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Role of P-Glycoprotein in the Disposition of Macrocyclic Lactones: A Comparison between Ivermectin, Eprinomectin, and Moxidectin in Mice

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ABSTRACT:

Macrocyclic lactones (MLs) are lipophilic anthelmintics and substrates for P-glycoprotein (P-gp), an ATP-binding cassette transporter involved in drug efflux out of both host and parasites. 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 high-performance liquid chromatography. The intestinal excretion rate after 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), whereas the moxidectin AUC was unchanged. Ivermectin and to a greater extent eprinomectin were both excreted by the intestine via a P-gp-dependent pathway, whereas 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 controlled mainly by P-gp efflux, more so than that of ivermectin, whereas 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 behavior of these drugs.

P-glycoprotein (P-gp) is a plasma membrane protein belonging to the ATP-binding cassette superfamily. First identified as a factor involved in multidrug resistance in mammalian tumor 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-dependent manner (Schinkel and Jonker, 2003). Physiologically expressed on 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, MLs must reach an effective concentration during a suitable length of time in target tissues. P-gp has been clearly identified as the main factor that controls the concentration of these drugs by affecting 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 homologs are overex-

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ABBREVIATIONS: P-gp, P-glycoprotein; ML, macrocyclic lactone; HPLC, high-performance liquid chromatography; AUC, area under the plasma concentration-time curve; MRT, mean residence time; wt, wild type; BCRP, breast cancer resistance protein.

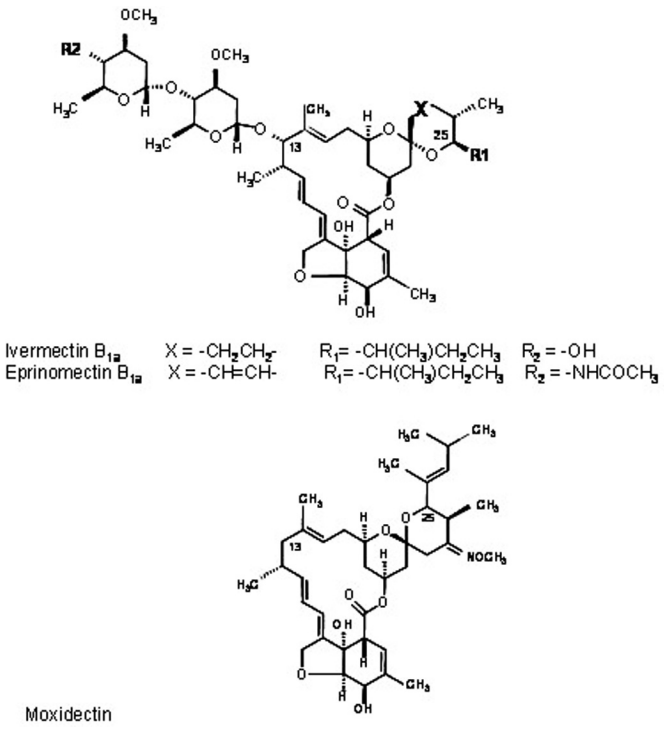

FIG. 1. Chemical structures of ivermectin, eprinomectin, and moxidectin. For ivermectin and eprinomectin, the dry compound is a mixture of B1a (substituent butyl on C25) and B1b (substituent isopropyl on C25) forms. The majority (more than 90%) of the drug is present as the B1a form.

pressed in ivermectin resistance (Prichard and Roulet, 2007). In the context of the emergence of MLs resistance in target parasites, the inhibition of P-gp is considered to be 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 seems clear that the kinetic behavior 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, whereas 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 affect 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 behavior of structurally different molecules of the ML family. The *mdr1ab*(−/−) mice developed by Schinkel et al. (1994), lacking the two P-gp products of the *mdr1a* and *mdr1b* genes are a pertinent model to investigate the role of P-gp in drug pharmacokinetics in vivo. We have studied and compared the behavior 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 (Fort Dodge, IA) and Merial France (Lyon, France), respectively. Ivermectin, trifluoroacetic anhydride, and *N*-methyl imidazole of analytical reagent grade were purchased from Sigma-Aldrich Chimie (St. Quentin Fallavier, France). Acetonitrile and methanol [high-performance liquid chromatography (HPLC) grade] were obtained from Thermo Fisher Scientific (Loughborough, UK). Glacial acetic acid (10%) was obtained from Merck (Clevenot, Chelles, France). PIC B7 low UV reagent was purchased from Waters (Guyancourt, France). Solid-phase extraction was done using Sulpelclean LC18 cartridges (100 mg, 1 ml) obtained from Supelco (Bellefonte, PA).

