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Role of P-glycoprotein in the disposition of macrocyclic lactones: a comparison between ivermectin, eprinomectin and moxidectin in mice

Solange Kiki-Mvouaka, Cécile Ménez, Christiane Borin, Faouri Lyazrhi, Magali Foucaud-Vignault, Jacques Dupuy, Xavier Collet, Michel Alvinerie and Anne Lespine

INRA UR66, F-31027 Toulouse, France (S.K., C.M., C.B., M.F., J.D., M.A., A.L.)

ENVT/INRA UMR 181, F-31076 Toulouse, France (F.L.)

ENFA, 2 route de Narbonne, F-31326 Castanet-Tolosan (C.B.)

INSERM U563, CPTP, IFR150 and Université Paul Sabatier, F-31024 Toulouse (X.C.)

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Corresponding author:

Anne Lespine, INRA-UR66, Laboratoire de Pharmacologie-Toxicologie, BP 93173, 31027 Toulouse Cedex 3, France

Phone : 33-561285387 - Fax : 33-561285310

E-mail : anne.lespine@toulouse.inra.fr

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Abbreviations

ABC: ATP-Binding Cassette. AUC: Area under the plasma concentration-time curve. EPR: eprinomectin. IVM: ivermectin. MLs: macrocyclic lactones. MOX: moxidectin. MRT: Mean Residence Time. *Per os*: oral administration.

Abstract

Macrocyclic lactones (MLs) are lipophilic anthelmintics and substrates for P-glycoprotein (P-gp), an ABC transporter involved in drug efflux out of both host and parasites. In order to evaluate the contribution of P-gp to the *in vivo* kinetic disposition of MLs, the plasma kinetics, brain concentration and intestinal excretion of three structurally different MLs (ivermectin, eprinomectin and moxidectin) were compared in wild-type and P-gp deficient (mdr1ab$^{-/-}$) mice. Each drug (0.2 mg/kg) was administered orally, intravenously or subcutaneously to the mice. Plasma, brain and intestinal tissue concentrations were measured by HPLC. The intestinal excretion rate following intravenous administration was determined at different levels of the small intestine by using an *in situ* intestinal perfusion model. P-gp deficiency led to a significant increase in the area under the plasma concentration-time curve (AUC) of ivermectin (1.5 fold) and eprinomectin (3.3 fold) while the moxidectin AUC was unchanged. Interestingly, ivermectin, and to a greater extent eprinomectin, were both excreted by the intestine *via* a P-gp-dependent pathway while moxidectin excretion was weaker and mostly P-gp independent. The three drugs accumulated in the brains of the mdr1ab$^{-/-}$ mice but eprinomectin concentrations were significantly lower. We concluded that eprinomectin disposition in mice is mainly controlled by P-gp efflux, more so than ivermectin, while moxidectin disposition appears to be mostly P-gp-independent. Given that eprinomectin and ivermectin have higher affinity for P-gp than moxidectin, these findings demonstrated that the relative affinity of MLs for P-gp could be predictive of the *in vivo* kinetic behaviour of these drugs.

Introduction

P-glycoprotein (P-gp) is a plasma membrane protein belonging to the ATP-Binding Cassette (ABC) super-family. First identified as a factor involved in multidrug resistance in mammalian tumour cells (Juliano and Ling, 1976), P-gp was subsequently shown to pump a broad range of structurally and functionally unrelated compounds out of cells in an energy-dependant manner (Schinkel and Jonker, 2003). Physiologically expressed in the apical surface of epithelial cells (Thiebaut et al., 1987), P-gp promotes the efflux of potentially toxic compounds, from the blood into the gut lumen, or from the brain into the blood (Bodo et al., 2003), but also limits the availability of a large number of drugs in many organisms.

Macrocyclic lactones (MLs) are a large family of broad spectrum antiparasitic drugs widely used for the treatment of internal and external parasites in veterinary and human medicine (Hennessy and Alvinerie, 2002). The mechanism of action of MLs in parasites is based on their high affinity for glutamate-gated chloride channels, and in mammals they bind to γ-aminobutyric acid-gated chloride channels that are confined to the central nervous system. To achieve therapeutic activity, an effective concentration of MLs during a suitable length of time is needed in target tissues. P-gp has been clearly identified as the main factor that controls the concentration of these drugs by impacting on their *in vivo* absorption, distribution and elimination in the host (Schinkel et al., 1996; Kwei et al., 1999). MLs are poorly metabolized (Chiu et al., 1987; Alvinerie et al., 2001) and are good substrates and potent inhibitors of P-gp (Didier and Loor, 1996; Pouliot et al., 1997; Lespine et al., 2007). P-gp protects the brain from MLs by limiting their penetration across the blood-brain-barrier and thus their subsequent neurotoxicity (Schinkel et al., 1994; Lankas et al., 1997; Roulet et al., 2003), and it contributes to the elimination of ivermectin *via* intestinal excretion (Laffont et al., 2002; Ballent et al., 2006). Moreover, P-gp mediated drug efflux has been suggested as a protection mechanism in nematodes and several P-gp homologues are over-expressed in

ivermectin resistance (Prichard and Roulet, 2007). In the context of the emergence of ML resistance in target parasites, the inhibition of P-gp is considered as a valuable strategy to increase the efficacy of MLs (Lespine et al., 2008).

There are two main families of MLs: avermectins and milbemycins, distinguishable by specific substituents and physicochemical properties such as lipophilicity (Fig 1). From the large number of studies performed in the bodies of hosts, it appears clear that the kinetic behaviour of these drugs varies markedly from one molecule to another (Hennessy and Alvinerie, 2002) and seems to be governed by their physicochemical properties such as lipophilicity. The milbemycin, moxidectin, is characterized by wide distribution in tissues and a remarkable persistence in the organism, while avermectins, such as ivermectin and eprinomectin, show lower volumes of distribution and shorter mean residence times (Lanusse et al., 1997; Baoliang et al., 2006). In addition, moxidectin seems to have a different impact on parasites (Prichard and Roulet, 2007) and it has been suggested that it may select less strongly for resistance than ivermectin (Ranjan et al., 2002).

The aim of this study was to determine the contribution of P-gp to the *in vivo* behaviour of structurally different molecules of the MLs family. The mdr1ab$^{-/-}$ mice developed by Schinkel et al. (Schinkel et al., 1994) lacking the two P-gp products of the mdr1a and mdr1b genes are pertinent model to investigate the role of P-gp in drug pharmacokinetics *in vivo*. We have studied and compared the behaviour of three MLs: ivermectin, eprinomectin and moxidectin, selected on the basis of their different structure and physicochemical properties, in wild-type and in mdr1ab$^{-/-}$ mice. Three different areas were explored: the plasma kinetics, and the blood-brain-barrier and the intestinal tract, which are two major barriers in the organism where P-gp plays a crucial role. By comparing the in *vivo* plasma kinetics, the brain concentration and the intestinal excretion and accumulation of these molecules in mice, we evaluated the role of P-gp in the disposition of ivermectin, eprinomectin and moxidectin and

demonstrated a close relationship between their body distribution and their relative affinities for P-gp.

