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Interaction of macrocyclic lactones with P-glycoprotein: Structure–affinity relationship

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ABSTRACT

P-glycoprotein (P-gp) is involved in the ATP-dependant cellular efflux of a large number of drugs including ivermectin, a macrocyclic lactone (ML) endectocide, widely used in livestock and human antiparasitic therapy. The interactions of P-gp with ivermectin and other MLs were studied. In a first approach, the ability of ivermectin (IVM), eprinomectin (EPR), abamectin (ABA), doramectin (DOR), selamectin (SEL), or moxidectin (MOX) to inhibit the rhodamine123 efflux was measured in recombinant cells overexpressing P-gp. Then, the influence of these compounds on the P-gp ATPase activity was tested on membrane vesicles prepared from fibroblasts overexpressing P-gp. All the MLs tested increased the intracellular rhodamine123. However, the potency of MOX to inhibit P-gp function was 10 times lower than the other MLs. They all inhibited the basal and decreased the verapamil-stimulated P-gp ATPase activity. But SEL and MOX were less potent than the other MLs when competing with verapamil. According to the structural specificity of SEL and MOX, we conclude that the integrity of the sugar moiety is determinant to achieve the optimal interaction of macrocyclic lactones with P-gp. The structure–affinity relationship for interaction with P-gp is important information for improving ML bioavailability and reversal of multidrug resistance (MDR).

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1. Introduction

The MDR1 gene product P-glycoprotein (P-gp) is a membrane protein that belongs to the superfamily of the ATP-binding cassette (ABC) transporters. Its main function is the ATP-dependent efflux of various structurally unrelated exogenous compounds, coupling ATP hydrolysis and drug transport (Gottesman and Pastan, 1993). It belongs to the multidrug resistance (MDR) transporters family because it is overexpressed in cancers in response to chemotherapy and severely

restricts antitumoral drug effectiveness (Borst et al., 1999). Located in the intestine, liver, kidney and in blood–tissue barriers, P-gp exerts a protective function against xenobiotic toxicity but also, by limiting drug bioavailability (Bodo et al., 2003), it may reduce drug efficacy. In addition, due to its broad substrate specificity, P-gp plays a clinically relevant role in drug–drug interactions (Ho and Kim, 2005).

The macrocyclic lactones (MLs) belong to a large family of structurally related compounds widely used for the treatment of both internal and external parasites (McKellar and

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Abbreviations: ABA, abamectin; ABC, ATP-binding cassette; DOR, doramectin; EPR, eprinomectin; IVM, ivermectin; ML, macrocyclic lactone; MOX, moxidectin; P-gp, P-glycoprotein; Rho123, rhodamine 123; SEL, selamectin; VSP, valsopodar
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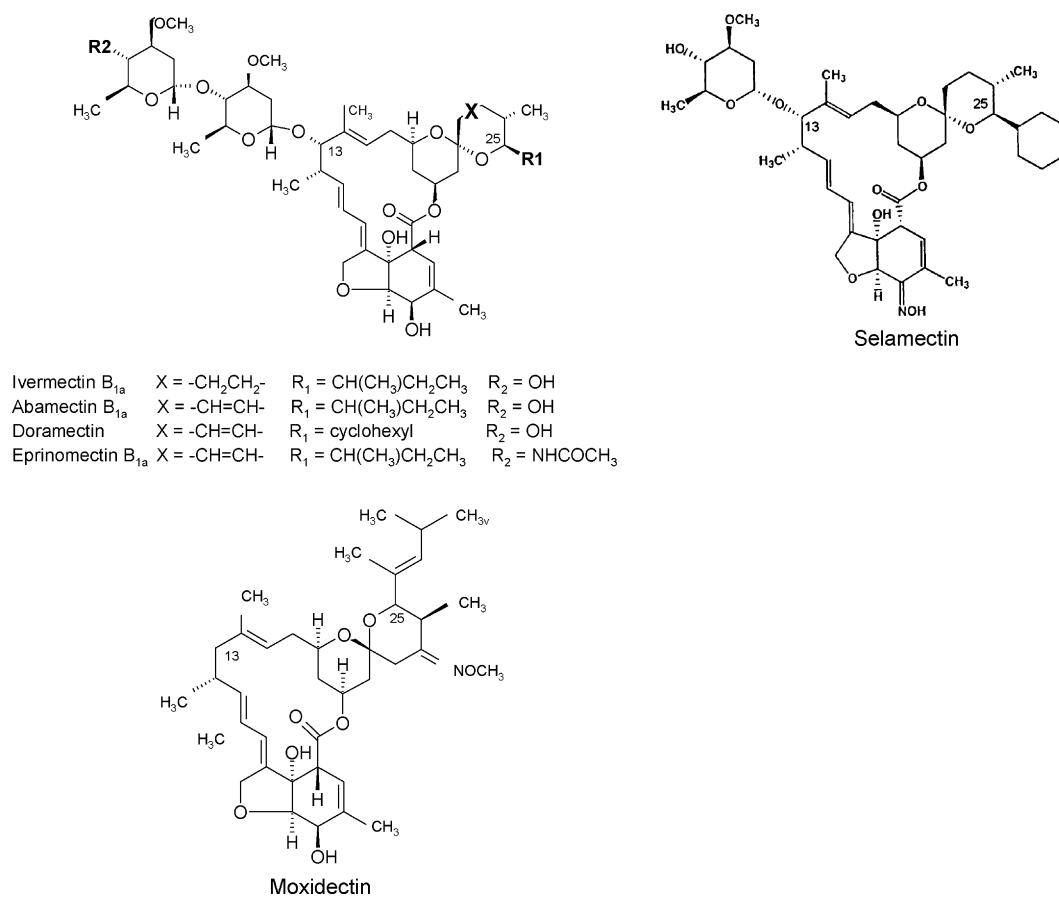


Fig. 1 – Structures of macrocyclic lactones. For ivermectin, the dry compound is mixture of 22,23 dihydro-avermectin B1a (substituent isobutyl on C25) and B1b (substituent isopropyl on C25) forms. Abamectin, eprinomectin, doramectin are also mixture of B1a and B1b forms. The majority (over 90%) of the drug is present as the B1a form.

