The Veterinary Journal 192 (2012) 422–427

Contents lists available at SciVerse ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

In vivo and ex vivo assessment of the interaction between ivermectin and danofloxacin in sheep

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ARTICLE INFO

Article history:
Accepted 8 September 2011

Keywords:
Ivermectin
Danofloxacin
P-glycoprotein
Intestinal transport
Pharmacokinetics
Sheep

ABSTRACT

The impact of an efflux pump-related interaction between ivermectin and danofloxacin on their intestinal transport (ex vivo) and disposition kinetics (in vivo) was assessed. Eighteen male Corriedale sheep were randomly assigned to one of three groups. Animals in Group A received 0.2 mg/kg ivermectin by SC injection, those in Group B were given 6 mg/kg danofloxacin SC on two occasions 48 h apart and those in Group C were treated with both compounds at the same rates. Plasma concentrations of ivermectin and danofloxacin were measured by HPLC using fluorescence detection. Ex vivo intestinal drug transport activity was measured by the use of the Ussing chamber technique.

Plasma concentrations of ivermectin in the first 6 days after injection tended to be higher in Group C than Group A. Contemporaneous treatment with ivermectin significantly increased systemic exposure to danofloxacin (AUC values were 32–35% higher) and prolonged the elimination half-life of danofloxacin (40–52% longer). Ex vivo, incubation with ivermectin significantly decreased the efflux transport of rhodamine 123, a P-glycoprotein substrate, in sheep intestine, but no significant effect of danofloxacin on transport activity was observed. Evaluation of the interaction of danofloxacin with the breast cancer resistance protein (BCRP) showed that pantoprazole and ivermectin significantly decreased danofloxacin secretion in the rat intestine. Thus, the ivermectin-induced reduction of danofloxacin efflux transport observed in this study may involve BCRP activity but the involvement of P-glycoprotein cannot be ruled out.

Introduction

Transport proteins can substantially modify both the disposition and pharmacological effect of many therapeutic agents. Several in vitro approaches have been established for testing drug–drug interactions with ATP-binding-cassette (ABC) transporters (Stephens et al., 2001) and a number of in vitro and in situ methodologies have been used to characterize the interaction between the macrocyclic lactone, ivermectin and P-glycoprotein (P-gp) (Schinkel et al., 1995; Laffont et al., 2002; Lespine et al., 2007). The contribution of drug transporter proteins to the disposition kinetics of ivermectin has been demonstrated in vivo in several different animal species (Lifschitz et al., 2004, 2010a; Ballent et al., 2007). The interaction of ivermectin with other ABC transporters such as multidrug resistance-associated proteins (MRPs) and breast cancer resistance protein (BCRP) has been described in vitro (Lespine et al., 2006; Real et al., 2011; Jani et al., 2011), but the impact of these multiple affinities with different ABC transporters on the in vivo pharmacokinetics of ivermectin remains unclear.

Danofloxacin is a synthetic fluoroquinolone antibiotic developed for veterinary use for which there is published pharmacokinetic data for sheep (McKellar et al., 1998; Aliabadi et al., 2003; Escudero et al., 2007). The interaction of danofloxacin with P-gp and MRP2 has been demonstrated in vitro using Caco-2 cells (Schrickx and Fink-Gremmels, 2007). Additionally, transepithelial efflux mediated by ABC transporters has been proposed as an important elimination mechanism for fluoroquinolones (Griffiths et al., 1993; Lowes and Simmons, 2002). The influence of ivermectin on secretion of danofloxacin into the milk has recently been described (Real et al., 2011), but the underlying interaction

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doi:10.1016/j.tvjl.2011.09.006

mechanisms and their impact on the pharmacokinetics of danofloxacin in vivo have not been fully explored.

Concurrent administration of different drugs is a frequent clinical practice in veterinary and human medicine which can lead to modifications in the pharmacological activity of therapeutically relevant active ingredients. Thus sheep may be treated with a parasiticide such as ivermectin at the same time as being treated with an antibiotic such as danofloxacin. The present study was designed to gain further insights into transporter-mediated drug interactions in ruminant species by evaluating the effect of combining treatment with ivermectin with danofloxacin on the pharmacokinetics of the two compounds. Additionally, in order to better understand the pharmacological basis of this interaction, complementary ex vivo assays using the Ussing chamber system were also undertaken.

