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Pharmacological knowledge and sustainable anthelmintic therapy in ruminants

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ABSTRACT

Considering the increasing concern for the development of anthelmintic resistance, the use of pharmacology-based information is critical to design successful strategies for the future of parasite control in livestock. Integrated evaluation of the available knowledge on pharmacological features is required to optimize the activity and to achieve sustainable use of the existing anthelmintic drugs. The assessment of the drug disposition in the host and the comprehension of the mechanisms of drug influx/efflux/detoxification in different target helminths, has signified a relevant progress on the understanding of the pharmacology of anthelmintic drugs in ruminant species. However, additional scientific knowledge on how to improve the use of available and novel molecules is required to avoid/delay resistance development. Different pharmacokinetic-based approaches to enhance parasite exposure and the use of mixtures of drugs from different chemical families have been proposed as valid strategies to delay the development of anthelmintic resistance. The rationale behind using drug combinations is based on the fact that individual worms may have a lower degree of resistance to a multiple component formulation (each chemical with different mode of action/resistance) compared to that observed when a single anthelmintic is used. However, the limited available information is unclear on the potential additive or synergistic effects occurring after co-administration of two (or more) drugs with different mode of action. This review article contributes to the topic with some pharmacology-based data emerging from the assessment of combined anthelmintic preparations. The activity against multi-drug-resistant isolates based on novel modes of action is a highly favorable element to judge the future of some of the recently developed anthelmintic compounds. More specific knowledge on the basic host–parasite kinetic behavior as well as a highly responsible use of those novel compounds will be necessary to secure their maximum lifespans. Overall, the outcome from integrated pharmaco-parasitological research approaches has greatly contributed to optimize drug activity, which seems relevant to preserve existing and particularly novel active ingredients as useful tools for parasite control in livestock animals.

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1. Introduction

Helminth parasites of ruminants produce the greatest infectious disease problem in grazing livestock systems worldwide (Perry and Randolph, 1999). Despite promising research results, non-chemical parasite control strategies (biological control, vaccines, *etc.*) are not yet available for

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routine commercial use. Thus, parasite control in livestock still relies on the use of antiparasitic drugs, which comprise the largest sector of the animal pharmaceutical industry (Waller, 2006). The integration of available information on the host–parasite–environment relationship, with the understanding of the pharmacological properties of existing antiparasitics has contributed to more efficient parasite control. The excellent broad spectrum efficacy, good tolerability and low costs of the available anthelmintics have accounted for the extended use of chemical control in livestock animals during the past five decades. However, inappropriate use has led to therapeutic failures and to the widespread development of parasite resistance. Among others, some factors responsible for that failure are: (a) inadequate integration between management strategies and chemotherapy, (b) incorrect use of anthelmintic drugs due to insufficient knowledge of their pharmacological features, (c) insufficient understanding of the relationship between pharmacological properties, and several host-related factors that could lead to modifications on the pharmacokinetic behavior and to a decreased antiparasite efficacy of the chosen drug. The time of parasite exposure to active drug concentrations determines the efficacy and/or persistence of activity for most of the anthelmintic drugs used in ruminants. The development of highly efficient analytical techniques to quantify drug/metabolites concentrations in various host tissues and target parasites has significantly contributed to the knowledge of the pharmacokinetic and metabolic features of the available anthelmintics. The characterization of drug concentration profiles in tissues of parasite location and within target parasites provided a basis for understanding the differences in therapeutic and preventive efficacies observed for the different chemical families of anthelmintic compounds.

Integrated evaluation of the available knowledge on the pharmacological features is required to optimize anthelmintic activity and to achieve sustainable use. Anthelmintic resistance in human and animal pathogenic helminths has been spreading in prevalence and severity. Multi-drug resistance is becoming a widespread problem in farm animals. Considering the increasing concern for the development of resistance, the use of pharmacology-based information is critical to design successful future strategies. A more complete understanding of the pharmacological properties of existing and novel antiparasitic drugs should assist with more efficient parasite control. Detailed knowledge on the relationship between physicochemical properties and host tissues disposition kinetics for the most widely used broad-spectrum anthelmintics in ruminants, benzimidazoles (BZD) and macrocyclic lactones (ML) (extensively reviewed in the literature), has provided the scientific basis to generate novel research approaches on the field of drug therapy. Some complementary available pharmacological knowledge useful to achieve sustained parasite control is reviewed in the current article. The summarized data emphasizes on how the use of pharmacology-based information for existing and novel molecules may be critical to design successful strategies for the future of parasite control in livestock.

2. Pharmacology-based strategies to optimize anthelmintic efficacy

2.1. Basic pharmacokinetic principles applied to anthelmintics

The pharmacokinetics of an anthelmintic drug involves the time course of drug absorption, distribution, metabolism and elimination from the host, which, in turn, determines the concentration of the active drug reaching the parasite location. Knowledge of the processes of drug/metabolites diffusion into different target parasites, together with the available kinetic information, has been relevant to elucidate the mechanism of drug penetration and the pharmacological activity of most anthelmintics. Both *in vivo* and *ex vivo* studies have shown that transcuticular/tegumental diffusion is the predominant pathway for drug entrance into helminths (including blood-sucker parasites) (Mottier et al., 2006a). Determining the capability of different helminths to biotransform anthelmintic drugs (Solana et al., 2001; Vokřál et al., 2013) is another crucial step for the overall interpretation of their pharmacological activity.

The time of parasite exposure to active drug concentrations determines the efficacy and/or persistence of activity for most of the anthelmintics used in ruminants. The characterization of drug concentration profiles in tissues of parasite location and within target parasites provides a basis for understanding the differences in therapeutic and preventive efficacies observed for the different chemical families. To maximize the efficacy of anthelmintic compounds against parasites difficult to control in human and veterinary medicine whilst preserving an adequate margin of safety, a complete understanding of their pharmacokinetic and metabolic patterns in the host is necessary. Additionally, knowledge of the differential drug pharmacologic behavior among animal species and identification of different factors affecting drug activity is relevant for achieving optimal parasite control and avoiding selection for drug resistance.

Due to the great efforts and difficulties implicit in the development of new anthelmintic molecules, optimization of the existing compounds has been a high-priority for research in the field. The main strategies to optimize the use of existing anthelmintic drugs, as well as secure an extended use of the novel ones, to control resistant parasites are either a pharmacokinetic-based enhancement of parasite exposure or the combined use of anthelmintics with different mode of action/resistance (Fig. 1), as it is described below.

3. Pharmacokinetic optimization: enhanced drug exposure

Most fields of chemotherapy benefit from *in vitro* test systems that can be used to accurately predict drug concentrations required for efficacy *in vivo*. However, it has been difficult to develop a culture system for nematodes useful to determine *in vitro* potency for anthelmintics (Geary et al., 1999). This limitation to estimate the active drug concentration required to achieve optimal *in vivo* activity

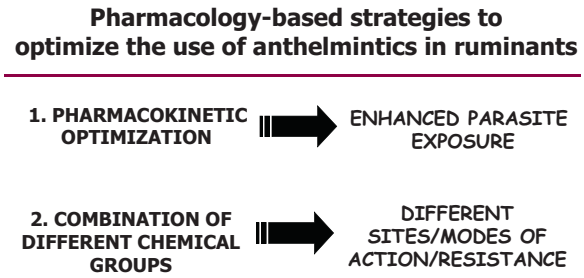

Fig. 1. Currently available pharmacological strategies to achieve a sustainable use of existing and/or novel anthelmintic compounds in parasite control. Experimental data focusing on those main topics is provided in the current review article.

has discouraged further development on the field. However, the progress made on the comprehension of the kinetic behavior and pharmacodynamic mechanisms of drug action/resistance, has been sufficient to achieve a deep understanding on the pharmacology of the main chemical families.