Benchaoui, 1996). Their remarkable broad spectrum activity and their safety profile put these drugs at the cornerstone of modern anthelmintic therapy in livestock. Several million humans are also treated with ivermectin (IVM) for the control of onchocerciasis and lymphatic filariasis (Molyneux et al., 2003). MLs are large hydrophobic molecules characterized by the macrocyclic lactone ring as a common structural feature. IVM is the semi-synthetic derivative of the natural product avermectin B1 abamectin (ABA). Eprinomectin (EPR), doramectin (DOR) and selamectin (SEL) are synthetic derivatives from avermectin family, developed for use in veterinary medicine. Moxidectin (MOX) is a member of the milbemycin family currently under development for use in humans (Cotreau et al., 2003), and differs structurally from the avermectins by the lack of the sugar moiety attached to the C13 of the macrocyclic ring (Fig. 1).

MLs distribute throughout the body in the blood and lymph circulations (Lespine et al., 2006a). An effective concentration of drug for a suitable length of time in the target tissues, which correlates with the systemic concentration, determines the antiparasitic activity of the drugs (Lifschitz et al., 2000; Craven et al., 2002; Hennessy and Alvinerie, 2002). Among the factors controlling the disposition of MLs, the lipophilicity of these drugs is certainly a major determinant in modulating the rate of exchange between the tissues and the blood stream

(Craven et al., 2002; Bassissi et al., 2004). Although it has been shown recently that IVM interacts with the multidrug resistance protein MRP1 (Lespine et al., 2006b), P-gp appears as the ABC transporter playing the major role in IVM transport (Pouliot et al., 1997) and body disposition. As it is located on the blood-brain barrier, P-gp protects mammals against the penetration of IVM into the brain and its subsequent neurotoxicity (Kwei et al., 1999; Roulet et al., 2003; Schinkel et al., 1994). P-gp is also present on the surface of the intestinal epithelium and bile canalicules, and thus contributes to the high faecal elimination of MLs (Laffont et al., 2002), and it modulates IVM and MOX bioavailability in the whole organism and the cells (Kwei et al., 1999; Alvinerie et al., 1999a; Dupuy et al., 2001, 2003). Moreover, a P-gp homologue is expressed in parasites where it expels IVM, reducing drug activity and favoring the development of resistance to MLs (Sangster et al., 1999; Xu et al., 1998). In a wider range of therapeutic applications, recent data have revealed the ability of MLs to reverse multidrug resistance in tumor cells (Korystov et al., 2004). All these data point to P-gp being an important player in the modulation of ML pharmacokinetics.

The objective of the current study is to characterize the interaction of ivermectin and other structurally related compounds with P-gp. We combined cellular and subcellular approaches in an attempt to establish a relationship between

the structure of MLs and their affinity for P-gp. In the first approach, the avermectins, IVM, EPR, ABA, DOR, SEL, and the milbemycin MOX were tested for their ability to inhibit the P-gp mediated rhodamine 123 (Rho123) transport function in recombinant cell lines overexpressing P-gp. In the second approach, membrane vesicles were prepared from a hamster cell line highly overexpressing P-gp and the interactions of the MLs with P-gp were studied by measuring the modulation of its ATPase activity. A structure–affinity relationship was then drawn up and discussed in the context of the two important pharmacological applications of improving ML bioavailability and reversal of multidrug resistance.

2. Material and methods

2.1. Chemicals

Eprinomectin (EPR) and Ivomec® were generous gift from Merial (Lyon, France). Moxidectin came from Fort-Dodge Santé Animale (Tours, France). Doramectin and selamectin were from Pfizer (Orsay, France). Abamectin, ivermectin, dimethyl sulfoxide (DMSO), sodium dodecyl sulphate (SDS), rhodamine 123 (Rho123), verapamil and trypsin-EDTA were purchased from Sigma Chimie (Saint-Quentin Fallavier, France). Bovine serum albumin (BSA, fraction V, fatty acid free) was from MP Biochemicals (Illkirch, France). SDZ PSC-833 or valspodar (VSP) was a generous gift from Novartis (Basel, Switzerland).

Medium 199, phosphate saline buffer (PBS 1×), foetal calf serum, Hanks' balanced salt solution (HBSS) without phenol red, penicillin, streptomycin and gentamicin (G418) were obtained from Invitrogen (Cergy Pontoise, France). Culture flasks and 24-well plates were from Sarstedt France (Orsay, France). The bicinchoninic acid (BCA) kit was from Interchim (Montluçon, France). Acetonitrile and methanol, RS grade for HPLC, were obtained from Carlo Erba (Milan, Italy). All the other chemicals and solvents used were of the highest grade commercially available. Ultra-pure water (Milli Q A10, Millipore SA, Saint-Quentin, France) was used in all studies.

2.2. Assay for the inhibition of P-gp mediated transport

2.2.1. Cell culture

The transport function of P-gp was measured by following the intracellular accumulation of fluorescent rhodamine 123 (Rho123) in a cell line overexpressing P-gp. The pig kidney epithelial cell line LLC-PK1 transfected with the murine *mdr1a* gene (LLC-PK1-*mdr1a*) was a gift of Dr A. Schinkel (The Netherlands Cancer Institute, Amsterdam). Cells were cultured in medium 199 supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), 10% of fetal calf serum and G418 (400 mg/l) as selecting compound. Before the experiment, the cells were plated in 24-well plates in G418-free medium until confluence. The cells were then cultured in HBSS, 1% BSA containing 10 µM Rho123 without or with VSP (0.01–10 µM), verapamil (0.01–10 µM), IVM (0.05–15 µM), EPR (0.05–15 µM), ABA (0.05–15 µM), DOR (0.05–15 µM), SEL (0.05–15 µM) or MOX (0.10–15 µM). Similarly, LLC-PK1 transfected with human P-gp

gene (LLC-PK1-MDR1) were cultivated in presence of VSP or of increasing concentration of IVM, EPR or MOX.