Animals. Wild-type and *mdr1ab*(−/−) mice were from Taconic Farms (Germantown, NY). Experiments were carried out on 8- to 10-week-old mice (25–30 g). Male littermates of 7 to 12 weeks were randomly allocated to two comparison groups: control (6–10 mice) and *mdr1ab*(−/−) (6–10 mice). 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, 1997). Animals were kept under controlled temperature with a 12:12 h light/dark cycle. They received a standard diet and normal water ad libitum. All procedures adhered to the *Guide to the Care and Use of Experimental Animal Care* (Canadian Council on Animal Care, 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) 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% to administer to each mouse a dose of 0.2 mg/kg b.wt. in 100 μl. Blood samples were collected over 48 h after drug administration at 2, 5, 8, 24, and 48 h post-treatment. For intravenous kinetics, 5 mM ivermectin, moxidectin, or eprinomectin stock solutions in dimethyl sulfoxide were diluted in mouse plasma to a final concentration of 0.006%, and 100 μl corresponding to 0.2 mg/kg b.wt. 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, and 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 b.wt. with ivermectin, moxidectin, or eprinomectin formulated in propylene glycol/formaldehyde (60:40, v/v). Two 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 described previously (van der Velde et al., 2007). Mice were anesthetized by intraperitoneal administration of a xylazine and ketamine cocktail (53 and 10 mg/kg b.wt., 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- to 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, Les Ulis, 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, and 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) 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 were 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 milliliter per hour per kilogram body weight.

Drug Extraction and Analytical Procedures. ML concentrations were determined in plasma, brain, intestinal perfusate, and intestinal tissue by 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 homogenized in acetonitrile (1:1, v/v or 1:2, v/w, respectively). Samples were centrifuged at 2000*g*, and the supernatant was applied to a Supelco C18 cartridge (Supelco) by using automated solid-phase extraction. The extraction recoveries for the three 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), and RF-551 fluorescence detector (Shimadzu, Kyoto, Japan)]. For eprinomectin, the separation was performed in a stainless steel analytical column (250 × 4.6 mm i.d. packed with Suplex pKb-100, 5 μm) with a mobile phase containing acetic acid (0.4% in water), PIC B7, and acetonitrile (42:0.4:57, v/v/v). For ivermectin and moxidectin a Supelcosil LC18 column (250 × 4.6 mm, 5 μm; Supelco) was used with acetic acid (0.2% in water)-methanol-acetonitrile (4:40:56, v/v/v) as the mobile phase.

Pharmacokinetic Analysis. Data were analyzed using a noncompartmental approach with version 4.2 of the Kinetica computer program (InnaPhase, Philadelphia, PA). 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 intravenously administered dose divided by $AUC_{i.v.\ 24\,h}$; *F*, the bioavailability of the drug after oral administration was calculated as follows: ($AUC_{oral}/AUC_{i.v.}$) × 100.

Statistical Analysis. The experimental values are expressed as the mean ± S.D. Statistical analyses were performed using a three-way factorial analysis of variance model. A three-way factorial analysis of variance model with repeated measures was used when more than one observation came from the same animal. Multiple comparisons of means were performed with Tukey's test. Statistical significance was accepted as $p < 0.05$.

Results

Kinetics of MLs in Mouse Plasma after Oral and Intravenous Administration. The plasma kinetics of each drug was determined after intravenous 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 b.wt.) 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 intravenous administration revealed major differences in the kinetic behavior between the three drugs. The partial AUC calculated from t_0 to $t_{24\,h}$ after oral administration was in the same range for the three molecules. However, the MRT of ivermectin, which represents the time one molecule will stay in the organism, taking into account the absorption, diffusion, and elimination phases, was different from one molecule to another. It was shorter for eprinomectin (10.9 ± 2.6 h, not significant) and longer for moxidectin (54.2 ± 16.6 h, $p < 0.001$) (Table 1), compared with ivermectin (18.7 ± 4.7 h). The oral bioavailability and the total plasma clearance calculated after intravenous 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).

