Modulation of the P-glycoprotein-mediated intestinal secretion of ivermectin: 

\textit{in vitro and in vivo} assessments

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Abstract

The everted gut sac method was used to assess the role of the P-glycoprotein (P-gp) on the intestinal secretion of ivermectin (IVM), an antiparasitic widely used in human and veterinary medicine. The work included the evaluation of two different P-gp modulators (itraconazole and PSC833) used at equimolar doses in the rat. Furthermore, the influence of both P-gp modulator agents on the disposition kinetics of IVM in plasma, liver and gastrointestinal tissues was characterised. For the in vitro experiments, ileal sacs were incubated with IVM (3 µM) in the presence or absence of either itraconazole (ITZ) (10 µM) or PSC833 (10 µM). In the in vivo experiments, male Wistar rats were randomly allocated in three groups (n=18) and subcutaneously treated with IVM (200 µg/Kg⁻¹) alone and co-administered with ITZ (5 mg, two doses) or PSC833 (8.6 mg, two doses). Animals were sacrificed between 6 and 96 h. Blood, liver and gastrointestinal samples were collected. IVM concentrations were determined by HPLC. The rate of IVM accumulation in the intestinal wall of everted sacs was significantly higher after its incubation with ITZ (0.115 nmol/g/min) and PSC833 (0.238 nmol/g/min) than that obtained after the incubation without the P-gp modulators (0.016 nmol/g/min). In agreement with the in vitro experiment, the presence of ITZ and PSC833 induced an enhancement in the concentrations of IVM in plasma and gastrointestinal tissues. The results obtained in the current work, both under in vivo and in vitro conditions, confirm the relevance of P-gp-mediated transport on the intestinal secretion of IVM.
Introduction

P-glycoprotein (P-gp) is a transmembrane protein associated with a phenotype of multidrug resistance (MDR) to certain anticancer drugs in mammalian cancer cells, which is able to pump a broad range of structurally and functionally unrelated compounds out of the cell by an ATP-dependent process (Lin, 2003). P-gp is physiologically expressed in a number of tissues including liver, blood-brain barrier and intestine (Thiebaut et al., 1987). An enormous effort has been made to interact with the P-gp-mediated drug transport, which led to the development of pharmacologically active inhibitors. These compounds, also known as P-gp modulators, appear to inhibit P-gp activity by competing with the P-gp binding site and/or through inhibition of ATP hydrolysis (Garrigos et al., 1997). Modulator agents from the first generation of modulators are compounds commonly used in therapeutics with capacity to reversal the MDR phenotype. Verapamil, a calcium channel blocker (Tsuruo et al., 1981) was one of the first identified P-gp modulator agents. The main disadvantage for first generation of modulators was that the inhibition is produced at concentrations higher than those uses for therapeutic purposes, which increases their cytotoxic effects (Lampidis et al., 1990). Itraconazole is a fungistatic/ fungicidal agent widely used in human and veterinary medicine and has been described as a potent P-gp and cytochrome P450 3A (CYP3A) inhibitor (Cooper et al., 2003). The search for a second generation of non-toxic modulators was addressed to the development of more effective and less toxic compounds. PSC833 (Balspodar, Novartis Pharma AG), a non immunosuppressive Cyclosporin A analogue is one of the most effective modulator agent with capacity to reverse the P-gp-mediated multidrug resistance in clinical trials (Fisher, 1995).
Ivermectin (IVM) is a broad-spectrum anthelmintic compound extensively used in human and veterinary medicine. IVM is largely excreted in bile and faeces as the parent drug in different animal species (Chiu et al 1990, Lifschitz et al., 2000). Ivermectin was shown to be a potent P-gp inhibitor in vitro (Didier and Loor, 1996). The active intestinal secretion of IVM in the rat has been demonstrated (Laffont et al., 2002) using the intestinal close-loop model. Co-administration of IVM with loperamide (a P-gp modulator) resulted on changes on the pattern of IVM bile-faecal excretion, which accounted for an enhanced availability of the antiparasitic compounds in tissues of parasite location (Lifschitz et al., 2004).