All drugs were dissolved in DMSO and diluted in the medium (final DMSO concentration < 0.2%). After 2 h incubation, the medium was discarded and the cells washed with PBS and lysed by adding PBS/0.5% SDS. The lysates were stored at –20 °C until analysis.

2.2.2. Cellular accumulation of Rho123

The intracellular fluorescence corresponding to Rho123 accumulation was measured by using a fluorimeter (Perkin-Elmer LS50B, λ_{max} excitation = 507 nm; λ_{max} emission = 529 nm, mechanical slit = 3.5 nm). Values were normalized to the protein content per well determined by the method of Bradford, using a Bio-Rad protein assay kit with bovine serum albumin as the standard. In order to compare the effect obtained in presence of different MLs, we selected valspodar as reference of our transport inhibition assay because it was the chemical able to induce a maximal Rho123 intracellular accumulation by the maximal inhibition of P-gp, in our experimental conditions. The results obtained were expressed as percent of total VSP inhibition as follows:

$$\% \text{ VSP} = \frac{(f_x/F_o) - 1}{(F_{\text{VSP}}/F_o) - 1} \times 100$$

where f_x was the normalized fluorescence in cells treated with the compound of interest (x); F_o , the mean normalized fluorescence in the control (Rho123 alone); F_{VSP} , the mean normalized fluorescence in cells treated with VSP. The data generated were fitted with Scientist software (Micromath Research, Saint Louis, USA) according to Michaelis–Menten equation.

2.2.3. Statistical analysis

Mean and standard deviation (S.D.) were calculated for all the investigated parameters. All the data were subjected to statistical analyses using the PLSD Fischer test performed with Statview software (Abacus Concepts, Berkeley, CA, USA). In all cases, a value of $p < 0.05$ was considered significant.

2.3. Preparation of P-gp-containing membrane vesicles

Inside-out membrane vesicles were prepared as previously described (Garrigues et al., 2002b) from MDR cells DC-3F/ADX selected from spontaneously transformed DC-3F Chinese hamster lung fibroblasts on the basis of their resistance to actinomycin D (Biedler and Riehm, 1970), and overexpressing the *pgp1* gene. Inside-out membrane vesicles were also prepared from DC-3F cells, their sensitive parental counterparts, according to the previously described techniques (Garrigues et al., 1993), except that the final pellet of membrane vesicles was homogenized in phosphate buffer containing 2 mM MgCl_2 and 1 mM dithiothreitol, by passage through a 25 gauge needle. The membrane protein concentration was measured by the method of Bradford. It is reported that in vesicles prepared from DC-3F/ADX cells, P-gp accounted for about 12–15% of membrane proteins, whereas in control vesicles prepared from sensitive DC-3F cells, P-gp level was undetectable with the monoclonal antibody C219 (Garrigues et al., 1993).

2.4. Determination of the interaction of MLs with P-gp by measuring ATPase activity

The kinetic analysis of changes in P-gp ATPase activity can be described according to a simple Michaelis–Menten model using MgATP as the substrate, with one or two ligands modulating the catalytic reaction (Garrigos et al., 1997). In the presence of a modulator, the ATP hydrolysis rate increases or decreases. The rate of ATP hydrolysis after the addition of this modulator will be referred to as “stimulated activity”, in contrast to the basal activity measured in the absence of any added drug. EC₅₀ is the concentration that gives 50% modulation. Specificity with respect to P-gp of the observed effects on ATPase activity is checked by using the P-gp-devoid control vesicles prepared from the DC-3F cells.

The ATPase activity of the membrane vesicle suspension was determined at 37 °C using a coupled enzyme assay comprising an ATP-regenerating system and the continuous spectrophotometric detection of NADH absorbance at 340 nm (Garrigos et al., 1997). The reaction medium consisted of 30 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol and 1 mM MgATP with 0.1 mg/ml pyruvate kinase, 0.1 mg/ml lactate dehydrogenase, 1 mM phosphoenolpyruvate and 0.5 mM NADH. The assay medium was supplemented with 10 mM sodium azide, 0.5 mM ouabain and 1 mM EGTA to inhibit the ATPase activity of the major ion pumps. The membrane vesicles were diluted in the reaction medium. At that point, the final phosphate concentration in the enzymatic assay medium was about 100 μM. Since we were measuring ADP levels in a NADH-coupled assay, such phosphate concentration did not interfere with the assay and was not a concern as a possible inhibition of ATPase activity. The compounds tested were added to the reaction medium from stock solutions prepared in DMSO, or from diluted solutions prepared in water. DMSO never exceeded 0.4% (v/v), dilution without effect per se on P-gp ATPase activity. Experiments were also performed with membrane vesicles prepared from Sf9 insect cells transfected with human MDR1 gene (SOLVO biotechnology, Hungary). Data were obtained from four different membrane vesicle preparations. The accuracy of the ATPase activity measurement, evaluated from the accuracy of the slope of the curve of absorbance versus time, was about ±10 nmol/mg/min (i.e. relative accuracy of about 5%). Curves were fitted using SigmaPlot (Systat Software, Roissy, France).

We used verapamil, a well-known P-gp substrate that stimulates its ATPase activity to evaluate the ML affinity for P-gp. For each ML the inhibition constant (K_i) for the inhibition of the verapamil stimulation was determined:

$$K_i = \frac{(I)K_{act}^{\circ}}{K_{act} - K_{act}^{\circ}}$$

where (I) was the concentration of the MLs used in the inhibition assay, K_{act}° the concentration of the reference activator (verapamil) that gave half-maximal ATPase activation when alone in the medium, K_{act} was the concentration of the reference activator (verapamil) that gave half-maximal ATPase activation in the presence of the MLs at the given concentration (I).

