

MODULATION OF THE P-GLYCOPROTEIN-MEDIATED INTESTINAL SECRETION OF IVERMECTIN: 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 (ITZ) and valsopodar (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 characterized. For the *in vitro* experiments, ileal sacs were incubated with IVM (3 μM) in the presence or absence of either ITZ (10 μM) or PSC833 (10 μM). In the *in vivo* experiments, male Wistar rats were randomly allocated to three groups ($n = 18$) and subcutaneously treated with IVM (200 $\mu\text{g}/\text{kg}^{-1}$), alone and coadministered 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 high performance liquid chromatography. 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 to the intestinal secretion of IVM.

P-glycoprotein (P-gp) is a transmembrane protein, associated with a phenotype of multidrug resistance to certain anticancer drugs in mammalian cancer cells, that 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, seem 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 the capacity to reverse the multidrug resistance phenotype. Verapamil, a calcium channel blocker (Tsuruo et al., 1981), was one of the first identified P-gp modulator agents. The main disadvantage for the first generation of modulators was that the inhibition was produced at concentrations higher than those used 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 CYP3A inhibitor (Cooper et al., 2003). The search for a second generation of nontoxic modulators was addressed by the development of more effective and less toxic compounds. PSC833 (valsopodar, Novartis Pharma AG), a nonimmunosuppressive cyclosporin A analog, is one of the most effective modulator agents, with the capacity to reverse the P-gp-mediated multidrug resistance in clinical trials (Fisher and Sikic, 1995).

Ivermectin (IVM) is a broad-spectrum anthelmintic compound extensively used in human and veterinary medicine. IVM is largely excreted in bile and feces 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 closed-loop model. Coadministration of IVM with loperamide (a P-gp modulator) resulted in changes in the pattern of IVM bile-fecal 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 trans-epithelial passage of different P-gp substrates (Fricker et al., 1996). However, the correlation between this *in vitro* model and *in vivo*

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ABBREVIATIONS: P-gp, P-glycoprotein; PSC833, valsopodar; IVM, ivermectin; ITZ, itraconazole; HPLC, high performance liquid chromatography; AUC, area under the concentration-time curve.

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: 1) to assess *in vitro* the involvement of P-gp in the intestinal secretion of IVM using the everted gut sac technique; 2) 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, 3) 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 4) 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 to 300 g were starved overnight. Under anesthesia, the intestine was rapidly removed and washed with buffer solution (1 mM $\text{PO}_4\text{H}_2\text{NaH}_2\text{O}$, 2.5 mM $\text{Cl}_2\text{Ca}_2\text{H}_2\text{O}$, 4.7 mM KCl, 1.1 mM $\text{Cl}_2\text{Mg}_6\text{H}_2\text{O}$, 0.004 mM EDTA, 11 mM glucose, 119 mM NaCl, 25 mM Co_3Na , 0.11 mM ascorbic acid). The intestine was immediately placed in warm (37°C) oxygenated (O_2/CO_2 , 95%:5%) Medium 199 (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°C 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°C.

Incubation Assays. To study the tissue uptake and serosal transfer of IVM in the 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°C with O_2/CO_2 (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 was 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°C 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). Because 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 TC 199 medium into the chambers in the absence and presence of IVM (3 μM) and P-gp modulators (10 μM), maintaining the same conditions as in 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 follows: 20 μl of sample was incubated at 37°C for 10 min with a reagent that consists of 50% distilled water, 5% 4-aminofenazona (25 mM solution in Tris buffer, 0.92 mol/l), 5% fenol solution (55 mM), and 0.3% glucose oxidase (1000 U/ml)/peroxidase (120 U/ml). Finally, the absorbance was measured in the spectrophotometer at 505 nm.

