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Basic and clinical pharmacology contribution to extend anthelmintic molecules lifespan

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ABSTRACT

The correct use of pharmacology-based information is critical to design successful strategies for the future of parasite control in livestock animals. Integrated pharmaco-parasitological research approaches have greatly contributed to optimize drug activity. In an attempt to manage drug resistance in helminths of ruminants, combinations of two or more anthelmintics are being used or promoted, based on the fact that individual worms may have a lower degree of resistance to a multiple component formulation, when each chemical has a different mode of action compared to that observed when a single compound is used. However, as emphasized in the current review, the occurrence of potential pharmacokinetic and/or pharmacodynamic interactions between drug components highlights the need for deeper and integrated research to identify the advantages or disadvantages associated with the use of combined drug preparations. This review article provides integrated pharmacokinetic/pharmacodynamic and clinical pharmacology information pertinent to preserve the traditional and modern active ingredients as practical tools for parasite control. Novel pharmacological data on derquantel and monepantel, as representatives of modern anthelmintics for use in livestock, is summarized here. The article also summarizes the pharmaco-parasitological knowledge considered critical to secure and/or extend the lifespan of the recently available novel molecules.

1. Introduction

Evaluation of the available knowledge on the pharmacological features of anthelmintics is required to optimize activity and to achieve sustainable use. It is well known that inadequate use of anthelmintics has led to therapeutic failures and to the widespread development of parasite resistance. Anthelmintic resistance in human and animal helminths has been spreading in prevalence and severity with multi-drug resistance becoming a widespread problem in farm animals. With this in mind, the use of pharmacology-based information is critical to design successful future strategies. Earlier work contributed to detailed knowledge on the relationship between physico-chemical properties and host/parasite tissues disposition kinetics for the most widely used broad-spectrum anthelmintics in ruminants (*i.e.* benzimidazoles, macrocyclic lactones), and this provided the scientific basis to generate novel research approaches on the field of drug therapy. 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 still key research areas within the pharmacology of antiparasitic drugs.

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 extended efficacy of the novel ones, to control drug resistant parasites are either a pharmacokinetic-based enhancement of parasite exposure or the combined use of anthelmintics with different modes of action/resistance (Lanusse et al., 2014). The current review article provides integrated basic and clinical pharmacology information on existing and novel anthelmintic drugs. The pharmacological basis of drug–drug interactions and the rationale behind the use of combined anthelmintic treatments is addressed. Understanding the information compiled here is crucial to extend the lifespan of the recently available modern molecules with alternative modes of action and well demonstrated activity against multiple drug resistant nematodes.

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2. Combination of traditional anthelmintic drugs. Exploring drug–drug pharmacological interactions

There is a long history related to the use of drug combinations in treating diseases. For instance, the use of drug combinations in cancer chemotherapy was early explored (Goldin and Mantel, 1957). Concerning parasite control, drug combination has been successfully used as a strategy to expand efficacy spectrum (*i.e.* ivermectin-clorsulon). Additionally, in an attempt to overcome/manage anthelmintic resistance in ruminants, combinations of two or more anthelmintics are being used in most geographic regions (Geary et al., 2012). The combination of nematodicidal drugs could be achieved by the combination of two or more pharmaceutical formulations containing a different active principle each, or alternatively by the use of combined veterinary medicinal products (two or more active substances in the same preparation) named as "fixed combination products" (EMEA, 2006). The main goal behind the use of two or more drugs with different modes of action is to increase the treatment efficacy. Other possible favourable outcomes include: (a) the use of lower doses to avoid toxicity (reaching equivalent or even higher efficacy) and, (b) minimizing or slowing down drug resistance. The rationale behind using combined anthelmintic preparations 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) compared to that observed when a single anthelmintic compound is used (Anderson et al., 1988; Barnes et al., 1995; Leathwick et al., 2009). Thus, several pharmaceutical formulations combining either two or three chemical entities have been developed. Preparations combining the actives from the main available chemical groups (benzimidazoles, macrocyclic lactones, imidazothiazoles, salycilanilides) have been introduced into the veterinary pharmaceutical market in many countries. However, pharmacokinetic (PK) and/or pharmacodynamic (PD) interactions between drug components may occur and need to be addressed (see Fig. 1). A potential drug interaction refers to the possibility that one drug may alter the intensity of the pharmacological effects of another drug that is 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 (PK interaction), or from a change in the relation between drug concentration and response of the organism to the drug (PD interaction). It is well established that when two or more drugs are combined, they can behave like a third drug with several pharmaco-toxicological uncertainties (Chou, 2010). Consequently, the occurrence of potential PK and/or PD interactions between drug components highlights the need for further pharmacological-based research to identify the advantages or disadvantages of the use of combined preparations for anthelmintic control in livestock. The pharmaco-parasitological consequences derived from PK and/or PD drug to drug interactions after the combined use of nematodicidal compounds or nematodicidal plus non-nematodicidal compounds are briefly outlined here.