3. Results

3.1. Influence of MLs on Rho123 accumulation in LLC-PK1-*mdr1a* or -MDR1 cells

LLC-PK1 pig kidney epithelial cells are characterized by a low drug transport activity attributable to endogenous P-gp or MRPs, making them suitable for the overexpression of drug efflux transporters of interest (Goh et al., 2002). Transfection with the murine *mdr1a* gene guarantees a high level of P-gp expression as previously reported (Schinkel et al., 1995). We measured the accumulation of a fluorescent dye substrate Rho123 typically used to study P-gp functionality (van der Sandt et al., 2000). Fig. 2A showed the intracellular accumulation of Rho123 in LLC-PK1 and LLC-PK1-*mdr1a* cells. Under our experimental conditions with no inhibitor added, control non-transfected LLC-PK1 cells accumulated twice the amount of Rho123 when compared to control P-gp overexpressing cells. In LLC-PK1-*mdr1a*, IVM induced an accumulation of intracellular Rho123 comparable to the one observed in non-transfected cells with a maximal inhibition efflux at 5 μM (Fig. 2A). In LLC-PK1 cells no significant increase was observed in presence of increasing concentration of IVM. Similar effects were obtained with 5 μM of valsopodar, a cyclosporin A analogue used as a positive control, which led to a massive increase of intracellular Rho123 in LLC-PK1-*mdr1a* cells, with only a marginal, non-significant effect in LLC-PK1 when compared to the respective controls (Fig. 2B). Fig. 2C shows the comparative effect of IVM, EPR and MOX on Rho123 accumulation in P-gp-overexpressing cells compared with 5 μM of VSP. The three compounds induced an increase of intracellular Rho123 accumulation in a concentration dependent manner. The IVM and EPR effects were very similar with a maximum effect (E_{max}) at concentrations above 1 μM, and the half-maximal effect (IC₅₀) obtained at 0.4 and 0.5 μM, respectively (Table 1). ABA, DOR and SEL were also able to induce

Table 1 – Influence of MLs on P-gp transport activity in LLC-PK1-*mdr1a* cells

Drug	E_{max} (% of VSP effect)	IC ₅₀ (μM)
Reference P-gp inhibitors		
Valsopodar	100	0.11 ± 0.03
Verapamil	64 ± 6	3.2 ± 0.1
Macrocyclic lactones		
Ivermectin	86 ± 2	0.44 ± 0.07
Eprinomectin	75 ± 2	0.50 ± 0.13
Abamectin	83 ± 2	0.11 ± 0.01
Doramectin	84 ± 2	0.31 ± 0.06
Selamectin	98 ± 3	0.60 ± 0.06
Moxidectin	88 ± 6	4.4 ± 0.6

LLC-PK1-*mdr1a* cells were cultured for 2 h with 10 μM Rho123 as the reference P-gp substrate with increasing concentrations of valsopodar, verapamil, or different macrocyclic lactones. IC₅₀: concentration needed for half-maximal effect. E_{max} : maximal effect as the percent of the maximal effect obtained in the presence of 5 μM valsopodar (100%). Values are mean ± S.D. of 6–9 experimental points out of three experiments.

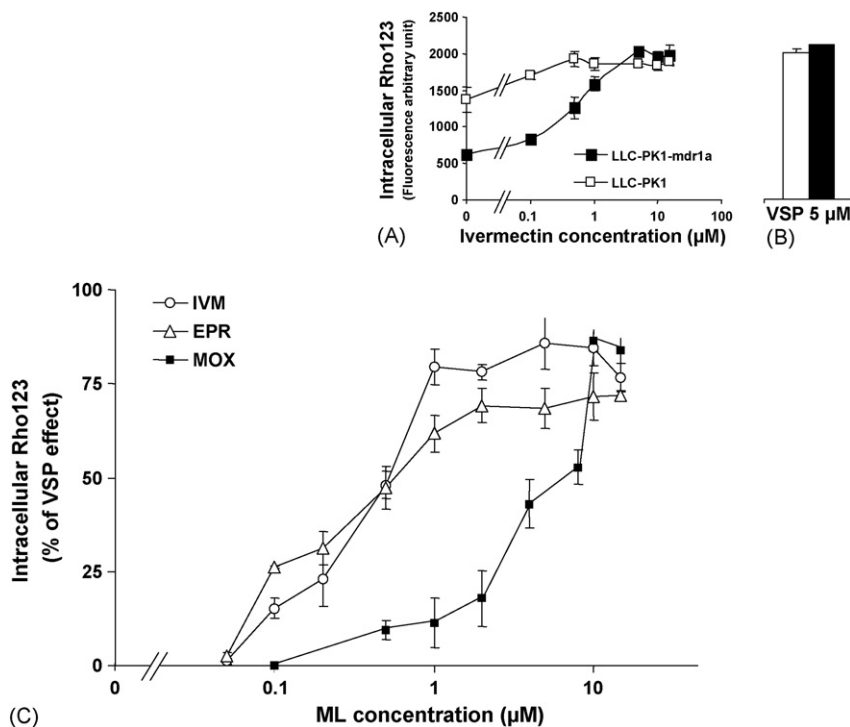


Fig. 2 – (A) Influence of IVM on intracellular accumulation of Rho123 in control and *mdr1a* transfected cells. LLC-PK1 or LLC-PK1-*mdr1a* cells were incubated without or with increasing concentration of ivermectin. Results are expressed as arbitrary units of fluorescence per mg protein. **(B)** Influence of valsopodar on intracellular accumulation of Rho123 in control and *mdr1a* transfected cells. Cells were incubated with 5 μM valsopodar. The y-axis was the same than in A. Values are mean \pm S.D. of three experiments. **(C)** Influence of MLs on Rho123-P-gp mediated transport in *mdr1a* cells. Cells were incubated without or with increasing concentration of MLs. Results were expressed as percent of the VSP effect at 5 μM (values are mean \pm S.D. of 6–9 experimental points out from 3 experiments).