New in vitro models have been developed to predict in vivo P-gp activity (Stephens et al., 2001). Caco-2 cell monolayers, derived from a human colonic adenocarcinoma, are widely used to estimate transepithelial passage of different P-gp substrates (Fricker et al., 1996). However, the correlation between this in vitro model and in vivo studies is rather poor (Yee, 1997). The use of everted gut sacs has been proposed as a new in vitro model for quantification of P-gp-mediated intestinal efflux for different drugs (Barthe et al., 1998). The goals of the current work were: a) to assess in vitro the involvement of P-gp on the intestinal secretion of IVM using the everted gut sac technique; b) to evaluate the comparative effects of two different generation P-gp-modulating agents (itraconazole and PSC833) used at equimolar doses on the IVM ileal transport, c) to characterize the influence of both P-gp-modulating agents on the in vivo plasma and gastrointestinal disposition kinetics of IVM in Wistar rats, and to correlate these results with those from the in vitro assays.
Materials and Methods

Experimental animals, treatment and sampling

In vitro experiment

Gut sac preparation

The everted gut sac method was performed following a technique previously described by Barthe et al, (1998). Male rats weighing 250-300 g were starved overnight. Under anaesthesia the intestine was rapidly removed and washed with buffer solution (PO₄H₂NaH₂O 1 mM; Cl₂Ca₂H₂O 2.5 mM; CIK 4.7 mM; Cl₂Mg₆H₂O 1.1 mM; EDTA 0.004 mM; glucose 11 mM; ClNa119 mM, Co₃Na 25 mM; Ac. Ascorbic 0.11mM). The intestine was immediately placed in warm (37 ºC) oxygenated (O₂/CO₂, 95%/5%) 199 medium (Sigma- Aldrich, Dorset, UK) and then gently everted over a glass rod of 2.5 mm in diameter. One end was clamped and tied with a silk braided suture before filling it with medium at 37 ºC using a 1 ml plastic syringe. The intestine segment was then sealed with a second tie using a braided silk suture. Sacs (5 cm in length) were placed in individual incubation chambers containing 6 ml of pregassed oxygenated media at 37 ºC.

Incubation assays

To study the tissue uptake and serosal transfer of IVM in presence or absence of the modulator agents, IVM (3 µM), ITZ and PSC833 (10 µM) were added to the chambers. Sacs were incubated in a water bath for 5, 10, 15, 25, 45 and 60 min. The solution was maintained at 37 ºC with O₂/CO₂ (95%/5%) throughout the experiment. At the defined time points, sacs were removed, washed in buffer solution and blotted dry. The sacs were cut open and the serosal fluid drained into small tubes. Each sac was weighed before and
after fluid collection to calculate accurately the volume inside the sac. All samples were conserved at -20 ºC until analysis.

**Gut sac viability**

To verify the integrity of the gut sacs, glucose concentrations were measured both in the incubation medium and in the sac contents using a commercial test (Wiener®, Argentina). As glucose is actively transported by the small intestine, healthy metabolically active sacs that are not leaking, will concentrate glucose in the serosal medium. The sacs were incubated in the TC 199 medium into the chambers in absence and presence of IVM (3 µM) and P-gp modulators (10 µM) maintaining the same conditions as the experimental assay. The gut sacs were incubated from 20 to 70 min. Samples of incubation medium and content of the sacs were collected and stored until glucose determination by spectrophotometry. Glucose determination was carried out as a follow: 20 µl of sample was incubated at 37 ºC for 10 min with a reagent which consists of 50% distilled water, 5% of 4-aminofenazona 25 mmol/l solution in buffer Tris 0.92 mol/l; 5% fenol solution 55 mmol/l and 0.3% glucose oxidadase (1000 U/ml)/peroxidase (120 U/ml). Finally, the absorbance was measured in spectrophotometer at 505 nm.