Increasing drug bioavailability is a well established pharmacological tool that may cooperate to delay the development of anthelmintic resistance. An enhancement on drug systemic availability may account for higher drug concentrations reaching the parasite location for sufficient time to improve the antiparasitic effect, particularly against worms carrying resistance genes. The progressive reduction on the efficacy of BZD compounds due to the development of nematode resistance, particularly in sheep and goats, has stimulated the search for strategies to increase drug efficacy. Compromised drug availability in the bloodstream and target tissues, resulting in a sub-therapeutic exposure of those individuals carrying resistant alleles, may facilitate the progressive development of resistance. As mentioned above, several host-related factors may affect the kinetics and resultant efficacy of anthelmintics in livestock. Manipulation of the pharmacokinetic/metabolic patterns and the comprehension of factors modulating them have been considered as useful alternatives to improve the use of different anthelmintic molecules in ruminants (Lanusse, 2003). Some novel pharmaco-parasitological findings with practical significance on parasite control are described here.

3.1. Oral versus parenteral anthelmintics against resistant nematodes

The potency of most anthelmintics is not only dependent on their affinity for a specific receptor (site of action) but also on the kinetic properties that facilitate achievement of effective drug concentrations at the site of action. A remarkable amount of work on the kinetic behavior of the most widely used broad-spectrum anthelmintics in ruminants is now available. The complex connections among route of administration, formulation, drug physicochemical properties and the resultant kinetic behavior need to be understood to optimize drug efficacy. The macrocyclic lactones (MLs) are broad-spectrum antiparasitic drugs widely used to control endo and ecto-parasites. After many years of extensive overuse, resistance to ivermectin and to other related MLs, is widespread in nematodes from small ruminants and it is becoming a serious concern in cattle nematodes (Kaplan, 2004; Demeler et al., 2009). The appearance of ML resistant nematode strains introduced a new scenario, which stimulated research to determine the *in vivo* concentrations required to kill the different nematode species at the gastrointestinal tract.

The choice of the administration route for MLs in ruminants is based on either management practice reasons or influenced by the technical marketing of the pharmaceutical companies. In the early days, shortly after ivermectin introduction into the market, nematode susceptibility was high and equivalent efficacy patterns were observed against abomasal parasites after parenteral and oral treatments in sheep/goats. A similar pattern was later on described for other ML from both the avermectin (abamectin) and milbemycin (moxidectin) families. A slightly improved ivermectin efficacy against sheep intestinal nematodes was observed after its oral administration compared to the parenteral treatment (Borgsteede, 1993). However when the efficacy was assessed against ivermectin-resistant nematodes, a significant greater pharmacological activity was observed after the oral administration of both abamectin and moxidectin, compared to their subcutaneous (SC) injections in lambs (Gopal et al., 2001; Alka et al., 2004). The highest efficacy against resistant *Trichostrongylus colubriformis* in sheep was obtained after the oral administration of abamectin and moxidectin. On the other hand, an equivalent efficacy against a susceptible strain of *T. colubriformis* was obtained after ivermectin administration by both routes in goats (Lespine et al., 2005). Thus, the pharmacological basis underlying the observed differential efficacy patterns after the MLs administration by both routes was recently investigated in our laboratory (Lloberas et al., 2012). The simultaneous measurement of drug concentrations in the bloodstream, in the gastrointestinal tissues containing parasites, and within resistant target worms (*Haemonchus contortus*), was performed in infected lambs. Increased ivermectin plasma concentrations were obtained after the SC treatment compared to the oral administration, which accounts for the improved efficacy against ectoparasites observed after parenteral treatment. Additionally, the described longer mean residence time and elimination half-life observed for ivermectin after its SC administration account for the persistent antiparasitic activity (over 10 days) against *H. contortus* in sheep (Borgsteede, 1993). It is also likely that the concentrations of the dissolved drug attained at the lumen of the gastrointestinal tract may be critical to the pharmacological activity against worms living in the abomasum and small intestine, particularly if they have a reduced susceptibility to the drug. The anthelmintic action depends on the ability of the active drug to reach its specific receptor within the target parasite. Thus, drug entry and accumulation into target helminths are important issues when considering the achievement of optimal clinical efficacy (Alvarez et al., 2007). It seems that the transcuticular diffusion is the main route of access for different substances in nematodes and the drug lipophilicity is the major determinant of the rate of transfer across the nematode cuticle (Thompson et al., 1993; Mottier et al.,

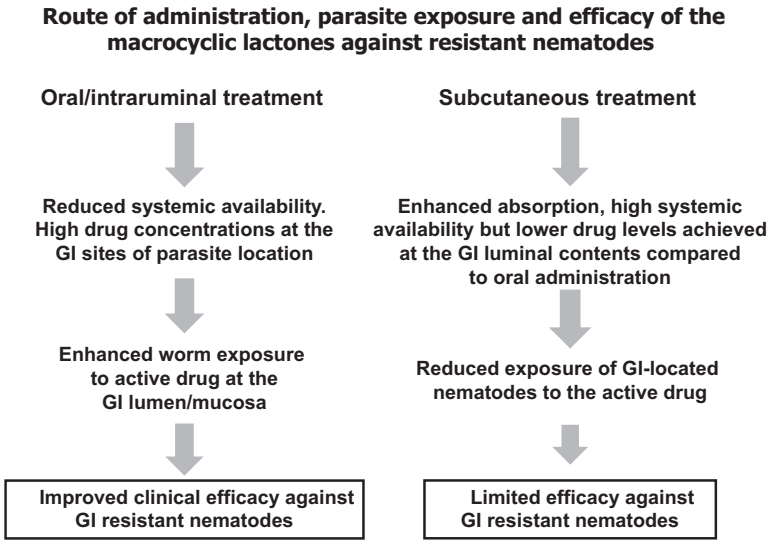

Fig. 2. Influence of the route of drug administration on the pharmacokinetics and efficacy of the macrocyclic lactones against gastrointestinal nematodes of reduced susceptibility.

2006a,b). Lipophilic drugs such as ivermectin, may reach the target parasite from the gastrointestinal contents (transcuticular route) or from plasma (oral ingestion) if the nematodes (*H. contortus*) feed on host blood. Markedly lower ivermectin concentrations were recovered in the abomasal contents after the SC injection of the drug in infected lambs (Lloberas et al., 2012). Consistently, early studies with ivermectin showed that its SC administration at ten (10) times (2 mg/kg) the therapeutic dose to sheep resulted in very low concentrations at the abomasal content (Bogan and McKellar, 1988). This phenomenon may indicate that the ivermectin concentrations achieved in the adult *H. contortus* after the parenteral treatment may be mainly determined by the drug coming from the bloodstream. Significantly higher ivermectin concentration profiles in the abomasal content were measured after the intraruminal (IR) treatment compared to SC injection in sheep (Lloberas et al., 2012). The higher concentrations measured in the abomasal content after the IR (equivalent to the oral) administration of ivermectin, accounted for the greater amount of drug measured within adult *H. contortus* recovered from treated sheep. Those enhanced ivermectin concentrations explain the lower number of adult *H. contortus* specimens recovered after treatment and the enhanced efficacy obtained after the IR treatment in spite of the observed high level of resistance. Interestingly, a recent trial in cattle also confirmed those findings (Leathwick and Miller, 2013). Few comparative efficacy studies have included oral administration of MLs in cattle. The efficacy of moxidectin was evaluated after its oral, SC and pour-on administrations. Whereas a high moxidectin efficacy against *Ostertagia* spp. was observed by all the administration routes, the activity of the drug against *Cooperia oncophora* was significantly higher after the oral treatment. The overall moxidectin efficacy measured as the reduction in the numbers of eggs in feces (FECRT) was 91.1 (oral) and 55.5% (SC treatment) in cattle (Leathwick and Miller, 2013). Altogether, these findings show that the administration of MLs by the SC injection may achieve lower efficacy against nematodes located at the gastrointestinal tract compared to the oral treatment. These differences on drug efficacy attributed to the administration route may only be evidenced if the parasite population has a reduced susceptibility. The improved efficacy obtained in sheep and cattle after the oral administration of the MLs may be based on the enhanced drug exposure of the worms located at the lumen of the abomasums and/or small intestine. As it can be appreciated in Fig. 2, the route of drug administration may have a marked impact on drug response, which is based on basic pharmacokinetic principles accounting for enhanced parasite exposure to the drug. This type of finding may have direct impact on the practical use of the MLs in ruminant species accounting for marked improvement in the control of ML-resistant nematodes in the field, at least in the early stages of resistance development.