Materials and methods

Chemicals

Moxidectin and eprinomectin were a generous gift from Fort-Dodge International and Merial France, respectively. Ivermectin, trifluoacetic anhydride and N-methyl imidazole of analytical reagent grade were purchased from Aldrich (Sigma, Aldrich Chimie, St Quentin Fallavier, France). Acetonitrile and methanol (HPLC grade) were obtained from Fisher Scientific (Loughborough, UK). Glacial acetic acid (10%) was obtained from Merk (Clevenot, Chelles, France). Pic B7 low UV was purchased from Waters (Guyancourt, France). Solid phase extraction was done using sulpelclean LC18 cartridges (100 mg, 1ml) obtained from Supelco (Bellefonte, PA. USA).

Animals

Wild-type and mdr1ab$^{-/-}$ mice were from Taconic (NY, USA). Experiments were carried out on 8-10 week-old mice (25-30 g). Male littermates of 7-12 weeks were randomly allocated to 2 comparison groups: control (6-10) and mdr1ab$^{-/-}$ (6-10). The controls were the FVB strain wild-type and the P-gp deficient mice were mdr1ab$^{-/-}$ mice deficient for the two forms of rodent P-glycoprotein mdr1a and mdr1b (Schinkel et al., 1994; Schinkel et al., 1997). Animals were kept under controlled temperature with a 12/12 h light/dark cycle. They received *ad libitum* a standard diet and normal water. All procedures adhered to the “Guide to the Care and Use of Experimental Animal Care” (Canadian Council on Animal Care guidelines, 1984) and the protocol was approved by the local animal ethics committee.

Drug administration for drug plasma kinetic and brain accumulation

For the oral drug kinetics, both groups received by gavage a solution prepared from commercial ivermectin (0.1% Oramec, Merial, France) and moxidectin (0.1% Cydectin, Fort Dodge International) and from a 0.1% eprinomectin oral formulation prepared in the laboratory. Before administration, all stock solutions were diluted in water to a final

concentration of 0.006 % in order to administer to each mouse a dose of 0.2 mg/kg body weight (bw) in 100 µl. Blood samples were collected over 48 h after drug administration at 2, 5, 8, 24 and 48 h post-treatment. For *i.v.* kinetics, 5 mM ivermectin, moxidectin or eprinomectin stock solutions in DMSO were diluted in mouse plasma to a final concentration of 0.006 % and 100 µl corresponding to 0.2 mg/kg bw were injected into the orbital sinus artery. Blood samples were collected over 24 h after drug administration at 0.08, 0.25, 0.5, 1, 2, 4, 8, 24 h post-treatment.

For drug accumulation in the brain, wild-type (n=3) and mdr1ab$^{-/-}$ (n=3) mice were injected subcutaneously at a dose of 0.2 mg/kg bw with ivermectin, moxidectin or eprinomectin formulated in propylene glycol/formaldehyde (60:40 v/v). 2 or 24 h after administration, plasma was collected and the mice were sacrificed for the brain collection. Heparinized blood samples were collected from the orbital sinus vein under methoxyflurane anesthesia. Blood was centrifuged at 1500 g for 10 min and plasma was stored at -20°C until analysis. The brains were removed, washed in saline solution and frozen at -20°C until analysis.

***In situ* open intestinal perfusion**

The perfusion was performed as previously described (van der Velde et al., 2007). Mice were anesthetized by intraperitoneal administration of a xylazine and ketamine cocktail (53 and 10 mg/kg bw, respectively). Ivermectin, eprinomectin or moxidectin were administered intravenously at the standard dose of 0.2 mg/kg and the mice were placed on a heated operating table to maintain the body temperature at 37°C. The abdomen was opened by a midline longitudinal incision and the intestinal segment to be studied (6-8 cm duodenum, jejunum or ileum segment) was isolated and cannulated at both ends with a polyethylene catheter. The intestinal segment was continuously perfused with thermostated buffer using an infusion pump (constant flow of 3 ml/h, Harvard Apparatus, 91952 Les Ulis Cedex-France). The composition of the perfusion solution was 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4,

1.2 mM $MgSO_4$, 15 mM HEPES, 10 mM L-Glutamine, 1.3 mM $CaCl_2$ (pH 7.4). Just before the experiment, micelles were formed in this aqueous media by the addition of taurocholic acid (4 mM), cholesterol (0.1 mM) and phosphatidylcholine (2 mM) in order to ensure the solubility of MLs in the perfusate. The perfusion started 40 min after drug administration. Samples of the intestinal perfusate were collected from the distal cannula every 30 min up to 180 min. At the end of the experiment, blood was collected and the animals were killed. The small intestine segment was excised, rinsed and blotted dry and the length and weight recorded. The excretion rate corresponded to the amount of test molecule excreted per hour in the whole intestinal segment of interest taking into account the weight of each segment for an average length of 7, 24, and 8 cm for duodenum, jejunum and ileum, respectively in 30 g mice. The weight and the length of intestine were closely correlated in our experimental conditions. The intestinal clearance was the excretion rate divided by the plasma concentration at the end of experiment expressed per ml/h per kg bw.

Drug extraction and analytical procedures

ML concentrations were determined in plasma, brain, intestinal perfusate and intestinal tissue by high performance liquid chromatography (HPLC) with fluorescence detection according to previously described and validated methods (Alvinerie et al., 1998; Sutra et al., 1998; Lifschitz et al., 2000). In brief, plasma and tissues were homogenised in acetonitrile (1:1 v/v or 1:2 v/w, respectively). Samples were centrifuged at 2000g and the supernatant applied to a Supelco C18 cartridge (Supelco Inc., Bellefonte, PA, USA) by using automated solid phase extraction (SPE). The extraction recoveries for the 3 molecules were 0.95 for plasma and intestine and 0.65 for brain. The eluate was evaporated and the dry extract was processed to obtain a fluorophore derivative by dissolving it in 1*N*-methylimidazole and trifluoroacetic anhydride solutions. Samples were injected into the HPLC system (PU980 pump, Jasko, Tokyo, Japan; 360 automatic injector, Kontron, Paris, France; RF-551 fluorescence detector,

Shimadzu, Kyoto, Japan). For eprinomectin, the separation was carried out in a stainless steel analytical column (250 x 4.6 mm i.d. packed with Suplex PKB 100, 5 µm) material with a mobile phase containing acetic acid (0.4% in water), PicB7 and acetonitrile (42:0.4:57 v/v/v). For ivermectin and moxidectin a Supelcosil LC18 column (250 x 4.6 mm, 5 µm, Supelco, Bellefonte, PA, USA) was used with acetic acid (0.2% in water):methanol:acetonitrile (4:40:56, v/v/v) as mobile phase.

