Macrocyclic Lactones and Cellular Transport-Related Drug Interactions: A Perspective from *In Vitro* Assays to Nematode Control in the Field

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Abstract: Macrocyclic lactones (MLs) are antiparasitic drugs used against endo-ectoparasites. Regarding the wide use of MLs in different species, it is likely that drug-drug interactions may occur after their co-administration with other compounds. A new paradigm was introduced in the study of the pharmacology of MLs during the last years since the interactions of MLs with ATP-binding cassete (ABC) transporters have been described. The current review article gives an update on the available information concerning drug-drug interactions involving the MLs. The basis of the methodological approaches used to evaluate transport interactions, and the impact of the pharmacology-based modulation of drug transport on the MLs disposition kinetics and clinical efficacy, are discussed in an integrated manner. A different number of *in vitro* and *ex vivo* methods have been reported to study the characterization of the interactions between MLs and ABC transporters. The production of the ABC transporters knockout mice has provided valuable *in vivo* tools to study this type of drug-drug interaction. *In vivo* trials performed in different species corroborated the effects of ABC transporter modulators on the pharmacokinetics behaviour of MLs. Important pharmacokinetic changes on plasma disposition of MLs have been observed when these compounds are co-administered with P-glycoprotein modulators. The modulation of the activity of P-glycoprotein was evaluated as a strategy not only to increase the systemic availability of MLs but also to improve their clinical efficacy. The understanding of the MLs interactions may supply relevant information to optimize their use in veterinary and human therapeutics.

Keywords: ABC transporters, drug combinations, drug-drug interactions, macrocyclic lactones.

INTRODUCTION

Macrocyclic lactones (MLs) are broad-spectrum antiparasitic drugs from the avermectin and milbemycin families, widely used in veterinary and human medicine. The avermectins include a series of natural and semisynthetic molecules, such as abamectin (ABM), ivermectin (IVM), doramectin (DRM), selamectin (SLM) and eprinomectin (EPM), which share some structural and physicochemical properties [1]. Moxidectin (MXD) belongs to the milbemycin family showing distinctive pharmacokinetic and pharmacodynamic characteristics compared to the avermectin compounds [2-4]. IVM, introduced into the veterinary market in 1981, rapidly become in a leading veterinary drug in annual sales [5]. Additionally, millions of humans have received annual doses of IVM since 1985 for the treatment of the river blindness, a parasitic disease caused by *Onchocerca volvulus* [6].

An extensive literature body addressing the pharmacological features of the ML compounds is currently available. Their pharmacokinetic behaviours have been studied in different animal species. Several research groups have focused their activity on the characterization of the disposition kinetics of the MLs, and on the factors affecting the absorption, distribution and elimination of these antiparasitic compounds in different animal species [2, 7-19]. From all these studies, it has been corroborated that pharmacokinetics affects the drug concentrations reaching the site of action, and therefore impact on the observed pharmacological response. However a new paradigm was introduced into the study of the pharmacology of MLs during the last few years. The influence of cell transporter systems in the pharmacokinetic behaviour of different compounds has been described [20]. The interaction of MLs with different cell transporters has been corroborated [21-23]. Thus, concomitant administration of multiple drugs, as is often done in current veterinary and human clinical practice, may drastically induce changes to the disposition kinetics and pharmacological activity of different drugs used in therapy. Considering the wide use of the MLs in different animal species, it is likely that drug - drug interactions may occur after their co-administration with a large variety of drug compounds. In fact, combined formulations including MLs with other different antiparasitic drugs are commercially available to be used in livestock and companion animals in different countries. In addition to the early published work on the pharmacokinetic features of the MLs, some further kinetic information [24] as well as the interaction of MLs with ATP-binding cassete (ABC) transporters [25-27], has been recently reviewed. The current review article gives an update on the available information concerning drug-drug interactions involving the MLs. The assessment of the drug cellular transport mechanism is approached all the way from *in vitro* molecular assays to its application in nematode con-

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trol in field trials. The basis of the methodological approaches used to evaluate transport interactions, and the impact of the pharmacology-based modulation of drug transport on the MLs disposition kinetics and clinical efficacy, are discussed in an integrated manner.

MACROCYCLIC LACTONES PHARMACOKINETIC PROPERTIES

The main pharmacokinetic characteristics of the MLs are briefly described here to understand the pharmacological basis of the drug-drug interactions that may affect these compounds. MLs are large molecules relatively insoluble in water [28]. Moxidectin shows a lipophilicity 100 times higher than IVM [29] with greater affinity for fat tissue. The pharmacokinetic profiles of MLs in cattle have been shown to be substantially affected by the composition of the administered formulation [30, 31]. Oral administration of MLs both in ruminants [32-35] and horses [36] results in a lower absorption compared to that observed after parenteral treatment. The pour-on formulations offer considerable practical advantages compared to other routes of drug administration. [37]. However, low systemic availability and high variability in the plasma kinetic behaviour was observed after the topical administration of MLs in cattle [38-45]. The high lipophilicity determines the extensive tissue distribution of MLs in the body [7-8]. Liver metabolism of MLs is rather low (< 15 %) and they are largely excreted in bile and faeces as the unchanged parent drugs [8, 14-15, 46-47]. MLs are also considerably eliminated by milk in different species [10, 35, 48]. Eprinomectin was introduced to the veterinary market due to its limited distribution to milk, compared to other MLs, based on changes introduced to its chemical structure [49]. The involvement of different ABC transporters in the secretion process of MLs in bile, intestine and milk has been recently confirmed [50-52]. Thus, drug-drug interactions between MLs and substrates of cellular transporter proteins may occur in different organs of body.