Materials and methods

All animal procedures and management protocols were approved by the Ethics Committee according to the Animal Welfare Policy (act 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina.

Animals

The study was conducted in clinically healthy and parasite-free sheep. Eighteen adult male Corriedale sheep weighing 45–50 kg were used. Sheep were grazed ad libitum on a lucerne/red clover pasture during the whole experimental period with free access to water. The health of the animals was monitored by clinical examination throughout the experimental period.

The 18 sheep were randomly allocated into three groups of six. Animals in Group A received a single subcutaneous (SC) injection of 0.2 mg/kg ivermectin (Ivosint, Biogénesis). Sheep in Group B were injected with 6 mg/kg of 18% danofloxacin (Advocin 180, Pfizer) SC on two occasions 48 h apart. For Group C, the two treatments were combined, with the first danofloxacin injection given at the same time as the ivermectin. Jugular blood samples were collected into heparinized Vacutainer tubes (Becton, Dickinson) for ivermectin quantification at 0, 3, 6, 9, 12 h and 1, 2, 3, 4, 5, 7, 9, 12, 15 days (Groups A and C only). Samples were taken for danofloxacin analysis at 0, 15, 30, 45 min, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48 h after the first danofloxacin dose, and at 1, 2, 3, 4, 6, 8, 12, 24, 36, 48 h after the second danofloxacin dose (Groups B and C only). Blood samples were centrifuged at 2000 *g* for 20 min, and plasma stored at −20 °C until analysis by high performance liquid chromatography (HPLC).

Ivermectin and danofloxacin analysis

The extraction of ivermectin from plasma samples was carried out following the technique described by Alvinerie et al. (1993), as modified by Lifschitz et al. (1999) and using the derivatization described by De Montigny et al. (1990). After completion of the reaction, a 100 μL aliquot was injected directly into the chromatographic system. The measurement of ivermectin concentrations was carried out using the Shimadzu 10A HPLC system (Shimadzu Corporation) following the technique described by Lifschitz et al. (2000).

Danofloxacin and the internal standard marbofloxacin (5 μg/mL) were extracted directly from 0.2 mL of plasma by liquid phase extraction using a method modified from Aliabadi et al. (2003). HPLC analysis for danofloxacin was undertaken as described by González et al. (2006). Separation of danofloxacin and marbofloxacin was undertaken on a Shimadzu LC system comprising an LC-10AS liquid chromatogram with an RF-10A spectrofluorometric detector (Shimadzu Corporation).

All analytical procedures, including chemical extraction and HPLC analysis of ivermectin and danofloxacin in plasma were validated. Linearity was established to express the concentration-detector response relationship, as determined by injection of ivermectin or danofloxacin spiked standards at different concentrations (three replicates). Calibration curves were established using least squares linear regression analysis and determination coefficients (r^2) were calculated. The intra-assay precision was estimated by processing replicate aliquots of pooled plasma samples containing known ivermectin and danofloxacin concentrations and calculating the coefficient of variation (CV). Drug recovery was estimated by comparison of the peak area from spiked plasma standards at different concentrations, with the peak areas resulting from direct injections of ivermectin or danofloxacin standards in methanol. The limit of quantification was established as the lowest concentration measured with a recovery higher than 70% and a CV < 20%. The accuracy was the closeness of the measured value to the true value calculated as a percentage.

Intestinal efflux assays: Studies using the Ussing chamber system

The potential interaction of ivermectin and danofloxacin with P-gp was assayed across sheep intestine placed in diffusion chambers, with rhodamine 123 as the substrate. For each set of experiments, the entire gastrointestinal tract was immediately removed from sacrificed sheep and the ileocaecal valve identified. The first 60 cm of small intestine proximal to the ileocaecal valve was defined as ileum. The ileum was cut open along the mesenteric border and the resulting flat sheets were mounted into Ussing chambers (K. Mussler Scientific Instruments) providing an exposed area of 1 cm^2.

