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Role of ABCG2 in secretion into milk of the anti-inflammatory flunixin and its main metabolite: in vitro-in vivo correlation in mice and cows

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Running title: flunixin and ABCG2 interaction

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Abbreviations: ABC, ATP- binding cassette; ABCG2, ATP-binding cassette subfamily G2; FLU, flunixin; 5OH-FLU, 5-hydroxyflunixin; NSAID, nonsteroidal anti-inflammatory drug; MDCKII, Madin-Darby canine kidney epithelial cell; HPLC, High performance liquid chromatography; UPLC-qTOF-MS, ultra-performance liquid chromatography quadrupole time of flight mass spectrometry.

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ABSTRACT

Flunixin meglumine is a nonsteroidal anti-inflammatory drug (NSAID) widely used in veterinary medicine. It is indicated to treat inflammatory processes, pain and pyrexia in farm animals. In addition, it is one of the few NSAIDs approved for use in dairy cows, and consequently gives rise to concern regarding its milk residues. The ABCG2 efflux transporter is induced during lactation in the mammary gland and plays an important role in the secretion of different compounds into milk. Previous reports have demonstrated that bovine ABCG2 Y581S polymorphism increases fluoroquinolone levels in cow milk. However, the implication of this transporter in the secretion into milk of anti-inflammatory drugs has not yet been studied. The objective of this work was to study the role of ABCG2 in the secretion into milk of flunixin and its main metabolite, 5-hydroxyflunixin, using Abcg2$^{(-/-)}$ mice, and to investigate the implication of the Y581S polymorphism in the secretion of these compounds into cow milk. Correlation with the in vitro situation was assessed by in vitro transport assays using MDCKII cells overexpressing murine and the two variants of the bovine transporter. Our results show that flunixin and 5-hydroxyflunixin are transported by ABCG2 and that this protein is responsible for their secretion into milk. Moreover, the Y581S polymorphism increases flunixin concentration into cow milk, but it does not affect milk secretion of 5-hydroxyflunixin. This result correlates with the differences in the in vitro transport of flunixin between the two bovine variants. These findings are relevant to the therapeutics of anti-inflammatory drugs.

Keywords: ABCG2, Flunixin, 5-hydroxiflunixin, milk, Y581S polymorphism

Introduction

The ATP-binding cassette (ABC) sub-family G member 2 (ABCG2) membrane efflux transporter is responsible for excretion from the cells of a wide variety of endogenous compounds, xenobiotics and drugs. It is located in the apical membrane of epithelial cells of several tissues and organs, including the intestine, liver and mammary gland (van Herwaarden and Schinkel, 2006). This protein transporter therefore participates in drug-drug interactions, drug adverse effects and even drug efficacy (Mealey, 2013; Lee et al., 2015; Robey et al., 2018).

In addition to the drug physico-chemical properties related to membrane diffusion, ABCG2 is considered an important factor affecting drug transfer into milk (Ito et al., 2015). Its induced expression in mammary gland during lactation plays an important role in the active secretion into milk of different compounds (Jonker et al., 2015) such as antibiotics (Otero et al., 2013; 2016), carcinogens (van Herwaarden and Schinkel, 2006), antiparasitics (Mahnke et al., 2016) and natural compounds (Miguel et al., 2014; Garcia-Mateos et al., 2017) among others.

Genetic variants of ABCG2 can alter the pharmacokinetics and bioavailability of its substrates (Mealey et al., 2013; Rocha et al., 2018). In the human species, there are some ABCG2 polymorphisms associated with a lower function of this transporter. They are related with diseases and adverse drug effects in the population, such as Q141K (Tu et al., 2018) or V12M (Tamura et al., 2012). In the veterinary field, the bovine ABCG2 Y581S single nucleotide polymorphism is widely spread in the Holstein population (Ron et al 2006). It is gain-of-function polymorphism (Merino et al., 2009) that also affects milk production and fat, protein and lactose content of milk (Cohen- Zinder et al., 2005; Sanchez et al., 2017; Lopdell et al., 2017). Our previous in vitro studies using transduced cell models have revealed differential antimicrobial transport activity depending on the genetic variant (Real et al., 2011). In vivo pharmacokinetic studies with Y/Y homozygous and Y/S heterozygous cows have shown differences in secretion into milk of fluoroquinolones such as danofloxacin, enrofloxacin and ciprofloxacin between both groups of animals (Otero et al., 2013; 2015; 2016).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in human and veterinary medicine for their pharmacological effects such as analgesic, anti-inflammatory and

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antipyretic actions. These drugs inhibit cyclooxygenase enzymes (COX) in the arachidonic acid cascade, which are involved in the biosynthesis of prostaglandins (Landoni et al., 1995; Cheng et al., 1998; Lees et al., 2004).

Flunixin (FLU) is the most important NSAIDs used in cattle (Fajt et al., 2011). It is the only anti-inflammatory agent authorized in the USA for use in milking cows (FDA, 2004) to treat pyrexia and endotoxemia associated with mastitis and it is one of the few NSAIDs labeled for intravenous route authorized in lactating cows in the European Union (EMEA, 2000). However, NSAIDs are drugs under regulation in food animals due to the presence of residues that pose a serious risk for consumers (Smith et al., 2008). In the case of FLU therapeutics, 5-hydroxyflunixin (5OH-FLU) was identified as the main FLU metabolite and the marker residue in bovine milk (Feely 2002) with a maximum residue limit (MRL) of 40 μg/kg established by EMEA (2000) and of 2 ppb by FDA (2004). In fact, 5OH-FLU levels are higher than FLU levels in cow milk (Feely, 2002), probably due in part to their different physico-chemical properties and extensive liver metabolism of FLU. Nevertheless, some studies have shown that alteration of the dose or route of administration, or the presence of disease processes, could affect the presence of violative residues even after withdrawal periods are finished (Kissell et al., 2015; Smith et al., 2015; Shelver et al., 2016).

Knowledge of the factors affecting the presence of residues of these compounds into milk is therefore relevant for therapeutics and human health.

The objective of our study was to investigate the role of ABCG2 in the pharmacokinetics and secretion into milk of FLU and its main metabolite, 5OH-FLU, using Abcg2$^{(-/-)}$ mice, and to research the effect of the bovine ABCG2 Y581S polymorphism in FLU and 5OH-FLU plasma and milk levels. Correlation of our in vivo data with in vitro cells studies involving murine Abcg2 and the two bovine ABCG2 variants (Y581 and S581) was also assessed.