Rho123 accumulation with high efficiency (E_{max} of 83, 84 and 98% of VSP effect, respectively) and potency similar to that of IVM (IC_{50} of 0.1, 0.3, 0.6 μM , respectively, Table 1). Interestingly, MOX also induced Rho123 accumulation with high efficiency ($E_{\text{max}} = 88\%$ of VSP effect), but a higher concentration was required to achieve the maximal effect (10 μM). The MOX half-maximal effect was reached at $4.4 \pm 0.6 \mu\text{M}$, which was 10 to 40-fold higher than the IC_{50} obtained for the other MLs. In parallel, we also used the Rho123 transport inhibition assay with LLC-PK1-MDR1, cells overexpressing human P-gp. In these cells, IVM and EPR inhibited the Rho123 transport with IC_{50} of 1.0 ± 0.2 and $0.8 \pm 0.1 \mu\text{M}$, respectively, and with E_{max} of 95 ± 5 and $78 \pm 3\%$ of valsopodar effect, respectively. Moxidectin was clearly less potent than ivermectin in inhibiting human P-gp, with an IC_{50} of 3 μM and an E_{max} of 50% (data not shown).

3.2. Influence of MLs on the basal ATPase activity of P-gp

The basal ATPase activity of P-gp was measured using membrane inside-out vesicles prepared from MDR cells DC-3F/ADX overexpressing P-gp. The ATPase activity of these vesicles, in the absence of added drugs, was around 75–130 nmol/mg of total membrane protein/min, which corresponded to the inherent ATPase activity of P-gp with variations from one

preparation to another. In the presence of increasing concentrations of IVM or MOX, basal P-gp ATPase activity was decreased (Fig. 3). Fifty percent inhibition was observed at concentration of 2 and 10 μM for IVM and MOX, respectively. The other MLs tested were also able to inhibit basal ATPase activity, with concentrations for 50% inhibition of 0.3, 0.2, 0.5 and 3.0 μM for EPR, ABA, DOR and SEL, respectively (Table 2). In the control membrane vesicles devoid of P-gp and prepared from the parental sensitive DC-3F cells, a residual ATPase activity ranging between 15 and 30 nmol/mg protein/min was measured. Increasing concentration of IVM or MOX (Fig. 3) or the other MLs (data not shown) had no effect on this residual ATPase activity.

3.3. Modulation of the verapamil-stimulated ATPase activity of P-gp

We then explored the interaction of drugs with P-gp by investigating the mutual effects on ATPase activity of MLs and the typical P-gp transport substrate, verapamil, used as a reference P-gp ATPase activator. When alone, verapamil displays a typical bell-shaped modulation curve characterized by a stimulation of ATPase activity from 0.1 to 20 μM followed by an inhibition at higher concentrations (control curve in Fig. 4). Changes induced by a compound of interest on this verapamil-stimulated ATPase activity profile provide information about

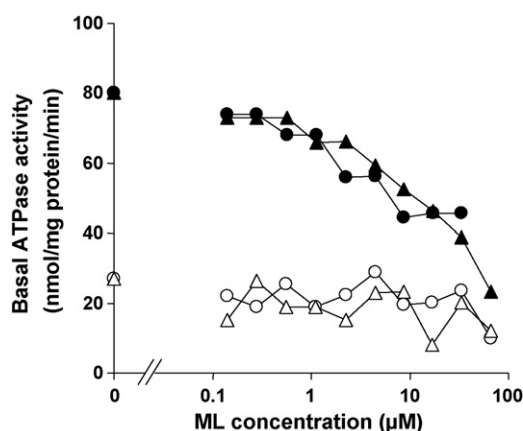


Fig. 3 – Modulation by IVM or MOX of basal P-gp ATPase activity. Membrane vesicles prepared either from MDR cells DC-3F/ADX overexpressing P-gp (closed symbols) or parental sensitive DC-3F cells (open symbols), were incubated in presence of increasing concentrations of IVM (circles) or MOX (triangles).

the interaction between the compound and P-gp (Garrigues et al., 2002a).

IVM modified the verapamil control curve by a competitive mechanism, as shown by the increase in the half-activation concentration of verapamil (K_{act}) in the presence of increasing concentrations of IVM (Fig. 4). For this experiment, the K_{act} for verapamil without any added drug was $0.6 \mu\text{M}$ and was increased to 2, 4 and 30 in presence of 0.1, 0.3 and $5 \mu\text{M}$ IVM, respectively. The linear regression was drawn between IVM concentrations and the apparent K_{act} of verapamil ($R^2 = 0.99$) according to the competition model assuming a 1/1 exclusion stoichiometry. The inhibition constant calculated for IVM, was thus about $0.05 \mu\text{M}$ (Table 3). The commercial formulation of IVM, Ivomec[®], had a similar competitive effect on verapamil-induced P-gp ATPase activation, giving a K_i of $0.04 \mu\text{M}$.

Table 2 – Modulation of basal P-gp ATPase activity by macrocyclic lactones

Macrocyclic lactones	EC_{50} (μM)
Ivermectin	2.0
Ivomec [®]	2.5
Eprinomectin	0.3
Abamectin	0.2
Doramectin	0.5
Selamectin	3.0
Moxidectin	10.0

The interaction of MLs with P-gp was evaluated by measuring the ability of the drug to modulate the ATPase activity of P-gp-containing membrane vesicles. EC_{50} corresponds to the concentration inducing 50% inhibition, calculated after subtraction of the background ATPase activity measured in control DC-3F cells non-expressing P-gp (one experiment representative of 2 for ivermectin, selamectin and moxidectin; one experiment was performed for each other ML).

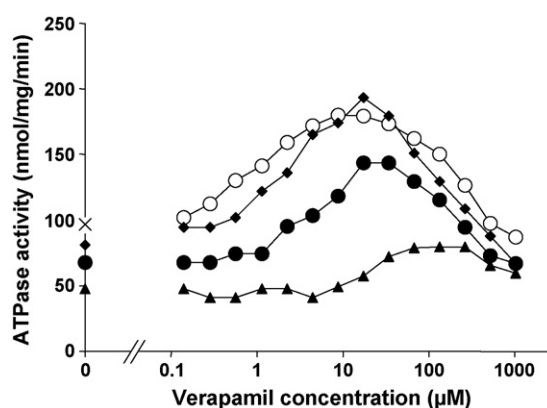


Fig. 4 – Modulation by IVM of the P-gp ATPase activity stimulated by verapamil. Membrane vesicles prepared from MDR cells DC-3F/ADX overexpressing P-gp were incubated in absence (x) or in the presence of increasing concentrations of verapamil without (O) or with IVM at $0.1 \mu\text{M}$ (◊), $0.3 \mu\text{M}$ (●), $5 \mu\text{M}$ (▲); one experiment representative of 2.