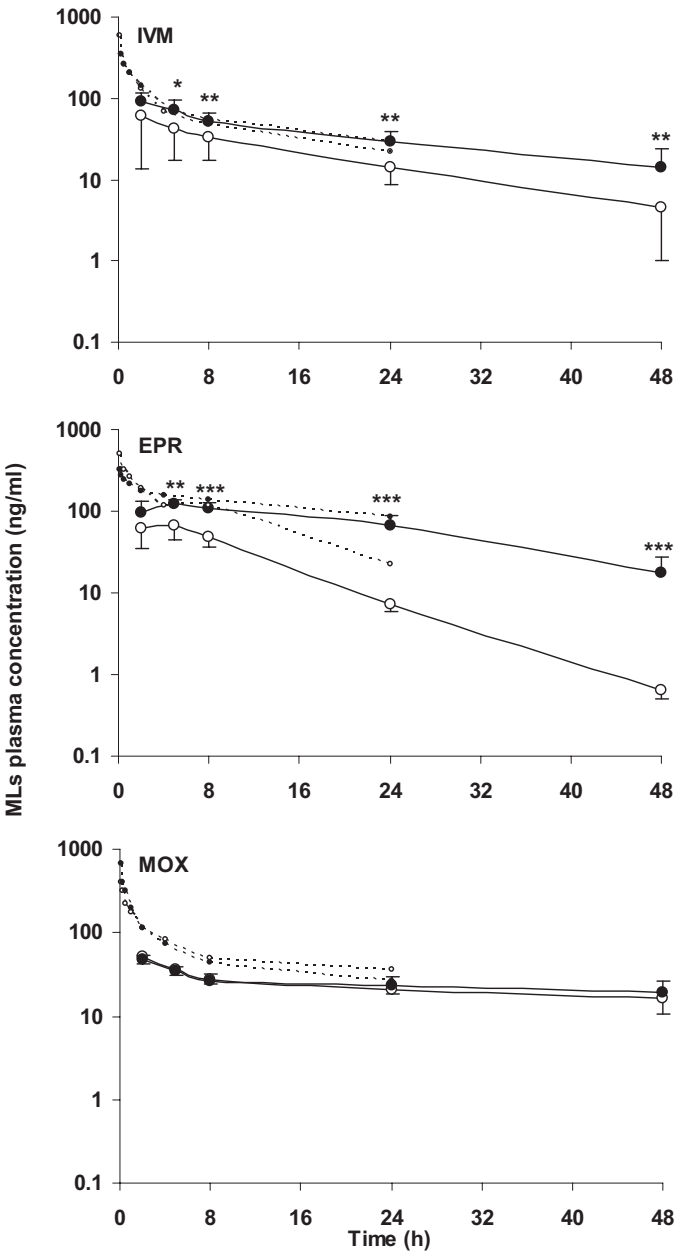

FIG. 2. Influence of P-glycoprotein on the plasma kinetic of MLs. Ivermectin (IVM), eprinomectin (EPR), or moxidectin (MOX) was administered orally (——) or injected intravenously (· · · · ·) in wild-type (○) and *mdr1ab*(−/−) mice (●) at 0.2 mg/kg b.wt.. Blood was collected at 2, 5, 8, 24, and 48 h after oral administration and at 0.08, 0.25, 0.5, 1, 2, 4, 8, and 24 h after intravenous administration. Drug concentration was measured in plasma by HPLC as described under *Materials and Methods*. Data are means ± S.D. (n = 6–11 animals/group). Statistical analyses are shown for oral administration: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus wild-type mice.

Regarding the *mdr1ab*(−/−) mice, Fig. 2 clearly shows that oral plasma kinetics of ivermectin and eprinomectin were both modified by the P-gp deficiency, whereas the kinetics of moxidectin were unchanged. Indeed, ivermectin and eprinomectin concentrations in

TABLE 1

Kinetic parameters of MLs after intravenous and oral administration in mice

$n = 6$ to 11.