Both mucosal (M) and serosal (S) sides of the chamber were filled with 11 mL of pre-warmed and oxygenated Krebs buffer (pH 7.4) which was maintained at 37 °C. In order to ensure oxygenation and agitation, a mixture of 95% O_2 and 5% CO_2 was bubbled through each compartment. Measurement of transepithelial electrical resistance (Rt) was conducted prior to the beginning and at the end of the transport studies to assess the integrity of the intestinal tissue. A maximum decrease of 30% of the initial Rt measurements was fixed as the criteria for integrity. After a 20 min equilibration period, rhodamine 123 in a final concentration of 5 μM was added to the M or S side of the chambers. For inhibition studies, either ivermectin or danofloxacin at equimolar concentrations (10 μM) were added to both the donor and acceptor sides. One milliliter volume samples were taken from the acceptor chambers at intervals of 30 min.

To evaluate better the mechanism of BCRP-mediated intestinal efflux, the transport of danofloxacin was evaluated in rat intestine. Intestinal flat sheets from the ileum of male Wistar rats were mounted into the Ussing chamber system with an exposed area of 0.85 cm^2. Bidirectional (S–M and M–S) transport of danofloxacin (50 μM) was studied after its incubation either alone or in the presence of pantoprazole or ivermectin at equimolar concentrations of 20 μM. At intervals of 15 min, 1 mL aliquots of buffer were taken from acceptor chambers.

The concentration of rhodamine 123 was measured by a fluorescent spectrophotometer RF-5301PC (Shimadzu Corporation) set at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The concentration of danofloxacin was measured using the RF-5301PC set at a wavelength of 275 nm (excitation) and 443 nm (emission).

Pharmacokinetic analysis

The time curves of plasma concentration obtained after each treatment were fitted using PK Solutions 2.0 software. Pharmacokinetic parameters were determined using a non-compartmental model method (Gibaldi and Perrier, 1982).

Intestinal efflux analysis

Unidirectional transepithelial effective permeability values (P_{eff}) (cm/s) for each chamber were calculated using the following equation:

$$P_{eff} = (dC/dt) \cdot [1/(A \cdot C_o)]$$

The appearance rate in the receiving compartment (dC/dt) was calculated from the slope of the concentration against time curve over a time period of 240 min. A was the exposed area of the tissue in the Ussing chamber and C_o was the initial drug concentration in the donor compartment. The efflux ratio was calculated as follows:

$$\text{Efflux ratio} = \frac{\text{mean } P_{eff} \text{ S—M}}{\text{mean } P_{eff} \text{ M—S}}$$

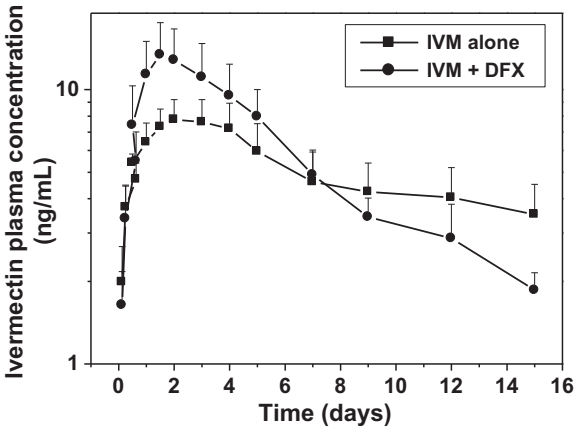

Fig. 1. Mean (±SEM) (n = 6) ivermectin (ivermectin) plasma concentrations (ng/mL) obtained after SC administration (0.2 mg/kg), either alone or co-administered with danofloxacin (danofloxacin) (6 mg/kg, two doses every 48 h, SC) to sheep.

Table 1
Mean (±SEM) ($n = 6$) kinetic parameters for ivermectin (ivermectin) and danofloxacin (danofloxacin) in plasma obtained after their subcutaneous administration either alone at 0.2 mg/kg (ivermectin) (Group A), at 6 mg/kg, two times every 48 h (danofloxacin) (Group B) or co-administered (Group C) to sheep.