CHARACTERIZATION OF THE ABC TRANSPORTERS

The members of the ABC transporters use the energy of the ATP hydrolysis to transport a wide variety of substrates out of cells against a concentration gradient leading to a decreased intracellular concentration [49]. This family includes the members of the ABCB1 (P-glycoprotein, P-gp), ABCC1-5, (Multidrug resistance proteins, MRPs) and ABCG2, (breast cancer resistant protein, BCRP) [54-55]. Among all the identified cell transporters, P-gp has been the most studied. P-gp was initially described due to its capacity of preventing the intracellular accumulation and cytotoxic effects of antineoplasic drugs by actively removing them from the cell membrane before they reach their intracellular target. Besides tumor cells, P-gp has also been detected in healthy tissues and particularly in organs involved in drug pharmacokinetics [56-58]. P-gp is a complex membrane glycoprotein with a molecular weight of 170 to 180 kDa. P-gp is 1280 aminoacids long with 12 predicted transmembrane domains consisting of two homologous parts. Each half contains a hydrophilic domain for ATP binding [59]. P-gp actively removes substrates from the cell against concentration gradient impeding intracellular drug accumulation [53]. P-gp is located in tissues and particularly in organs involved in the processes of drug absorption (e.g. mucosa of the small and large intestine), distribution (e.g. brain-blood barrier) and elimination (luminal surface of hepatocytes and ducts cells, kidney tubules and enterocytes) [60]. In placenta, P-gp may also transport substances required for fetal maturation [61]. The anatomical distribution of ABC transporters suggests that they may perform different functions such as limiting intestinal drug absorption, excrete diverse chemical compounds toward the extracellular space (regulating intracellular and tissue levels of endogenous and exogenous molecules), and prevent drug entrance into the central nervous system.

MRPs tissue distribution is similar to that of P-gp, being located in the liver (bile canaliculi), membrane of erythrocytes, heart, kidney (renal tubules), intestinal epithelium, lung, and in certain tumor cell lines [62-64]. Multidrug resistance protein also possesses wide substrate specificity: a) MRP1 possesses a high specificity for leukotriene C4 (LTC4), (b) MRP2 would be responsible for the secretion of glucuronide bilirubine into bile (c) MRP4 and MRP5 would be related to nucleotides and nucleosides transport and (d) MRP6 to peptides transport [64-66]. MRPs transport different glutathione, sulphate and glucuronide conjugative metabolites and anionic drugs (e.g. methotrexate) [67].

There is an increasing interest on the latest discovered members of the ABC-transporter family: the breast cancer resistant protein (BCRP) [68] initially isolated from a breast cancer cell line [69]. As opposed to the general structure of ABC transporters which usually comprises 12 transmembrane segments split into two halves, each with a nucleotide binding domain, BCRP is a so called half transporter [70].

BCRP is not only expressed in cancer cells, but is also present in many normal tissues, such as placenta, brain, colon, small intestine, breast tissue, testis, ovary, liver and prostate [69, 71]. The number of BCRP substrates and/or inhibitors has been steadily expanding since its discovery. Among the transported substrates are sulfated hormones and metabolites of the anticancer drugs mitoxanthrone, topoisomerase 1 inhibitors (topotecan or irinotecan), tyrosin kinase inhibitors (imatinib and gefitinib) [72-73]. The involvement of BCRP in the milk secretion of different drugs used in veterinary and human medicine has been recently studied. BCRP facilitates the excretion of xenobiotics in the milk of mice and sheep [74-76]. Since high concentrations of MLs are excreted by milk, the potential interactions of these compounds with BCRP may have consequences on both their residue levels in milk and on the clinical use of MLs in lactating animals.

As previously mentioned, one of the most important aspects of ABC transporters is that a single membrane protein can recognize a wide range of chemically unrelated compounds. Based on this feature, speculation on the important consequences of co-administering drug substrates/inhibitors or inducers can be made. The induction of the activity of this transport system, for example at intestinal level, will lead to the reduction of the bioavailability of orally administered P-gp substrates, while an increment on the bioavailability would be observed when an inhibitor is co-administered with a P-gp substrate. The impact of induction/inhibition of the

transport proteins on the systemic availability of drug substrates of the ABC transporters are schematically summarized in Fig. (**1**). Fig. **2** summarizes the main sites of potential interactions between MLs and ABC transporters.