A similar type of ATPase modulation was observed for EPR, ABA and DOR, which were thus able to compete with verapamil-induced activation, as shown by the increased verapamil K_{act} by 6–9-fold (from 1.6 to $10 \mu\text{M}$, $10 \mu\text{M}$ and $15 \mu\text{M}$ for EPR, ABA and DOR, respectively, tested at a concentration of $0.3 \mu\text{M}$), which gave inhibition constants very close to that of IVM (0.02 , 0.02 , $0.03 \mu\text{M}$, respectively, Table 3). MOX and SEL were also able to compete with verapamil for ATPase activation. However, SEL and MOX at $0.3 \mu\text{M}$ did not change the K_{act} value for verapamil and higher concentrations were required when compared with the other MLs. At $1 \mu\text{M}$ SEL or MOX, the verapamil K_{act} was shifted from 1.6 to $2.5 \mu\text{M}$ for SEL and from 0.6 to $2.5 \mu\text{M}$ for MOX (Fig. 5), leading to calculated K_i of 1.0 and $0.5 \mu\text{M}$, respectively (Table 3).

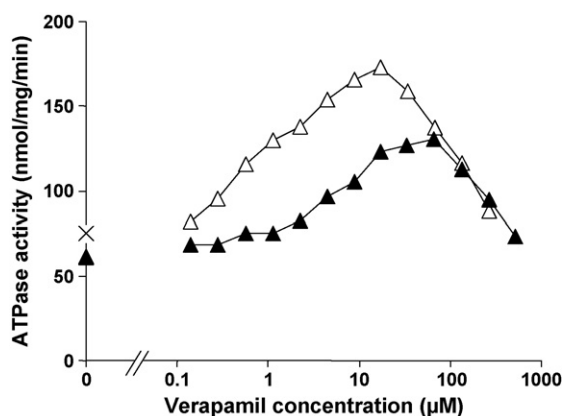


Fig. 5 – Modulation by MOX of the P-gp ATPase activity stimulated by verapamil. Membrane vesicles prepared from MDR cells DC-3F/ADX overexpressing P-gp were incubated in the absence (x) or in the presence of increasing concentrations of verapamil without (Δ) or with MOX at $1 \mu\text{M}$ (▲); one experiment representative of 2.

Table 3 – Modulation of verapamil-stimulated P-gp ATPase activity by macrocyclic lactones (MLs)

	MLs concentration (μM)	Apparent K_{act} of verapamil (μM)			Calculated K_i (μM)
		Experiment 1	Experiment 2	Experiment 3	
Without MLs	0	0.6	1.3	1.6	
Ivermectin	0.1	2			0.05
	0.3	4			
	5.0	30			
Ivomec [®]	0.3			13	0.04
Eprinomectin	0.3			10	0.02
Abamectin	0.3			10	0.02
Doramectin	0.3			15	0.03
Selamectin	0.3			2	1.0
	1.0			2.5	
	3.0			6.8	
Moxidectin	1.0		4.0		0.5

The ability of the MLs to modulate P-gp ATPase activity in P-gp-containing membrane vesicles was measured on verapamil-stimulated ATPase. One experiment was performed for Ivomec[®], EPR, ABA, DOR and one experiment was shown representative of 2 for SEL, MOX, and IVM.

4. Discussion

Although a number of lines of evidence have shown that IVM is among the most potent inhibitors and substrates of P-gp (Pouliot et al., 1997; Didier and Loor, 1996; Lespine et al., 2003; Griffin et al., 2005), the precise interactions between MLs and P-gp and particularly the basis for the high affinity need to be further documented. We describe here two complementary approaches combining the inhibition of P-gp transport function and competition with verapamil-stimulated ATPase activity. We clearly show that besides IVM, other structurally-related compounds from the ML family also interact with P-gp and that the chemical structure of the MLs influences their affinity for P-gp.

At the cellular level, we studied the capacity of IVM to restore the retention of Rho123, a fluorescent P-gp substrate, in P-gp overexpressing cells. The cellular model used here was transfected with murine *mdr1a* gene. While there is only one gene *MDR1* corresponding to one P-gp isoform involved in the multidrug resistance phenotype in humans, there are two genes in mice: *mdr1a* and *mdr1b*. Murine *mdr1a* is the most relevant for our study because it encodes for the protein expressed at intestinal, liver epithelium and blood-brain barrier level and that was shown to modulate drug bioavailability (Schinkel et al., 1994, 1995).

We confirmed that IVM is a potent and strong inhibitor of the efflux of Rho123 mediated by P-gp, in the same range as VSP and largely better than verapamil, which are reference inhibitors for P-gp transport. Similar potent inhibitory effect on P-gp was reported for cyclosporin A (Eneroth et al., 2001). The other compounds belonging to avermectin family tested, EPR, ABA, DOR, were also strong inhibitors of P-gp transport function. Only MOX showed a major difference with similar efficiency to induce Rho123 accumulation but with a lower potency, requiring 10 times higher concentration to reach the half-maximal effect when compared with the other MLs. Similar results have been recently obtained for MOX on Rho123 transport inhibition in Caco2 cells (Griffin et al., 2005).

The interactions of IVM, EPR and MOX with human *MDR1* were also evaluated in LLC-PK1-*MDR1* cells: their potencies to inhibit Rho123 transport were comparable in *MDR1* and *mdr1a* transfected cells. This validates the use of P-gp from different animal origins for studying the structure-affinity relationship of MLs with P-gp.