	$AUC_{i.v.}$[a]	Clearance[b]	AUC_{oral}[a]	MRT[c]	F[d]	Ratio F[e]	$AQ_{in\ vivo}$[f]
	ng · h/ml	*ml/kg/h*	*ng · h/ml*	*h*	*%*		
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		

** $p < 0.01$ versus wild-type mice.
*** $p < 0.001$ versus wild-type mice.
[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 oral administration from 0 to 48 h.
[d] Oral bioavailability $F = (AUC_{oral}/AUC_{i.v.}) \times 100$.
[e] Ratio $F = F_{(-/-)}/F_{wt}$.
[f] $AQ_{in\ vivo}$: in vivo absorptive quotient $= \frac{(AUC_{oral(-/-)}/AUC_{i.v.(-/-)}) - (AUC_{oral\ wt}/AUC_{i.v.\ wt})}{AUC_{oral(-/-)}/AUC_{i.v.(-/-)}}$

mdr1ab(−/−) mouse 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 AUCs after oral administration of ivermectin and eprinomectin were significantly higher (1.6-fold, from 711.7 ± 305.0 to 1140.7 ± 225.5 ng · h/ml, $p < 0.01$; 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, whereas as expected, the AUC of moxidectin in *mdr1ab*(−/−) mice was not significantly different from that of the control mice. Moreover, it is interesting to note that the MRTs of ivermectin and moxidectin were unchanged, whereas MRT 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 intravenous 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 that in wild-type mice. Nevertheless, after intravenous 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 the 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, whereas it was unchanged for moxidectin (ratio F = 1.1). The $AQ_{in\ vivo}$ values 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 the plasma kinetics of eprinomectin was more dramatically affected in the case of P-gp deficiency than that of ivermectin or moxidectin strongly suggests that its elimination rate must be more affected than those of these other two MLs.

Intestinal Excretion of MLs. Because parenterally administered MLs are excreted considerably into the feces via P-gp, we suggest that P-gp-mediated intestinal efflux is involved in the kinetic behavior of these drugs. 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 measurement of 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. Figure 3 clearly shows that in wild-type mice the clearance was much higher for eprinomectin at least in the two 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 two upper parts of the intestine, which was of different amplitudes from one molecule to another. More precisely, the decrease in the intestinal clearance of eprinomectin in *mdr1ab*(−/−) mice compared with that in 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 b.wt.). The decrease in intestinal clearance induced by P-gp deficiency was lower for ivermectin than for 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 in the duodenum or in the jejunum. In addition, in the ileum part of the intestine, no change in clearance values was observed for

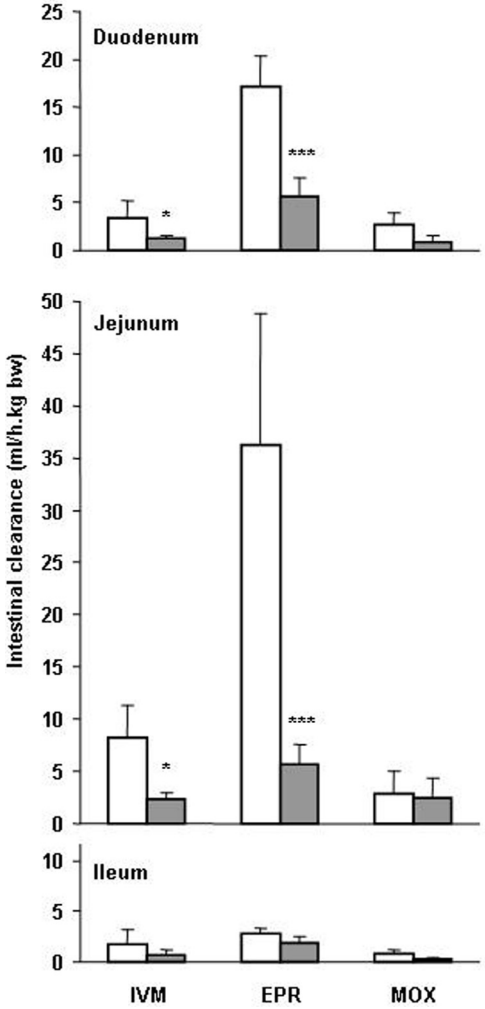

FIG. 3. Influence of P-glycoprotein on the intestinal clearance of MLs. Ivermectin (IVM), eprinomectin (EPR), or moxidectin (MOX) was injected intravenously in wild-type (□) and *mdr1ab*(−/−) (▨) mice at 0.2 mg/kg b.wt. One hour after injection, the upper, medium, or lower part (5–7 cm) of the small intestine corresponding, respectively, to the duodenum, the jejunum, or the ileum was perfused during 3 h. Drug concentration was measured in plasma and perfusates by HPLC as described under *Materials and Methods*. Intestinal clearance was the excretion rate/mean plasma concentration during the perfusion. Data are means ± S.D. of three to six animals. *, $p < 0.05$; ***, $p < 0.001$ versus wild-type mice.

the three molecules in *mdr1a/b*(−/−) compared with those in wild-type mice.