In Vivo Experiments. Fifty-four male Wistar rats weighing 250 to 300 g were used 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 to three groups of 18 animals each. Animals in group A (IVM alone) received IVM at 200 $\mu\text{g}/\text{kg}^{-1}$ (Ivomec; Merial, Whitehouse Station, NJ) by s.c. 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 coadministered with ITZ (ethanol/propylene glycol 25:75) at 5 mg (two doses with a 12-h interval) injected intraperitoneally. Animals in group C (IVM + PSC833) received IVM at the same dose rate coadministered with PSC833 formulated in ethanol/propylene glycol (25:75) at a dose equimolar with ITZ (two doses of 8.6 mg with a 12-h interval), and administered by oral route. Under superficial ether anesthesia, 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 2000g for 20 min, and the recovered plasma was kept in labeled vials. Plasma and tissue 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, 10 ng/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) over 15 min, sonicated in an ultrasonic bath for 10 min (Transsonic 570/H; Laboratory Line Instruments Inc., Melrose Park, IL), and the solvent-sample mixture was centrifuged at 2000g 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). After washing with 1 ml of deionized water followed by 1 ml of water/methanol (4:1 v/v), the cartridges were dried off for 5 min and the sample was eluted with 1.5 ml of methanol, which was collected. After solid phase extraction, the eluate 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 C_{18} column (Selectosil; Phenomenex, Torrance, CA) (5 μm , 4.6 mm \times 250 mm). IVM detection was performed using a fluorescence detector (spectrofluorometric detector RF-10; Shimadzu) set at an excitation wavelength of 365 nm and an emission wavelength of 475 nm. The abamectin/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; GraphPad Software Inc., San Diego, CA) 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 tissue IVM spiked standards at different concentrations (three replicates). Calibration curves were established using least-squares linear regression analysis, and correlation coefficients (r) and coefficients of variation (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 analyzed. 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 analyzed. Coefficients of variation <7% were obtained when the interday 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 analyzed were fitted using the Pk Solutions 2.0 computer software (Summit Research Services, Ashland, OH). Pharmacokinetic parameters were determined using a noncompartmental method. Peak concentration (C_{max}) and time to peak concentration (T_{max}) were read from the plotted concentration-time curves. The area under the concen-

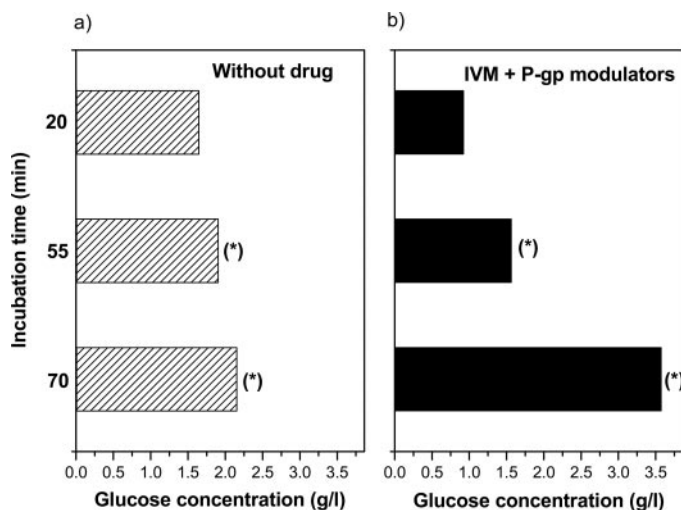


FIG. 1. Mean ratio between glucose concentration (g/l) into the everted gut sac content and that in the incubation medium ($n = 4$) as an indicator of intestinal viability. a, incubation of gut sacs without drug; b, incubation of gut sacs in the presence of ivermectin ($3 \mu\text{M}$) and P-gp modulators ($10 \mu\text{M}$). Glucose concentration ratios are statistically different from those obtained at time 0 (ratio = 1) at $P < 0.05$.

tration-time curve (AUC) was calculated by the trapezoidal rule (Gibaldi and Perrier, 1982). Mean pharmacokinetic parameters for IVM obtained after the administration of IVM, alone or coadministered with either ITZ or PSC833, were statistically compared by analysis of variance (Instat 3.0; GraphPad Software Inc.). 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 (Fig. 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 ileal wall was 0.016 nmol/g/min . Higher IVM concentrations in the intestinal wall were measured after the incubation with ITZ and PSC833, compared with the incubation with IVM alone. IVM concentrations in the ileal 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 Fig. 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 ileal wall is shown in Fig. 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 with 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, coadministration with both P-gp modulators enhanced IVM concentrations in the intestinal tissues and in the liver. The mean IVM concentrations measured in the jejunal wall after its coadministration with ITZ and PSC833 compared with the control group are shown in Fig. 4. The comparison of the IVM AUCs obtained in plasma and digestive tissues in the control and coadministered experimental groups is shown in Table 2. Figure 5 summarizes the percentage of enhancement on IVM peak concentration (C_{max}) 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 coadministration of PSC833 induced a marked decrease on the IVM concentration measured in the intestinal content. At 6 h after 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 coadministration with PSC833 in jejunum, ileum, and colon contents, respectively. Consequently, a significantly higher ratio between the C_{max} at the intestinal content and the C_{max} at the intestinal wall was obtained after IVM alone compared with IVM coadministered treatments. The ratio of C_{max} intestinal content/ C_{max} intestinal wall in the coadministered groups as a percentage from the control group is shown in Fig. 6.