Pharmacokinetic analysis

Data were analysed using a non-compartmental approach with version 4.2 of the Kinetica Tm computer program (InnaPhase, Philadelphia, USA). The partial area under the plasma concentration-time curve (AUC) from t_0 to $t_{24\,h}$ was calculated by the linear trapezoidal rule. The mean residence time (MRT) after oral administration of the drug was calculated using the linear trapezoidal rule without extrapolation to infinity (from 0 to 48 h) using the formula: $MRT = AUMC/AUC_{48\,h}$, where AUMC is the area under the momentum curve. The plasma clearance was calculated from the ratio of *i.v.* administered dose divided by AUC *i.v.* $_{24h}$; F, the bioavailability of the drug after oral administration (*per os*), was calculated as follows: (AUC *per os* /AUC *i.v.*) X 100.

Statistical analysis

The experimental values are expressed as mean ± standard deviation (s.d.). Statistical analyses were performed using a three-way factorial ANOVA model. A three-way factorial ANOVA model with repeated measures was used in the case where more than one observation came from the same animal. Multiple comparisons of means were performed with Tukey test. Statistical significance was accepted as $p<0.05$.

Results

Kinetics of MLs in mouse plasma after oral and *i.v.* administration

The plasma kinetics of each drug was determined after *i.v.* and oral administration. The plasma concentration profiles of ivermectin, eprinomectin and moxidectin over 48 h of wild-type and mdr1ab$^{-/-}$ mice treated orally with a single dose of ML (0.2 mg/kg bw) are shown in Fig 2, and the calculated pharmacokinetic parameters are given in Table 1. As shown in Fig 2, in wild-type mice, the plasma kinetic profiles after oral and *i.v*. administration revealed major differences in the kinetic behaviour between the three drugs. The partial area under the plasma concentration-time curve (AUC) calculated from t_0 to $t_{24\,h}$ after oral administration was in the same range for the three molecules. However, the mean residence time (MRT) of ivermectin, which represents the time one molecule will stay in the organism taking account the absorption, diffusion and elimination phases, was different from one molecule to another. It was shorter for eprinomectin (10.9 ± 2.6 h, NS) and longer for moxidectin (54.2 ± 16.6 h, $p<0.001$, Table 1), when compared with ivermectin (18.7 ± 4.7 h). The oral bioavailability and the total plasma clearance calculated after *i.v.* administration, which reflects the capacity of the whole organism to eliminate the drug, were in the same range for the three molecules (35-46% for bioavailability and 97-131 ml/kg/h for the clearance, Table 1).

As far as the mdr1ab$^{-/-}$ mice were concerned, Fig 2 clearly shows that oral plasma kinetics of ivermectin and eprinomectin were both modified by the P-gp deficiency while the kinetics of moxidectin were unchanged. Indeed, ivermectin and eprinomectin concentrations in mdr1ab$^{-/-}$ mice plasma were significantly higher all along the curve from 5 to 48 h (Fig 2). However, it is interesting to note that, the increases in plasma concentrations of eprinomectin were much higher than those observed for ivermectin. Table 1 shows that, in accordance with the plasma concentration profiles, the AUC after *per os* administration of ivermectin and eprinomectin were significantly higher (1.6 fold, from 711.7 ± 305.0 to 1140.7 ± 225.5 ng.h/ml, $p<0.01$;

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and 2.7 fold, from 777.5 ± 192.1 to 2123.4 ± 230.2 ng.h/ml, $p<0.001$, respectively) in mdr1ab$^{-/-}$ mice compared with the wild-type mice, indicating that P-gp influenced the whole kinetic disposition of both drugs, while as expected, the AUC of moxidectin in mdr1ab$^{-/-}$ mice was not significantly different from the control mice. Moreover, it is interesting to note that the MRTs of ivermectin and moxidectin were unchanged while it tended to be increased for eprinomectin in the case of P-gp deficiency (Table 1).

In contrast to what was observed after oral administration, the AUC determined after *i.v.* administration of ivermectin was similar in control and in P-gp deficient mice. Subsequently, the whole body clearance of ivermectin was unchanged in mdr1ab$^{-/-}$ mice compared with wild-type mice. Nevertheless, after *i.v.* administration of eprinomectin to mdr1ab$^{-/-}$ mice, the plasma AUC was significantly increased (1.5 fold, $p<0.001$) and the clearance was decreased (1.5 fold, $p<0.01$). It has to be noted that these parameters determined for moxidectin in P-gp deficient mice were not significantly different from those in control mice (Table 1).

The ratio between oral bioavailability (F) of mdr1ab$^{-/-}$ and wild-type mice (ratio F) and the relative apparent attenuation of oral bioavailability also called *in vivo* absorptive quotient ($AQ_{in\ vivo}$) were calculated as potent indicators of the contribution of P-gp in the intestinal absorption and/or excretion (Table 1, del Amo et al., 2009). The oral bioavailability was increased for ivermectin and eprinomectin by P-gp deficiency in mice as revealed by the ratio F of 1.7 and 1.9 respectively, while it was unchanged for moxidectin (ratio F=1.1). The $AQ_{in\ vivo}$ of 0.39 and 0.45 for ivermectin and eprinomectin, respectively, revealed the important contribution of P-gp in the absorption and/or elimination processes of these two drugs. On the contrary, the low $AQ_{in\ vivo}$ of 0.12 for moxidectin reflected a very slight contribution of P-gp in these processes.

Taken together, these results demonstrate that P-glycoprotein influences the plasma kinetics of ivermectin and eprinomectin but not that of moxidectin. The fact that plasma kinetics of eprinomectin was more dramatically affected in the case of P-gp deficiency than that of

ivermectin or moxidectin strongly suggest that its elimination rate must be more affected than these other two MLs.

Intestinal excretion of MLs

Because parentally administered MLs are considerably excreted into the faeces via P-gp, we suggest that P-gp-mediated intestinal efflux is involved in the kinetic behaviour of these drugs. In order to demonstrate this point, we have used an open-perfusion model commonly used to study the intestinal secretion of drugs in wild-type and mdr1ab$^{-/-}$ mice. It enables the measurement of an overall intestinal excretion by keeping the reabsorption negligible and by maintaining the integrity of the mesenteric vessels. The segments of the small intestine were perfused with a drug-free medium at three anatomical levels corresponding to the duodenum, jejunum and ileum, after intravenous administration of ivermectin, eprinomectin or moxidectin. By measuring the drug concentration in the plasma and the amount of drug secreted into the perfusate, we were able to determine the excretion rate and the intestinal clearance for each drug, which represents the capacity of the intestine to eliminate the drug. The intestinal clearance for each drug at the duodenum, jejunum and ileum levels is shown in Fig 3. In wild-type mice, the three drugs were excreted into the lumen all along the intestine and the clearance calculated for the whole segment was higher in the jejunum than in the duodenum and was low in the ileum. Fig 3 clearly shows that, in wild-type mice, the clearance was much higher for eprinomectin at least in the tow upper parts of the small intestine, than for ivermectin and moxidectin, reflecting the high intestinal efflux of this drug.