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 in 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 to the intestinal clearance was different from one drug to another. Indeed, eprinomectin, with a wild-type/*mdr1ab*(−/−) clearance ratio of approximately 4.3, was found to be quite significantly excreted into the lumen via a P-gp-dependent mechanism, whereas ivermectin (clearance ratio 3.1) and to a less extent moxidectin (clearance ratio 1.7), were excreted with less efficiency by P-gp at the intestinal level. This finding is also reflected in the low absolute values obtained for the P-gp-mediated clearance of

TABLE 2

Total small intestine clearance of MLs in wild-type and mdr1ab(−/−) mice

	Total Small Intestine Clearance[a]	P-gp Contribution to Clearance[b]	Ratio Wild-Type/*mdr1ab*(−/−)[c]
	ml/kg/h	*ml/kg/h*	
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 segment.
[b] Intestinal clearance$_{wt}$ − intestinal clearance$_{(-/-)}$.
[c] Intestinal clearance$_{wt}$/intestinal clearance$_{(-/-)}$.

TABLE 3

Influence of P-glycoprotein on MLs accumulation in the intestinal segments

n = 3–10.

	Drug Concentration in Intestinal Segment		
	Duodenum	Jejunum	Ileum
	ng/g		
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

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

ivermectin and moxidectin compared with that for 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 and less so in the ileum. Moreover, these results show that quantitatively P-gp eliminates more eprinomectin than ivermectin, whereas 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 because of the large variability of the data. Again, the eprinomectin concentration in the intestinal tissue was more affected by P-gp deficiency than the ivermectin concentration with an increase in drug accumulation of 1.8- versus 1.3-fold, respectively, whereas moxidectin tissue accumulation was not different except slightly in the duodenum.

Accumulation of MLs in the Brain. Because 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, whereas 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 performed an in vivo experiment with the aim of determining the role of P-gp at the blood-brain barrier by measuring the concentration of the

TABLE 4

Influence of P-gp on plasma and brain concentrations of MLs in mice

$n = 3$.

	Drug Concentration in Plasma		Drug Concentration in Brain	
	2 h	24 h	2 h	24 h
	ng/ml		*ng/g*	
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**

** $p < 0.01$ versus wild-type mice.
*** $p < 0.001$ versus wild-type mice.

three 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 b.wt. 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 after administration and 27-, 297-, and 11-fold 24 h after 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 experiment. Two hours after 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), whereas 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 24 h after administration with brain concentrations for ivermectin and moxidectin at 64.7 ± 9.1 and 65.7 ± 23.6 ng/ml, respectively, that were lower than the eprinomectin concentration (29.7 ± 12.1 ng/ml, $p < 0.001$ and $p < 0.01$, compared with ivermectin and moxidectin concentrations, respectively).

To compare the brain uptake of each ML, the brain/plasma concentration ratio was calculated. The results after 2 h of treatment are shown in Fig. 4. In wild-type mice, the brain/plasma concentration ratio 2 h after administration was in the same range for ivermectin and moxidectin (0.03 and 0.08, respectively), whereas it was approximately 10 times lower for eprinomectin (0.005), suggesting 1) higher protection of the brain from eprinomectin entry or 2) better efflux of the drug out of the brain. In *mdr1ab*(−/−) mice, the brain/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/plasma concentration ratio of eprinomectin was lower than that of ivermectin and moxidectin (0.10 ± 0.02) (Fig. 4), suggesting that the low eprinomectin concentration in brain could not be explained only by a significant P-gp-mediated efflux at the blood-brain barrier. The $K_{(-/-)/wt}$ ratio shown in Fig. 4, calculated according to Chen et al. (2003), represents the enhancement of the brain/plasma concentration ratio between *mdr1ab*(−/−) and wild-type mice. Figure 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 by the high $K_{(-/-)/wt}$ ratio (27 and 21, respectively). This confirms an important contribution of P-gp in the efflux of these two drugs of the brain. The lower ratio of approximately 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 of treatment were in the same range of values (31, 20, and 7 for ivermectin, eprinomectin, and moxidectin, respectively; data not shown).