A PK interaction takes place when an altered drug concentration of either one or both molecules administered in combination is observed. PK drug–drug interactions are mainly related to metabolic enzyme induction or inhibition, competition with drug transport proteins and/or competition by plasma protein binding, which determine that the extent and duration of the pharmacological activity may be extended/decreased as a consequence of the PK interaction. Available data indicates that PK interactions among anthelmintic molecules may be more common than expected. While the ivermectin plasma area under the concentration *vs* time curve (AUC) obtained after its intravenous co-administration with albendazole was 88% higher ($P < 0.05$) compared to the treatment with ivermectin alone, their subcutaneous (ivermectin)/intraruminal (albendazole) co-administration only increased plasma availability of albendazole metabolites (Alvarez et al., 2008). A different situation has been described after oral administration of albendazole, levamisole and ivermectin, each administered either alone or as a combined formulation in parasitized lambs (Suarez et al., 2014). Although the overall levamisole plasma disposition kinetics was unaffected, significantly lower (61%) albendazole sulphoxide and higher (71%) ivermectin systemic availabilities were obtained after administration of the combined formulation in comparison to those obtained after treatment with each drug alone. Here, all PK changes could be attributed to a combined effect on metabolism and drug efflux transporter interactions among albendazole/albendazole sulphoxide, ivermectin and levamisole (Alvarez et al., 2008; Suarez et al., 2014).

In an effort to improve control of highly resistant nematodes (*i.e. Haemonchus contortus*) different drug combinations have been assessed. The combined administration of closantel and moxidectin did not markedly alter their disposition kinetics with the exception of an increment (47%, $P < 0.05$) in the closantel elimination half-life after its co-administration with moxidectin (both subcutaneously administered) to lambs (Suarez et al., 2013). Additionally, the ivermectin plasma AUC obtained after its oral co-administration with fenbendazole was 50% higher, compared to that observed when ivermectin when administered alone (Luque et al., 2015). The overall data obtained in different PK studies in sheep indicate that the route of administration would determine the "magnitude" of the

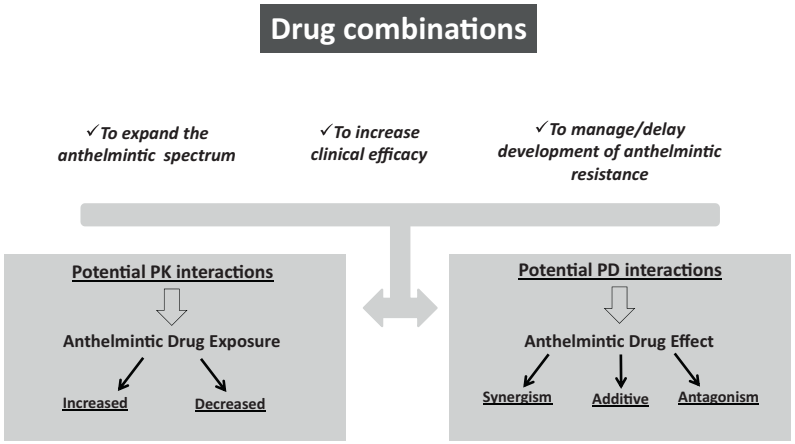


Fig. 1. Anthelmintic drug combinations: rationale behind their therapeutic use in Veterinary Medicine. Potentially occurring pharmacokinetic (PK) and pharmacodynamic (PD) drug to drug interactions after combined treatments.

drug–drug interaction. Consequently, potential changes on plasma drug exposure derived from a PK interaction may depend on the route of administration used for the "fixed" mixture formulation, or for each constituent included in the combined treatment (when different commercial formulations are simultaneously administered).