As shown in Fig 3, when P-gp was deficient, there was a reduction in the intestinal clearance in the tow upper parts of the intestine which was of different amplitudes from one molecule to another. More precisely, the decrease of the intestinal clearance of eprinomectin in mdr1ab$^{-/-}$ mice compared with wild-type mice was markedly pronounced in the duodenum (3.0 fold, $p<0.001$) and in the jejunum (6.4 fold, from 36.2 ± 12.6 down to 5.7 ± 1.9 ml/kg/h bw). The decrease of intestinal clearance induced by P-gp deficiency was lower for ivermectin than for

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eprinomectin but was still significant in the duodenum and in the jejunum (2.6 and 3.6 fold, respectively, $p<0.05$). P-gp deficiency did not affect the intestinal clearance of moxidectin neither in the duodenum nor in the jejunum. In addition, in the ileum part of the intestine, no change in the clearance values was observed for the three molecules in mdr1a/b$^{-/-}$ compared with wild-type mice.

In order to estimate more precisely and compare the absolute contribution of the P-gp to the small intestinal clearance for each drug, the total intestinal clearance, calculated by summing the clearance of each intestinal segment, in mdr1ab$^{-/-}$ mice was subtracted from that obtained in wild-type mice. The ratio of the clearances wild-type/mdr1ab$^{-/-}$ was also calculated and represents the relative contribution of P-gp in the intestinal clearance. These results are presented in Table 2. The wild-type/mdr1ab$^{-/-}$ clearance ratio revealed that the contribution of P-gp in the intestinal clearance was different from one drug to another. Indeed, eprinomectin, with a wild-type/mdr1ab$^{-/-}$ clearance ratio of about 4.3, was found to be quite significantly excreted into the lumen via a P-gp-dependant mechanism, whereas ivermectin (3.1) and to a less extent moxidectin (1.7), were excreted with less efficiency by P-gp at the intestinal level. This is also reflected in the low absolute values obtained for the P-gp-mediated clearance of ivermectin and moxidectin when compared to eprinomectin (9.1 and 2.7 versus 43.1 ml/kg/h, respectively).

Taken together, these results showed that P-gp was clearly involved in the intestinal efflux of eprinomectin and ivermectin mainly at the level of the duodenum and the jejunum level and less so in the ileum. Moreover, these results show that quantitatively P-gp eliminates more eprinomectin than ivermectin while the involvement of P-gp in moxidectin clearance was discrete and occurred only in the duodenum.

At the end of the *in situ* intestinal perfusion experiment, ML concentrations were determined in the intestinal segments (Table 3). The concentrations of the MLs in the intestinal tissues

were consistent with the results presented above: ivermectin and eprinomectin accumulated more in duodenal and jejunal tissues of mdr1ab$^{-/-}$ mice compared with the controls. These differences were significant for the three molecules in the duodenum ($p<0.05$) but not in the jejunum or in the ileum due to the large variability of the data. Again, eprinomectin concentration in the intestinal tissue was more affected by P-gp deficiency than ivermectin with an increase in drug accumulation of 1.8-fold versus 1.3-fold respectively, while moxidectin tissue accumulation was not different except slightly in the duodenum.

Accumulation of MLs in the brain

Since differences in the influence of P-gp deficiency on plasma kinetics and intestinal excretion of MLs were obvious, we then investigated the ability of MLs to cross the blood-brain-barrier, where P-gp has been reported to play a key role in limiting the penetration of MLs and therefore their neurotoxicity (Schinkel et al., 1994; Roulet et al., 2003). We carried out an *in vivo* experiment aimed at determining the role of P-gp at the blood-brain-barrier by measuring the concentration of the 3 MLs in the brain of wild-type and P-gp deficient mice. The ML concentrations were measured in the plasma and brain 2 and 24 h after a subcutaneous administration of each drug at 0.2 mg/kg bw in wild-type and in mdr1ab$^{-/-}$ mice. The results are shown in Table 4. As expected, in wild-type mice the brain concentration of MLs was very low, in accordance with the protective role of the blood-brain-barrier. It is interesting to note that 24 h after treatment, eprinomectin concentration in brain of wild-type mice was extremely lower than that of ivermectin and moxidectin (24- and 60-fold, respectively, $p<0.001$,). When P-gp was deficient, there was considerable accumulation of the three drugs in the brain. Although the ML concentration in mdr1ab$^{-/-}$ brain was very high compared with the controls (14-, 16- and 4-fold 2 h post-administration and 27-, 297- and 11-fold 24 h post-administration for ivermectin, eprinomectin and moxidectin, respectively, $p<0.001$), it has to be noted that no signs of neurotoxicity were observed during the entire

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experiment. Interestingly, 2h post administration, the concentration of eprinomectin and moxidectin in the brains of mdr1ab$^{-/-}$ mice was quite similar (16.4 ± 4.1 and 15.7 ± 2.5 ng/g, respectively) while the concentration of ivermectin was significantly higher (twice as high at 38.4 ± 13.7 ng/g, $p<0.001$ and $p<0.05$ compared with eprinomectin and moxidectin respectively). This pattern was changed at 24h post administration with brain concentration for ivermectin and moxidectin at 64.7 ± 9.1 and 65.7 ± 23.6 ng/ml, respectively, that were lower that eprinomectin concentration (29.7 ± 12.1 ng/ml, $p<0.001$ and $p<0.01$ compared with ivermectin and moxidectin respectively).

