the milk Cmax (0.88 ± 0.32 versus 1.76 ± 0.74 µg/ml, P < 0.05), AUC0–24h values (6.53 ± 1.41 versus 12.73 ± 5.16 µg.h/ml, P < 0.05), and AUC milk/plasma ratio (4.09 ± 1.07 versus 8.81 ± 3.69, P < 0.05) were 2-fold higher in the animals carrying the

Fig. 1. Plasma concentrations of danofloxacin after its intramuscular administration at a dosage of 1.25 mg/kg to Y/Y 581 homozygous and Y/S 581 homozygous lactating cows. The inset shows a semilog plot of the data. Plasma samples were collected at several points throughout 48 hours. Plasma levels of danofloxacin were determined by HPLC. Concentrations at 48 hours were undetectable. The results are presented as means; error bars indicate standard deviation (n = 6).
and greater persistence in milk need to be performed to elucidate the
withdrawal time for danofloxacin is set at 48 hours. Experiments with
reported a higher danofloxacin transport ratio in cells transduced with
relative V\textsubscript{max} values (Merino et al., 2009). Moreover, we recently
showed a 2-fold greater capacity of Y/S animals to transfer the
polymorphism. In addition, the elimination parameters mean residence
time (6.30 ± 0.32 versus 5.72 ± 0.52 hours, P < 0.05) and
elimination half-life (4.36 ± 0.22 versus 3.96 ± 0.36 hours, P < 0.05)
were significantly lower in the Y/S heterozygous animals, as com-
pared with 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 of this
antibiotic into milk. Our findings are in agreement with our previ-
ously published in vitro data showing the greater in vitro capacity of the
SS81 variant compared with the Y581 variant in terms of higher
relative V\textsubscript{max} values (Merino et al., 2009). Moreover, we recently
reported a higher danofloxacin transport ratio in cells transduced with
the SS81 variant in comparison with Y581 cells (Real et al., 2011a).
Withdrawal time for danofloxacin is set at 48 hours. Experiments with
other veterinary ABCG2 substrates with longer withdrawal periods
and greater persistence in milk need to be performed 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 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 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,
impermanent exposure of consumers of dairy products to low levels of
fluoroquinolones 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 with 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 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 the expression of Y581S as
a heterozygous allele, previous in vitro results have been corroborated,
showing a 2-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.

Acknowledgments
The authors thank Dr. Gregorio Alcántara (FEFRICALE), Dr. Juan C.
Boixo (CENSYRA), Dr. Juan J. Arranz (Animal Production Department,
University of Leon), Borja Barrera (Biomedical Sciences Department,
University of Leon), Ana González, and Garfi SAT farm (Santa Maria del
Monto del Condado, Province of Leon, Spain) for technical assistance and support. The authors
are grateful to James McCue and University of Leon Professional Translation
Services for assistance in language editing.

Department of Biomedical Sciences-
Physiology (J.A.O., R.R., A.F., J.G.P.,
A.I.A., G.M.), Instituto de Desarrollo
Ganadero y Sanidad Animal (J.A.O.,
M.M., A.I.A., G.M.), and Instituto de
Biomedicina (J.G.P.), University of
León, Campus de Vegazana, León,
Spain

Jon A. Otero
Rebeca Real
Álvaro de la Fuente
Julio G. Prieto
Margarita Marqués
Ana I. Álvarez
Gracia Merino

Authorship Contributions
Participated in research design: Merino, Marqués, Álvarez, Prieto.
Conducted experiments: Otero, Real, de la Fuente.
Performed data analysis: Otero, Merino.
Wrote or contributed to the writing of the manuscript: Otero, Álvarez,
Marqués, Merino.

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Table 1
Mean ± S.D. 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>
<thead>
<tr>
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<th>Y/Y 581</th>
<th>Y/S 581</th>
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<tbody>
<tr>
<td>Plasma</td>
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<tr>
<td>AUC\textsubscript{0-\infty} (\mu g/ml)</td>
<td>1.59 ± 0.29</td>
<td>1.44 ± 0.12</td>
</tr>
<tr>
<td>C\textsubscript{max} (\mu g/ml)</td>
<td>0.19 ± 0.02</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>5.95 ± 1.29</td>
<td>5.42 ± 0.33</td>
</tr>
<tr>
<td>T\textsubscript{max} (h)</td>
<td>1.33 ± 0.47</td>
<td>1.50 ± 0.50</td>
</tr>
<tr>
<td>T\textsubscript{1/2, el} (h)</td>
<td>4.12 ± 0.89</td>
<td>3.75 ± 0.23</td>
</tr>
<tr>
<td>Milk</td>
<td></td>
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<tr>
<td>AUC\textsubscript{0-\infty} (\mu g/ml)</td>
<td>6.53 ± 1.41</td>
<td>12.73 ± 5.16*</td>
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<tr>
<td>C\textsubscript{max} (\mu g/ml)</td>
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<tr>
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</tr>
<tr>
<td>AUC milk/plasma</td>
<td>4.09 ± 1.07</td>
<td>8.81 ± 3.69*</td>
</tr>
</tbody>
</table>

AUC, area under the curve; MRT, mean residence time; T\textsubscript{1/2, el}, elimination half-life; T\textsubscript{max},
time to peak concentration.
* P < 0.05, significantly different between the two groups of animals.

Fig. 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 throughout 48 hours.
Milk levels of danofloxacin were determined by HPLC. Concentrations at 48 hours
were undetectable. The results are presented as means; error bars indicate standard
deviation (n = 6).

TABLE 1
Mean ± S.D. 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

the bovine ABCG2 gene with a major effect on the QTL on chromosome 6 affecting milk yield and composition in Holstein cattle. *Genome Res* **15**:936–944.


**Address correspondence to:** Dr. Gracia Merino, Department of Biomedical Sciences-Physiology, University of Leon, Campus de Vegazana 24071, Leon, Spain, E-mail: gmeri@unileon.es