**In vivo Experiments**

Fifty-four male Wistar rats weighing 250-300 g were use in this trial. The management of experimental animals was performed in accordance with institutional and internationally accepted welfare guidelines (Canadian Council on Animal Care, 1980; American Veterinary Medical Association, 2001). The animals were kept under controlled conditions
of temperature and cycles of darkness/light. The rats were randomly allocated in three groups of 18 animals each. Animals in Group A (IVM alone) received IVM at 200 µg/Kg⁻¹ (Ivomec®, Merial) by subcutaneous (SC) injection. The original IVM formulation was diluted in propylene glycol to fit the low dose volumes. Rats in Group B (IVM + ITZ) received IVM at the same dose rate co-administered with ITZ (ethanol/propylene glycol 25/75) at 5 mg (two doses with 12 h interval) injected intraperitoneally. Animals in Group C (IVM + PSC833) received IVM at the same dose rate co-administered with PSC833 formulated in ethanol/propylene glycol (25:75) at a dose equimolar with ITZ (two doses of 8.6 mg with 12 h interval), and administered by oral route. Under superficial ether anaesthesia, three animals from each experimental group were killed at 6, 12, 24, 48, 72 and 96 h post-treatment. Blood, liver, wall tissue and luminal content of jejunum, ileum and colon were collected. Blood samples were centrifuged at 2000 g for 20 min and the recovered plasma kept in labelled vials. Plasma and tissues samples were rapidly cooled and stored at -20 °C until analysis.

The extraction of IVM from plasma, liver and intestinal wall and luminal content was carried out following the technique earlier described by Alvinerie et al, (1993) and adapted by Lifschitz et al, (2000). Samples from both in vivo and in vitro assays, including plasma, liver tissue, intestinal wall and content (0.250 ml or g) were fortified with 10 ng of abamectin (used as internal standard, 10ng/10 µl) and 0.25 ml of acetonitrile plus 70 µl of deionized water were added to each sample. The preparation was mixed (Multi Tube Vortexer, VWR Scientific Products, West Chester, PA, USA) over 15 min, sonicated in an ultrasonic bath for 10 min. (Transsonic 570/H, Laboratory Line Instruments Inc., Melrose Park, IL, USA) and the solvent-sample mixture was centrifuged at 2000 g for 10 min. The
supernatant was manually transferred into a tube and the procedure was repeated once more for liver and intestinal tissue samples. The supernatant was applied to a conditioned Supelclean LC 18 cartridge (Supelco, Bellefonte, PA, USA). After washing with 1 ml deionised water followed by 1 ml water-methanol (4:1 v/v), the cartridges were dried off for 5 min and the sample eluted with 1.5 ml methanol, which was collected. After solid phase extraction, the elute was evaporated to dryness under a gentle stream of dry nitrogen at 60 °C in a water bath. The sample was subjected to a procedure described by De Montigny et al, (1990). After completion of the reaction, an aliquot (100 µl) of each sample was injected directly into the chromatographic system.
Ivermectin analysis and validation procedures

The measurement of IVM concentrations in plasma and tissues was carried out by HPLC (Shimadzu 10 A HPLC system, Shimadzu Corp. Kyoto, Japan). The HPLC analysis was performed following the technique described by Lifschitz et al. (2000). The chromatographic conditions included a mobile phase of acetic acid (0.2 % in water)-methanol-acetonitrile (5:40:55 v/v/v) pumped at a flow rate of 1.5 ml/min through a reverse phase C18 column (Selectosil, Phenomenex, Torrance, USA) (5 μm, 4.6 mm x 250 mm). IVM detection was performed using a fluorescence detector (Spectrofluorometric detector RF-10, Shimadzu, Kyoto, Japan) set at an excitation wavelength of 365 nm and an emission wavelength of 475 nm. The ABM/IVM peak area ratio was used to estimate IVM concentration in spiked (validation of the analytical method) and experimental samples. There was no interference of endogenous compounds in the chromatographic determinations.