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Materials and methods

Reagents and Chemicals. FLU, 5OH-FLU, diclofenac sodium, niflumic acid and Lucifer Yellow were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Ko143 was obtained from Tocris (Bristol, United Kingdom). For the pharmacokinetic studies, flunixin meglumine (Finadyne® 50 mg/ml) was obtained from MSD Animal Health SL (Salamanca, Spain).

Cell culture. MDCK-II (Madin-Darby canine kidney epithelial cell) parental cells and its Abcg2 murine transduced subclone were supplied by Dr. Schinkel (The Netherlands Cancer Institute). Generation and characterization of MDCK-II subclones transduced with the Y581 and S581 variants have been reported previously by our research group (Real et al., 2011). Cell culturing was performed as described elsewhere (Gonzalez-Lobato et al., 2014).

Transport studies. Transepithelial transport assays were carried out as previously described with minor modifications (Pavek et al., 2005; Real et al., 2011) using parental MDCK-II, Abcg2 murine-transduced subclones and bovine ABCG2-transduced subclones. At the end of the experiment, confluence of the monolayer was checked with Lucifer Yellow permeability assays (Oltra-Noguera et al., 2015).

At the beginning of the experiment (t=0) medium was substituted in apical or basolateral compartment with fresh medium including 20 µM of FLU or 10 µM of 5OH-FLU, with or without Ko143. Concentrations were chosen based on sensitivity of HPLC analysis. Hanks´ Balanced Salt solution (Sigma- Aldrich, St. Louis, Missouri, USA) supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES (25 mM) pH 7.4 was used as transport medium. Aliquots of 100 µl were sampled each hour up to 4 h from the opposite compartment and the same volume of culture media was replaced each time. Samples were kept at -20ºC until analysis.

Active transport across MDCK-II cells was reported as the relative transport ratio, determined as the concentration permeated towards apical compartment (basolateral to apical transport) divided by the concentration permeated towards basolateral compartment (apical to basolateral transport) at 4 h.

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Animal Experiment. European legislation (2010/63/EU) was applied to animal handling. Experimental methods were approved by the Animal Care and Use Committee of University of Leon and Junta de Castilla y Leon (ULE_011_2016 and ULE_002_2017).

Milk Secretion Studies in Mice. Lactating female Abcg2$^{(-/-)}$ and wild-type mice (N =3-7, 9-13 weeks of age) were supplied by Dr. Schinkel (The Netherlands Cancer Institute). They were maintained in an environment controlled for temperature and light/dark cycle.

Pups around 10 days old were removed from their mothers 4 h before the experiment. For i.v. administration of FLU or 5OH-FLU (4 mg/kg), 150 µl of drug solution/30 g of bw was administered into the tail of mice under anesthesia with isoflurane. Drug solution consisted of 10% (v/v) Finadyne® and 90% (v/v) saline solution for FLU experiments. In the case of 5-OH FLU, the compound was dissolved in 10% (v/v) ethanol, 40% (v/v) polyethylene glycol 400 and 50% saline. Milk and plasma samples were collected using the method reported by Miguel et al., (2014).

Pharmacokinetic Studies on Dairy Cows. Cows were kept on a private farm near Leon, Spain. Twelve lactating Holstein cows weighing ~ 800 kg were used. Milk yield was 41.96 ± 6.67 kg/d. The Y581S genotypes were determined in accordance with Komisarek and Dorynek (2009). Accordingly, two groups of six Y/Y581 homozygous and six Y/S581 heterozygous cows were defined for experimental design. No differences were found in age, weight or milk yield between the two sets of cows. Both groups were treated i.m. with 2.2 mg/kg of flunixin meglumine (Finadyne®, MSD Animals Health SL, Salamanca, Spain). Blood samples (5 ml) from the tail vein were obtained at 1, 3, 6, 14, 24, 30 and 38 h after administration. Samples of milk were manually taken at 3 h and using an automatic milking machine during normal milking at 6, 14, 24, 30, 38, 48 and 54 h. No more sampling points were possible because of the private nature of the farm. Plasma was obtained by centrifugation and samples were kept at -20ºC until analysis.

HPLC Analysis. Determination of concentration of FLU and its main metabolite 5OH-FLU in plasma and milk samples from mouse experiments and samples of transport assays were based on previously published methods (Gallo et al., 2008) with some modifications using

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diclofenac as an internal standard (IS). Mouse samples were cleaned-up method described by Odensvik and Johansson (1995) with some modifications. Samples of culture medium were analysed without processing. A 4µm particle 250 X 4.6 mm 80 A Synergi column (Phenomenex, Torrance, CA, USA) was used for the chromatographic separation. In transepithelial transport assays, LOD (limit of detection) and LOQ (limit of quantification) obtained for FLU were 0.01 µg/ml and 0.03 µg/ml and 0.008 µg/ml and 0.02 µg/ml for 5OH-FLU, respectively.

In the mouse experiments LOD and LOQ obtained for FLU in plasma were 0.15 µg/ml and 0.39 µg/ml and in milk 0.05 µg/ml and 0.11 µg/ml. For 5OH-FLU LOD and LOQ plasma were 0.05 µg/ml and 0.12 µg/ml and, 0.03 µg/ml and 0.07 µg/ml respectively in milk.

UPLC-QTOF-MS analysis. Determination of concentration of FLU and its main metabolite 5OH-FLU in plasma and milk samples from pharmacokinetics studies in dairy cattle were based on previously published methods (Rubies et al., 2016) with some modifications.

Plasma and milk samples must be processed before injection into the UPLC system following the clean-up method previously described by Kissell et al., (2015) using niflumic acid as an IS. Analyses were performed using a Waters ACQuity UPLC H-Class system coupled to a ACQuity TQD- Tandem quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). A Phenomenex Kinetex C18 column (2.6 µm, 100 x 3.00 mm) was used for the chromatographic separation. Experiments were performed in positive ion mode at 313, 297 and 283 m/z for 5OH-FLU, FLU and niflumic acid respectively. Masslynx™ software (version 4.1, Waters Corporation) was used for data acquisition and processing.

Plasma LOD was 2.84 ng/ml for FLU and 4.83 ng/ml for 5OH-FLU, whereas the LOQs were 7.67 ng/ml and 6.68 ng/ml for FLU and 5OH-FLU, respectively. The milk LODs were 0.17 ng/ml and 1.42 ng/ml for FLU and 5OH-FLU and the LOQs were 0.41 ng/ml and 3.96 ng/ml for FLU and 5OH-FLU, respectively.

Pharmacokinetic calculations and statistical analysis. Pharmacokinetic parameters were determined as reported elsewhere (Otero et al., 2018).