Discussion

The pharmacological effects of a given drug compound are highly influenced by its pharmacokinetic behavior. Physicochemical 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 in regulating the kinetic disposition of several drugs (Hochman et al., 2002). In addition, clinically relevant drug 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 and 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 the absorption process for a given P-gp substrate from in vitro assays could not be correlated to the in vivo results obtained when coadministration with a P-gp modulator is performed (Yee, 1997). This outcome 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 (Schinkel 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 the presence or absence of verapamil or quinidine, obtaining a high

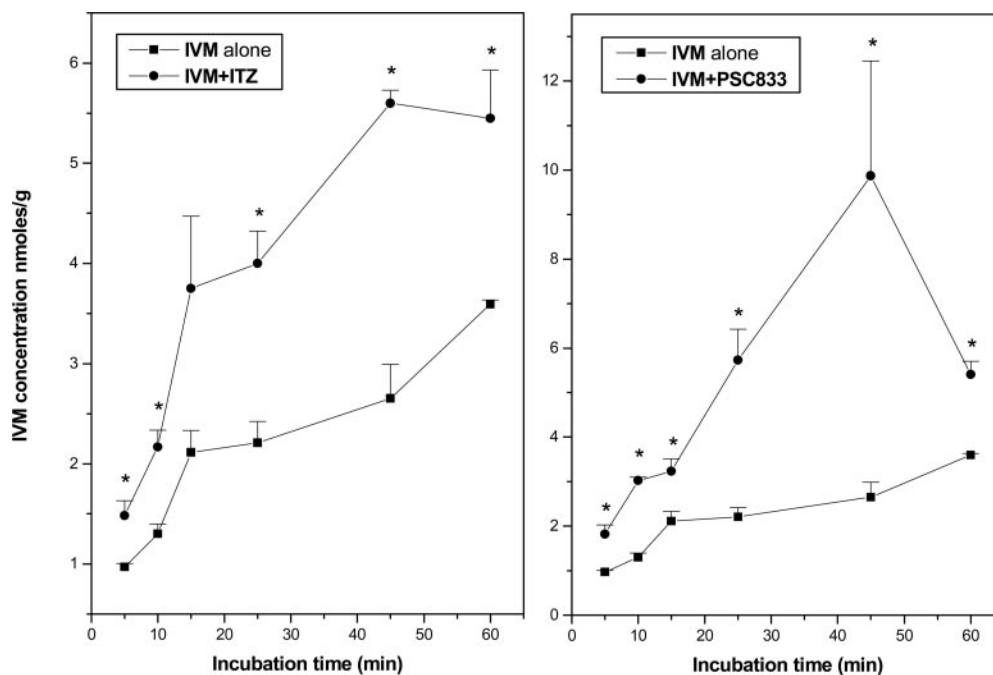


FIG. 2. IVM accumulation in the ileal wall of the everted gut sacs after its incubation alone (3 μ M) or with ITZ (10 μ M) (left) or PSC833 (10 μ M) (right). *, values are statistically different from those obtained after IVM-alone incubation at $P < 0.05$. Each point represents the mean \pm S.E.M. of four experiments.