3.2. Modulation of anthelmintic drug excretion. Drug transporters in host and target parasite tissues

The influence of cell transporter systems in the pharmacokinetic behavior of different drug compounds is considered as a new paradigm within the pharmacology of the MLs. Among all the identified cell transporters, p-glycoprotein (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 identified in several healthy tissues and particularly in organs actively involved on drug pharmacokinetics (Schinkel, 1997). P-gp is located in different tissues implicated in the processes of drug absorption (*i.e.* small/large intestine mucosa), tissue distribution (*i.e.* brain-blood barrier, enterocytes) and excretion

(luminal surface of hepatocytes and ducts cells, kidney tubules and enterocytes) (Lin, 2003).

The interaction of MLs with different P-gp has been well demonstrated (Lespine et al., 2007) and different aspects of that interaction have been recently reviewed in the literature (Lespine et al., 2012; Lifschitz et al., 2012). Considering the wide use of the MLs in different animal species, it is likely that some kind of drug–drug interactions may occur after their co-administration with a large variety of drug compounds.

The interaction between drug compounds at the transport proteins level is now considered as a key pharmacological issue with a variety of potential therapeutic implications. For instance, the induction of P-gp activity at the intestinal level may lead to a decreased absorption and systemic availability of orally administered P-gp substrates, while enhanced drug bioavailability may be observed when an inhibitor is co-administered with a P-gp substrate. A number of different *in vitro* and *ex vivo* methods have been reported to characterize the interactions between MLs and cell transporters. The everted sac technique was corroborated as a useful system for studying the P-gp-mediated efflux of extremely lipophilic molecules such as the MLs. The ivermectin accumulation rate at the intestinal wall was significantly higher after its incubation with the P-gp inhibitors itraconazole and PSC833 than that obtained after its incubation alone (Ballent et al., 2006). The strong ivermectin interaction with sheep intestinal P-gp was demonstrated with an Ussing chambers system (Ballent et al., 2012). Whereas different avermectin compounds increased the intracellular Rhodamine-123 (a P-gp subtrate) accumulation with a similar potency, moxidectin appears to have a different P-gp efflux potential, with a half-maximal inhibitory effect (IC_{50}) approximately 10 times higher than that reported for ivermectin (Griffin et al., 2005; Lespine et al., 2007). This reduced moxidectin P-gp-mediated efflux compared to that observed for ivermectin, may account for its preserved clinical efficacy against nematode resistant to ivermectin in the field.

There is an increasing interest on the latest discovered cell transporter: the breast cancer resistant protein (BCRP) (Allen and Schinkel, 2002) initially isolated from a breast cancer cell line. 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 (Fetsch et al., 2006). The interaction of the MLs with BCRP has been investigated (Muenster et al., 2008; Pérez et al., 2009). However, there is a long way to go to understand the impact of this drug-transporter interaction both in the pattern of tissue distribution and milk excretion of the different ML compounds.

In vivo trials performed on different animal species provided 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 were co-administered with P-gp modulating agents. The effect of verapamil (a P-gp modulator) on ivermectin plasma disposition kinetics after pour-on treatment in rats (Alvinerie et al., 1999) and after oral administration to sheep (Molento et al., 2004) has been evaluated. Significantly higher moxidectin plasma concentrations were observed when moxidectin was co-administered with loperamide (an opiod derivative acting as P-gp substrate), compared to plasma concentrations measured after giving moxidectin alone (Lifschitz et al., 2002). In lambs quercetin (Dupuy et al., 2003), itraconazole (Ballent et al., 2007) and ketoconazole (Alvinerie et al., 2008) produced a significant increase on moxidectin and/or ivermectin systemic exposure.

Combined formulations including MLs with other different antiparasitic drugs are commercially available in countries such as Australia, New Zealand, Uruguay, *etc.*, to be used in livestock and companion animals. However, potential interaction with transport proteins should be evaluated before new combined antiparasitic preparations are developed. Despite the fact that *in vitro* studies indicated that closantel modulated the P-gp transport in cell lines (Dupuy et al., 2010), a similar plasma disposition was observed after the co-administration of ivermectin-closantel compared to that described after the treatment with each anthelmintic compound alone in cattle (Cromie et al., 2006). Recent work in our lab investigated the potential pharmacokinetic interaction between closantel and moxidectin in sheep (Suarez et al., unpublished observations). Any adverse pharmacokinetic interaction was observed between both compounds administered by either the SC or the oral route.

The basis of the pharmacological interaction between ivermectin and triclabendazole, a worldwide (except USA) available anthelmintic combined formulation, has been assessed *in vitro* and *in vivo*. The ability of triclabendazole and its metabolite triclabendazole sulphoxide to interfere with P-gp transport in cell lines over-expressing P-gp was shown (Dupuy et al., 2010). Further work demonstrated that triclabendazole enhances the ivermectin intestinal accumulation after their co-incubation with everted gut sacs (Lifschitz et al., 2009). The *in vivo* co-administration of ivermectin and triclabendazole in sheep resulted in a significant change on the kinetic disposition of both molecules. The ivermectin systemic availability was 3.13-fold higher in the presence of triclabendazole. Additionally, the co-administration of triclabendazole with ivermectin resulted in higher triclabendazole sulphoxide plasma concentration profiles (Lifschitz et al., 2009). Based on this evidence, triclabendazole and its metabolite would play a role in the modulation of the intestinal or biliary cell-transporter mediated elimination of ivermectin in sheep. Although ivermectin has no activity on *Fasciola hepatica* an interesting effect was observed after its co-incubation with triclabendazole (Mottier et al., 2006b). The *in vitro* influx/efflux balance for triclabendazole and triclabendazole sulphoxide in susceptible and resistant flukes in the presence/absence of ivermectin as substrate of the drug transporter P-gp, was assessed. The ivermectin -induced modulation of P-gp activity decreased triclabendazole efflux from the resistant flukes and higher concentrations of triclabendazole and triclabendazole sulphoxide were recovered from the resistant *F. hepatica* in the presence of ivermectin (Mottier et al., 2006b). Despite the observed *in vitro* and *in vivo* interactions, the co-administration of ivermectin + triclabendazole and the metabolic inhibitor methimazole failed to reduce the number of adult resistant

F. hepatica in infected sheep (Ceballos et al., 2010). Further work to adjust the dosing regimen may be necessary to translate the observed *in vitro*/*in vivo* pharmacological interaction between triclabendazole and ivermectin into a change on the flukicidal efficacy in the infected host.

The majority of the studies on drug interactions mediated by cell transporters has been addressed to modulate/inhibit their activity, and thus, to increase the absorption or delay the elimination of therapeutically relevant drugs. However, the effect of potential inducers of the transport proteins on the kinetic behavior of MLs has not been investigated as thoroughly. Recent work reported that ivermectin can also induce P-gp expression and function through mRNA stabilization in murine hepatic cells (Ménez et al., 2012). Although numerous therapeutic agents can induce P-gp expression under *in vitro* conditions, the relevance of these observations to P-gp induction *in vivo* is not entirely clear. The effect of the inducer agent phenobarbital, on both plasma and gastrointestinal disposition of ivermectin, was examined in our laboratory (Ballent et al., 2010). The measured ivermectin area under the concentration *vs* time curve (AUC) values were significantly lower in plasma, intestine and liver tissue in rats pre-treated with phenobarbital. Additionally, it has been shown that chronic administration of dexamethasone in sheep produces a decrease in P-gp expression along the small intestine compared to the untreated control animals (Ballent et al., 2013). Thus, a better understanding of the factors regulating P-gp and other cell transporters expression is needed to elucidate the clinical implications of drug–drug interactions in pharmaco-therapy in livestock animals. More specifically, this is an open field for the future of the MLs as antiparasitic agents which must be necessarily addressed if the combination of anthelmintic molecules turns into an alternative for parasite control in resistant populations.