The analytical procedures, including chemical extraction and HPLC analysis of IVM in plasma, liver, intestinal wall and luminal contents were validated. The statistical program (Instat 3.0, Graph Pad Software Inc., San Diego, CA, USA) was used for linear regression analyses and linearity tests. Calibration curves were prepared in a range between 1 and 80 ng/ml (plasma) and ng/g (tissues). Linearity was established to express the concentration-detector response relationship, as determined by injection of plasma and tissues IVM spiked standards at different concentrations (three replicates). Calibration curves were established using least squares linear regression analysis and correlation coefficients (r) and coefficient of variations (CV) were calculated. Drug recovery was estimated by comparison of the peak area...
from spiked plasma and tissue standards at different concentrations, with the peak areas resulting from direct injections of IVM 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 linear regression lines showed correlation coefficients ranging between 0.997 and 0.999. The mean recoveries of IVM were in a range between 74 and 77 % for the different tissues analysed. The limit of quantification was established at 0.2 ng/ml for the everted sac content and at 1 ng/g for the different gastrointestinal tissues analysed. Coefficients of variation < 7 % were obtained when the inter-day precision of the chromatographic method was evaluated for the different biological matrices under investigation.
Pharmacokinetic and statistical analyses of the data

The concentration versus time curves obtained for each fluid and tissue analysed were fitted using the PkSolution 2.0 computer software (Ashland, OH, USA). Pharmacokinetic parameters were determined using a non-compartmental method. Peak concentration (Cmax) and time to peak concentration (Tmax) were read from the plotted concentration-time curves. The area under the concentration-time curves (AUC) was calculated by the trapezoidal rule (Gibaldi and Perrier, 1982). Mean pharmacokinetic parameters for IVM obtained after the administration of the IVM alone or co-administered with either ITZ or PSC833 were statistically compared by ANOVA (Instat 3.0, Graph Pad software Inc., San Diego, USA). The Bonferroni’s test was used to indicate the level of significance among the three groups. A value of P < 0.05 was considered statistically significant.
Results

In vitro experiments

The ability of everted sacs to concentrate glucose by active transport across the intestine wall was used as an indicator of tissue viability. Both in the absence and presence of IVM and the P-gp-modulating compounds, the ratios between the glucose concentration measured in the sac content and incubation medium increased gradually with time up to 70 min of incubation (Figure 1), which indicates an adequate viability of the everted intestinal tissue.

IVM was accumulated into the intestinal tissue of the sacs during the 60 min incubation time. The rate of IVM accumulation in the ileum wall was 0.016 nmol/g/min. Higher IVM concentrations in the intestinal wall were measured after the incubation with ITZ and PSC833, compared to the incubation with IVM alone. IVM concentrations in the ileum wall were 81 % (ITZ) and 159 % (PSC833) higher in the presence of P-gp modulators. The IVM concentration profiles measured in the intestinal wall after its incubation alone or in the presence of either ITZ or PSC833 are shown in Figure 2. The IVM accumulation rate in the intestinal wall was also significantly higher after its incubation with ITZ (0.115 nmol/g/min) and PSC833 (0.238 nmol/g/min) than that obtained after the incubation without the P-gp modulators. The comparison of IVM accumulation rates in ileum wall is shown in Figure 3.

IVM was recovered from the contents of the everted gut sacs. The IVM concentrations measured into the sacs were markedly lower than those recovered in the intestine wall tissue. Both P-gp modulators increased the IVM serosal transfer into the sacs. Table 1
shows the IVM concentrations into the everted sacs after its incubation in the presence of both P-gp modulators.

**In vivo experiments**

ITZ and PSC833 were used as P-gp modulators at equimolar doses to study their influence on the IVM *in vivo* plasma and gastrointestinal disposition. No acute toxic effects were observed either in the control or in the experimental groups. IVM was recovered in the bloodstream and all tissues investigated, from 6 h up to 96 h post-treatment. The presence of ITZ and PSC833 induced a marked enhancement in the IVM plasma concentrations, which was between 2.3 and 3-fold higher (at 72 h) compared to that obtained after its administration alone. The IVM plasma AUC values were 89 and 93% higher in the presence of ITZ and PSC833, respectively, suggesting a decreased clearance of the antiparasitic compound. Also, co-administration with both P-gp modulators enhanced IVM concentrations in the intestinal tissues and in the liver. The mean IVM concentrations measured in the jejunum wall after its co-administration with ITZ and PSC833 compared to the control group are shown in Figure 4. The comparison of the IVM area under the concentration vs. time curves (AUC) obtained in plasma and digestive tissues in the control and co-administered experimental groups is shown in Table 2. Figure 5 summarises the percentage of enhancement on IVM peak concentration (Cmax) in the different tissues obtained after the use of both P-gp modulator compounds.