Kinetic parameters	Ivermectin		Danofloxacin			
	Group A	Group C	Group B (1st dose)	Group C (1st dose)	Group B (2nd dose)	Group C (2nd dose)
C_{max}	8.88 ± 1.40	14.1 ± 3.74	0.88 ± 0.07	0.97 ± 0.007	0.91 ± 0.09	1.00 ± 0.04
T_{max}	2.15 ± 0.63	2.50 ± 0.84	3.00 ± 0.26	2.50 ± 0.41	2.50 ± 0.43	2.67 ± 0.21
AUC_{total}	75.6 ± 17.0	88.0 ± 19.3	6.59 ± 0.64	8.90 ± 0.84*	5.78 ± 0.18	7.64 ± 0.49*
$AUMC_{total}$	487 ± 131	459 ± 78.7	39.3 ± 5.78	67.8 ± 8.65	32.3 ± 1.99	50.9 ± 4.26
MRT	6.1 ± 0.53	5.46 ± 0.63	5.81 ± 0.35	7.51 ± 0.27*	5.61 ± 0.33	6.63 ± 0.18*
$T_{½\ el}$[a]	7.15 ± 6.49	4.37 ± 2.10	3.70 ± 0.41	5.22 ± 0.21*	3.73 ± 0.17	5.64 ± 0.32*

C_{max} (ng/mL; μg/mL): peak plasma concentration. T_{max} (days; hours): time to peak plasma concentration. AUC_{total} (ng day/mL; μg h/mL): area under the concentration vs. time curve extrapolated to infinity. $AUMC_{total}$ (ng day^2/mL; μg h^2/mL): area under the first-moment concentration vs. time curve extrapolated to infinity. MRT (days; hours): mean residence time. $T_{½\ el}$ (days; hours): elimination half-life.

* Values are statistically different from those obtained in Group B at $P < 0.05$.

[a] Harmonic mean ± pseudo standard deviation.

Statistical analysis

All analyses were undertaken using Instat 3.0 (Graph Pad). The plasma concentrations of ivermectin and danofloxacin and all estimated pharmacokinetic parameters are reported as means ± SEM. Mean pharmacokinetic parameters for ivermectin and danofloxacin obtained after their administration either alone or co-administered were statistically compared using Student's *t* test or a non-parametric Mann–Whitney *U* test depending on whether the standard deviations were similar across groups. For permeability studies, mean P_{eff} were statistically compared by ANOVA or a non-parametric Kruskal–Wallis test depending on whether the standard deviations were similar across groups.

Results

A complete validation of the analytical procedures for extraction and quantification of ivermectin and danofloxacin in plasma was performed before starting the analysis of experimental samples. The linear regression lines showed determination coefficients to be >0.99 for both compounds. The recoveries from plasma were >73% (ivermectin) and >79% (danofloxacin). The limit of quantification was established at 0.2 ng/mL and 0.005 μg/mL for ivermectin and danofloxacin, respectively, with a precision of 7% (ivermectin) and 3.15% (danofloxacin) and an accuracy of 5.5% (ivermectin) and 7.8% (danofloxacin).

Fig. 1 shows the change in time in the plasma concentration of ivermectin for the first 15 days after its administration in animals treated with ivermectin alone (Group A) and treated with both danofloxacin and ivermectin (Group C). The pharmacokinetic parameters obtained for ivermectin in these two groups are summarized in Table 1. The mean plasma concentration of ivermectin tended to be higher for the first 6 days after administration in sheep in Group C, but this was not significant ($P = 0.18$).

Fig. 2 shows the change in time in the plasma concentration of danofloxacin in animals treated with danofloxacin alone (Group B) or treated with both danofloxacin and ivermectin (Group C). Co-administration with ivermectin prolonged the period over which danofloxacin was detected. In Group B danofloxacin was detected up to 24 h after administration, whereas in Group C it was detected for 36 h post-treatment. Additionally, systemic exposure of danofloxacin was enhanced between 32% and 35% ($P = 0.04$ and 0.03) in Group C compared to Group B, and the elimination half-life was lengthened (5.75 ± 0.16 days vs. 3.25 ± 0.19 days, respectively ($P = 0.001$).