Results are reported as the mean ± standard deviation (SD). Normal distribution of data was tested by the Shapiro-Wilk test. Data with normal distribution was analyzed by the two-tailed Student´s test. Data with non-normal distribution were tested using Mann-Whitney U test. Differences were considered statistically significant when $P < 0.05$. Statistical analyses were carried out with the SPSS 24 software.

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Results

In vitro interaction of FLU and its main metabolite with the ABCG2 transporter. To study the in vitro interaction between ABCG2 and NSAID FLU and its main metabolite 5OH-FLU, transepithelial transport assays were carried out using parental MDCKII and their subclones transduced with murine and two variants of bovine ABCG2 (Figs. 1 and 2).

In the parental MDCKII cells, apical to basolateral directed translocation was equal to basolateral to apical translocation in FLU and 5OH-FLU assays (Figs. 1A and 2A). We observed basolateral to apical preferential transport of FLU and 5OH-FLU in murine Abcg2 and bovine ABCG2 transduced cells compared with the parental cells (Figs. 1B-D and 2B-D). Relative efflux transport ratios at 4 h (r) were significantly higher for FLU (34.00 ± 7.44 in murine Abcg2, 1.47 ± 0.05 in bABCG2 Y581 and 2.09 ± 0.33 in bABCG2 S581) compared to parental cells (1.08 ± 0.26) ($P < 0.05$). In the case of OH-FLU, these ratios were also significantly increased (4.30 ± 2.69 in murine Abcg2, 2.19 ± 0.46 in bABCG2 Y581 and 2.14 ± 0.65 in bABCG2 S581) compared with control cells (0.89 ± 0.14) ($P < 0.05$). When the selective ABCG2 inhibitor Ko143 was added the basolateral to apical preferential transport was completely reversed in all ABCG2-transduced subclones (Figs. 1E-H, 2E-H). Furthermore, in the case of FLU the relative efflux transport ratio (r) was significantly higher in the S581 bovine variant compared to the Y581 variant (Figs. 1C and D, 2.09 ± 0.33 vs. 1.47 ± 0.05, respectively) ($P < 0.05$), indicating that the S581 variant was more efficient in FLU transport. In contrast to the FLU results, no significant differences between the two both bovine ABCG2 variants were found for 5OH-FLU (Figs. 2C and D).

These results show that murine and bovine ABCG2 play an important role in the active efflux transport of the NSAIDS FLU and its main metabolite 5OH-FLU. Moreover, FLU is transported more efficiently by the bovine S581 variant compared to the Y581 variant. This difference was not reported for 5OH-FLU.

Plasma and milk levels of FLU and 5OH-FLU in Abcg2$^{(-/-)}$ knockout mice. To study the involvement of Abcg2 in plasma and milk levels of these compounds, we administered FLU

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or 5OH-FLU to wild-type and Abcg2$^{(-/-)}$ mice (Fig. 3). No 5OH-FLU was detected in plasma and milk after FLU administration.

No significant differences were observed in plasma concentration of FLU and 5OH-FLU after treatment in wild-type and Abcg2$^{(-/-)}$ mice. However, milk concentrations were more than 2.5-fold higher in wild-type mice compared with Abcg2$^{(-/-)}$ mice for FLU (1.85 ± 0.29 μg/ml vs. 0.72 ± 0.18 μg/ml, $P < 0.01$) and approximately 8-fold higher in wild-type compared to Abcg2$^{(-/-)}$ mice for 5OH-FLU (1.82 ± 0.90 μg/ml vs. 0.23 ± 0.17 μg/ml, $P < 0.01$). Milk-to-plasma ratio of FLU in wild-type mice was more than 3-fold higher than in Abcg2$^{(-/-)}$ mice (0.43 ± 0.17 vs. 0.14 ± 0.02, $P < 0.05$). In the case of 5OH-FLU, milk-to-plasma ratio was 5-fold higher in wild-type compared to Abcg2$^{(-/-)}$ lactating mice (0.58 ± 0.25 vs. 0.11 ± 0.08, $P < 0.01$). Our results clearly show that Abcg2 plays an important role in the active secretion into milk of FLU and 5OH-FLU.

Plasma pharmacokinetics and secretion into milk of FLU and 5OH-FLU in dairy cows carrying the Y581S polymorphism. Effect of the Y581S polymorphism on plasma levels and secretion into milk of the NSAID FLU and its main metabolite 5OH-FLU was studied using Y/Y581 homozygous and Y/S581 heterozygous lactating cows (Figs. 4 and 5), after the administration of a single dose of Finadyne® at 2.2 mg/kg b.w. by intramuscular administration. Plasma profile was dominated by the parental drug FLU. No significant differences in plasma levels (Fig. 4) and in plasma pharmacokinetics parameters (Table 1) were observed between the two groups of animals.

Milk profile was dominated by 5OH-FLU (Fig. 5). No significant differences were obtained in milk levels and milk pharmacokinetics parameters for 5OH-FLU between the two groups of cows. Only half-life ($t_{1/2}$) was significantly different (Table 2). However, significant differences for the secretion of FLU into milk between both groups of animals were shown (Fig. 4). Milk levels for FLU were significantly higher for Y/S581 compared with Y/Y581 animals at 6, 14 and 24 h ($P < 0.05$). In addition, area under de curve ($AUC_{0-\infty}$) and C_{max} values were approximately 1.5-fold higher in Y/S581 animals than in Y/Y581 animals (104.67 ± 25.86 vs. 69.53 ± 9.61 ng·h/ml for $AUC_{0-\infty}$ and 10.42 ± 2.99 vs. 7.15 ± 1.43 ng/ml for C_{max}, Table 2).

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MRT was around 1.2-fold higher in cows carrying Y581S polymorphism ($P < 0.05$) (9.15 ± 0.88 vs. 7.82 ± 0.52 h) (Table 2). In addition, significant differences were observed between two groups in $AUC_{0-\infty}$ milk-to-plasma ratios for FLU.

Our results show that the bovine Y581S ABCG2 polymorphism increases the milk secretion of the parental compound FLU in dairy cattle; however, it does not affect secretion of 5OH-FLU into milk.

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Discussion

Several pharmacokinetics and residue researches and surveys have been previously undertaken with FLU and 5OH-FLU on different farm species such as dairy cows (Jedziniak et al., 2009, 2013; Deyrup et al., 2012; Kissell et al., 2012; 2013; 2015), sheep (Marini et al., 2016) and goats (Köningsson et al., 2003). However, this is the first study to explore the in vivo role of ABCG2 transporter in these processes for an anti-inflammatory drug in farm animals.