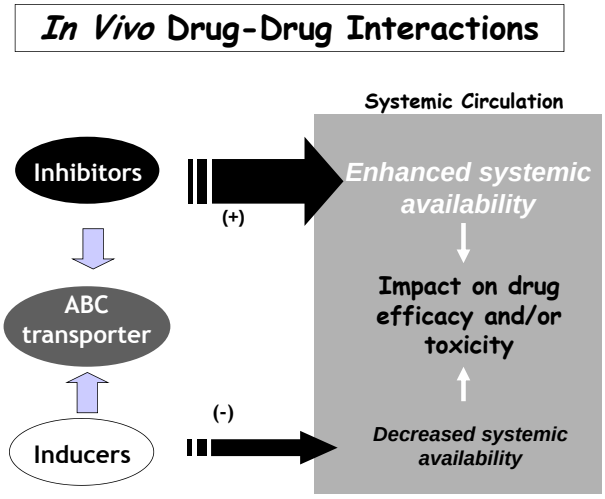

Fig. (1). Impact of inhibitors and/or inducers of the ABC transporter systems on the systemic availability of different drug substrates.

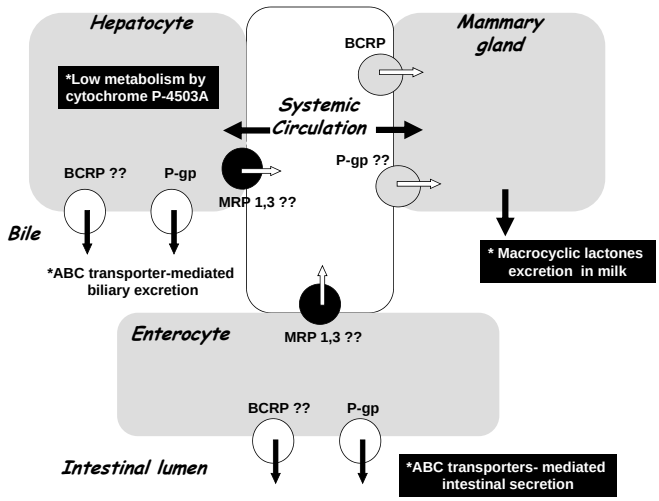

Fig. (2). Schematic representation of the main excretion routes for macrocyclic lactones and potential targets for ABC transporters-mediated drug-drug interactions.

IN VITRO AND EX VIVO EXPERIMENTAL APPROACHES

Several *in vitro* systems have been developed for testing drug-drug interactions with ABC transporters. These *in vitro* models are used to predict the *in vivo* activity of ABC transporters [77]. Under *in vivo* conditions, orally administered drug molecules have to cross different barriers to get absorbed (enterocytes), distributed (blood-brain barrier endothelial cells), or eliminated (hepatocytes, proximal tubule epithelial cells). This transcellular movement is modeled in cellular monolayer efflux ('vectorial transport') assays [78]. The *in vitro* assays using cells over-expressing ABC proteins or membranes proteins isolated from the same cells are widely used to estimate transepithelial passage of different P-gp drug substrates [79]. A number of different *in vitro* and *ex-vivo* methods have been reported to characterize the interactions between MLs and ABC transporters. The first *in vitro* studies reported that IVM was actively secreted by cells transfected with the gene coding for P-gp in mice [56] and by multidrug-resistant tumor cells [22]. The interaction of MXD with the ABC transporters was demonstrated in cultured rat hepatocytes. Ketoconazole, quercetin and fumagillin increased the quantity of 14C-moxidectin in hepatocytes [80, 81]. It has been recently shown that affinity by P-gp may differ among different ML molecules [23]. The different MLs were tested for their ability to inhibit the P-gp mediated rhodamine 123 (Rho123) transport function in recombinant cell lines over-expressing P-gp. The different avermectins (IVM, eprinomectin, abamectin, doramectin and selamectin) increased the intracellular Rho123 accumulation with a similar potency. It is interesting to note that MXD appears to have different P-gp efflux potential, with a half-maximal inhibitory effect (IC_{50}) approximately 10 times higher than that reported for IVM [23, 82].

An other interesting methodological approach to measure the P-gp activity is the use of vesicles prepared from cells that over-express this transport protein. The efflux activity of ABC transporters usually increases the rate of ATP hydrolysis. Therefore, ATPase activity can be estimated by quantifying ATP consumption, ADP release or the liberation of inorganic phosphate [83]. In the presence of transported substrates, the ATPase activity of the transporter increases. However, the inhibitors and/or compounds transported at a lower rate inhibit the ATPase activity of the stimulated transporter [78]. The MLs inhibited the basal P-gp ATPase activity but MXD required higher concentrations (between 5 and 50-fold) to achieve half-maximal effect [23]. Although IVM has been shown to have high affinity by P-gp, it has been also reported that IVM may interact with other cell transport proteins such as multidrug resistance proteins (MRP1, MRP2 and MRP3). P-gp may be involved in the IVM efflux at low drug concentration, whereas at the micromolar concentration range, which may saturate the Pgp function, MRP1 and to a lesser extent MRP2 and 3, could participate to transport IVM across physiological barriers [84]. The impact that the affinity of MLs by multiple ABC transporters may have on the *in vivo* pharmacokinetic behaviour of these compounds is unknown. The *in vivo* involvement of multiple transporters suggests a complex interplay between MLs and the different ABC transporter proteins, which could affect their systemic disposition.