The ratio between IVM concentrations in the luminal content and intestine wall was used as an estimator of the capacity of inhibition of both P-gp modulators on the gastrointestinal secretion of IVM. The co-administration of PSC833 induced a marked decrease on the IVM
concentration measured in the intestinal content. At 6 h post-administration of IVM alone, its concentrations in the luminal content were 1.53, 1.14 and 9.4-fold higher than those obtained after the co-administration with PSC833 in jejunum, ileum and colon contents, respectively. Consequently, a significantly higher ratio between the Cmax at the intestinal content and the Cmax at the intestinal wall was obtained after the IVM alone compared with the IVM co-administered treatments. The ratio of Cmax intestinal content / Cmax intestinal wall in the co-administered groups as a percentage from control group is shown in Figure 6.
Discussion

The pharmacological effects of a given drug compounds are highly influenced by its pharmacokinetic behavior. Physico-chemical properties are critical to the absorption, distribution, metabolism and excretion of different xenobiotic compounds. However, it has now become apparent that different transport proteins play an important role regulating the kinetic disposition of several drugs (Hochman et al., 2002). Additionally, clinically drug relevant interactions may occur after the concomitant administration of different compounds. The use of P-gp inhibitors to enhance drug systemic and tissue availabilities has been applied in vitro (Song et al., 1999) and in vivo (Mayer et al., 1997; Kwei et al., 1999). The ultimate goal of the interaction studies between P-gp substrate/modulator has been addressed to identify the pharmacokinetic consequences and to predict the clinical outcome.

Several approaches are available to assess "in vitro" the role of the specific proteins involved in the intestinal drug transport processes. However, the inference from the quantitative information obtained in the in vitro models to the "in vivo" situation may be limited. Thus, the prediction of absorption process for a given P-gp substrate from "in vitro" assays could not be correlated to the "in vivo" results obtained where a co-administration with a P-gp modulator is performed (Yee, 1997). This could be due to differences in the length of drug exposure, drug concentrations, concomitant induction of metabolic pathways and extent of P-gp inhibition (Fricker and Miller, 2002).
Previous *in vitro* studies reported that IVM was actively secreted by multidrug-resistant tumor cells (Poulliot et al., 1997) and by cells transfected with the gene coding for P-gp in the mouse (Shinkel et al., 1995). In addition, Barthe et al, (1998) described the everted gut sac as a simple method to study intestinal absorption of digoxin in presence or absence of verapamil or quinidine, obtaining a high correlation with the "*in vitro*" Caco-2 cells (Cavet, 1996) and "*in vivo" methods using knockout mice (Mayer et al., 1997). The results of the current trial confirm that the everted sac technique is a useful *in vitro* model system for studying the P-gp-mediated efflux of extremely lipophilic molecules such as IVM. Differential P-gp transport properties between IVM and other close related macrocyclic lactone compounds (moxidectin, eprinomectin, etc) have been previously suggested (Lespine *et al.* 2003; Molento et al., 2004). Thus, the application of the everted sac technique described here may result as a straightforward *in vitro* method to further characterize the comparative intestinal transport of the different IVM-related compounds as well as the impact of its modulation, which may have a remarkable relevance in antiparasitic therapy. The satisfactory results obtained with the use of the everted sac model to assess the P-gp-mediated modulation of IVM transport may be relevant to achieve reproducible results between the *in vitro* and *in vivo* assays, avoiding also potential side effects of the P-gp-modulating agents under evaluation.