The use of knockout mice as a first step in the study of in vivo interaction with drug transporters has been validated (Giacomini et al., 2010) and it is a widely used model to test the in vivo relevance of some transporters such as P-glycoprotein or ABCG2 in drug pharmacokinetics and secretion into milk (Vlaming et al., 2009). Therefore, as a preclinical set up, our results with knockout mice show that Abcg2 is implicated in the secretion of FLU and 5OH-FLU into milk with higher secretion in wild-type mice than in Abcg2$^{(-/-)}$ mice (Fig. 3). The effect of compensatory changes in the expression of proteins that participate in metabolism in the Abcg2$^{(-/-)}$ mice cannot be completely excluded. However, evidence of such problematic effects in previous studies has never been shown.

With this outcome we have added the anti-inflammatory FLU and its main metabolite 5OH-FLU to the list of compounds with an Abcg2-mediated secretion into milk. These results are in accordance with our in vitro findings, which report that FLU and 5OH-FLU were also effectively transported by murine and bovine ABCG2 (Figs. 1 and 2). The very high relative transport ratio in the case of murine Abcg2-mediated transport of FLU is noteworthy (34.00 ± 7.44). All these data suggest that ABCG2 active transport is involved in the secretion of these compounds into milk.

Regarding the effect of the bovine Y581S ABCG2 polymorphism in the secretion into milk of these compounds, a pharmacokinetic study using cows carrying this polymorphism was designed. Our results confirm that the predominant compound detected in plasma was FLU (Odensvik and Johansson, 1995; Jedziniak et al., 2007; Kissel et al., 2015). Plasma pharmacokinetics parameters (Table 1) in both groups of lactating cows were similar to those obtained in previous studies in dairy cattle (Rantala et al., 2002; Jedziniak et al., 2007).

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However, no significant differences in both groups of animals were obtained for FLU and 5OH-FLU in plasma levels and pharmacokinetics parameters (Fig. 4 and Table 1).

Moreover, our data regarding milk levels are in good agreement with previous publications that describe 5OH-FLU as the main FLU metabolite presented into milk (Feely et al., 2002; Ngoh et al., 2003; Daeseleire et al., 2003; Kissell et al., 2012, 2015; Jedziniak et al., 2013). A large amount of 5OH-FLU appeared rapidly in cow milk following administration of FLU. 5OH-FLU milk levels presented a fast rate of elimination after 24 h of treatment (Ngoh et al., 2003; Jedziniak et al., 2009; 2013, Kissell et al., 2012, 2015). This decrease is more pronounced in intravenous route than in other routes (Kissell et al., 2012). We obtained significant differences between both groups of cows in milk secretion of the parental drug FLU at 6, 14 and 24 h post-treatment and in its milk pharmacokinetics parameters C_{max} and $AUC_{0-\infty}$, with 1.5-fold higher levels in cows carrying the polymorphism than in non-carrier animals (Fig. 5 and Table 2). In addition, the elimination parameter MRT significantly increased in the Y/S581 heterozygous animals compared to the Y/Y581 homozygous animals. These results suggest that milk levels of FLU were higher in the Y/S581 animals and that drug persistence in the milk may be longer, indicating that higher concentrations of this NSAID are secreted in the Y/S581 animals for a longer period of time. These results are in good agreement with previous studies by our group in which we demonstrated higher secretion into milk of several antibiotics such as danofloxacin, ciprofloxacin and enrofloxacin by cows carrying the Y581S polymorphism (Otero et al., 2013; 2015; 2016). However, this is the first time that such an effect has been observed for an anti-inflammatory drug. This contrasts with our results in plasma where no effect of the Y581S polymorphism was observed, probably due to the potential influence of several additional mechanisms at systemic level. In fact, Miyazaki et al., (2001) and Horii et al., (2004) reported that the OATP-2 active membrane transporter was involved in the bioavailability and biodisposition of FLU in rabbits and cats. However, ABCG2 is the only ABC transporter induced in the mammary gland during lactation (Jonker et al., 2005). Organic cation transporters have also been related with drug transfer in mice (Ito et al., 2014). Otero et al., (2013) also reported that no significant differences were found in plasma levels between

Y/Y581 and Y/S581 cows after danofloxacin administration at 1.25 mg/kg b.w., although these authors observed higher milk secretion of this antimicrobial in cows carrying the Y581S polymorphism than in non-carriers.

The difference observed between the two bovine genotypes regarding secretion of FLU into milk is in agreement with our in vitro data, where significant differences in the relative transport ratio of FLU at 4 h were found between cells transduced with Y581 and S581 variants, with higher apically directed transport in the polymorphic variant (Figs. 1C and D). However, no differences in the basolateral to apical transport of 5OH-FLU between the two bovine ABCG2 variants were observed (Figs. 2C and D) which is in agreement with the lack of difference in the secretion of 5OH-FLU into milk between the two groups of cows (Fig. 5).

All these data indicate that in vitro studies using MDCKII cells are strong and useful tools to research and predict in vivo results. Previous studies have reported the use of in vitro systems for prediction and monitoring the concentration of drug residues in ruminant milk mediated by ABCG2 (Real et al., 2011; Wassermann et al., 2013; Gonzalez-Lobato et al., 2014).

Our results show that ABCG2 is implicated in the secretion of FLU and 5OH-FLU into milk. This knowledge will help to understand and to manage the presence of anti-inflammatory drugs and their metabolite residues in milk. Although there is no available information, to the best of our knowledge, about the pharmacological activity of 5-OH-FLU, food residues of these compounds are deemed a potential risk to consumers and MRLs for them in food products of animal origin, including milk, were established (EMEA, 2000; FDA, 2004).

Apart from the genetic Y581S polymorphism, several factors influence the activity of ABCG2, including gender, diet compounds and coadministration with drugs that interact with the transporter. In fact, ABCG2-mediated secretion of drugs into milk can be greatly diminished by administration of ABCG2 inhibitors present in the diet such as flavonoids or lignans. This effect has been shown with ABCG2 substrates such as fluoroquinolones and nitrofurantoin in ruminants. This antimicrobial secretion into milk was decreased using flavonoids (soy-enriched diet) or lignans (flaxseed-enriched diet) (Pulido et al., 2006; Pérez et al., 2013; Otero et al.,

2018). Regarding drug modulation of milk residues, the milk secretion of the antimicrobial danofloxacin was reduced by coadministration of the macrocyclic lactone ivermectin, an ABCG2 inhibitor (Real et al., 2011). Moreover, the coadministration of the anthelmintic triclabendazole, another ABCG2 inhibitor, with moxidectin reduced milk levels of this macrocyclic lactone (Barrera et al., 2013). Therefore, active transport of FLU and its main metabolite into milk by ABCG2 may have implications in the coadministration of FLU with other drugs or natural feed components, which also interact with ABCG2, in the treatment of dairy cows, because it may affect milk levels of drug residues with an important health risk for human consumption.