The expression of P-gp has been described not only in mammalian tissues but also in different helminth parasites. Modifications on the pattern of P-gp expression have been observed in resistant nematodes recovered from lambs treated with MLs (Prichard and Roulet, 2007). The up-regulation of P-gp in *H. contortus* recovered at 1 day post-administration was reported after the ivermectin treatment but also in a lesser degree, after the moxidectin administration (Prichard and Roulet, 2007). Recent work demonstrates that ivermectin treatment significantly increases P-gp2 expression in resistant *H. contortus* recovered from treated lambs at 0.5 and 1 days post-treatment compared to those parasites recovered from untreated animals (Lloberas et al., 2013). However, treatment with moxidectin did not induce any significant modification on the pattern of the drug transporter expression in the nematode (Lloberas et al., 2013). It has been clearly shown, as recently reviewed by Prichard et al. (2012), that the chemical structure differences between moxidectin and ivermectin account for their differential pharmacokinetic, pharmacodynamic and P-gp interaction patterns.

P-gp-mediated drug efflux has been proposed as a potential resistance mechanism for MLs in different helminthes (Xu et al., 1998; Kerboeuf et al., 2002). The modulation of P-gp activity has been assayed as a pharmacology-based strategy not only to increase the systemic availability of the MLs at the host animal but also to produce a drug–drug interaction at the parasite level, which would account for improved clinical efficacy. *In vitro* assays were performed to assess the impact of modulation on P-gp activity. The modulation of P-gp increased the *in vitro* activity of ivermectin against ivermectin-sensitive and resistant larvae of *T. circumcincta* and *H. contortus* (Bartley et al., 2009). Further evidence was demonstrated when the P-gp substrate verapamil increased the *in vitro* ivermectin activity against susceptible and resistant isolates of *Cooperia* spp. The *in vitro* activity of ivermectin against *Cooperia* spp. was increased between 10 and 100-fold after its co-incubation with the P-gp modulator in the larval development and larval migration inhibition tests (Demeler et al., 2013). Although a modification of 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 described P-gp modulation. In an *in vivo* trial, the presence of the P-gp modulator pluronic 85 did not improve ivermectin efficacy against resistant *H. contortus* (Bartley et al., 2012). The efficacy of both ivermectin and moxidectin against resistant *Cooperia* spp. in a cattle field trial tended to increase after their co-administration with loperamide used as a P-gp modulator (Lifschitz et al., 2010a). Similarly, a significant increase in ivermectin efficacy against resistant nematodes of sheep together with enhanced ivermectin systemic availability was obtained in the presence of loperamide (Lifschitz et al., 2010b). The interaction at the parasite tissues level was specifically investigated using *Caenorhabditis elegans* as a model system. It was corroborated that various P-gp isoforms protect *C. elegans* from ivermectin toxicity and the interaction of P-gp with different modulator agents enhances susceptibility to ivermectin depending on the drug concentration used (Ardelli and Prichard, 2013). Therefore, it is evident that a P-gp-mediated drug to drug interaction increases the MLs systemic exposure in the host. However, such type of interaction may also occur at the target worm, which would decrease the P-gp-mediated efflux transport overexpressed in target resistant nematodes (see Fig. 3).

In conclusion, drug transport modulation has deserved great attention in recent years (see extensive review articles on the topic by Lespine et al., 2008, 2012 and Lifschitz et al., 2012). Different pharmacological approaches to delay the bile/intestinal secretions and to extend the plasma-intestine recycling time of ML molecules in the host have been investigated. The involvement of the efflux-transport protein P-gp (and perhaps, other drug transporters) on both the pharmacokinetic disposition (host) and resistance mechanisms (target parasites) to different anthelmintic chemical groups has been outlined here. The potential side effects and changes to the pattern of tissue residues induced by the P-gp modulating agents should be carefully investigated (Lespine et al., 2008). However, the search of specific P-gp modulators with high affinity by parasite transport proteins may help to identify useful pharmacological tools to extend the lifespan of the MLs in veterinary medicine. Further work in the field is required to assess the practical pharmaco-parasitological implication of the

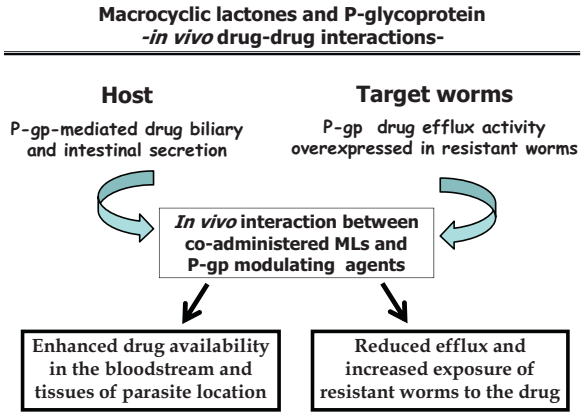

Fig. 3. Schematic representation of the *in vivo* interaction between P-glycoprotein (P-gp) and the macrocyclic lactone compounds. *In vivo* drug–drug interactions at the host and target worm tissues may account for enhanced drug efficacy.

chemical modulation of these cell efflux pump systems on antiparasitic therapy.

3.3. Assessment of the relationship between enhanced systemic exposure and anthelmintic efficacy against resistant nematodes

The biochemical basis of drug resistance includes different mechanisms: (i) insufficient intracellular drug concentration, (ii) enhanced inactivation of the active drug, (iii) decreased conversion of the drug into a more active compound, (iv) increased concentration of a metabolic product that antagonizes drug action, (v) altered availability of functional target receptors, and vi) altered receptor structure affecting drug affinity (Pratt, 1990). While the pharmacokinetic-based resistance includes mechanisms described from (i) to (iv), those in (v) and (vi) correspond to pharmacodynamic-based resistance mechanisms. For the main anthelmintic classes currently used to control nematode parasites (BZD, imidazothiazoles and MLs), decreased intracellular drug level and reduced affinity of target receptors appear to be the most common mechanisms involved in anthelmintic resistance. Mutations on the β-tubulin isotype 1 gene have been shown to encode amino-acid changes that result in a poor binding of BZD compounds (Kwa et al., 1995; Lubega and Prichard, 1990). Resistance to levamisole seems to be associated with a genetic mutation in the nicotinic Acetyl-choline receptor subunits (Boulin et al., 2011). Finally, the main mechanisms involved in nematode resistance to MLs implicate changes on multidrug ABC transporters activity (reviewed by Lespine et al., 2012) and/or changes in the glutamate-gated chloride channel (Blackhall et al., 1998; Njue and Prichard, 2004).

All the described anthelmintic resistance mechanisms could theoretically be overcome by increasing the active drug concentrations at the target receptor location commonly named "biophase". This enhanced exposure may be achieved as a result of either pharmacokinetic manipulation (above described), drug to drug pharmacokinetic interactions or merely by increasing the dosing rates. Recent work undertaken in our laboratory showed that albendazole activity against resistant nematodes in sheep highly correlates with the systemic availability of the active molecules (Alvarez et al., 2008, 2012a,b). The higher plasma availability observed following the intravenous treatment compared to the IR administration of albendazole to sheep correlated with the improved efficacy patterns obtained following the intravenous treatment (Entrocasso et al., 2008). However, a different trial dealing with a different resistant population showed that a 68% increase in the albendazole sulphoxide AUC value obtained after albendazole treatment in fasted sheep, did not improve the efficacy against a resistant *Haemonchus* spp. (Alvarez et al., 2010). Only when the albendazole sulphoxide systemic exposure (expressed as AUC) increased (treatment with a high dose) up to 18-fold (Alvarez et al., 2012a,b), the clinical efficacy against these recalcitrant *Haemonchus* spp. specimens changed from 16% up to 94% (Barrère et al., 2012). Additionally, there was a negative correlation between AUC/C_{max} values and nematode counts in treated lambs (Alvarez et al., 2012a,b). In the same way, after increasing the ivermectin dose rates administered by the IR route to infected lambs, clinical efficacies ranging from 48 (0.2 mg/kg) up to 96 (dose × 5), and 98% (dose × 10) were observed (Alvarez et al., unpublished observations). An enhanced parasite exposure to the drug at the abomasal level given by the increased dosing may explain the improved efficacy against this resistant *Haemonchus* spp. strain observed only after the ivermectin IR administration at 5 and 10-fold the therapeutic dosage. Both, the BZD resistance mechanism based on a reduced drug affinity at its target β-tubulin receptor and/or the decreased MLs intracellular concentrations due to an increased drug pumping efflux (over-expression of drug transport proteins), may be overcome by increasing the active drug concentrations at the biophase. Although doses as high as 9 (albendazole) to 10 (ivermectin) fold the therapeutic dosage were necessary to reach an acceptable efficacy level against a highly resistant *Haemonchus* spp. strain under experimental conditions, the search for kinetic-based strategies to enhance parasite exposure should not be ruled out to extend the lifespan of the existing and novel anthelmintic drugs. It is evident that a huge increment on systemic drug exposure must be achieved to obtain a significant improvement on drug efficacy against highly resistant nematodes, particularly when the homozygous resistance genotypes are predominant within the resistant populations. However, pharmacokinetic-based optimization of parasite exposure may always have a beneficial impact in the early stages of drug resistance development.