Interestingly, the interaction of MLs with BCRP was recently described. IVM inhibited BCRP-mediated albendazole sulphoxide transport in culture cells [85]. The IVM concentrations needed to interact *in vitro* with BCRP were higher compared to those used to inhibit P-gp transport. It seems, therefore, that IVM is a relatively potent P-gp and rather weak BCRP inhibitor [85]. The interaction between MXD and BCRP was corroborated using cellular transport assays and pharmacokinetic studies in BCRP1 (−/−) and wild type mice [86]. In this study, MXD was identified as a BCRP substrate and its milk BCRP-mediated secretion was demonstrated. The involvement of ABC transporters in the milk secretion of MLs may play a relevant role in the clinical

use of these compounds in lactating animals due to the presence of residues in milk and derived subproducts.

Whereas the bile secretion was initially proposed as the main route of elimination of MLs as parent drug, the *in vivo* intestinal secretion of IVM in rats using an *in situ* model of intestinal perfusion (closed loop) has been studied. In this study the importance was demonstrated of the intestinal P-gp-mediated secretion of IVM, principally along the jejunum. The use of *ex-vivo* approaches is an important tool to study the role of ABC transporters on the intestinal secretion of MLs. The everted gut sacs have been proposed as a simple model for quantification of P-gp-mediated intestinal efflux for different drugs [87]. The everted sac technique was corroborated as a useful system for studying the P-gp-mediated efflux of extremely lipophilic molecules such as MLs [51]. The IVM accumulation rate in the intestinal wall was significantly higher after its incubation with the P-gp inhibitors itraconazole (0.115 nmol/g/min) and PSC833 (0.238 nmol/g/min) than that obtained after its incubation alone (0.05 nmol/g/min) [51]. New methodological approaches are required to evaluate the physio-pharmacological features of the intestinal drug secretion process in ruminant species. The Ussing chamber technique has been previously validated to assess transmembrane transport of different xenobiotics [88]. In veterinary therapeutics, the Ussing chambers have been used to estimate the extent of oral absorption for drugs administered to horses [89]. The apparent permeability coefficient, per unit of membrane surface area (Peff) (cm/s), of Rho123, a P-gp substrate, was significantly increased (68 %) from the mucosa to the serosal side of sheep intestine in the presence of IVM [90]. The Ussing chamber technique offers a great potential to improve the comprehension of the transport mechanisms involved in the absorption/excretion processes for drugs therapeutically important in veterinary medicine such as MLs. Table **1** summarizes the different *in-vitro/ex- vivo* assays performed to study the interaction of different MLs with ABC transporters.

IN VIVO PHARMACOKINETIC INTERACTIONS

Interactions at the ABC transporters level may be defined as the modulation of the pharmacology of a substrate drug by another co-administered compound that interact with these proteins [91]. These interactions have been categorized as intentional modulation of the function of the ABC transporters (drug-drug interactions) and unintentional modulation of ABC transporters (unexpected toxic drug-drug interactions) [92]. The interplay between a transporter and either drug-metabolizing enzymes or other transporters adds difficulties to interpret the *in vivo* activity of these proteins due to a broad overlapping of substrate specificities [93-94].

The production of the ABC transporter knockout mice has provided valuable tools to study this type of drug-drug interactions since these knockout animals proved to be hypersensitive, as a result of a profound difference in the tissue distribution of ABC-substrates [78]. The first report on the interaction of IVM with P-gp was related to toxicological effects. *In vivo* studies using multi-drug resistant-1 gene (MDR-1) knockout mice treated with therapeutic doses of IVM revealed an increased potency of the drug, reflected as neurotoxicity and death [95]. A 4-base pair deletion in the MDR-1gene that encodes for P-gp produces the synthesis of a non-functional protein [96]. The clinical consequences of mutations in the MDR-1 gene may be observed in some dog breeds such as Collies and Old British Shepard [97]. The avermectin-induced neurotoxicity that occurs in the affected dog breeds is due to P-gp genetic polymorphism comparable to the MDR-1 knockout mice [58]. Identification of other dog breeds or animal species susceptible to the action of MLs, as a consequence of interaction with cell transporters such as P-gp, opens a new field of research in veterinary medicine [98].

Table 1. Summary of the *In Vitro* and *Ex Vivo* Methodological Approaches Used to Assess the Interaction between Macrocyclic Lactones (MLs) and ABC Transporters

Methodology	Outstanding Outcome
Culture of cells over expressing ABC transporters -Drug accumulation assays -Drug uptake assays -Drug transport assays	**-Differential affinity of MLs by P-gp [26]** **-Interaction of IVM with MRPs [23]** **- MXD affinity to BCRP [86]**
Membrane Vesicles -ATPase activity determination	**Characterization of IVM affinities by P-gp and MRPs [23]**
Loop intestinal technique **Everted gut sac technique** -Drug intestinal transport -Lumen/wall drug accumulation assays	**Involvement of P-gp on IVM intestinal secretion [50, 51]**
Using Chamber technique -Drug transport assays in host tissues -Drug influx/efflux in parasite tissues	**Studies on P-gp mediated transport in sheep intestine [90]**

IVM: ivermectin. MXD: moxidectin. P-gp: P-glycoprotein. MRP: Multidrug resistance protein. BCRP: breast cancer resistant protein.