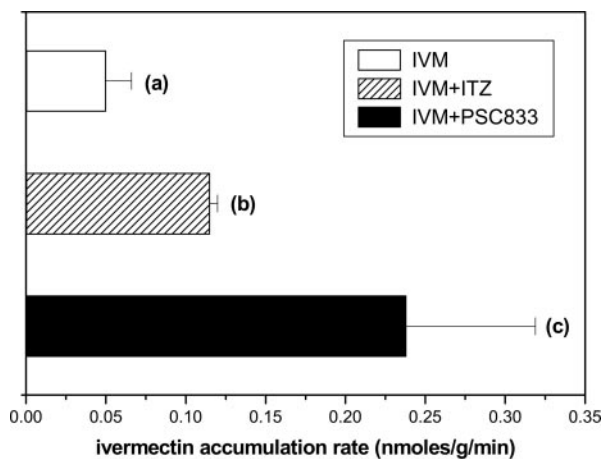


FIG. 3. IVM accumulation rate (expressed as nmol/g/min) (IVM) in the wall (ileum) of the everted gut sacs after its incubation alone (3 μ M) or with ITZ (10 μ M) and PSC833 (10 μ M). Data are expressed as mean \pm S.E.M. ($n = 4$). Values lacking a common letter are significantly different at $P < 0.05$.

correlation with the *in vitro* Caco-2 cells (Cavet et al., 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 closely related macrocyclic lactone compounds (moxidectin, eprinomectin, etc.) have been suggested previously (Lespine et al., 2003; Molento et al., 2004). Thus, the application of the everted sac technique described here may result in 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

TABLE 1

Serosal transfer of IVM (measured as drug concentration within the everted gut sac) obtained after its incubation in the presence/absence of the P-glycoprotein modulators, ITZ and PSC833

Values represent mean \pm S.E.M. ($n = 4$). Within a row, mean IVM concentration values lacking a common superscript are significantly different at $P < 0.05$.

| Incubation Time | IVM Concentration | | |
|-----------------|------------------------------|------------------------------|------------------------------|
| | IVM Alone | IVM + ITZ | IVM + PSC833 |
| | <i>nM</i> | | |
| 5 min | 2.08 \pm 0.35 ^a | 3.83 \pm 0.41 ^b | 8.64 \pm 2.32 ^c |
| 10 min | 3.01 \pm 0.25 ^a | 4.72 \pm 0.91 ^a | 8.41 \pm 1.87 ^b |
| 15 min | 5.63 \pm 1.49 ^a | 10.7 \pm 4.87 ^a | 11.9 \pm 4.31 ^a |
| 25 min | 7.10 \pm 2.78 ^a | 14.4 \pm 7.77 ^a | 13.9 \pm 0.86 ^a |

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 with 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 with 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 than ITZ in modulating the P-gp-mediated IVM intestinal secretion. Under *in vitro* conditions, PSC833 modulator activity was significantly higher compared with 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 with ITZ. The rate of IVM accumulation in the intestinal

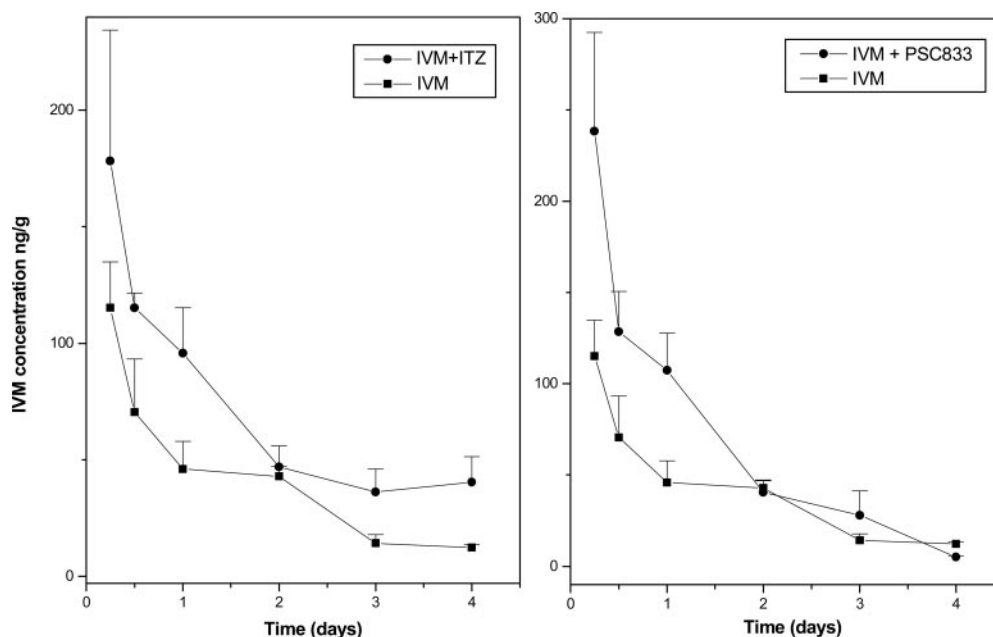


FIG. 4. Mean (\pm S.E.M.) IVM concentration profiles (ng/g) measured in the jejunal wall after its subcutaneous administration (200 μ g/kg) alone or coadministered with ITZ (left) and PSC833 (right) to Wistar male rats (in vivo experiments).