Active transport of rhodamine 123 across the ileum of sheep was seen in both the mucosal to serosal (M–S) and serosal to mucosal (S–M) directions. The secretion (P_{eff} S–M) of rhodamine 123 was 549% (95% CI 407–691) higher than the absorption (P_{eff} M–S). The presence of ivermectin and danofloxacin increased the absorption of rhodamine 123. The P_{eff} M–S for rhodamine-123 was higher ($P = 0.005$) in the presence of either ivermectin ($6.87 \times 10^{-7} \pm 0.47 \times 10^{-7}$ cm/s) or danofloxacin ($9.01 \times 10^{-7} \pm 2.41 \times 10^{-7}$ cm/s) than it was when the ileum samples were incubated with rhodamine 123 alone ($3.95 \times 10^{-7} \pm 0.45 \times 10^{-7}$ cm/s).

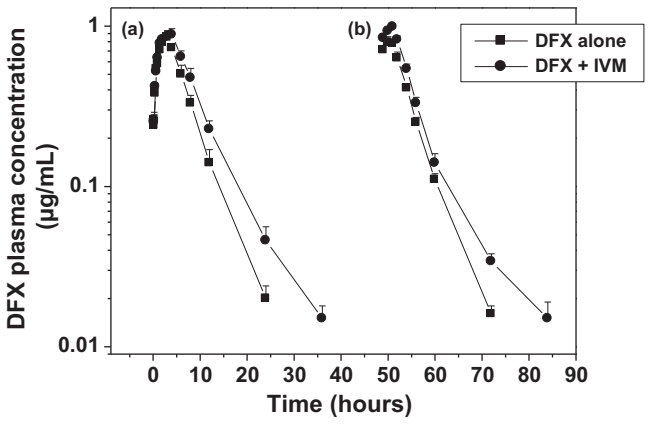

Fig. 2. Mean (±SEM) ($n = 6$) danofloxacin (danofloxacin) plasma concentrations (μg/mL) obtained after its (a) first and (b) second SC administration (6 mg/kg), either alone or co-administered with ivermectin (ivermectin) (0.2 mg/kg, SC) to sheep.

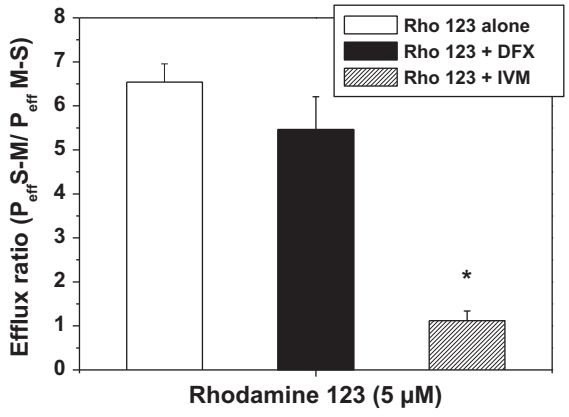

Fig. 3. Efflux ratio (P_{eff} S–M/P_{eff} M–S) of rhodamine 123 in sheep intestine (ileum) (mean ± SEM) after its incubation either alone (5 μM) or with danofloxacin (danofloxacin) (10 μM) or ivermectin (ivermectin) (10 μM). Each value is an average of at least five (5) measurements. (*) Values are statistically different from those obtained after the incubation of rhodamine 123 alone at $P < 0.05$.