In order to compare the brain uptake of each ML, the brain-to-plasma concentration ratio was calculated. The results after 2 h treatment are shown in Fig.4. In wild-type mice, the brain-to-plasma concentration ratio 2 h post administration was in the same range for ivermectin and moxidectin (0.03 and 0.08, respectively), while it was approximately ten-times lower for eprinomectin (0.005), suggesting (i) a higher protection of the brain from eprinomectin entry or (ii) a better efflux of the drug out of the brain. In mdr1ab$^{-/-}$ mice, the brain-to-plasma concentration ratio was increased compared with the control value and tended to be close to 1 for ivermectin (0.83 ± 0.35) and lower for moxidectin (0.33 ± 0.04). Consistent with the values obtained in wild-type mice, the brain-to-plasma concentration ratio of eprinomectin was lower than ivermectin and moxidectin (0.10 ± 0.02, Fig 4), suggesting that the low eprinomectin concentration in brain could not be explained by only a significant P-gp-mediated efflux at the blood-brain-barrier. The $K_{-/-/wt}$ ratio shown in Fig 4, calculated according to Chen (Chen et al., 2003), represents the enhancement of the brain-to-plasma concentration ratio between mdr1ab$^{-/-}$ and wild-type. Fig 4 shows that the contribution of P-gp in the prevention of drug penetration into the brain was in the same range for ivermectin and eprinomectin as shown be the high $K_{-/-/wt}$ ratio (27 and 21, respectively). This confirms an important contribution of P-gp in the efflux of these two drugs out of the brain. The lower

ratio around 5 calculated for moxidectin 2 h post-treatment showed that the drug is less effluxed out of the brain than the other two. It has to be noted that the $K_{-/-/wt}$ ratio values calculated after 24 h treatment were in the same range of values (31, 20 and 7 for ivermectin, eprinomectin and moxidectin, respectively, data not shown).

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Discussion

Given the strong interaction of MLs with P-gp (Lespine et al., 2007), our study aimed at evaluating the contribution of P-gp to their pharmacokinetic behaviour in mice. We have compared the *in vivo* plasma pharmacokinetics of ivermectin, eprinomectin and moxidectin in wild-type and P-gp deficient mice.

P-gp deficiency affected the oral bioavailability of ivermectin in agreement with previous data (Kwei et al., 1999; Geyer et al., 2009), but did not modify the clearance, suggesting that P-gp plays a greater role in the oral absorption of ivermectin than in its elimination. Concerning eprinomectin, the marked increase in the AUC after oral and *i.v.* administrations, and the decrease in the clearance, indicated that P-gp was highly involved not only in the absorption but also in the elimination of this drug. This is supported by the strong increase of plasma concentration observed only for eprinomectin 24h after *s.c.* administration in P-gp deficient mice while ivermectin and moxidectin plasma concentrations were not significantly affected (Table 4). As no major ML metabolites were detected, confirming that biotransformation is a minor elimination pathway for MLs (Chiu et al., 1987), these results support that P-gp was affecting the absorption of both ivermectin and eprinomectin, while the low $AUC_{i.v.}$ in wild-type mice shows that P-gp was also involved in the elimination of eprinomectin. On the contrary, for moxidectin, the contribution of P-gp in these processes was weak as shown by the lack of change of moxidectin plasma kinetics in mdr1ab$^{-/-}$ mice.

P-gp is known to be partly responsible for MLs elimination by the faecal route *via* intestinal excretions (Laffont et al., 2002; Ballent et al., 2006). Thus, we have compared the capacity of the intestine to eliminate these drugs. In wild-type mice**,** the three MLs were excreted into the perfusate all along the small intestine with eprinomectin being the highest. The intestinal clearance of ivermectin and eprinomectin were both reduced in mdr1ab$^{-/-}$ mice and, interestingly, the absolute clearance due to P-gp was higher for eprinomectin than for

ivermectin (Table 2). By comparing with the plasma clearance of eprinomectin for which 30 % was due to P-gp (Table 1), it is clear that intestinal P-gp efflux is the major pathway of eprinomectin elimination. On the contrary, the intestinal capacity to excrete moxidectin was low and mostly P-gp-independent. Accordingly, ivermectin, but not moxidectin, was shown to be excreted by P-gp at the apical side of Caco-2 cell monolayers (Griffin et al., 2005). Together, these data suggest that overall ivermectin and moxidectin are not as efficiently effluxed by the intestinal P-gp as eprinomectin, which explains the delay in the elimination of the two drugs *in vivo* by comparison with eprinomectin.

In our experiment the maximal MLs excretion was measured in the jejunum. Our results could therefore conflict with the observations that P-gp is more expressed and active in the distal part of the intestine (Stephens et al., 2002; Lacombe et al., 2004; MacLean et al., 2008). However, the intestinal P-gp activity can be modulated by many factors, such as the physicochemical properties of the substrate and the composition and pH of the lumen for basic drugs (Varma and Pachangnula, 2005). Since MLs are large hydrophobic non-ionic compounds, their interaction with P-gp is certainly more influenced by their hydrogen bond acceptor pattern, their amphiphilic character and steric hindrance.

By measuring drug concentration in the brain, we clearly show that P-gp at the blood-brain-barrier was able to efflux not only ivermectin and eprinomectin but also moxidectin. High $K_{-/-/wt}$ ratios for ivermectin (27, Fig.4) and eprinomectin (21) reveal the considerable contribution of P-gp in preventing their penetration into the brain while moxidectin had a low $K_{-/-/wt}$ ratio of 5, demonstrating the relatively lower contribution of P-gp. However, the lower brain-to-plasma concentration ratio for eprinomectin in wild-type mice suggests that it is more efficiently expelled out of the brain than ivermectin or moxidectin. In P-gp deficient mice, the ivermectin brain-to-plasma concentration ratio was close to 1, confirming the essential role of P-gp at the blood-brain-barrier in effluxing ivermectin. Interestingly, this ratio was much

lower for eprinomectin. Furthermore, selamectin, a good P-gp substrate, accumulated less than ivermectin in the mdr1ab$^{-/-}$ mouse brain (Geyer et al., 2009). These results are consistent with the broader safety margin of eprinomectin compared with ivermectin, which could be due to its lower capacity to enter the brain. These data show that MLs have different abilities to enter the brain. However, other transporters or lipophilicity/amphiphilicity of the drug should be also considered in limiting the drug entry into the brain. Furthermore, brain concentrations of the three MLs in P-gp deficient mice were higher 24h than 2h after treatment, suggesting that they tended to be sequestered into the brain in agreement with their high lipophilicity drugs.

Taken together these results show differences in the efficiency of intestinal P-gp to efflux the MLs that can be clearly related to the P-gp contribution to the drug disposition *in vivo*. Indeed, the plasma kinetics of eprinomectin is highly affected by P-gp deficiency which correlates with its high rate of P-gp-dependent efflux into the intestinal lumen and out of the brain. P-gp is able to effectively efflux moxidectin at the blood-brain-barrier but not at the intestinal level and it has little impact on the overall plasma disposition of the drug. Ivermectin shows an intermediate behaviour with the plasma kinetics determined by a P-gp dependent absorption mechanism and efficient efflux by P-gp out of the brain.