As ABC transporters have a relevant role on the overall drug kinetic behavior, *in vivo* trials performed in different species provide information on the action of different P-gp modulators on the MLs pharmacokinetic disposition. Important changes to the plasma disposition of the MLs have been observed when these compounds are co-administered with P-gp modulators. The effect of verapamil (VRP), a P-gp modulating agent, was demonstrated on IVM plasma disposition kinetics after pour-on treatment in rats [99]. The peak plasma concentration (Cmax) was significantly lower in the group treated with IVM alone than that observed in the animals receiving IVM+VRP. The influence of loperamide (LPM) on the plasma disposition and fecal excretion of MXD after its intravenous and subcutaneous administration was studied in cattle [100]. Significantly higher MXD plasma concentra-

tions were observed after the MXD+LPM co-administration compared with MXD alone. This pattern was independent of the administration route and it is explained by a reduction on MXD total body clearance induced by LPM. Other interesting interactions have been reported in adult sheep and lambs. The oral co-administration of VRP+IVM induced a significant increase on the IVM absorption in sheep [34]. In lambs the co-administration of quercetin, a derived flavonoide present in vegetables, with MXD produced a significant increase on MXD plasma concentrations [80]. A significant increment in the systemic availability of IVM was obtained after its intraruminal administration together with the antifungal drug itraconazole [101]. More recent work demonstrated that the drug-drug interactions produced after the co-administration of IVM and ketoconazole (a well-known inhibitor of both cytochromes P4503A and P-gp) in sheep [102] and dogs [103] does not seem to be related to inhibition on IVM metabolism by the antifungal compound. The concentration ratios for IVM parent compound to its metabolites in the bloodstream were equivalent in dogs and sheep receiving IVM alone or co-administered with ketoconazole. Therefore, a ketoconazole-P-gp interaction may explain the changes observed on IVM disposition in both animal species.

The measurement of IVM concentrations attained at the gastrointestinal tract after the *in vivo* modulation of P-gp has supplied useful data confirming the relevance of the intestinal secretion process. The co-administration of IVM+LPM in rats leads to higher IVM concentration profiles not only in plasma but also in liver and small intestine mucosa [104]. The differential affinities of MLs by P-gp were recently assayed using the knockout mice model. P-gp deficiency led to a significant increase in the systemic availability of IVM (1.5-fold) and eprinomectin (3.3-fold), whereas the MXD availability remained unchanged. IVM, and to a greater extent eprinomectin, were both excreted by the intestine via a P-gp-dependent pathway, whereas MXD excretion was lower compared to the avermectin-type MLs [105]. Animal studies have shown that the changes on drug tissue concentrations caused by inhibition/modulation of transporter activity are much greater than the modifications observed in plasma levels [94].

The *in vivo* interaction of MXD with BCRP was recently confirmed. MXD accumulation in proximal and distal intestinal content was significantly higher in male wild-type compared to BCRP1-/- mice, indicating that BCRP could participate in the secretion to the intestinal lumen [86]. Although the interaction of IVM with BCRP was demonstrated *in vitro* [85], no differences in IVM brain and/or other tissues concentrations were observed in BCRP-deficient knockout compared to wild-type mice [106].

The majority of the studies on drug interactions mediated by ABC transporters has been addressed to obtain the inhibition of these proteins, and thus, to increase the absorption or delay the elimination of therapeutically relevant drugs. However, the effect of potential inducers of the transporter proteins on the kinetic behavior of MLs is not fully understood. Although numerous therapeutic agents can induce P-gp expression under *in vitro* systems, the relevance of these observations to P-gp induction *in vivo* is not entirely clear. The effect of phenobarbital on both plasma and gastrointestinal disposition of IVM was recently examined [107]. The IVM area under the concentration *vs* time curve (AUC) values were 2.2 (plasma), 2.5 (jejunum) and 2.4 (liver)-fold lower in animals pre-treated with phenobarbital than those measured in control animals. The IVM intestinal secretion was increased in the phenobarbital treated animals. An enhanced P-gp-mediated intestinal transport activity in pre-treated induced rats may account for the drastic changes observed on IVM disposition [107]. Further studies are necessary to clarify the impact of specific inhibitor or inducer molecules of different ABC transporters, on the disposition of MLs in animal species and humans. The influence of different transport modulating agents on the systemic exposure of MLs in different species is shown in Fig. (**3**).