Working under the described experimental conditions, viability of the intestinal tissue was assumed as it was shown by the glucose uptake assay. The high lipophilicity of IVM accounted for its greater concentrations in the ileal tissue compared to those obtained in the fluid content of the sacs. The IVM concentration profiles measured in the ileal wall and content of the sacs was markedly modified by the presence of ITZ and PSC833. The IVM
tissue accumulation at 45 min of incubation was 2.1 (ITZ) and 3.7 (PSC833) –fold higher compared to the incubation of IVM alone. The same pattern of increase in IVM concentrations was observed in the serosal transfer, where the drug concentration in the sac contents at 5 min was 1.84 (ITZ) and 4.15 (PSC833) –fold higher than that measured when IVM was incubated alone. The comparison of the inhibitory effect between both P-gp modulators clearly indicated that PSC833 is more effective in modulating the P-gp-mediated IVM intestinal secretion than ITZ. Under in vitro conditions, PSC833 modulator activity was significantly higher compared to that of ITZ at equimolar doses. After 10 min of incubation, IVM concentration was 2.31-fold higher in intestinal wall and 1.78-fold higher in the serosal transfer in the presence of PSC833 compared to ITZ. The rate of IVM accumulation in the intestinal wall was significantly higher in the presence of PSC833 (0.016 nmol/g/min) than those obtained either in presence of ITZ (0.115 nmol/g/min) or IVM alone (0.238 nmol/g/min). Thus, these results demonstrated that the everted sac technique was useful to compare the P-gp inhibition by modulators from different generation used at equimolar doses. Besides, previously reported work done with everted sacs used a high molar ratio between the P-gp modulator and the substrate under study. 

Enhancement in the tissue accumulation of anticancer drugs and digoxin was obtained using molar ratios between 10 (Barthe et al., 1998) and 80 (Carreño-Gómez and Duncan, 2000). In the currently reported trial a marked increase on IVM ileal tissue accumulation was observed using a P-gp modulators/IVM molar ratio of 3.3.

Interestingly, the results reported in our in vitro experiments were directly related to the observed in vivo changes on the pharmacokinetic behaviour of IVM after the co-administration with both P-gp modulators. This is a relevant issue considering that in
previous studies was not always possible to reproduce \textit{in vivo}, the results observed \textit{in vitro}. For instance, whereas ketoconazole appeared as the most valuable tool for increasing the intracellular quantity of 14C moxidectin in rat hepatocyte cell cultures, the compound had no effect on the \textit{in vivo} pharmacokinetics of moxidectin in lambs (Dupuy et al., 2003).

Although Kwei et al, (1999) did not find a correlation between IVM plasma availability and its tissue disposition after the i.v. administration of verapamil as a modulator, under our experimental conditions, concomitant administration of IVM and ITZ or PSC833 increased the antiparasitic compound availability in the bloodstream and all intestinal tissues analysed. These results are in agreement with those obtained by Lifschitz et al, (2004), where the plasma and tissue disposition of IVM was markedly modified after its co-administration with loperamide, a P-gp modulator. A more pronounced increment on the IVM ileal availability (AUC between 91 and 149 \%) was obtained after its co-administration with ITZ and PSC833 compared to that obtained after the loperamide treatment (60 \%).

As observed in our \textit{in vitro} assays, the pharmacokinetic study showed that IVM intestinal concentrations were significantly higher in the presence of both P-gp modulators. The extent of interaction of several drugs with P-gp along the cephalocaudal axis of the intestine in relation to drug absorption has been demonstrated. Previous studies have noted significant regional differences in P-gp expression at the mRNA level with expression increasing from the small intestine to the colon (Fojo et al., 1987; Fricker et al., 1996). In the current work, the greatest response to the co-administration of the modulators was observed in the ileum wall, where the IVM Cmax was 2.94 (ITZ) and 4.41 (PSC833) -fold
higher compared to administration of IVM alone. The regional distribution of P-gp activity observed here is consistent with the results reported by Stephens et al, (2002), where P-gp expression was higher in ileum and distal colon of mice.

In agreement with the data collected from the everted sac study, some differences were observed between the \textit{in vivo} effect of the P-gp modulators on IVM pharmacokinetics. The effect of PSC833 was greater compared to ITZ particularly at the intestinal level where PSC833 induced a significant reduction on the IVM concentrations measured in the intestinal contents compared to the IVM+ITZ and IVM alone groups. Therefore, the ratio of peak concentration in intestinal content and intestinal wall may be considered as a measurement of the inhibition of the intestinal secretion process. The inhibitory effect of P-gp modulators on the IVM intestinal secretion accounted for a reduction on the ratio between the Cmax in intestinal content and Cmax in the intestinal wall of 43 \% (ITZ) and 77 \% (PSC833) compared to the control group.