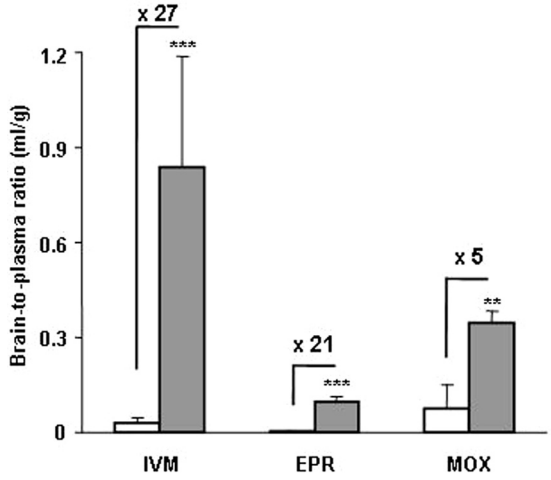

FIG. 4. Brain/plasma concentration ratio of MLs. Ivermectin (IVM), eprinomectin (EPR), or moxidectin (MOX) was administered subcutaneously in wild-type (□) and *mdr1ab*(−/−) (▨) mice at 0.2 mg/kg b.wt. Three mice in each group were sacrificed at 2 h after treatment. Drug concentrations were determined in plasma and brain as noted in Table 4, and the brain/plasma concentration ratios were calculated. The values shown on the figure are the $K_{(-/-)/wt}$ values calculated as follows: [brain/plasma concentration ratio$_{(-/-)}$]/[brain/plasma concentration ratio$_{wt}$]. Data are means ± S.D. of three animals. **, $p < 0.01$; ***, $p < 0.001$ versus wild-type mice.

Discussion

Given the strong interaction of MLs with P-gp (Lespine et al., 2007), the aim of our study was to evaluate the contribution of P-gp to their pharmacokinetic behavior 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 intravenous administration 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 finding is supported by the strong increase in plasma concentrations observed only for eprinomectin 24 h after subcutaneous administration in P-gp deficient mice, whereas ivermectin and moxidectin plasma concentrations were not significantly affected (Table 4). Because no major ML metabolites were detected, which confirms that biotransformation is a minor elimination pathway for MLs (Chiu et al., 1987), these results support the fact that P-gp was affecting the absorption of both ivermectin and eprinomectin, whereas 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 fecal 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 clearances 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). Compared with the plasma clearance of eprinomectin, 30% of which 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 in comparison with that of 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 Panchagnula, 2005). Because MLs are large hydrophobic nonionic compounds, their interaction with P-gp is certainly more influenced by their hydrogen bond acceptor pattern, amphiphilic character, and steric hindrance.

By measuring drug concentration in the brain, we clearly showed 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, whereas moxidectin had a low $K_{(-/-)/wt}$ ratio of 5, demonstrating the relatively lower contribution of P-gp. However, the lower brain/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/plasma concentration ratio was close to 1, which confirms the essential role of P-gp at the blood-brain barrier in effluxing ivermectin. 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 that of 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 at 24 h than at 2 h after treatment, suggesting that they tended to be sequestered into the brain in agreement with their high lipophilicity.

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 behavior with 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 behavior 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 seems to be directly related to the logP 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 ($K_i = 0.02\ \mu M$, logP = 4), which results in less attraction to the adipose tissue and hence lower persistence in the plasma (MRT) and lower entrance into the brain. Conversely, the higher lipophilicity and lower affinity for P-gp of moxidectin (logP = 6, $K_i = 0.5\ \mu M$) favor attraction to fatty tissue to the detriment of P-gp efflux. Moxidectin kinetics may therefore be determined more by lipophilicity/amphiphilicity, resulting in a delay in elimination and a prolonged MRT. Finally, ivermectin (logP = 4.8, $K_i = 0.05\ \mu M$) displays an intermediate behavior 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 and Landwojtowicz, 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, whereas 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. More recently, moxidectin was shown to be eliminated in milk through BCRP-mediated efflux but not effluxed by cells overexpressing BCRP (Perez et al., 2009). This discrepancy in drug-transporter interaction may be due, at least partly, to 1) the drug concentration, 2) the transporter expression level, 3) saturation with endogenous substrates of the pumps that are specific to the tissues, 4) the influence of the lipid environment, and 5) 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 has 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 does not appear to be a relevant carrier for ivermectin and

selamectin (Geyer et al., 2009). However, we cannot exclude the possibility that BCRP, also present at the blood-brain barrier and overexpressed 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 behavior 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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