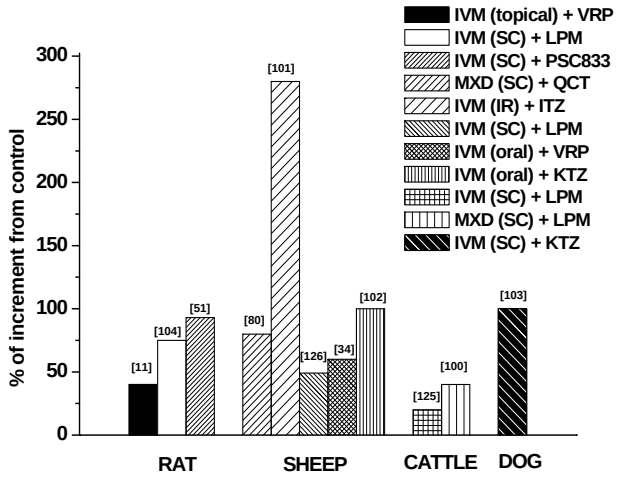

Fig. (3). *In vivo* effects of the P-glycoprotein modulating agents on the systemic exposure of the macrocyclic lactones in different animal species. The values represent the changes observed in plasma area under the concentration *vs* time curve (AUCs) in comparison to control animals treated with either IVM or MXD alone.
IVM: ivermectin. MXD: moxidectin. VRP: verapamil. LPM: loperamide. QCT: quercetin. ITZ: itraconazole. KTZ ketoconazole. SC: subcutaneous. IR: intraruminal.

DRUG EFFLUX MODULATION AND FIELD ANTIPARASITIC ACTIVITY

The resistance to MLs was firstly recognized as a problem in small ruminant production systems [108]. However, anthelmintic resistance in bovine gastrointestinal nematodes has been reported worldwide [109-113]. P-gp has been described not only in mammals but also in parasites such as *Onchocerca volvulus* [114] and *H. contortus* [115-116]. Drug efflux mediated by P-gp in different parasites has been proposed as a potential resistance mechanism for different drugs [117-119]. Increased scientific evidence supporting this concept has been reported during the last few years. IVM and MXD induce over-expression of some P-gp isoforms in nematodes [116]. A modification on the P-gp gene expression pattern was described in IVM resistant compared to susceptible isolates of *Teladorsagia circumcincta* [120]. In this context, the modulation of the activity of P-gp has been assayed as a pharmacology-based strategy not only to increase the systemic availability of the MLs in the host animal but also to improve their clinical efficacy.

In vitro assays and clinical efficacy studies in laboratory animals were performed to assess the impact of modulation on P-gp activity. The combinations of VRP and CL347099 with either IVM or MXD significantly reduced worm counts of resistant strains of *Haemonchus contortus* [121]. Also, the modulation of P-gp increased the *in vitro* activity of IVM against IVM-sensitive and resistant larvae of *T. circumcincta* and *H. contortus* [122]. The presence of the P-gp modulators PSC833, VRP, ketoconazole and pluronic 85 in the larval feeding inhibition test enhanced the sensitivity of larvae to IVM between 56- and 77-fold (*T. circumcincta* resistant isolates) and between 15 and 57-fold (*H. contortus* resistant isolates) [122]. Further evidences were added after the revelation that VRP increased the *in vitro* IVM activity against susceptible and resistant isolates of *Cooperia spp*. [123]. Using the larval development test and the larval migration inhibition test, the *in vitro* activity of IVM against *Cooperia spp*. was increased between 10 and 100-fold after its co-incubation with the P-gp modulator.

Although a modification on MLs activity after P-gp modulation was confirmed *in vitro*, *in vivo* trials performed under field conditions are necessary to evaluate the clinical impact of the P-gp inhibition. The enhanced sensitivity of resistant larvae to IVM obtained after its co-incubation with pluronic 85 did not correlate with their *in vivo* co-administration to sheep. In the *in vivo* trial, the presence of pluronic 85 did not improve the efficacy against resistant *H. contortus* [124]. Work recently done in our laboratory has shown that the efficacy of both IVM and MXD against resistant *Cooperia spp*. in a cattle field trial tended to increase after their co-administration with LPM, as a P-gp modulator [125]. A low nematodicidal efficacy (measured by the faecal count reduction test) was observed for both IVM (23%) and MXD (69%) in cattle, *Cooperia spp*. was the most abundant nematode species recovered after the different drug treatments. The egg output reduction values increased from 23% to 50% (IVM) and from 69% to 87% (MXD) following their co-administration with LPM. Enhanced systemic concentrations and an altered disposition of both MLs in cattle, which correlates with a tendency to increased anthelmintic efficacy, were observed in the presence of LPM [125].

Further work looking for the clinical relevance of drug efflux modulation included the assessment of the same P-gp modulator (LPM) in a field trial in sheep naturally infected with resistant nematodes. A significant increment on IVM efficacy against resistant nematodes in sheep together with an enhancement of the systemic availability of the antiparasitic compound was obtained in the presence of the P-gp inhibitor [126]. The egg output reduction increased from 78.6% (IVM alone treatment) to 96% after the co-administration with LPM. A nematode population highly resistant to IVM was identified. The efficacy of IVM against *H. contortus* was 0 % and the percentage of reduction against intestinal nematodes such as *Trichostrongylus colubriformis* and *Nematodirus spp*. was 77.9 % and 85.5 %, respectively. The clinical efficacy against the resistant nematodes was enhanced in the presence of LPM, with percentages of reduction of 72.5% (*H. contortus*), 96.3% (*T. colubriformis)* and 93.0% (*Nematodirus spp*.) [126]. Thus, there is evidence that the P-gp-mediated drug-drug interaction increases the IVM systemic exposure in the host and it may also decrease the P-gp-mediated efflux transport over-expressed in target resistant nematodes. Table **2** summarizes the different *in-vitro/in vivo* assays performed to study the modulation of MLs antiparasitic activity by inhibitors of P-gp. The practical implications of this drug interaction should be carefully investigated. Potential side effects induced by the P-gp modulating agents, their relatively short persistence and changes to the pattern of tissue residues [26] are among issues to be addressed before any practical applications can be advised. However, the modulation of P-gp or other proteins involved in drug transport, may be a valid pharmacological approach to improve the activity and extend the lifespan of the MLs in veterinary medicine.