Because MLs are P-gp substrates and also lipophilic drugs, their *in vivo* behaviour is expected to be the result of two main driving forces: the P-gp efflux and the attraction to fat tissue. The balance of these two thus appears directly related to the log*P* of the MLs and inversely related to the drug affinity for P-gp. Our results clearly show that the higher the affinity of the ML for P-gp, the more efficiently the drug is effluxed *via* the transporter out of tissue or the organism. Given that ivermectin and eprinomectin have higher affinities for P-gp than moxidectin (Lespine et al., 2007), it is reasonable to assume that eprinomectin elimination is mainly due to its high affinity for P-gp and its lower lipophilicity (K_i=0.02 µM, Log*P*=4) which results in

less attraction to the adipose tissue, and hence lower persistence in the plasma (MRT) and the lower entrance into the brain. On the opposite, the higher lipophilicity and lower affinity for P-gp of moxidectin (Log*P*=6, K_i=0.5µM), favours attraction to fatty tissue to the detriment of P-gp efflux. Moxidectin kinetic may therefore be determined more by lipophilicity/amphiphilicity resulting in a delay in elimination and a prolonged MRT. Finally, ivermectin (Log*P*=4.8, K_i=0.05 µM) displays an intermediate behaviour certainly equally governed by both factors. However, if amphiphilicity/hydrophobicity dictates the partitioning into the lipid membrane, the hydrogen bonds formed between the substrate and the transporter (Seelig et al., 2000) and the molecule size also influence the interaction of the substrate with P-gp.

Moxidectin was shown to be mostly excreted via a P-gp independent pathway at the intestinal barrier while it was clearly effluxed by P-gp at the blood-brain-barrier. This result suggests tissue specificity in the efflux mechanisms at the transporter level. Recently, moxidectin was shown to be eliminated in milk through BCRP-mediated efflux, but not effluxed by cells over-expressing BCRP (Perez et al., 2009). This discrepancy in drug-transporter interaction may be due, at least partly, to (i) the drug concentration, (ii) the transporter expression level, (iii) saturation with endogenous substrates of the pumps that are specific to the tissues, (iv) the influence of the lipid environment and (v) the involvement of influx transporters expressed at the apical pole (or efflux transporters at the basal pole) of the enterocyte that enable MLs to "bypass" entering into the apical plasma membrane. Indeed, ivermectin or moxidectin have been shown to also interact with other transporters such as MRP or BCRP (Lespine et al., 2006; Muenster et al., 2008; Perez et al., 2009) but BCRP doesn't appear to be a relevant carrier for ivermectin and selamectin (Geyer et al., 2009). However, we cannot exclude that BCRP, also present at the blood-brain-barrier and over-expressed in mdr1ab$^{-/-}$ mice (Cisternino et al., 2004), contributes partly to the protection of the brain from

eprinomectin thus explaining the relatively low eprinomectin concentration in the brains of P-gp deficient mice. However, the interaction of eprinomectin with BCRP has not yet been explored.

In conclusion, this study emphasized a different contribution of P-gp to the disposition of MLs according to their affinities for P-gp and their physicochemical properties. Eprinomectin is more efficiently effluxed by P-gp which is consistent with its higher affinity for the transporter compared with ivermectin and moxidectin. These observations open new perspectives for using the affinity of MLs for P-gp, calculated from *in vitro* data, to predict the *in vivo* kinetic behaviour of the drugs. Our results may have important clinical implications for the prediction of not only the toxicity of MLs or derivatives, but also their efficacy and therefore the emergence of parasite resistance.

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Footnote

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Solange Kiki-Mvouaka, Cécile Ménez : These authors contributed equally to this manuscript.

Figure legends

Figure 1. Chemical structures of ivermectin; eprinomectin and moxidectin.

For ivermectin and eprinomectin, the dry compound is mixture of B1a (substituent butyl on C25) and B1b (substituent isopropyl on C25) forms. The majority (over 90 %) of the drug is present as the B1a form.

Figure 2. Influence of P-glycoprotein on the plasma kinetic of MLs.

Ivermectin (IVM), eprinomectin (EPR) or moxidectin (MOX) were administered orally (plain line) or injected intravenously (dotted line) in wild-type (empty circle) and mdr1ab$^{-/-}$ mice (filled circle) at 0.2 mg/kg bw. Blood was collected at 2, 5, 8, 24, 48 h after oral administration and at 0.08, 0.25, 0.5, 1, 2, 4, 8, 24 h after *i.v.* administration. Drug concentration was measured in plasma by HPLC as described in the "*Materials and Methods*" section. Data are mean ± S.D. (n=6 to 11 animals per group). Statistical analyses are shown for oral administration: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs*. wild-type mice.

Figure 3. Influence of P-glycoprotein on the intestinal clearance of MLs.

Ivermectin (IVM), eprinomectin (EPR) or moxidectin (MOX) were injected intravenously in wild-type (*white bar*) and mdr1ab$^{-/-}$ (*grey bar*) mice at 0.2 mg/kg bw. One hour after injection, the upper, medium or lower part (5 to 7 cm) of the small intestine corresponding respectively to the duodenum, the jejunum or the ileum was perfused during 3 hours. Drug concentration was measured in plasma and perfusates by HPLC as described in the "*Materials and Methods*" section. Intestinal clearance was the excretion rate/mean plasma concentration during the perfusion. Data are mean ± S.D. of 3 to 6 animals. * $p<0.05$, *** $p<0.001$ *vs*. wild-type mice.

Figure 4. Brain-to-plasma concentration ratio of MLs.

Ivermectin (IVM), eprinomectin (EPR) or moxidectin (MOX) were administered subcutaneously in wild-type (*white bar*) and mdr1ab$^{-/-}$ (*grey bar*) mice at 0.2 mg/kg bw. Three mice in each group were sacrificed at 2 h after treatment. Drug concentrations were determined in plasma and brain as described in Table 4 and the brain-to-plasma concentration ratios were calculated. The values shown on the figure is the $K_{-/-/wt}$ calculated as follow: brain-to-plasma concentration ratio$_{-/-}$/ brain-to-plasma concentration ratio$_{wt}$. Data are mean ± S.D. of three animals. ** p<0.01, *** p<0.001 *vs.* wild-type mice.

Table 1. Kinetic parameters of MLs after *i.v.* and *per os* administration in mice (n=6-11).