Our findings are a new step toward the identification of factors which alter drug exposure in livestock and which are involved in interindividual variability. This newly discovered factor involved in interindividual disposition variability of anti-inflammatory drugs could potentially affect treatment efficacy and contribute to milk residues.

In conclusion, we demonstrate that ABCG2 is involved in the secretion into milk of FLU and 5OH-FLU and that the bovine Y581S variant increases the levels of FLU in milk, but does not affect 5OH-FLU milk levels. Furthermore, our results obtained from the pharmacokinetics and milk secretion studies with mice and dairy cattle are in agreement with our in vitro assays.

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Authorship contributions

Participate in research design: Merino, Alvarez, Garcia-Mateos.

Conducted experiments: Garcia-Mateos, Garcia-Lino, De la Fuente, Alvarez-Fernandez, Blanco-Paniagua.

Performed data analysis: Merino, Garcia-Mateos.

Wrote or contributed to the writing of the manuscript: Merino, Alvarez, Garcia-Mateos.

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Footnotes

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Figures legends

Fig. 1. Transepithelial transport assay of FLU at 20 µM in parental MDCKII cells and its subclones transduced with murine Abcg2 and the two bovine variants; Y581 variant and S581, in the absence (A, B, C, D) or presence (E, F, G, H) of the specific ABCG2 inhibitor Ko143. The experiment was started (t=0) by replacing the medium in either the apical or basolateral compartment with fresh culture medium containing 20 µM of FLU. Aliquots of 100 µL were taken from the opposite compartment at 1, 2, 3 and 4 h and concentrations were measured by HPLC. Concentration permeated towards apical compartment represents basolateral to apical transport (●), concentration permeated towards basolateral compartment represents apical to basolateral transport (□). Results are represented as mean ± SD. "r" represents relative transport ratio, basolateral to apical transport divided by apical to basolateral transport at 4 h (n=3-6).

Fig. 2. Transepithelial transport assay of 5OH-FLU at 10 µM in parental MDCKII cells and its subclones transduced with murine Abcg2 and the two bovine variants; Y581 variant and S581, in the absence (A, B, C, D) or presence (E, F, G, H) of the specific ABCG2 inhibitor Ko143. The experiment was started (t=0) by replacing the medium in either the apical or basolateral compartment with fresh culture medium containing 10 µM of 5OH-FLU. Aliquots of 100 µL were taken from the opposite compartment at 1, 2, 3 and 4 h and concentrations were measured by HPLC. Concentration permeated towards apical compartment represents basolateral to apical transport (●), concentration permeated towards basolateral compartment represents apical to basolateral transport (□). Results are represented as mean ± SD. "r" represents relative transport ratio, basolateral to apical transport divided by apical to basolateral transport at 4 h (n=3-6).

Fig. 3. Plasma and milk concentration and milk-to-plasma ratio of FLU (A) and 5OH-FLU (B) in Abcg2$^{(-/-)}$ mice after their intravenous administration at a dose of 4 mg/kg b.w. Plasma and milk were collected 40 min after administration of FLU and 20 min after 5OH-FLU treatment. Milk and plasma levels were determined by HPLC. Results are means ± SDs. * $P < 0.05$ and ** $P < 0.01$ significant differences between both groups of mice. (n=3-6).

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Fig. 4. Plasma concentration of FLU and 5OH-FLU after IM administration of a single dose of Finadyne® at 2.2 mg/kg b.w. to Y/Y 581 homozygous and Y/S 581 heterozygous lactating cows. Plasma samples were collected at various times over 38 h. Plasma levels were determined by UPLC-MS/MS. The results are presented as means ± SDs. (n=6). * $P < 0.05$ significant differences between both groups of cows.

Fig. 5. Milk concentration of FLU and 5OH-FLU after IM administration of a single dose of Finadyne® at 2.2 mg/kg b.w. to Y/Y 581 homozygous and Y/S 581 heterozygous lactating cows. Milk samples were collected at various times over 54 h. Milk levels were determined by UPLC-MS/MS. Concentrations from 30 h were undetectable. The inset shows FLU concentrations alone. The results are presented as means ± SDs. (n=6). * $P < 0.05$ and ** $P < 0.01$ significant differences between both groups of cows.

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Table 1. Plasma pharmacokinetic parameters (means ± SD) in Y/Y 581 homozygous and Y/S581 heterozygous dairy cows after administration of a single dose of Finadyne® at 2.2 mg/kg (n=6).

Pharmacokinetic parameters	5OH-FLU		FLU	
	Y/Y 581	Y/S 581	Y/Y 581	Y/S 581
$AUC_{0-\infty}$ (ng·h/ml)	1396.3 ± 445.2	1039.3 ± 307.3	10949.3 ± 2133.3	11815.0 ± 2018.0
C_{max} (ng/ml)	79.25 ± 25.14	70.33 ± 14.06	1600.7 ± 330.4	1494.6 ± 359.2
T_{max} (h)	1.00 ± 0.00	1.33 ± 0.82	1.00 ± 0.00	1.33 ± 0.82
$T_{1/2\ el}$ (h)	12.20 ± 4.04	9.60 ± 1.60	4.60 ± 0.37	5.49 ± 1.06
MRT (h)	19.98 ± 5.12	14.98 ± 3.98	5.55 ± 0.80	7.13 ± 1.78

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Table 2. Milk pharmacokinetic parameters (means ± SD) in Y/Y 581 homozygous and Y/S581 heterozygous dairy cows after administration of a single dose of Finadyne® at 2.2 mg/kg (n=6).

Pharmacokinetic parameters	5OH-FLU		FLU	
	Y/Y 581	Y/S 581	Y/Y 581	Y/S 581
$AUC_{0-\infty}$ (ng·h/ml)	585.0 ± 134.4	645.2 ± 202.4	69.53 ± 9.61	104.7 ± 25.86*
C_{max} (ng/ml)	56.10 ± 13.39	54.83 ± 10.40	7.15 ± 1.43	10.42 ± 2.99*
T_{max} (h)	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
$T_{1/2\ el}$ (h)	5.42 ± 0.63	6.59 ± 1.03*	4.80 ± 0.84	4.98 ± 0.82
MRT (h)	9.97 ± 0.81	10.78 ± 1.81	7.82 ± 0.52	9.15 ± 0.88**
AUC milk/plasma	0.450 ± 0.168	0.684 ± 0.319	0.007 ± 0.001	0.009 ± 0.002*

* $P < 0.05$ and ** $P < 0.01$, significantly different from the Y/Y581 group.