Sufficiently high anthelmintic concentrations can surmount the lower receptor affinity and/or the decreased intracellular drug level. However other issues related to how the active drug reaches the adult *H. contortus* specimens with different abomasal location, may also contribute to the enhanced efficacy observed when the parasite exposure is optimized. The work reported by Alvarez et al. (2011) measured the accumulation of albendazole and its active albendazole sulphoxide metabolite, in *H. contortus* recovered from two different locations within the abomasum: worms attached to the abomasal mucosa and

worms unattached ("free") recovered from the luminal abomasal contents. The obtained data on drug accumulation within the target *H. contortus* was complemented with the assessment of albendazole/metabolites concentration profiles in plasma, abomasal fluid and mucosal tissue collected from the same infected lambs treated with different albendazole doses. Albendazole concentration profiles in unattached worms were between 185% (albendazole at 5 mg/kg) and 66% (albendazole at 15 mg/kg) higher than those measured in worms attached to the mucosal tissue. This indicates that a different partitioning process between worm/fluid compared to that obtained for the worm/mucosa may have facilitated the greater drug accumulation observed in unattached worms collected from the abomasal fluid. The observed different pattern of drug exposure according to worm location within the abomasum may have marked influence on the survival of resistant parasites after albendazole treatment. Due to the difficulties that underlie the development of new anthelmintic molecules, optimization of existing compounds should still be a high priority for research in the field. Overall, it is well established that increased drug bioavailability is a pharmacological tool that may cooperate with treatment optimization, delaying the development of anthelmintic resistance. The findings summarized here contribute to that direction revealing the need for further knowledge in the specific field, which may or may not reach a relevant impact for the available anthelmintics to which a high degree of resistance is widespread. However, these integrated pharmaco-parasitological research approaches are critical to extend the lifespan of the novel anthelmintic compounds recently introduced into the market.

4. Drug combinations: a pharmacology overview

Mixtures of drugs from different chemical families have been proposed as a valid strategy to delay the development of anthelmintic resistance (Anderson et al., 1988), and several combined preparations are now available in different countries such as Uruguay, Australia and New Zealand. Considering the growing widespread use of the combined anthelmintic therapy in some countries to deal with resistance, current research efforts should be made to understand the advantages/disadvantages of the use of drug mixtures in parasite control. The rationale behind using drug combinations is based on the fact that individual worms may have a lower degree of resistance to a multiple component formulation (each chemical with different mode of action/resistance) compared to that observed when a single anthelmintic molecule is used. However, the limited available information is unclear on the potential additive or synergistic effects occurring after co-administration of two (or more) drugs with different modes of action.

Nematode infection control in livestock has been largely based on the over-use of broad spectrum antiparasitic drugs. Thus, the anthelmintic resistance of sheep, goats, horses and cattle nematodes is an increasing economic problem worldwide (Kaplan, 2004; Wolstenholme et al., 2004; El-Abdellati et al., 2010). Several pharmaceutical formulations combining either two or three chemical entities have been developed and are available in the veterinary pharmaceutical market of important sheep producing countries such as Australia, New Zealand and Uruguay. Some broad spectrum antiparasitic combinations containing albendazole, ivermectin and levamisole (Triton®, Merial) or oxfendazole, abamectin and levamisole (Matrix®, Ancare) have been initially introduced into the Australian and/or New Zealand markets. A multi-combination drench for sheep, which combines albendazole, levamisole, closantel, and abamectin (Q-Drench®, Jurox) is approved for use in sheep in Australia. There will be always a low probability to find individual worms resistant to multiple drug components (each one with different mechanism of action), which may justify the use of combined formulations. In an ideal situation, if an anthelmintic treatment reaches 100% of efficacy, selection of anthelmintic resistance will never occur. To achieve the highest efficacy in treated animals while the few surviving parasites are diluted into a susceptible untreated nematode population, is a key principle for slowing the emergence of anthelmintic resistance in a real field situation (Dobson et al., 2001). Consequently, in farms where multiple-resistant nematode populations are present, the use of drug combinations may be a pharmacological tool to improve its chemical control. Anthelmintic combinations can be used for delaying anthelmintic resistance, for specific targeting of dose-limiting species and for managing existing resistance (Geary et al., 2012; Bartram et al., 2012). It has been reported that after the use of either a triple (levamisole + albendazole + ivermectin) combined treatment or ivermectin alone, a similar nematode control was observed (Suarez et al., unpublished observations). Thus, the use of drug combinations may not always be effective at controlling resistant parasites. Clearly under a number of field situations, the use of anthelmintic combinations in sheep production (where anthelmintic resistance is common) may have limited sustainability. However, in cattle production systems where individual molecules still maintain their highest efficacy, the combined use of anthelmintics may be an important tool to delay resistance.

Currently, combinations of two or more anthelmintics are primarily being used to manage anthelmintic resistance in ruminants (Geary et al., 2012). However, the occurrence of potential pharmacokinetic and/or pharmacodynamic interactions between drug components highlight the need of deeper pharmacological-based research to identify the advantages/disadvantages of the use of combined drug preparations for anthelmintic control in livestock. A potential drug interaction refers to the possibility that one drug may alter the intensity of the pharmacological effects of another drug given concurrently (Nies and Spielberg, 1996). The modified effect may result from a change on the concentration of either one or both drugs in the organism (pharmacokinetic interaction) or from a change in the relationship between drug concentration and response of the organism to the drug (pharmacodynamic interaction). The pharmaco-parasitological consequences derived from pharmacodynamic and/or pharmacokinetic drug to drug interactions after the combined use of nematodicidal compounds are briefly outlined here.