The influence of the P-gp modulators on the IVM elimination processes seems to be different in the intestinal tissue compared to that observed in the liver. Whereas the IVM plasma availability was increased 90-93 \% after its co-administration with ITZ and PSC833, the increase in liver AUC was between 59-61 \% compared to the control group. However, ITZ and PSC833 increased the IVM availability in ileum between 91 and 149 \% which indicates a potent modulation of the active P-gp- mediated IVM secretion process. The relationship between IVM availabilities in tissues and bloodstream (tissues/plasma ratio) may result useful to assess the influence of the modulators on the P-gp excretory activity. The ratio of AUC liver/plasma was lower after the co-administration of P-gp
modulators while at the ileal level, the ratio AUC ileum/plasma was similar and higher after ITZ and PSC833 modulation, respectively. These results are consistent with those reported by Laffont et al. (2002), where the amount of IVM parent drug actively secreted in the small intestinal lumen was five times higher than that eliminated in bile and, suggesting that the observed increase in the IVM plasma availability was largely due to an inhibitory effect on its intestinal secretion. The relative involvement of the biliary and intestinal excretion mechanisms for a P-gp substrate as well as IVM in different species needs to be elucidated.

In conclusion, the results from the studies reported here indicate that the plasma and tissue disposition of IVM was strongly affected by P-gp activity. Additionally, we have demonstrated that IVM disposition kinetics in the intestinal tissues was significantly modified by the presence of P-gp modulators, both under in vivo and in vitro conditions, which confirm the relevance of this cellular transporter on the intestinal secretion of IVM. The in vitro everted sac technique was an accurate model to evaluate the differential effects of P-gp-modulating agents belonging to different generations. The characterisation of the intestinal elimination pathway for IVM would be of therapeutic significance both in human and veterinary medicine. As a consequence of the inhibitory effect on P-gp intestinal activity, high IVM concentrations could be available at the target tissues, improving its clinical antiparasitic efficacy. Recently, the in vivo efficacy of the co-administration of IVM and a P-gp modulator (verapamil) was assayed in sheep infected with resistant nematodes. IVM given alone had a 0 % reduction in the number of parasite eggs in feces, confirming the strong resistance of those parasite strains. However, a 74.7 % reduction in the egg fecal counts was achieved after the co-administration of IVM with verapamil.
(Borges et al., 2005). The co-administration of IVM with a modulator agent could become a useful pharmacological tool to modify the kinetic behaviour of anthelmintic compounds, improving their antiparasitic efficacy and slowing down, in some cases, the development of resistance.
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References


Footnotes

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Legends for Figures

**Figure 1.** Mean ratio between glucose concentration (g/l) into the everted gut sac content and in the incubation medium (n=4) as an indicator of intestinal viability. a) Incubation of gut sacs without drug, b) Incubation of gut sacs in presence of ivermectin (3 µM) and P-gp modulators (10 µM). Glucose concentration ratios are statistically different from those obtained at time 0 (Ratio=1) at P<0.05.

**Figure 2.** Ivermectin (IVM) accumulation in the ileum wall of the everted gut sacs after its incubation alone (3 µM) or with a) itraconazole (ITZ) (10 µM) or b) PSC833 (10 µM). Values are statistically different from those obtained after IVM alone incubation at *P < 0.05.

Each point represents the mean ± SEM of four (4) experiments. (*) Values are statistically different from those obtained after the incubation with IVM alone at P<0.05.

**Figure 3.** Ivermectin (IVM) accumulation rate (expressed as nmol/g/min) (IVM) in the wall (ileum) of the everted gut sacs after its incubation alone (3 µM) or with itraconazole (ITZ) (10 µM) and PSC833 (10 µM). Data are expressed as mean ± SEM (n=4). Values lacking a common letter are significantly different at P<0.05.

**Figure 4.** Mean (± SEM.) ivermectin (IVM) concentration profiles (ng/g) measured in the jejunum wall after its subcutaneous administration (200 µg/Kg) alone or co-administered with a) itraconazole (ITZ) and b) PSC833 to Wistar male rats (*in vivo* experiments).
Figure 5. Comparative effect of P-glycoprotein modulators on ivermectin (IVM) peak concentration (Cmax) values in plasma, liver, jejunum and ileum wall obtained after its co-administration with itraconazole (ITZ) and PSC833 to male Wistar rats. The data are expressed as percentage of enhancement compared to control group (IVM alone treatment).