At the sub-cellular level, we studied the influence of MLs on the P-gp ATPase activity in inside-out membrane vesicles prepared from the *MDR* cells DC-3FADX overexpressing P-gp at high level, thus allowing convenient enzymatic measurements. In addition, this vesicular system has the advantage that drugs are exposed directly to the cytosolic side of the P-gp-containing membranes. With respect to the added drug in the bulk medium, the system avoids thus the transmembrane concentration gradient which occurred rapidly with lipophilic drugs in the whole cells. Firstly, we showed that all the IVM, EPR, ABA and DOR inhibited the basal P-gp ATPase activity, with comparable EC_{50} values ranging from 0.2 to 2 μM , while MOX required higher concentration ($EC_{50} = 10 \mu\text{M}$). In general, according to the energetic coupling existing between ATP hydrolysis and drug transport both catalyzed by P-gp, good substrates activate the transporter's basal ATPase activity, whereas slowly transported substrates rather inhibit it with respect to the basal activity measured in the absence of added substrate (and presumably due to the presence of endogenous substrates). In agreement with this observation, IVM has been shown to be a slowly transported substrate of P-gp (Pouliot et al., 1997; Schinkel et al., 1995; Griffin et al., 2005).

In addition, by following the inhibition of the P-gp-ATPase activity stimulated by verapamil, a reference ATPase activator, we showed that EPR, ABA and DOR were able to compete with the verapamil stimulation as efficiently as IVM. The commercial IVM, Ivomec[®] has similar effect than IVM on basal and verapamil-stimulated ATPase activity excluding some influence of the formulation. Moreover, MOX also competed with verapamil, but higher concentrations were required to achieve the same inhibition.

SEL appeared equivalent to the other avermectins in terms of Rho123 transport inhibition in P-gp-transfected cells, in

full agreement with recent work performed on Rho123 transport inhibition in Caco2 cells (Griffin et al., 2005). However, in terms of basal ATPase activity inhibition, the EC₅₀ of SEL was in the higher range when compared with the other avermectins. Moreover, when we consider the effect of SEL on the verapamil-stimulated ATPase curve, the calculated K_i was in the range of the MOX rather than the other avermectins.

In a previous study, ATPase assay was also performed using inside-out vesicles prepared from membranes of Sf9 insect cells transfected with human MDR1 gene. In this model, IVM competitively modulated the verapamil-stimulated ATPase activity with calculated K_i value of 0.06 μM (Lespine et al., 2006b). By using similar model and approach, very recent and still preliminary data allowed us to propose a K_i around 0.6 μM for moxidectin interaction with human P-gp. This data are in full agreement with those reported here with rodent P-gp. However, in our study, we have compared results obtained in cells overexpressing murine P-gp with data obtained in vesicles overexpressing Chinese hamster P-gp. Although both are rodents with strong homology for *mdr1a*, we cannot rule out a possible difference which may influence results. Indeed, species can influence the interaction of the substrate with ABC-transporters as previously shown for benzimidazole (Merino et al., 2005). Thus, more accurate approach would have consisted in using proteins from same species.

The strength of our results lies on the use of complementary approaches. The cells offer an integrate system which requires the drug diffusion across the membrane before reaching the target P-gp, while the membrane vesicles expose the P-gp directly to the drug thus avoiding intermediate events such as the drug transmembrane concentration gradient. This may explain the 10 time difference between IC₅₀ values obtained in cells (Table 1) and K_i calculated on membrane vesicles (Table 3). Nevertheless, with both systems we obtained similar differential potency and affinity of avermectins with MOX. Only SEL displayed a similar potency as avermectins in inhibiting Rho123 transport in cells while it was closer to MOX in terms of affinity for P-gp as determined on vesicular system. This may be due to an interaction of SEL with membranes closer to avermectins than MOX and suggests that the diffusion through the membrane is a key step conditioning the intracellular availability of the drug and its further interaction with P-gp.

The interaction parameters calculated with both approaches revealed that EPR, DOR and ABA behave comparably to IVM in terms of their ability to inhibit P-gp transport function and its ATPase activity. The four molecules share a disaccharide moiety on the C13 of the macrocycle as a common structural feature (Fig. 1). The original natural compound is ABA, while the other three compounds have been synthetically modified but none of the differences in their substituents significantly affected their interaction with P-gp in the systems studied here. MOX belongs to the milbemycin family and differs structurally from avermectins by lacking the disaccharide moiety on the C13 of the macrocycle. We showed that MOX required 10 times higher concentration for 50% inhibition of Rho123 transport and basal ATPase activity and showed a 10 times higher K_i for competition with verapamil, when compared with the avermectins. SEL is an avermectin which presents an intermediate structural feature with only one sugar residue instead of two for IVM, EPR, ABA

and DOR. The rest of the molecule contains a cyclohexyl substituent on C25 similar to DOR (Fig. 1).

Our results show that the integrity of the disaccharide moiety present on the molecule plays a determining role in the affinity for P-gp. The presence of the sugar moieties influences the degree of hydrophobicity of the molecule, and a relationship can be proposed between the octanol/water partition coefficient (logP) of the MLs and the affinity for P-gp. The logP, calculated by using atomic parameters (HyperChem 7.0, HyperCube, Inc. (Vellarkad et al., 1989)), are 6 and 6.3, respectively, for MOX and SEL, while for the other MLs they are in a lower range (4.8, 4.4, 5.3 and 5.6 for IVM, EPR, ABA, DOR, respectively). The hydrophobicity of the molecule dictates its partitioning within the lipid membrane, which is an obligatory first step for the interaction of the substrate with P-gp. The inverse relationship between the logP of MLs and the P-gp interaction parameters determined in this study showed clearly that the events observed are dictated by a direct interaction with P-gp and not due to a secondary effect of drug-membrane interaction. On the basis of the different ability of DOR and SEL to compete with verapamil on P-gp, we suggest that the second sugar unit plays an important role in the interaction with P-gp. These observations are in favour of a role of the sugar pattern in governing the ability to interact with P-gp drug binding sites, more markedly than the other substituents that characterize each avermectin.