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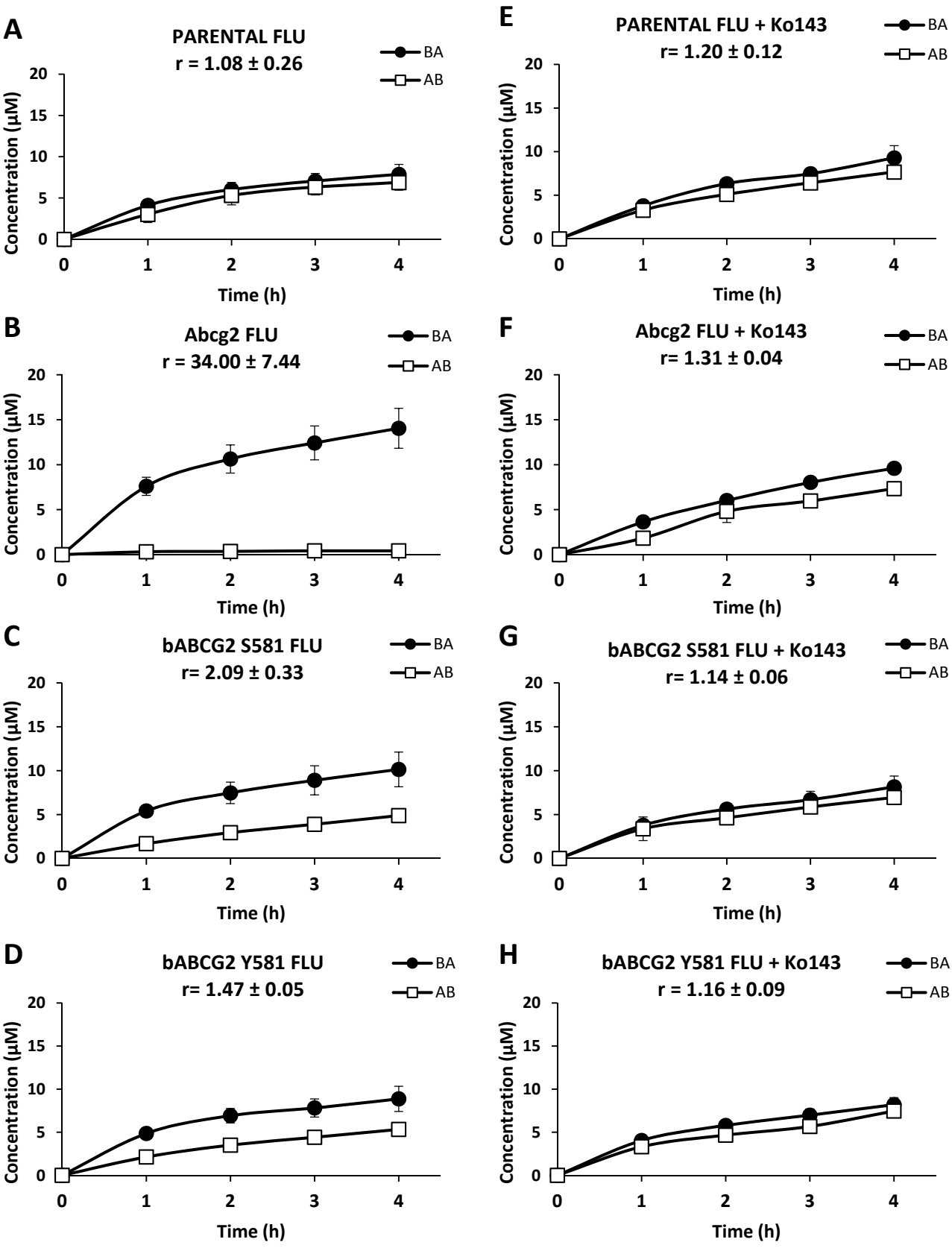

Fig. 1

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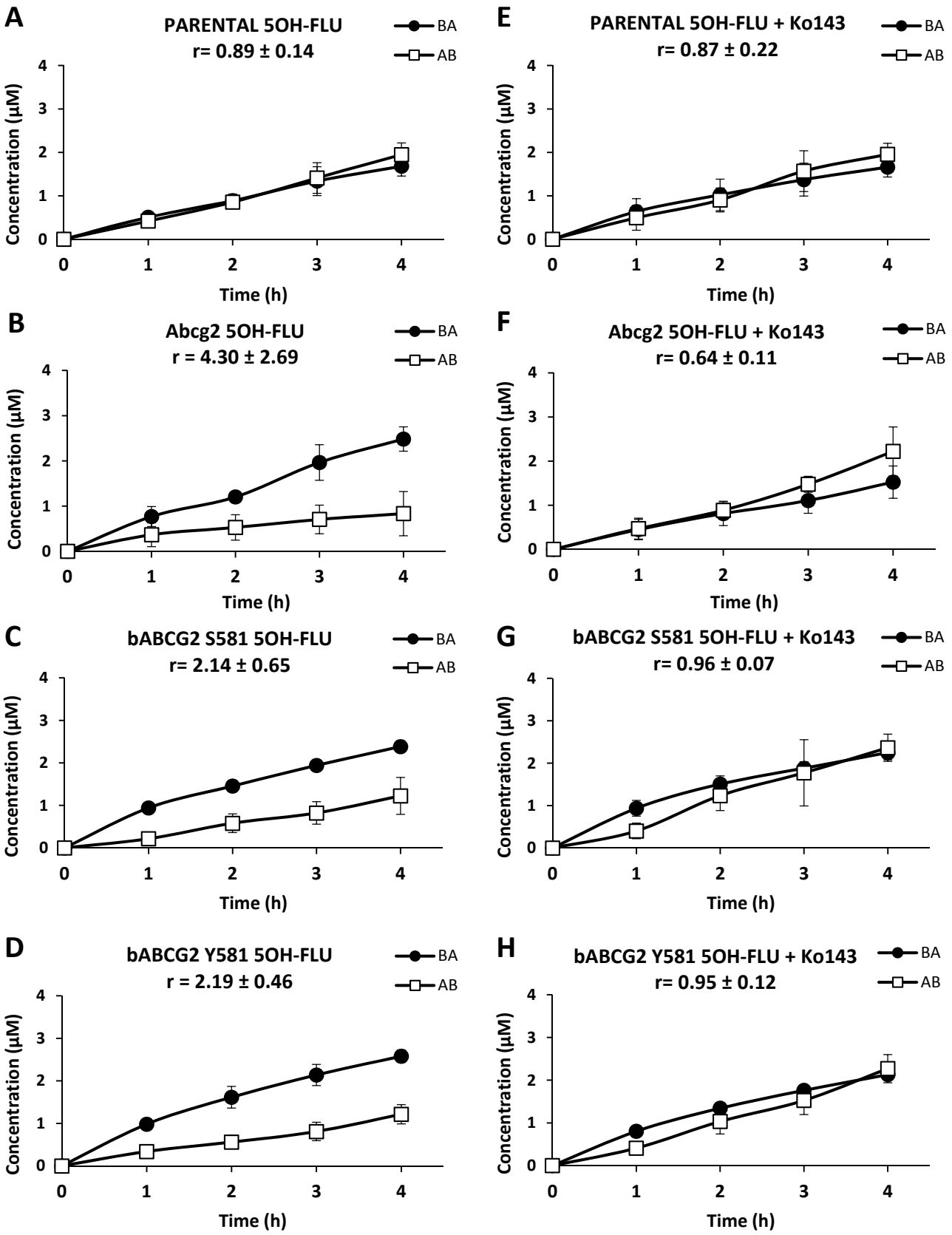