COMBINATION OF ANTHELMINTIC DRUGS: ASSESSMENT OF KINETIC/DYNAMIC INTERACTIONS

Different measures have been applied to maintain the efficacy of the existing antiparasitic molecules. The management of grazing and the presence of refugia of susceptible nematodes have been utilised in strategies to reduce the negative impact of parasitism on animal production. From a pharmacological point of view, it has been suggested that drug combinations may be efficacious against resistant nematode strains where the failure of individual drugs is documented. The use of combined formulations prepared with drug molecules acting by different mechanisms of action, has been proposed to reduce the frequency of treatments and to delay the development of resistance [127-128]. There are several drenches in the veterinary pharmaceutical market which combine benzimidazoles (BZD) and avermectin-type compounds. Some broad spectrum antiparasitic combinations contain albendazole (ABZ), IVM and levamisole or oxfendazole, IVM and levamisole. A multi-combination drench for sheep, which combines ABZ, levamisole, closantel, and abamectin, is approved for use in sheep in Australia. As IVM is not effective against trematodes [129], the combination of IVM with trematodicidal compounds is a commonly used strategy to extend its antiparasitic spectrum. Commercial formulations containing IVM combined with either clorsulon, nitroxynil or triclabendazole or MXD with triclabendazole, have been introduced into the veterinary market.

The use of combined anthelmintic preparations are based on a lower resistance in individual worms to a preparation with multiple components (each one with different mechanism of action) compared to the treatment with a formulation containing a single active component. However, the available information on the potential additive or synergistic anthelmintic effect occurring after co-administration of two drugs with different mode of action is limited. Potential pharmacokinetic and or pharmacodynamic interactions between components may occur, which may affect the resultant clinical activity. Among potential interactions are those related to cellular transport mechanisms, particularly if one considers that most the anthelmintic molecules used in the combined preparations are substrates for P-gp and/or other transporters.

For instance, despite the fact that *in vitro* studies indicated that closantel modulated the P-gp transport in cell lines

Table 2. Influence of P-Glycoprotein Modulation on the Activity/Efficacy of Different Macrocyclic Lactones (MLs) against Ivermectin Susceptible and Resistant Nematode Strains

ML Compound/ P-gp Modulator	Parasite/Animal Species	Modification of Activity	Reference
IVM + verapamil MXD + verapamil	*H. contortus* in jirds	Enhanced efficacy (from 1.71 to 2.69-fold)	[121]
IVM + ketoconazole IVM + PSC833 IVM + verapamil IVM + pluronic85	IVM-sensitive and resistant larvae of *T. circumcincta* and *H. contortus*	Increment on IVM *in vitro* activity (larval feeding inhibition test) from 10 up to 77-fold	[122]
IVM + verapamil	IVM-sensitive and resistant *Cooperia spp.* larvae	Enhancement on IVM *in vitro* activity (larval development and migration tests) between 10 and 100-fold	[123]
IVM + pluronic 85	Resistant *H.contortus* in sheep	No changes on efficacy compared to the IVM alone treatment	[124]
IVM + loperamide MXD + loperamide	Resistant *Cooperia spp.* in cattle	Increment on efficacy (FECRT) from 23 to 50 % (IVM) and from 69 to 87 % (MXD)	[125]
IVM + loperamide	Resistant *H. contortus* and *T. colubriformis* in sheep	Enhanced IVM efficacy (adult nematode counts) from 0 to 72.5 % (*H. contortus*) and from 77.9 to 96.3% (*T.colubriformis*)	[126]

IVM: ivermectin. MXD: moxidectin. FECRT: fecal eggs count reduction test.

[130], a similar plasma disposition was observed after the co-administration of IVM-closantel compared to that described after the treatment with each anthelmintic compound alone in cattle [131]. The basis of the pharmacological interaction between IVM and triclabendazole (TCBZ), a worldwide available anthelmintic combined formulation, was recently assessed *in vitro* and *in vivo*. The ability of TCBZ and its metabolite triclabendazole sulphoxide (TCBZSO) to interfere with P-gp transport in cell lines over-expressing P-gp has been demonstrated [130]. TCBZ and TCBZSO increased the intracellular concentration of Rho123, a P-gp substrate, with an IC_{50} of 41 (TCBZ) and 63 (TCBZSO) μM [129]. Recent work carried out in our laboratory demonstrated that TCBZ enhances the IVM intestinal accumulation after their co-incubation with everted gut sacs [132]. The *in vivo* co-administration of IVM and TCBZ in sheep resulted in a significant change on the kinetic disposition of both molecules. The IVM systemic availability was 3.13-fold higher in the presence of TCBZ. The elimination half-life and mean residence time for IVM were between 63 and 81% longer, respectively, after its co-administration with TCBZ ($p < 0.05$) [132]. Additionally, the co-administration of TCBZ with IVM resulted in higher TCBZSO plasma concentration profiles. Higher Cmax for TCBZSO was obtained after the co-administration of TCBZ with IVM (23.2 μg/ml) compared to those obtained after the TCBZ alone treatment (12.6 μg/ml) [132]. It is likely that TCBZ and its metabolite would play a role in the modulation of the intestinal or biliary ABC-transporter mediated elimination of IVM in sheep. Although IVM has not activity on *Fasciola hepatica*, an interesting effect was observed after its co-incubation with TCBZ. The *in vitro* influx/efflux balance for TCBZ and TCBZSO in susceptible and resistant flukes in the presence/absence of IVM as substrate of the drug transporter P-gp, was assessed [133]. The IVM-induced modulation of P-gp activity decreased TCBZ efflux from the resistant flukes and higher concentrations of TCBZ and TCBZSO were recovered from the resistant *F. hepatica* in the presence of IVM [133].