4.1. Pharmacodynamic interactions

Pharmacodynamic drug–drug interactions may occur at the receptor or effector levels. Assessing a pharmacodynamic interaction between combined drugs requires a valid quantitative measurement of a specific drug effect for the two (or more) drugs individually, as well as a quantitative measurement of the effect of the drugs administered as a combination. There are four possible types of pharmacodynamic interactions resulting on either additive, potentiating (synergistic or supra-additive), antagonism (infra-additive) or indifferent effects (Bourgeois, 2005). An additive effect arises when the combined effect of two drugs equals the sum of their independent activities measured separately. On the other hand, a potentiating effect is achieved when the combined effects of the drugs are significantly greater than the sum of the independent effects (Prescott, 2000). A potentiating effect obtained after the co-administration of two drugs with different mode of action would be an ideal situation to deal against resistant parasites, particularly in cases of simultaneous multiple resistance, characterized by the presence of one or more worm genera resistant to all the molecules included in the combination. Since levamisole, albendazole and ivermectin differ in their intrinsic anthelmintic mode of action, their co-administration may potentially induce a synergistic effect. Evidence of synergist action between the BZD compound fenbendazole and levamisole has been described (Miller and Craig, 1996). These authors reported a 62% of fecal egg count reduction after the combination of fenbendazole + levamisole compared to 1% and 23% of reduction when fenbendazole and levamisole were administered alone to goats naturally infected with *H. contortus.* Similar results were reported in sheep, where mebendazole and levamisole acted synergistically on *H. contortus* infection (Bennet et al., 1980). Similarly, a synergistic effect after the combined administration of pyrantel and febantel against *Heterakis spumosa* developed in mice has been observed (Mehlhorn and Harder, 1997). Furthermore, a synergistic interaction between the novel anthelmintic derquantel and abamectin appears to occur under *in vitro* laboratory conditions (Puttachary et al., 2013). However, most cases of pharmacodynamic interactions between nematodicidal drugs appear to be limited to an additive effect. When multiple resistance refers to the presence of different worm genera, each one resistant to one different anthelmintic chemical family, a greater anthelmintic efficacy could be obtained by using the combination compared to that achieved after the use of each component alone, since worms surviving one molecule could be eliminated by the other. In this situation, an additive effect is apparently involved in the increased efficacy observed for the combination of albendazole sulphoxide and levamisole against BZD-resistant nematodes (Anderson et al., 1991a,b). However, recent field work undertaken in Uruguay (Suarez et al., unpublished observations) showed an equivalent efficacy against multiple resistant *H. contortus* following the use of either a triple combined treatment (levamisole + albendazole + ivermectin) or ivermectin alone, indicating no advantageous effect of the triple combined preparation. The observed anthelmintic efficacy was 87% (combined treatment), 80% (ivermectin treatment), 72% (albendazole treatment) and 52% (levamisole treatment), showing that the combined treatment did not result in a clinically significant enhanced anthelmintic effect compared to ivermectin alone. Furthermore, it has been reported that in the presence of a *H. contortus* strain with simultaneous albendazole and ivermectin resistance ("bi-resistant" worms), the co-administration of both compounds did not result in a clinically significant enhanced anthelmintic effect, with the disadvantage of an increased resistance selection pressure over the parasite population (Entrocasso et al., 2008). In conclusion, drug combination use requires previous diagnosis of the resistance status and it should be avoided once resistance to all the combined molecules is present, since it may have limited success reversing the resistance status. Pharmacodynamic interactions occurring after the use of a combined formulation may depend in the resistance status of the nematode population, the molecules included in the combined product, the parasite prevalence related to seasonal and/or weather conditions, *etc.* Thus, evaluation of potential anthelminitc dynamic interactions in different farms faces many practical difficulties, and it may complicate the beneficial contribution emerging from drug combination uses.

4.2. Pharmacokinetic interactions

A pharmacokinetic interaction occurs when a drug compound modifies the absorption, distribution, biotransformation and/or elimination of other drug. As a consequence, the drug concentration at the biophase (the site of action) may be either increased or decreased. There is a strong relationship between drug concentration in the bloodstream and those measured in fluids/tissues of parasite location as well as in target parasites both for BZD (Alvarez et al., 1999, 2000, 2011) and ML anthelmintics (Lloberas et al., 2012, 2013). Since the higher the concentration achieved at the tissue where the parasite is located, the higher the amount reaching the target parasite receptor, pharmacokinetic interactions may determine a modified anthelmintic efficacy. Upon these circumstances, different pharmacokinetic interactions among nematodicidal drugs after its combined use in lambs have been reported (see Table 1). Alvarez et al. (2008) reported a significantly higher ivermectin plasma concentration measured after its co-administration with albendazole (both administered by the IV route) and a higher albendazole sulphoxide plasma availability obtained after the IR administration of albendazole co-administered with ivermectin given by the SC route. Although it is unclear at which level albendazole/metabolites and ivermectin may interact, two possible explanations may be based on an ivermectin-induced inhibition of albendazole metabolism and/or a drug–drug interaction *via* drug efflux transporters-mediated mechanisms (Alvarez et al., 2008). Both albendazole sulphoxide (Merino et al., 2003) and ivermectin (Didier and Loor, 1996; Pouliot et al., 1997) have been described as P-gp substrates. Thus, a drug–drug interaction at this level may help to explain the observed pharmacokinetic interaction between albendazole and ivermectin. Furthermore, increased ivermectin plasma concentrations were observed after the IR

Table 1
Assessment of pharmacokinetic drug-drug interactions occurring after the administration of albendazole (ABZ), ivermectin (IVM), levamisole (LEV) and triclabendazole (TCBZ) administered either alone or under different combined preparations to sheep. The impact of the different routes of administration on the systemic exposure is compared.

Anthelmintic combination	Assayed molecule	Route of administration	Systemic exposure expressed as AUC (μg ng h d/mL)		Pharmacokinetic drug–drug interaction
			Drug alone treatment	Combined treatment	
ABZ + IVM[1]	ABZ	IV	30.2 ± 5.31	33.9 ± 6.65	No interaction
	IVM	IV	112.3 ± 37.4	210.3 ± 80.6*	Positive interaction
ABZ + IVM[1]	ABZ	IR	19.8 ± 2.55	28.2 ± 3.72*	Positive interaction
	IVM	SC	131.1 ± 70.5	139.7 ± 28.6	No interaction
LEV + ABZ + IVM[2]	LEV	Oral	8.63 ± 5.22	10.5 ± 5.73	No interaction
	ABZ	Oral	30.7 ± 9.01	19.4 ± 7.90*	Negative interaction
	IVM	Oral	30.9 ± 11.6	51.6 ± 16.2*	Positive interaction
TCBZ + IVM[3]	TCBZ	IV	297 ± 74.3	319 ± 70.2	No interaction
	IVM	IV	14.4 ± 5.83	48.5 ± 46.6*	Positive interaction
TCBZ + IVM[4]	TCBZ	IR	654 ± 141	651 ± 123	No interaction
	IVM	SC	Not determined	Not determined	–

Data from [1]Alvarez et al. (2008); [2]Suarez et al. (unpublished); [3]Lifschitz et al. (2009); [4]Ceballos et al. (2010). IV, intravenous; IR, intraruminal; SC, subcutaneous. AUC: area under the plasma concentration *vs* time curve.

* Differences between the alone and combined treatments are statistically significant ($P < 0.05$). ABZ and TCBZ AUC values are refereed to their sulphoxide metabolites, ABZ-sulphoxide and TCBZ-sulphoxide, respectively.

administration of a levamisole + albendazole + ivermectin combined formulation in lambs (Suarez et al., unpublished observations), leading to an increase of 71% (AUC) and 58% (C_{max}) compared to the ivermectin alone treatment. In contrast to that described by Alvarez et al. (2008), after the triple levamisole + albendazole + ivermectin combination, albendazole sulphoxide systemic exposure (estimated as C_{max} and AUC values) was significantly reduced compared to that achieved after the albendazole alone administration. An ivermectin inhibition/competition in albendazole/albendazole sulphoxide transporter mediated-enteral absorption could account for the altered absorption pattern; however, the real contribution of such a mechanism is unknown. The lower ivermectin concentrations achieved at the gastrointestinal tract after the SC route compared to the oral/IR administration (see Fig. 2) (Lloberas et al., 2012) would determine a different relevance of the drug–drug interaction. It seems clear that the extension of the pharmacokinetic interaction depends on the route of administration of the drug compounds under assessment. Recently, the potential pharmacokinetic interaction between closantel and moxidectin in sheep has been assessed after their subcutaneous and oral administration (Suarez et al., unpublished observations). While the co-administration closantel + moxidectin in nematode infected lambs did not show any adverse pharmacokinetic interaction, their combined anthelmintic effect was sufficient to restore the maximum nematodicidal efficacy, which was not reached by the individual active ingredients. In fact, the administration of both molecules as a single active principle reached efficacy levels (estimated using the FECRT) ranging from 80% (oral moxidectin), 84% (oral closantel) up to 85% (SC closantel) and 92% (SC moxidectin), while the combined treatments given both orally and subcutaneously reached a 100% efficacy.