TABLE 2

Comparative IVM AUC in plasma, liver, and gastrointestinal wall tissues (jejunum, ileum, and colon) obtained after its subcutaneous administration (200 μ g/kg) either alone or coadministered with ITZ or PSC833 to Wistar rats (in vivo experiments)

Values represent mean \pm S.E.M. ($n = 3$). Within a row, mean AUC values lacking a common letter are significantly different at $P < 0.05$.

| Tissue/Fluid | Ivermectin AUC | | |
|--------------|------------------------------|------------------------------|------------------------------|
| | IVM Alone | IVM + ITZ | IVM + PSC833 |
| | ng · day/g | | |
| Plasma | 20.0 \pm 1.40 ^a | 37.9 \pm 4.88 ^b | 38.6 \pm 1.65 ^b |
| Liver | 153 \pm 15.9 ^a | 243 \pm 38.1 ^b | 246 \pm 21.9 ^b |
| Jejunal wall | 153 \pm 12.9 ^a | 259 \pm 18.1 ^b | 263 \pm 40.5 ^b |
| Ileal wall | 126 \pm 7.90 ^a | 241 \pm 57.7 ^a | 314 \pm 61.9 ^b |
| Colon wall | 146 \pm 29.0 ^a | 182 \pm 43.4 ^a | 174 \pm 15.3 ^a |

wall was significantly higher in the presence of PSC833 (0.238 nmol/g/min) than those obtained either in the presence of ITZ (0.115 nmol/g/min) or IVM alone (0.016 nmol/g/min). Thus, these results demonstrated that the everted sac technique was useful to compare the P-gp inhibition by modulators from different generations 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 in IVM ileal tissue accumulation was observed using a P-gp modulator/IVM molar ratio of 3.3.

Interestingly, the results reported in our in vitro experiments were directly related to the observed in vivo changes in the pharmacokinetic behavior of IVM after coadministration with both P-gp modulators. This is a relevant issue, considering that in previous studies, it was not always possible to reproduce in vivo the results observed in vitro. For instance, whereas ketoconazole seemed to be the most valuable tool for increasing the intracellular quantity of [¹⁴C]moxidectin in rat hepatocyte cell cultures, the compound had no effect on

the 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 analyzed. These results are in agreement with those obtained by Lifschitz et al. (2004), wherein the plasma and tissue disposition of IVM was markedly modified after its coadministration with loperamide, a P-gp modulator. A more pronounced increment on the IVM ileal availability (AUC between 91 and 149%) was obtained after its coadministration with ITZ and PSC833 compared with that obtained after the loperamide treatment (60%).

As observed in our 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 coadministration of the modulators was observed in the ileal wall, where the IVM C_{max} was 2.94 (ITZ)- and 4.41 (PSC833)-fold higher compared with administration of IVM alone. The regional distribution of P-gp activity observed here is consistent with the results reported by Stephens et al. (2002), in which 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 in vivo effect of the P-gp modulators on IVM pharmacokinetics. The effect of PSC833 was greater compared with ITZ, particularly at the intestinal level, where PSC833 induced a significant reduction in the IVM concentrations measured in the intestinal contents compared with 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 inhibi-