The glutamate and γ-aminobutyric acid (GABA)-agonist activity of the MLs are the mechanisms which lead to the paralysis and death of the treated parasites. The GABA-like action also mediates the mechanism of neurotoxicity in mammals. IVM and MOX bind irreversibly and with high affinity to the glutamate-gated chloride channel subunit (K_d of 0.11 and 0.18 nM, respectively (Forrester et al., 2002)). At much higher concentrations avermectins are also GABA-agonists. The similar low K_d determined for the interaction of IVM and MOX with the glutamate-gated chloride channel subunit testifies that the sugar moiety does not play a significant role in the strong interaction of these drugs with this type of receptors. This differential property with respect to P-gp may be used to obtain selectivity between these two targets, P-gp and glutamate-gated chloride channel, in order to develop a optimized strategy for MDR reversal by optimizing P-gp affinity, with limiting neurotoxic side effects by reducing GABA-receptors affinity. Indeed, avermectins have been shown to reverse multidrug resistance in tumor cells (Korystov et al., 2004), and our data strongly suggest that avermectins having a disaccharide moiety are better MDR reversal candidates than SEL or MOX. However, it needs to be taken into account the strong neurotoxicity of these compounds once in brain. Nevertheless, toxicity studies of ivermectin in cattle have established that the first signs of neurotoxicity appear at 8 mg/kg, with a therapeutic dose of 0.2 mg/kg, giving a good therapeutic index (Shoop and Soll, 2002). A large number of ML derivatives have been generated that need to be studied in order to select the most appropriate compounds that combine high affinity for P-gp with reduced affinity for glutamate or GABA-receptors, displaying thus a high safety margin as MDR reversal agent.

Cyclosporin A, a well-described cyclic undecapeptide with potent MDR reversing properties, shares a lot of similarities with IVM in terms of P-gp interactions. Indeed, both

compounds are strong P-gp inhibitors that are rich in hydrogen bonds which induces a slower transport (Pouliot et al., 1997; Saeki et al., 1993), possibly as a result of lower dissociation rates from the protein (Seelig and Landwojtowicz, 2000). Both interfere with calcein-AM transport in Caco2 cells with similar efficacy (Eneroth et al., 2001) and show similar competition with P-gp specific antibodies (Nagy et al., 2004). Cyclosporin A also competes with IVM on membranes from drug-resistant cells overexpressing P-gp (Pouliot et al., 1997). In membrane vesicles overexpressing P-gp, similar to those used in our study, cyclosporin A competed with verapamil displaying a K_i for P-gp of 0.015 μM (Garrigues et al., 2002a), which is comparable to the one calculated here for the avermectins. Altogether, these results strongly support the view that both types of drug share a P-gp binding site also common, at least partially, to verapamil. Based on an integrated approach using enzymatic analysis and *in silico* molecular modelling of some P-gp substrates, a two pharmacophore model has been proposed (Garrigues et al., 2002a), with at least two close hydrophobic pockets within P-gp able to bind specific compounds. The competitive effect of cyclosporin A on verapamil and on various other P-gp substrates may possibly be the consequence of its large molecular size leading to overlapping binding on the two P-gp recognition sites. Because of its large molecular size, IVM is also expected to compete with those P-gp substrates. It is likely that the macrolactone and the hexahydrobenzofuran unit which constitute the core of the MLs participate in their binding to P-gp while the disaccharide moiety is probably involved in an additional binding site, further increasing affinity. This property should be of special interest in order to inhibit the binding to P-gp of a large number of dissimilar drugs as it is desired for an efficient MDR-reversing activity.

The large number of pharmacokinetic studies available reveals that the body distribution of MLs varied from one molecule to another. When compared with IVM, MOX is characterized by a large volume of distribution, an extensive elimination in milk during lactation, and a remarkably long mean residence time in the host organism (Craven et al., 2002), that results in an interesting long-lasting efficacy of several weeks (Kerboeuf et al., 1995). The long persistence of MOX in the organism may be due to its low affinity for P-gp combined with its high affinity for adipose tissues, which results in a lower elimination rate when compared with IVM. Also, MOX is more extensively eliminated in milk when compared with IVM (Imperiale et al., 2004) or EPR (Alvinerie et al., 1999b). The role of P-gp has never been discussed in the context of drug secretion or reabsorption at the mammary gland level and it is likely that other ABC transporters such as Breast Cancer Resistance Protein (BCRP) are involved (Jonker et al., 2005).

P-gp at blood-brain barrier level effluxes IVM out of the brain, and protects thus the organism against the GABA-ergic action of the drug and its subsequent neurotoxicity. IVM become neurotoxic when the protective P-gp barrier is deficient such as in P-gp deficient mice (Schinkel et al., 1994) or in some Collie dogs (Roulet et al., 2003). The lower affinity of MOX or SEL for P-gp suggests that these two molecules will penetrate the brain more easily than IVM causing increased neurotoxicity. In agreement with this, a case report described an increased sensitivity to MOX of an Australian IVM-sensitive dog (Geyer et al., 2005). By contrast, SEL was claimed to have

a wider therapeutic index over IVM (Novotny et al., 2000), but this is not consistent with our conclusions. More data on toxicity of MOX or SEL need to be provided. Nevertheless, in parasite, the lower affinity of SEL for P-gp can be suggested to explain, at least partly, the higher concentration of SEL reported in brain of cat flea when compared to IVM (Phipps et al., 2005).

In this study, we provide evidences that besides IVM other avermectins also interact strongly with P-gp. P-gp certainly influences the absorption, distribution and elimination of the majority of marketed compounds from the MLs family. P-gp may thus be considered as the main target in the antiparasitic chemotherapy strategy aiming at increasing drug concentration. We have shown that the presence of the sugar moiety on the macrocycle determines the affinity of MLs for P-gp, leading to lower affinity for SEL and MOX. Based on our data, avermectins such as ivermectin appear as strong potential MDR reversing agents when compared with MOX and SEL. The challenge now is to select other MLs among the structurally different compounds that combine high affinity for P-gp with lower affinity for glutamate or GABA receptors. The information provided here on the interaction of MLs with P-gp is thus valuable for helping in the prediction of drug distribution and for the targeting of these drugs.

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