As a common feature, a drug to drug pharmacokinetic interaction can be observed after the co-administration of two or more anthelmintics, as it was previously described after the combined administration of albendazole + ivermectin (Alvarez et al., 2008), rafoxanide + ivermectin (El-Banna et al., 2008), levamisole + albendazole + ivermectin (Suarez et al., unpublished observations), However, there are some reports on the absence of pharmacokinetic interactions between anthelmintics used as combinations. For instance, no differences on plasma pharmacokinetic parameters were observed for ivermectin and closantel, administered by SC injection each alone or as a combined formulation to cattle (Cromie et al., 2006). This result indicates that neither the absorption nor the distribution of ivermectin or closantel were influenced by the presence of the other molecule. Interestingly, after the IV administration of ivermectin and triclabendazole given either separately or co-administered to sheep, ivermectin elimination was delayed and its plasma availability was 3-fold higher when co-administered with triclabendazole (Lifschitz et al., 2009). Once again, it seems clear that the occurrence of a pharmacokinetic interaction depends on the route of administration of the drugs used in the combination (see Table 1).

Some considerations for pour-on anthelmintic formulations intended for transdermal absorption of the actives included in the preparation may also be useful. MLs are commonly used as pour-on formulations in food animal practice to treat different endo- and ectoparasites. Furthermore, the combination of ivermectin + triclabendazole and abamectin + triclabendazole are currently registered in some countries for the treatment and control of ivermectin-sensitive gastrointestinal roundworms, lungworm, adult liver fluke and lice in beef cattle and dairy calves. There is a clear evidence that the natural grooming behavior of cattle influences the absorption and kinetic disposition of transdermally administered MLs (Sallovitz et al., 2005; Toutain et al., 2012), accounting for a highly

variable kinetic behavior. Additionally, drug–drug interactions between molecules included in a combined pour-on formulation (mainly at the absorption level) may increase the variability in plasma drug exposure observed after the use of topical formulations, which may influence their antiparasitic efficacy. Overall, animal licking behavior and the well described erratic percutaneous absorption pattern may drastically play against the successful use of combination of anthelmintic compounds topically administered to cattle.

The assessment of either the positive or negative (adverse) impact of pharmacokinetic interactions occurring between combined anthelmintic molecules needs to be elucidated before these drug mixtures are introduced into the market (see Fig. 4).

4.3. Drug combinations: impact on drug safety and withdrawal times

Changes (increase/decrease) on drug/metabolites concentrations in the bloodstream and/or tissues arising from drug to drug interactions may reach an order of magnitude (or even more) in comparison to concentrations measured when each drug was administered alone. As stated above, some beneficial impact on drug anthelmintic efficacy may arise from the pharmacokinetic interaction between combined molecules, particularly when enhanced systemic exposure is achieved. However, large positive changes on drug exposure can also alter the safety profile and the pattern of tissue residues depletion of a drug and/or its metabolites. Furthermore, even when the resulting plasma availability diminishes as a result of an interaction, it is likely that a modified distribution pattern may account for greater accumulation of the drug in tissues such as the liver. Once again, combination safety and/or the residue profiles of each drug compound in animal tissues intended for human consumption may be potentially altered. However, rarely the degree of interaction caused by a drug, or the degree to which other drugs alter its metabolism, can be such that it cannot be marketed safely. Further information on this issue may be obtained from FDA/CVM, 2006 (http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM052363.pdf). The magnitude of the increase of drug profiles in the bloodstream resulting from a drug–drug interaction does not seems to be sufficiently high to produce a toxic effect in the host, in comparison to the drug administered alone. Such statement can be affirmed for safe compounds such as the BZD and ML anthelmintics. These compounds have a large therapeutic index in the mammalian host, which would avoid toxic effects related to an increase in plasma drug exposure due to a pharmacokinetic interaction. In fact, after a huge increase on the plasma concentration of either albendazole sulphoxide (Alvarez et al., 2012a,b) or ivermectin (Alvarez et al., unpublished observations), related to dosing increments of 9 to 10-fold the therapeutic dose, no adverse events were observed in treated lambs. Additionally, the oral administration of oxfendazole at doses as high as 30, 90 and 150 mg/kg daily for three consecutive days did not significantly modify the health status of treated pigs (Alvarez et al., 2012b). However, levamisole has a narrow safety margin, particularly after its parenteral administration, and any potential enhancement on its systemic availability must be carefully evaluated. Fortunately, the presence of albendazole sulphoxide and/or ivermectin did not affect the plasma disposition kinetics of levamisole after administration of a triple combination to lambs (Suarez et al., unpublished observations). Additionally, neither albendazole nor ivermectin altered the plasma concentration of levamisole after its combined use (oral administration) in humans (Awadzi et al., 2004). Taken together, these data support the low toxicity risk of the most widely used triple anthelmintic preparation in sheep, which combines levamisole, albendazole and ivermectin. However, it should be noted that the therapeutic index in mammalian host may be species specific and safety information on drug combinations obtained in sheep may not be applicable to other animal species.

Data from target animal safety studies are required for registration of veterinary products, in order to provide information on the safety of a drug in the intended species under the proposed conditions of use (VICH, 2008). The target animal safety studies are obligatory in the approval of pharmaceutical products, and are generally required for new salts or formulations of a previously registered pharmaceutical product. Thus, and even for drugs with wide safety margins, anthelmintic combinations must be considered as new formulations. Consequently, if positive or negative pharmacokinetic interactions are observed, target animal safety studies should be performed. This implies a “paradoxical” situation. For most of the available anthelmintic combinations, even when a pharmacokinetic interaction may occur, the potential harmful effects on target animal species are very unlikely. However, if the pharmacokinetic interaction truly exists, the impact on animal safety needs to be confirmed by appropriate studies (see Fig. 4).

Another important concern regarding the use of combination formulations is related.to the impact of potential drug to drug kinetic interactions on the withdrawal times usually estimated for individual drug molecules. Most of the drugs used in helminth parasite control follow a first order kinetic disposition. In the first order kinetics, the change in concentration over a time interval is proportional to the available drug concentration (Gibaldi and Perrier, 1982). For example, the higher the plasma concentrations the greater is the amount of drug excreted per unit of time. Thus, an increase in systemic drug exposure due to a pharmacokinetic interaction between drugs included in the combined preparation may have a low impact on the pattern of tissue residues depletion. However, two pharmaceutical products (drug administered alone or in combination) may show the same plasma disposition profile but their tissue disposition kinetics may greatly differ (FDA, 2006). This could be particularly true when drug systemic clearance is affected by the drug to drug interaction, modifying the pattern of tissue residues depletion.

The involvement of the drug transport protein BCRP in the milk secretion of different drugs used in veterinary and human medicine has been recently studied. BCRP facilitates the excretion of xenobiotics into the milk of

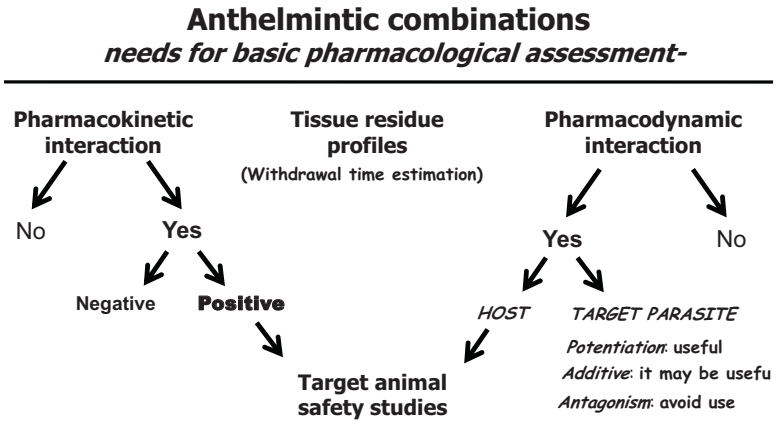

Fig. 4. Required pharmacological evaluation for combined anthelmintic preparations. Identification of either pharmacokinetic or pharmacodynamic drug to drug interactions is needed. The assessment of tissue residue profiles and withdrawal times is required for combined drug preparations. Animal safety data may be required when a potential interaction between combined drugs occurs (see explanation in the text).

mice and sheep (Merino et al., 2006). The interaction of MLs with BCRP has been described. Ivermectin inhibited BCRP-mediated albendazole sulphoxide transport in culture cells (Muenster et al., 2008). The interaction between moxidectin and BCRP was corroborated using cellular transport assays and pharmacokinetic studies in BCRP1 (−/−) and wild type mice (Pérez et al., 2009). In this study, moxidectin was identified as a BCRP substrate and its milk BCRP-mediated secretion was demonstrated. The involvement of cell transporters in the milk secretion of MLs, and perhaps other antiparasitic compounds, may play a relevant role in the clinical use of these compounds in lactating animals due to the presence of residues in milk and its derived subproducts. The interaction between BCRP and different anthelmintic drugs is a particularly relevant issue considering the use of combined formulation, where milk excretion and tissue residue patterns may be altered by changes/competition at the BCRP transport activity.