Fig. 2

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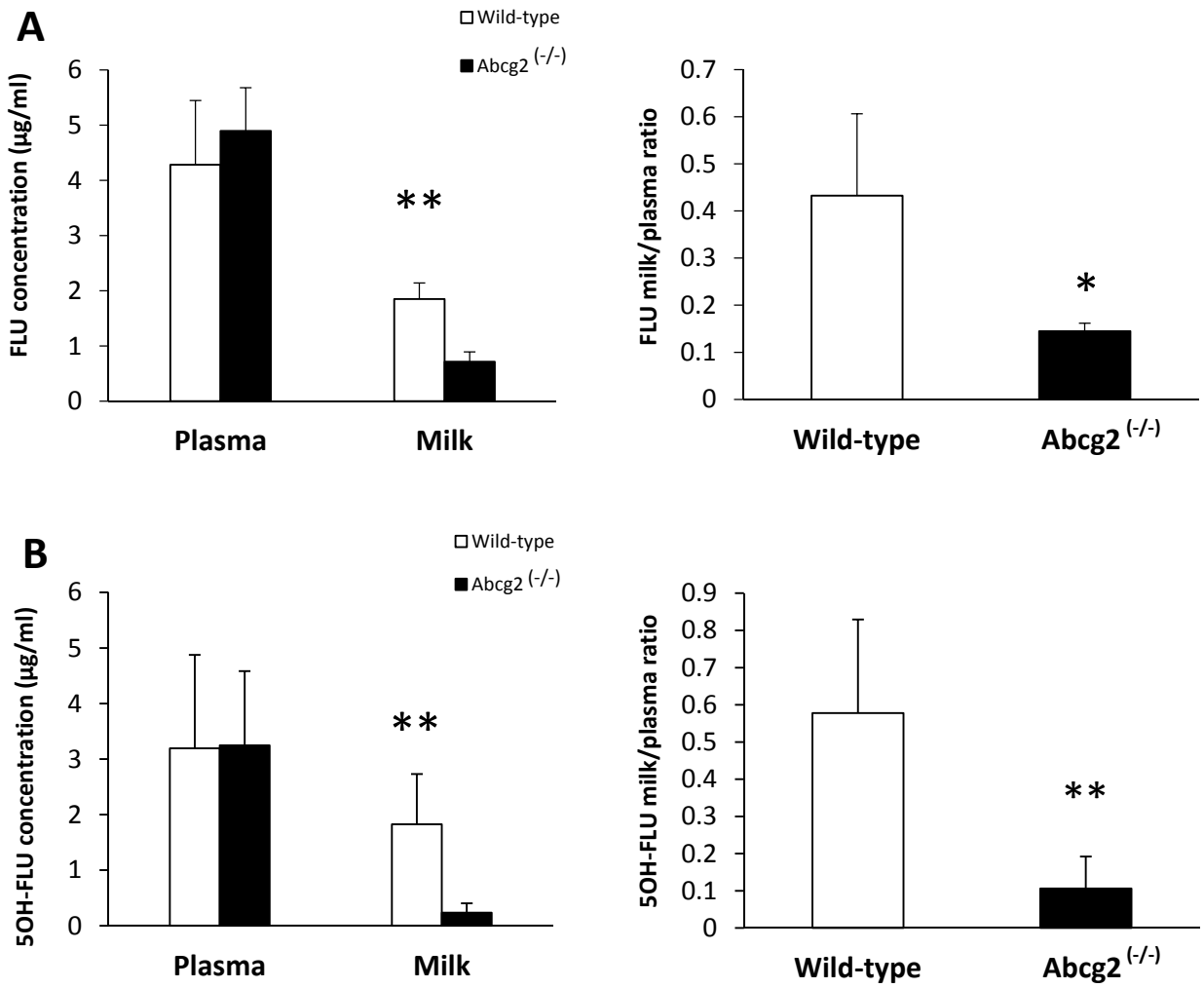