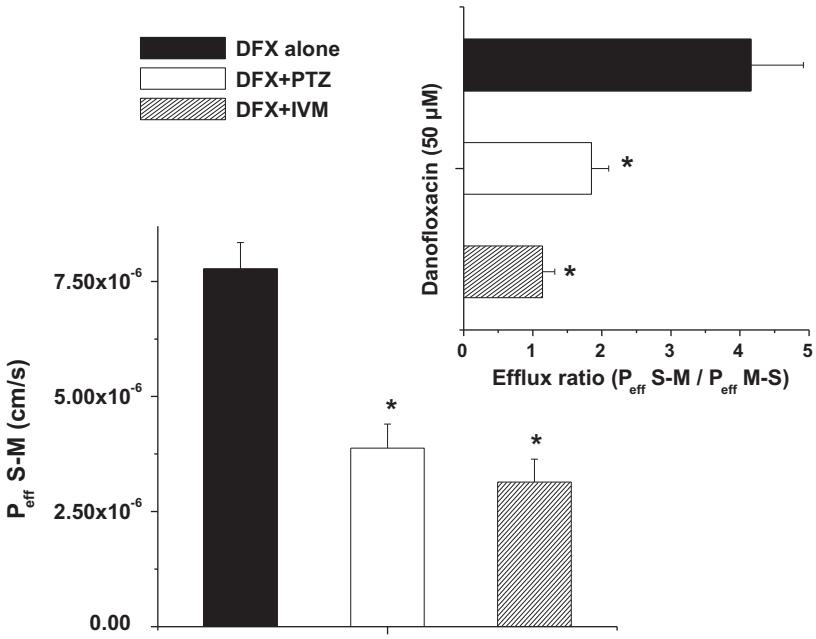

Fig. 4. Effective permeability (P_{eff} S–M) (mean ± SEM) of danofloxacin (danofloxacin) across rat intestine (ileum) after its incubation either alone (50 μM) or with pantoprazole (pantoprazole) (20 μM) or ivermectin (ivermectin) (20 μM). Inset shows the efflux ratio (P_{eff} S–M/P_{eff} M–S) of danofloxacin incubated either alone or in the presence of breast cancer resistance protein (BCRP) inhibitors. Each value is an average of at least five (5) measurements. (*) Values are statistically different from those obtained after the incubation of danofloxacin alone at $P < 0.05$.

However, the ex vivo intestinal secretion process of rhodamine 123 (S–M) was significantly affected only by ivermectin. The P_{eff} S–M of rhodamine 123 was 2.59×10^{-6} cm/s (control), 7.67×10^{-7} cm/s in the presence of ivermectin and 4.92×10^{-6} cm/s after the incubation with danofloxacin. The mean efflux ratios obtained after the incubation of rhodamine 123 either alone or in the presence of ivermectin or danofloxacin are shown in Fig. 3. The efflux ratio (P_{eff} S–M/P_{eff} M–S) decreased from 6.49 with rhodamine 123 alone to 1.12 when ivermectin was added ($P < 0.001$). No such effect was reported for danofloxacin ($P = 0.29$).

The P_{eff} S–M for danofloxacin and the efflux ratios obtained after its incubation either alone or co-administered with pantoprazole or ivermectin are shown in Fig. 4 for the rat model. The mean P_{eff} S–M for danofloxacin was fourfold higher ($P = 0.008$, 95% CI 2.58–5.74) than the mean P_{eff} M–S. The presence of pantoprazole and ivermectin did not modify P_{eff} M–S. For danofloxacin alone, mean P_{eff} M–S was $1.85 \times 10^{-6} \pm 0.42 \times 10^{-6}$ cm/s, for danofloxacin and pantoprazole it was $2.10 \times 10^{-6} \pm 1.03 \times 10^{-6}$ cm/s and for danofloxacin and ivermectin $2.76 \times 10^{-6} \pm 1.38 \times 10^{-6}$ cm/s. In contrast, P_{eff} S–M was reduced by incubation with either pantoprazole or ivermectin ($P = 0.016$ and 0.004 respectively), as a result the efflux ratio of danofloxacin was reduced by both pantoprazole (1.85) and ivermectin (1.14) ($P = 0.016$ and 0.004, respectively).

Discussion

The plasma concentrations of ivermectin seen in this study were similar to those reported previously (Pérez et al., 2008; Lifschitz et al., 2010b; Moreno et al., 2010), while the pharmacokinetics of danofloxacin were similar to that described by Escudero et al. (2007). There was no significant effect of danofloxacin on the pharmacokinetics of ivermectin. However, the high variability in the plasma concentrations of ivermectin meant that a biologically significant interaction with danofloxacin could not be ruled out.

However, marked changes in plasma concentration of danofloxacin were observed when it was administered with ivermectin; AUC was increased by 32–35% and its elimination half-life prolonged. In contrast, Real et al. (2011) reported that, in dairy sheep, co-administration of 0.2 mg/kg ivermectin with 1.25 mg/kg of danofloxacin did not significantly affect the plasma concentration of danofloxacin, although it did significantly reduce the milk concentration. Real et al. (2011) proposed that the reduced secretion of danofloxacin into milk was the result of a BCRP-mediated interaction. The difference in impact of co-administration of ivermectin on the pharmacokinetics of danofloxacin may be related to differences in dose rate, or the gender or physiological status of the experimental animals. However, current findings confirm that in vivo drug–drug interactions may occur after co-administration of ivermectin and danofloxacin to sheep.