Nevertheless, further understanding of potential modifications on the tissue residue profiles and on the required withdrawal times is needed before drug combined formulations are introduced into the pharmaceutical market. It seems reasonable to suggest that the withdrawal time of a combined product should be specifically estimated if a pharmacokinetic interaction between drug components has been identified. If this is the case, the tissue residue depletion study and the withdrawal time of the combined products need to be determined. This tissue residue study should at least include the drug molecule in the combination with the longest withdrawal time and/or the drug that more significantly increase/decrease its plasma concentration profiles due to the interaction phenomenon. For instance, in the triple levamisole/albendazole/ivermectin combination developed to be orally administered to sheep, the drugs with the longest meat withdrawal times (when they are administered alone by the oral route) are albendazole (7–14 days) and ivermectin (11–14 days). As discussed above, we have recently shown that a pharmacokinetic interaction occurs after the oral administration of this triple anthelmintic combination to sheep (Suarez et al., unpublished observations). This interaction enhances ivermectin and decreases albendazole sulphoxide plama profiles (systemic exposure). The decreased plasma concentrations of albendazole sulphoxide could be associated with an increased liver accumulation of albendazole/metabolites. Since the liver is the target tissue for albendazole/metabolites, human food safety studies may need to be undertaken for this compound. In conclusion, if a pharmacokinetic interaction between drugs included in the combined anthelmintic product occurs, human food safety studies must be performed at least for the molecule/s which demonstrated altered plasma exposure and/or with those requiring the longest withdrawal times. Overall, alterations in drug safety and/or tissue residues profiles are also relevant issues related to the use of drug mixtures in livestock animals (see Fig. 4). Although most of the individually used anthelmintic are safe compounds, their combined pharmaceutical preparations may require further safety demonstration.

5. Novel anthelmintic drugs

The remarkable high level of resistance has encouraged the search for strategies to improve the utilization of different compounds into rational control programs. However, the introduction of new molecules with different mechanisms of action into the veterinary pharmaceutical market is crucial for the control of parasitic diseases in different species. There is urgent need for new drugs which will not share mechanisms of resistance with existing anthelmintics, but the cost of developing a new class of broad-spectrum anthelmintics has rapidly become prohibitive. Despite intensive efforts over many years, only a few promising molecules with potent and unique pharmacological activity were identified. The diketopiperazines (*marcfortine* and *paraherquamide*), cyclic octadepsipetides (*PF1022, emodepside*), neuropeptides *(FaRP)*, artemisinins (*artesunate* and *artemether* are semi-synthetic derivatives with proved flukicidal efficacy), and the amino-acetonitrile derivatives (AADs) are among the novel anthelmintic chemical families. They represent encouraging classes of

novel drugs but only two compounds have emerged and reached the market for nematode control in sheep.

Derquantel is a semisynthetic derivative of paraherquamide (2-desoxoparaherquamide) that belong to the family of spiroindoles. Derquantel is well absorbed and extensively distributed into tissues after oral administration, showing excellent anthelmintic activity against *H. contortus* (adults stages), *T. colubriformis* and *Nematodirus* spp. (adults and L4 stages) (Little et al., 2011). It exerts its antiparasitic activity by blocking cholinergic transmission (nicotinic-acetylcholine receptor antagonist) and inducing flaccid paralysis in nematodes (Ruiz-Lancheros et al., 2011). This differential mode of action gives the possibility to act on nematode strains resistant to other chemical groups currently available in the market. In an attempt to optimize its anthelmintic activity and to decrease the selection pressure on resistant nematode strains, derquantel has been launched for use in combination with a ML (abamectin) in New Zealand and Australia (Little et al., 2011). Although comparative work carried out recently shows that derquantel + abamectin combination failed to reduce the burden of multi-resistant *H. contortus* (Kaminsky et al., 2011), there is a pharmacology-based evidence of a synergistic interaction between derquantel and abamectin (Puttachary et al., 2013). Some further parasitological studies may be required to extend the therapeutic benefits of this novel combination and to optimize its contribution in parasite control in livestock under the current complex scenario.

The recent discovery of the amino-acetonitrile derivatives (AADs) as a new chemical class of synthetic anthelmintics and the development of monepantel, are relevant steps forward in the field contributing to increase the available therapeutically options to control gastrointestinal nematodes (Kaminsky et al., 2008). A significant number of AAD compounds (about 700) were evaluated (Kaminsky et al., 2008) and finally monepantel was introduced into the veterinary pharmaceutical market. Monepantel was registered in Australia, New Zealand and in some South American countries. Monepantel is active against larval and adult stages of gastrointestinal nematodes of sheep and cattle. It is highly effective against nematode resistant to all the other available anthelmintic families. Monepantel effectiveness is based on a novel mechanism of action involving a unique, nematode specific clade of the nicotinic acetylcholine receptors, which allows overcoming existing resistance to the currently available anthelmintcs. Genetic studies conducted on the free-living nematode *C. elegans* and *H. contortus*, showed that monepantel acts as an agonist on the nicotinic acetylcholine receptor producing spastic paralysis and death of the worm (Kaminsky et al., 2008; Rufener et al., 2010). After its intravenous and oral administration to sheep, monepantel is rapidly converted into a monepantel sulphone metabolite (Karadzovska et al., 2009). It has been suggested that monepantel sulphone may have the same *in vitro* anthelmitic activity as monepantel parent drug (Karadzovska et al., 2009). Thus, the high sulphone concentration profiles measured in different sections of the gastrointestinal tract in monepantel treated sheep (Lifschitz et al., unpublished observations) may be relevant to the efficacy against different gastrointestinal nematodes. The activity against multi-drug-resistant isolates based in a novel mode of action, its good tolerability and low mammalian toxicity, are highly favorable elements to judge the future of monepantel for nematodes control. However, some specific knowledge on its pharmacological behavior as well as a highly responsible use will be necessary to secure its maximum lifespan. The emergence of new antiparasitic compounds into the veterinary pharmaceutical market makes it necessary to have a deep understanding of their pharmacological properties in order to avoid their misuse and therefore, delay the appearance of resistance.

6. Concluding remarks

The integrated assessment of the drug disposition kinetics in the host, the processes of drug influx/efflux in different parasites, their biotransformation/detoxification capacities, and the use of pharmaceutical technology to improve drug delivery at the site of infection, are key research areas within the pharmacology of antiparasitic drugs. Different pharmacokinetic-based approaches to enhance parasite exposure and the use of combinations of drugs from different chemical families, have been proposed as valid strategies to delay the development of anthelmintic resistance. The pharmacological basis of those therapeutic approaches has been summarized in the current article. The activity of the recently developed anthelmintic compounds against multi-drug-resistant isolates, which is based on novel mode of actions, is a highly favorable element to judge the future of nematode control. Overall, the outcome from integrated pharmaco-parasitological research approaches has greatly contributed to optimize drug activity, which seems relevant to preserve existing and particularly, novel active ingredients as useful tools for parasite control in livestock animals. The use of pharmacology-based information is critical to design successful strategies for the future of parasite control.

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