	AUC *i.v.* [a] (ng.h/ml)	Clearance [b] (ml/kg/h)	AUC *per os* [a] (ng.h/ml)	MRT [c] (h)	F [d] (%)	Ratio F [e]	$AQ_{in\ vivo}$ [f]
Ivermectin							
Wild-type	**1533.4** ± 108.7	**131.5** ± 14.4	**711.7** ± 305.0	**18.7** ± 4.7	**46**		
						1.7	**0.39**
Mdr1ab$^{-/-}$	**1489.9** ± 282.0	**137.9** ± 25.9	**1140.7** ± 225.5**	**22.8** ± 3.1	**76**		
Eprinomectin							
Wild-type	**2183.3** ± 575.4	**97.3** ± 27.0	**777.5** ± 192.1	**10.9** ± 2.6	**35**		
						1.9	**0.45**
Mdr1ab$^{-/-}$	**3236.2** ± 534.2***	**63.3** ± 12.4 **	**2123.4** ± 230.2***	**23.3** ± 7.1	**65**		
Moxidectin							
Wild-type	**1680.6** ±29.9	**119.5** ± 9.1	**643.7** ± 47.5	**54.2** ± 16.6	**38**		
						1.1	**0.12**
Mdr1ab$^{-/-}$	**1544.2** ±131.5	**130.2** ±10.4	**663.8** ± 128.9	**69.5** ±10.4	**43**		

[a] Partial area under the plasma concentration-time curve calculated from 0 to 24 h

[b] Clearance =dose/$AUC_{i.v}$

[c] Mean residence time calculated after *per os* administration from 0 to 48 h

[d] Oral bioavailability F = (AUC_{oral}/$AUC_{i.v.}$) x 100

[e] Ratio F = $F_{-/-}$ / F_{wt}

[f] $AQ_{in\ vivo}$: *in vivo* absorptive quotient = $\frac{(AUC_{oral\ -/-} / AUC_{i.v.\ -/-}) - (AUC_{oral\ wt} / AUC_{i.v.\ wt})}{AUC_{oral\ -/-} / AUC_{i.v.\ -/-}}$

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* wild-type mice.

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Table 2. Total small intestine clearance of MLs in wild-type and mdr1ab$^{-/-}$ mice.

	Total small intestine Clearance [a] (ml/kg/h)	**P-gp contribution to clearance** [b] (ml/kg/h)	**Ratio** [c] Wild-type/mdr1ab$^{-/-}$
Ivermectin			
Wild-type	**13.4**	**9.1**	**3.1**
Mdr1ab$^{-/-}$	**4.3**		
Eprinomectin			
Wild-type	**56.2**	**43.1**	**4.3**
Mdr1ab$^{-/-}$	**13.1**		
Moxidectin			
Wild-type	**6.3**	**2.7**	**1.7**
Mdr1ab$^{-/-}$	**3.6**		

[a] Sum of the clearance of each individual intestinal segments

[b] Intestinal clearance $_{wt}$ - Intestinal clearance $_{-/-}$

[c] Intestinal clearance $_{wt}$ / Intestinal clearance $_{-/-}$

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Table 3. Influence of P-glycoprotein on MLs accumulation in the intestinal segments (n=3-10).

	Drug concentration in intestinal segment (ng/g)		
	Duodenum	**Jejunum**	**Ileum**
Ivermectin			
Wild-type	**198.2** ± 8.8	**82.3** ± 24.3	**84.0** ± 33.9
Mdr1ab$^{-/-}$	**263.3** ± 26.3 *	**108.7** ± 60.1	**100.7** ± 34.2
Eprinomectin			
Wild-type	**48.0** ± 18.8	**98.6** ± 27.8	**86.6** ± 9.7
Mdr1ab$^{-/-}$	**66.1** ± 10.3 *	**180.8** ± 16.6	**109.0** ± 33.1
Moxidectin			
Wild-type	**58.6** ± 39.5	**158.4** ± 35.1	**61.0** ± 15.7
Mdr1ab$^{-/-}$	**108.6** ± 22.5 *	**108.3** ± 31.6	**88.5** ± 27.0

* $p<0.05$ *vs.* wild-type mice

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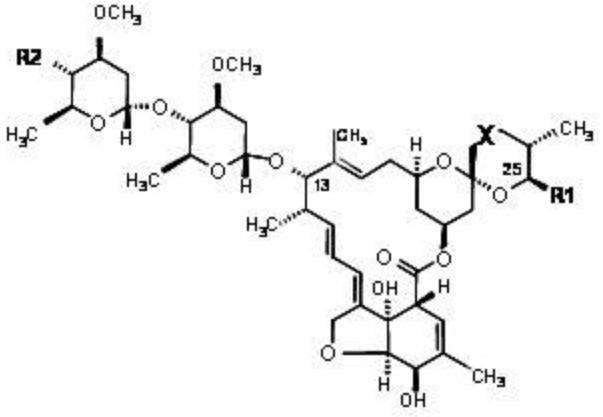
Table 4. Influence of P-gp on plasma and brain concentrations of MLs in mice (n=3).

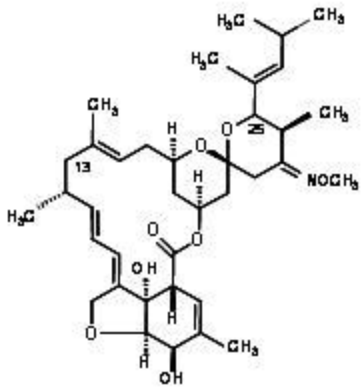
	Drug concentration in plasma (ng/ml)		**Drug concentration in brain** (ng/g)	
	2h	**24h**	**2h**	**24h**
Ivermectin				
Wild-type	**89.1** ± 11.9	**30.0** ± 9.0	**2.7** ± 1.3	**2.4** ± 1.0
Mdr1ab$^{-/-}$	**50.7** ± 20.1	**27.0** ± 2.2	**38.4** ± 13.7 ***	**64.7** ± 9.1 ***
Eprinomectin				
Wild-type	**226.9** ± 4.2	**10.9** ± 4.0	**1.0** ± 0.1	**0.1** ± 0.0
Mdr1ab$^{-/-}$	**172.9** ± 43.3	**158.0** ± 27.4 ***	**16.4** ± 4.1 ***	**29.7** ± 12.1 ***
Moxidectin				
Wild-type	**47.4** ± 5.9	**28.2** ± 8.1	**3.9** ± 3.9	**6.0** ± 5.2
Mdr1ab$^{-/-}$	**45.7** ± 7.6	**40.1** ± 10.9	**15.7** ± 2.5 **	**65.7** ± 23.6 **

** $p<0.01$, *** $p<0.001$ *vs* wild-type mice

Figure 1



Ivermectin B_{1a} X = $-CH_2CH_2-$ R_1= $-CH(CH_3)CH_2CH_3$ R_2 = -OH
Eprinomectin B_{1a} X = -CH=CH- R_1= $-CH(CH_3)CH_2CH_3$ R_2 = $-NHCOCH_3$