Figure 6. Changes on the ratio between ivermectin (IVM) peak concentration measured in intestinal content (lumen) and intestinal wall observed after its co-administration with itraconazole (ITZ) and PSC833 to male Wistar rats. The results express the percentage of reduction compared to the control group (IVM alone treatment). Values lacking a common letter are significantly different at P<0.05.
Table 1. Serosal transfer of ivermectin (IVM) (measured as drug concentration within the everted gut sac) obtained after its incubation in the presence/absence of the P-glycoprotein modulators, itraconazole (ITZ) and PSC833.

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>IVM alone</th>
<th>IVM + ITZ</th>
<th>IVM + PSC833</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.08 ± 0.35a</td>
<td>3.83 ± 0.41b</td>
<td>8.64 ± 2.32c</td>
</tr>
<tr>
<td>10</td>
<td>3.01 ± 0.25a</td>
<td>4.72 ± 0.91a</td>
<td>8.41 ± 1.87b</td>
</tr>
<tr>
<td>15</td>
<td>5.63 ± 1.49a</td>
<td>10.7 ± 4.87a</td>
<td>11.9 ± 4.31a</td>
</tr>
<tr>
<td>25</td>
<td>7.10 ± 2.78a</td>
<td>14.4 ± 7.77a</td>
<td>13.9 ± 0.86a</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM (n=4).

Within a row mean IVM concentration values lacking a common superscript are significantly different at P<0.05.
Table 2. Comparative ivermectin (IVM) area under the concentration vs time curve (AUC) in plasma, liver and gastrointestinal wall tissues (jejunum, ileum and colon) obtained after its subcutaneous administration (200 µg/kg) either alone or co-administered with itraconazole (ITZ) or PSC833 to Wistar rats (*in vivo* experiments)

<table>
<thead>
<tr>
<th>Tissue/ fluid</th>
<th>IVM alone</th>
<th>IVM + ITZ</th>
<th>IVM + PSC833</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>20.0 ± 1.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.9 ± 4.88&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.6 ± 1.65&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver</td>
<td>153 ± 15.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>243 ± 38.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>246 ± 21.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jejunum wall</td>
<td>153 ± 12.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>259 ± 18.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>263 ± 40.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ileum wall</td>
<td>126 ± 7.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>241 ± 57.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>314 ± 61.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colon wall</td>
<td>146 ± 29.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>182 ± 43.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>174 ± 15.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM (n=3). AUC in plasma is expressed in ng.d/ml

Within a row mean AUC values lacking a common letter are significantly different at P<0.05.
Figure 1

a) Without drug

- Incubation time (min)
  - 20
  - 55
  - 70

- Glucose concentration (g/l)
  - 0.0
  - 0.5
  - 1.0
  - 1.5
  - 2.0
  - 2.5
  - 3.0
  - 3.5

b) IVM + P-gp modulators

- Incubation time (min)
  - 20
  - 55
  - 70

- Glucose concentration (g/l)
  - 0.0
  - 0.5
  - 1.0
  - 1.5
  - 2.0
  - 2.5
  - 3.0
  - 3.5

(*)
Figure 2

[Graph showing the concentration of IVM alone and IVM+ITZ or IVM+PSC833 over time.]

* Indicates statistical significance.
Figure 3

The diagram shows the ivermectin accumulation rate (nmoles/g/min) for different treatments:

- **IVM**
- **IVM+ITZ**
- **IVM+PSC833**

(a) represents the control group, (b) shows a decrease in accumulation rate, and (c) indicates a significantly higher accumulation rate compared to the control.
Figure 5

Enhancement compared to control group (%)
Figure 6

% changes respect to control group (values are expressed as ratio Cmax_intestinal content/Cmax_intestinal wall)

- **Control**
- IVM + ITZ
- IVM + PSC833

**Legend**

- Jejunum
  - a
  - b
  - c

- Ileum
  - a
  - b

- Colon
  - a
  - b