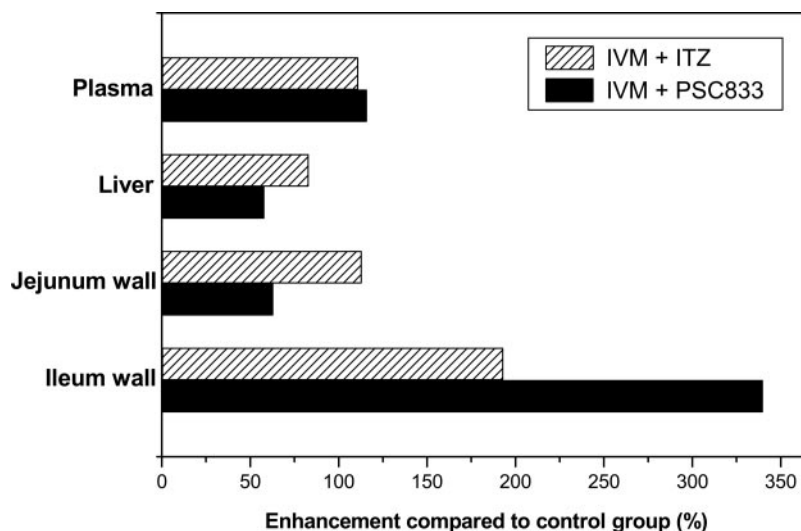


FIG. 5. Comparative effect of P-glycoprotein modulators on IVM peak concentration (C_{max}) values in plasma, liver, jejunal, and ileal wall obtained after its coadministration with ITZ and PSC833 to male Wistar rats. The data are expressed as percentage of enhancement compared with control group (IVM-alone treatment).

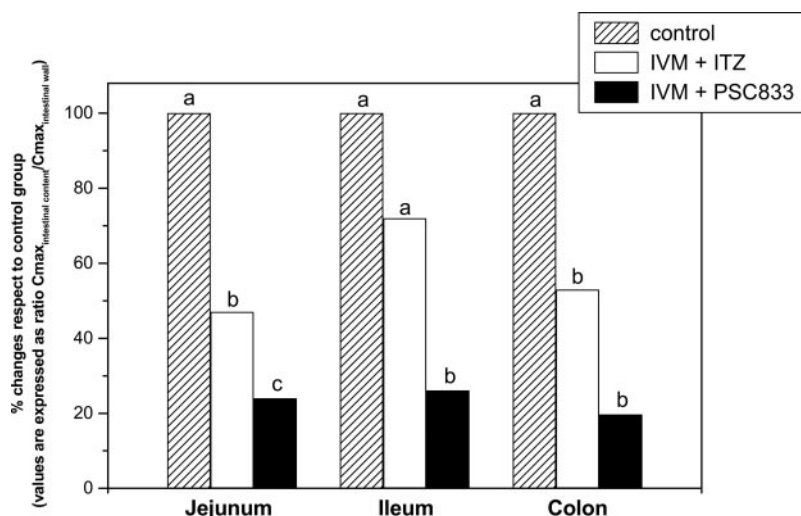


FIG. 6. Changes in the ratio between IVM peak concentration measured in intestinal content (lumen) and intestinal wall observed after its coadministration with ITZ and PSC833 to male Wistar rats. The results express the percentage of reduction compared with the control group (IVM-alone treatment). Values lacking a common letter are significantly different at $P < 0.05$.

tory effect of P-gp modulators on the IVM intestinal secretion accounted for a reduction in the ratio between the C_{max} in intestinal content and C_{max} in the intestinal wall of 43% (ITZ) and 77% (PSC833) compared with the control group.

The influence of the P-gp modulators on the IVM elimination processes seems to be different in the intestinal tissue compared with that observed in the liver. Whereas the IVM plasma availability was increased 90 to 93% after its coadministration with ITZ and PSC833, the increase in liver AUC was between 59 and 61% compared with 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 be useful to assess the influence of the modulators on the P-gp excretory activity. The ratio of AUC liver/plasma was lower after the coadministration of P-gp modulators, whereas 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), in which

the amount of IVM parent drug actively secreted in the small intestinal lumen was 5 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. In addition, we have demonstrated that IVM disposition kinetics in the intestinal tissues was significantly modified by the presence of P-gp modulators, under both in vivo and in vitro conditions, which confirms 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 characterization 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 concen-

trations could be available at the target tissues, improving its clinical antiparasitic efficacy. Recently, the *in vivo* efficacy of the coadministration 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 coadministration of IVM with verapamil (Borges et al., 2005). The coadministration of IVM with a modulator agent could become a useful pharmacological tool to modify the kinetic behavior of anthelmintic compounds, improving their antiparasitic efficacy and slowing down, in some cases, the development of resistance.

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