Moxidectin

Figure 2

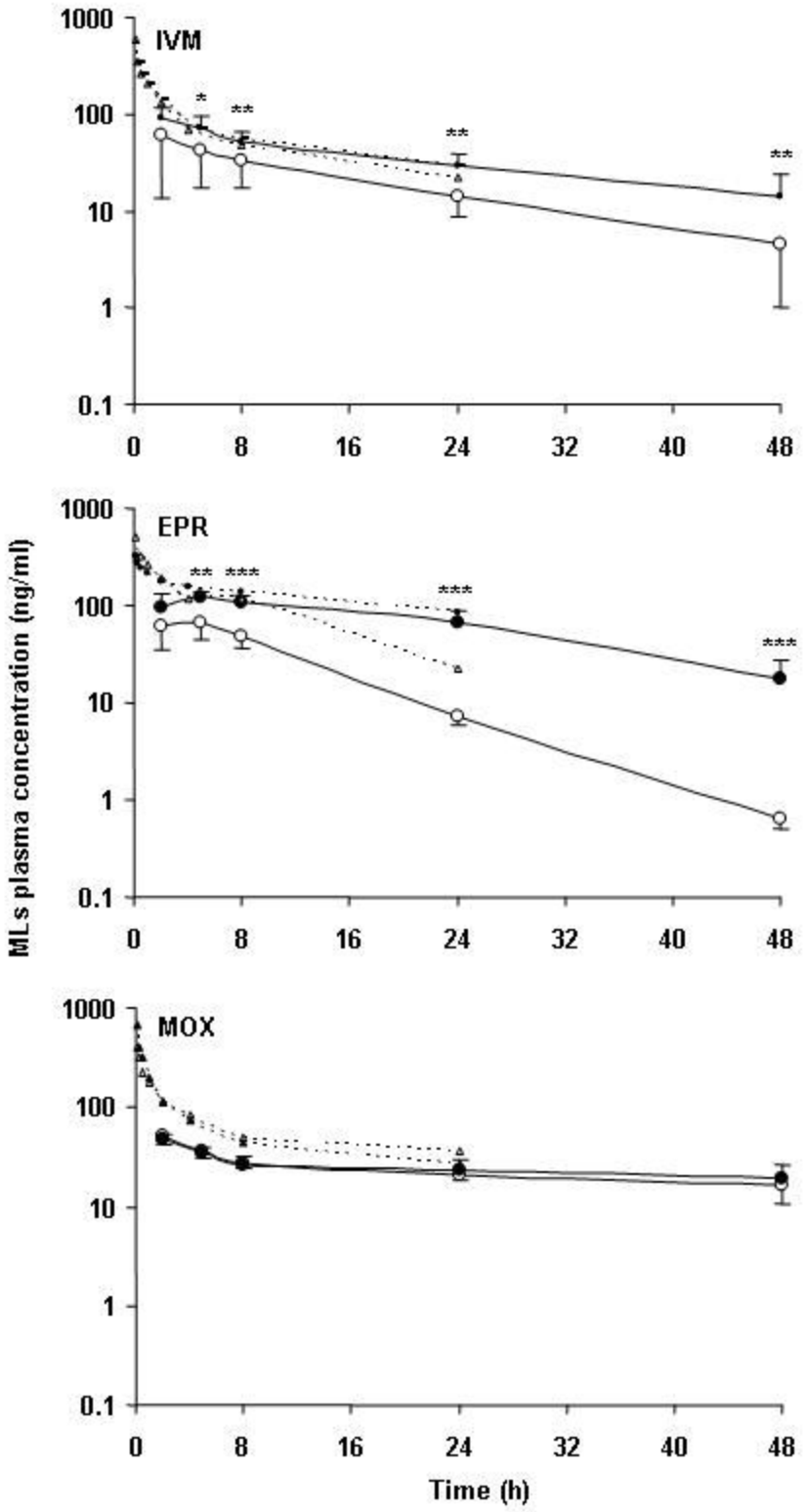

IVM
EPR
MOX
MLs plasma concentration (ng/ml)
Time (h)
1000
100
10
1
0.1
0
8
16
24
32
40
48
*
**
***

Figure 3

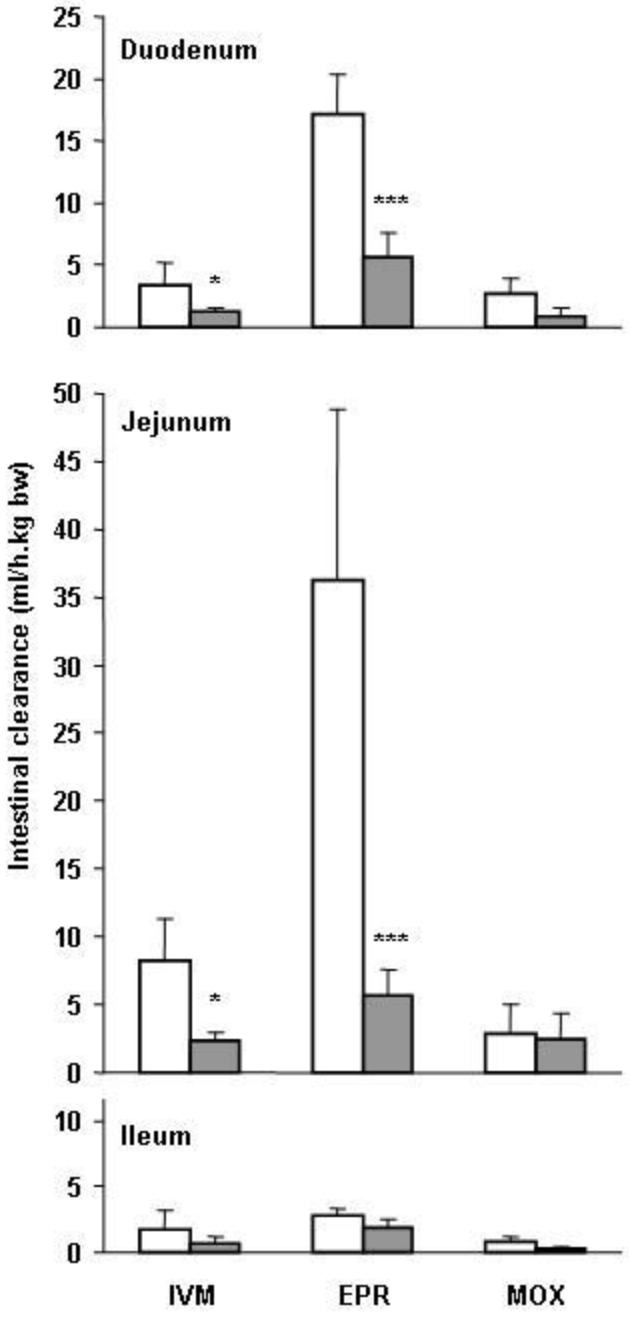

Duodenum
Jejunum
Ileum
Intestinal clearance (ml/h.kg bw)
25
20
15
10
5
0
50
45
40
35
30
25
20
15
10
5
0
10
5
0
*
***
*
***
IVM
EPR
MOX

Figure 4