The evaluation of drug-drug kinetic interactions between BZD and MLs has been recently studied. An increase on IVM plasma concentrations was observed in the presence of ABZ, after the intravenous co-administration of both compounds. The enhanced IVM plasma concentrations (87% higher AUC) measured after the intravenous coadministration of both anthelmintics is indicative of the occurrence of some degree of pharmacokinetic interaction [134]. The presence of IVM modifies the plasma pharmacokinetic behaviour of albendazole sulphoxide and sulphone (ABZSO and ABZSO$_2$). The systemic availability of the active ABZSO metabolite was significantly higher (> 42%) following the ABZ-IVM co-administration compared to that observed after ABZ alone (Table **3**). Whereas ABZ parent drug does not seem to interact with either P-gp [130, 135-136,], MRP2 or BCRP1 [136], a potential interaction between IVM and ABZSO sulphoxide mediated by BCRP may occur [85, 136]. A kinetic modification after the co-administration of two compounds does not always mean a clinically relevant interaction [137]. The impact of the pharmacokinetic modifications obtained after the coadministration of ABZ and IVM was studied in sheep infected with resistant nematodes [138]. The co-administration of both compounds did not result in a

clinically significant enhanced anthelmintic effect against multiple ABZ and IVM resistant nematodes, with the disadvantage of an increased resistance selection pressure over parasite populations. Therefore, further work is required to understand the potential pharmacokinetic/pharmacodynamic interactions among anthelmintics before drug combined formulations are developed to be introduced into the pharmaceutical market and any practical use can be advised. This may be of particular relevance in the light of the novel available knowledge related to the involvement of different transporter proteins/efflux mechanisms on the disposition kinetics of several anthelmintic drugs both in the host animal and in target parasites.

CONCLUSIONS

The MLs are widely used in veterinary and human medicine. The use of these compounds by practitioners has been threatened by the increasing reports of resistance of different endo and ectoparasites in different animal species. Therefore, the understanding of the pharmacology-based interactions involving the MLs may provide relevant information to optimize their therapeutic use. The affinity of MLs by different ABC transporters is determinant on their kinetic disposition in the host. The potential involvement of these transport proteins in the resistance mechanism of nematode parasites against MLs added a new scientific challenge in the evaluation of these therapeutically relevant compounds. Determining the drug efflux capacity of the different transporters and extrapolating those results to the assessment of the pharmacological impact of a given drug-transporter interaction, remain as a difficult task. Besides technical limitations, a major problem is the relevance of the extrapolation of *in vitro* data to the *in vivo* physiological role of ABC transporters. An important body of elegant results on the subject has been obtained by different research groups in the last few years. The overall comprehension of the interactions between MLs and ABC transporters has markedly improved on the basis of the different available *in vitro* assays. Besides, the characterization of the *in vivo* modulation of the cell transport activity was assessed in ruminant species. Interestingly enough, some field trials have provided exciting information on the impact of drug transport modulation on the MLs efficacy against resistant nematode in sheep and cattle. A major future challenge is to identify the appropriate and most convenient assay to validate these field efficacy results. The information generated should be of tremendous value to prolong the lifespan of MLs and, perhaps more importantly, to assess the transport features of novel antiparasite molecules in the early stages of drug development.

CONFLICT OF INTEREST

None declared.

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ABBREVIATIONS

ABM = Abamectin

ABC = ATP-binding cassette

ABZ = Albendazole

ABZSO = Albendazole sulphoxide

$ABZSO_2$ = Albendazole sulphone

AUC = Area under the concentration *vs* time curve

BCRP = Breast cancer resistant protein

BZD = Benzimidazoles

DRM = Doramectin

EPM = Eprinomectin

IC_{50} = Half-maximal inhibitory effect

IVM = Ivermectin

LPM = Loperamide

MDR-1 = Multi-drug resistant-1 gene

MLs = Macrocyclic lactones

MRPs = Multidrug resistance proteins

MXD = Moxidectin

P-gp = P-glycoprotein

Rho123 = Rhodamine 123

SLM = Selamectin

TCBZ = Triclabendazole

TCBZSO = Triclabendazole sulphoxide

VRP = Verapamil

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