To gain some further insight into the mechanism responsible for the in vivo interaction between ivermectin and danofloxacin, an ex vivo drug transport assay was set up. The everted gut sac technique has been proposed as a simple model for quantification of intestinal efflux for different lipophilic molecules such as ivermectin (Ballent et al., 2006), but new methodological approaches are required to evaluate the physiological and pharmacological features of the intestinal drug secretion process in ruminants. In this context, the use of ex vivo methodologies is an important tool to study the activity of ABC transporters in the intestinal secretion of drugs in the sheep. The Ussing chamber technique has been previously validated in the study of transepithelial transport processes for different xenobiotics (Lenernnäs, 1997). The work described here assessed the ex vivo interaction between ivermectin and danofloxacin with the intestinal ABC transporter proteins.

The efflux ratio of rhodamine 123 was significantly lower when it was co-incubated with ivermectin but not when it was co-incubated with danofloxacin, i.e. ivermectin has a marked inhibitory effect on P-gp activity but danofloxacin does not (Fig. 3). This inhibition of P-gp efflux is consistent with reports of previous in vitro studies (Lespine et al., 2007). Additionally, it has

been also reported that ivermectin may interact with other cell transport proteins such as multidrug resistance protein 1, 2 and 3 (MRP1, MRP2 and MRP3) (Lespine et al., 2006), and, in cell culture, with BCRP (Muenster et al., 2008; Jani et al., 2011; Real et al., 2011).

Fluoroquinolones have been shown to be substrates for multiple ABC cellular transport systems in several different animal species. The interaction of danofloxacin with multiple transport systems, including P-gp, MRP2 and BCRP has been confirmed in Caco-2 cells (Schrickx and Fink-Gremmels, 2007). Real et al. (2011) reported that danofloxacin was transported by murine and human BCRP-transduced cells and that the presence of ivermectin at 50 μM inhibited this transport. The involvement of multiple efflux transporters suggests that a complex interplay between ivermectin, danofloxacin and the efflux proteins could affect the disposition kinetics of danofloxacin.

After performing the ex vivo assays to evaluate the interaction of the ivermectin and danofloxacin with P-gp, we needed to understand the potential interaction of both compounds with the BCRP transporter. However, for ethical reasons, it was not possible to include more sheep in the current study and therefore this mechanism of interaction was evaluated using rat intestine. Our ex vivo assay showed that pantoprazole, a BCRP inhibitor, markedly reduced danofloxacin secretion in the Ussing chamber system (Fig. 4) suggesting this transporter protein is an important part of the intestinal efflux of danofloxacin. The presence of ivermectin also reduced the intestinal secretion of danofloxacin, with the efflux ratio of danofloxacin being reduced to close to 1. This means that ABC transporter-mediated secretion was strongly inhibited. As the efflux in the presence of pantoprazole (1.85) was higher than that in the presence of ivermectin this suggests that although intestinal secretion of danofloxacin is, probably, mainly due to BCRP activity, P-gp involvement cannot be ruled out as ivermectin, a BCRP and P-gp inhibitor, suppressed intestinal secretion of danofloxacin more effectively than pantoprazole, a BCRP inhibitor.

The clinical and practical consequences of the interactions reported here should be evaluated as a modification of the pharmacokinetics after the co-administration of two compounds is not always clinically relevant (Fuhr, 2007). In particular, the clinical consequences of the slower elimination of danofloxacin should be evaluated.

Conclusions

The study has confirmed that there are significant interactions between ivermectin and danofloxacin in sheep, with the systemic exposure of danofloxacin being significantly enhanced in the presence of ivermectin. The mechanisms involved in this type of kinetics interaction were studied for the first time under an ex vivo system supported by the Ussing chamber system. The ivermectin inhibition of the danofloxacin intestinal secretion seems to be mainly BCRP-mediated but the involvement of P-gp cannot be excluded. The in vivo influence of multiple ABC transporters should be carefully characterized for different therapeutically relevant drug–drug interactions in diverse animal species.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

This work was supported by CONICET (PIP 112-200801-01123) and Agencia Nacional de Promoción Científica y Técnica (ANPCyT) (PICT 1881), Argentina.

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