Fig. 3

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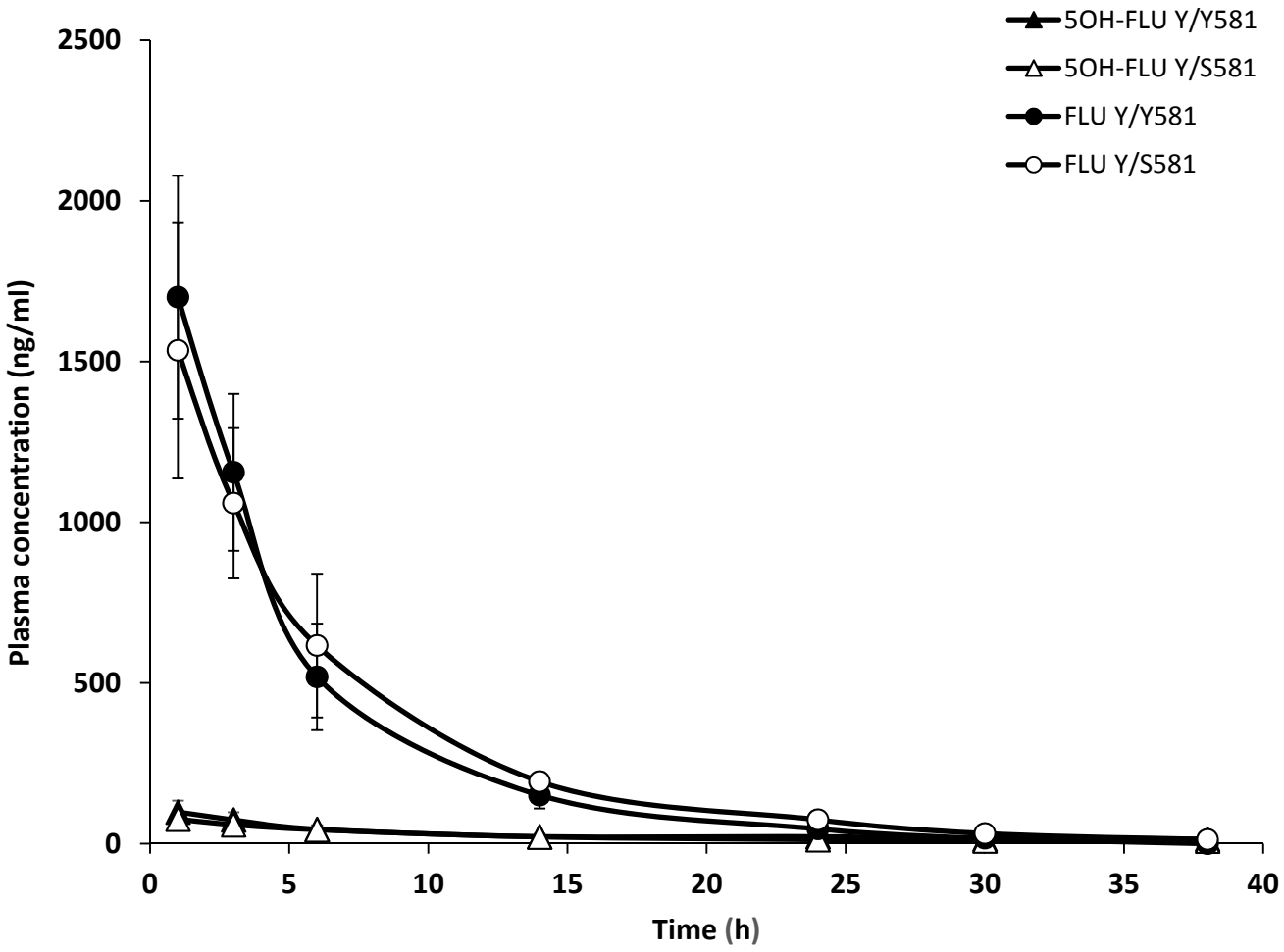

Fig 4.

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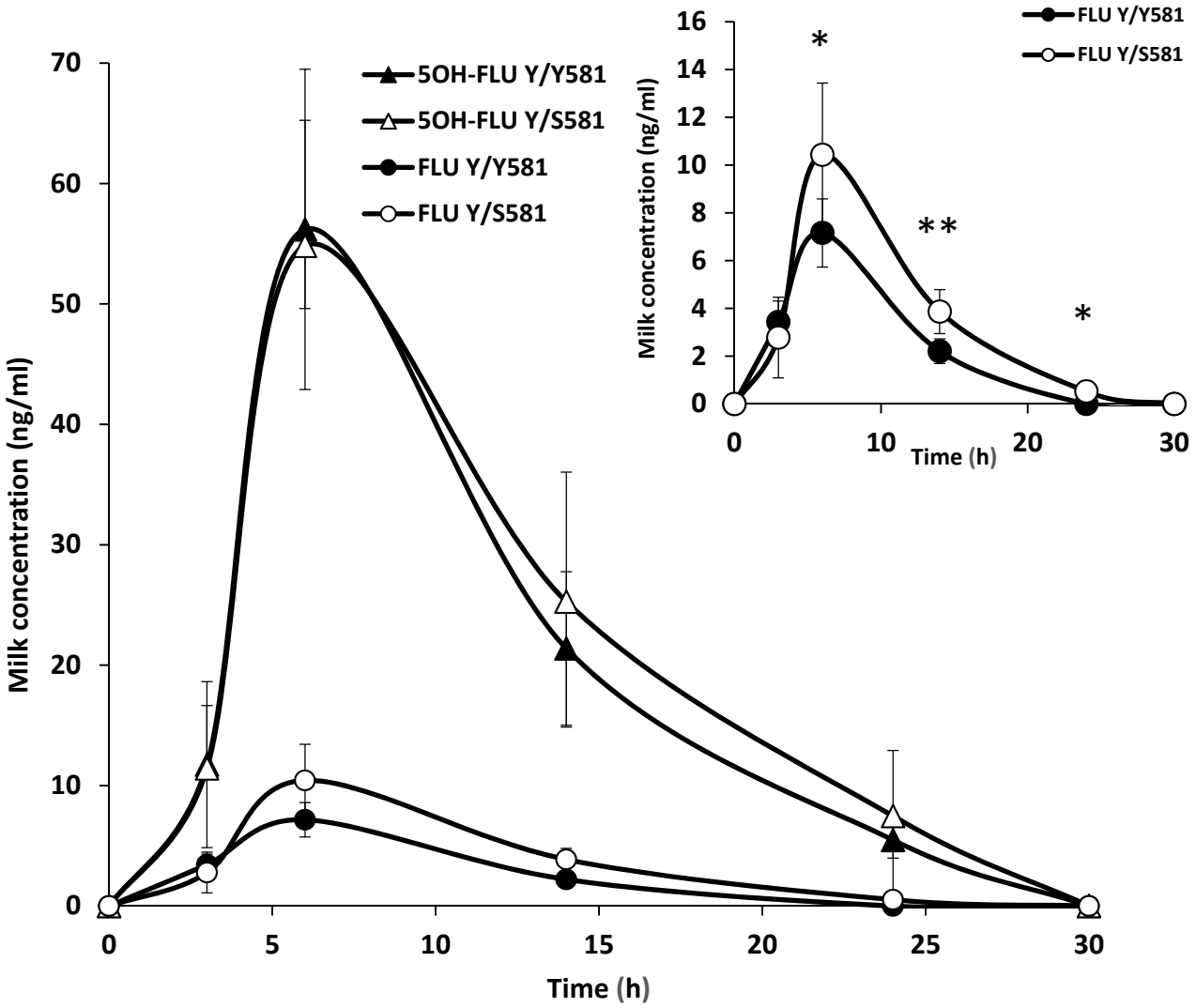

Fig 5