PK interactions between nematodicidal drugs have been investigated less in cattle. No significantly differences in the plasma PK behaviour of closantel and ivermectin (subcutaneous administration) were observed, indicating that neither the absorption nor the tissue distribution of both molecules is influenced by the presence of the other (Cromie et al., 2006). Similarly, no PK interactions were observed after the combined subcutaneous administration of ivermectin and ricobendazole in calves (Canton et al., 2014). Furthermore, the presence of levamisole did not modify the plasma PK behaviour of ricobendazole in cattle (Canton et al., 2015), with equivalent disposition kinetic variables obtained after the single-drug and combination-based strategies.

PD drug–drug interactions may occur at three different levels: at the receptor site, at signalling (*i.e.* second messenger) or at the effector level. This can lead to both enhanced (additive/synergic effect) and diminished (antagonism) drug responses. Overall, an additive effect is present when the combined activity of two drugs equal the sum of their independent activities measured separately. On the other hand, a synergistic effect is achieved when the combined effects of the drugs are significantly greater than the independent effects (Prescott, 2000). The presence of a pharmacological synergism implies a drug effect greater than additive. The effect achieved in the presence of antagonism is lower than additive (Chou, 2010). Synergism normally occurs between drugs that exert the same effect (*i.e.* anthelmintic) by different modes of action. However, exact knowledge of the mechanism of action is not required for the quantitative determination of synergism (Chou, 2010). Since levamisole, albendazole, monepantel, closantel and ivermectin are chemical entities which differ in their intrinsic anthelmintic mode of action, their co-administration may potentially induce a synergistic effect. On the other hand, when multiple resistance refers to the presence of different worm genera, each one resistant to a unique chemical group, worms surviving one compound could be killed by the other. In this case, the "additive" effect exerted by the combined product may allow the control of resistant nematodes.

PD interactions resulting in synergistic effects can be clinically relevant, and would represent an ideal situation to deal with resistant parasites. For example, a worm resistant to two different anthelmintics (bi-resistant worm) could be killed by the combined effect of the same two drugs acting synergistically. Due to the increasing anthelmintic resistance problem in the ruminant livestock production systems, as well as in horses, nematodicidal drug combinations appears to be potentially useful, particularly in delaying the emergence and spread of resistance and/or controlling parasite populations with existing resistance (Geary et al., 2012). Theoretically, if an anthelmintic treatment reaches 100% efficacy, selection of 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 anthelmintic resistance in the field situation (Dobson et al., 2001). Consequently, in farms where multiple-resistant nematode populations are present, the use of drug combinations may be an alternative to improve chemical control.

In human medicine, helminth control faced two main problems: a limited number of available approved drugs and low efficacy of these drugs administered as single doses; for example against a highly prevalent *Trichuris trichiura* (Keiser and Utzinger, 2008). In such conditions, the combined use of nematodicidal drugs showing synergistic effects could contribute to improve parasite control.

Information related to potential PD interactions between nematodicidal drugs is scarce, and includes data obtained from *in vitro* and/or *in vivo* experiments. The initial evaluation of PD interactions could be facilitated by *in vitro*/*ex vivo* experiments, where the dose/effect relationship of different drugs (*i.e.* A and B) allows the exploration of interactions between both compounds. Using the concept of "combination index" (CI) introduced by Chou and Talalay (1983), a synergism is demonstrated if the CI < 1, an additive effect when CI = 1, and CI > 1 indicates drug antagonism. The *in vitro* and *in vivo* PD interactions between the main available marketed human anthelmintics have been investigated using this approach. Keiser et al. (2012) reported that all possible drug–drug combinations established between albendazole, mebendazole, pyrantel pamoate, levamisole and ivermectin revealed an *ex vivo* synergistic (CI < 1) behaviour against *Trichuris muris*. A strong synergism (CI < 0.1) was observed for the albendazole-mebendazole, albendazole-ivermectin, mebendazole-ivermectin, mebendazole-levamisole and levamisole-pyrantel pamoate combinations. All these combinations were further investigated *in vivo* against the same parasite (*T. muris*) in artificially infected mice, demostrating that mebendazole-ivermectin, mebendazole-levamisole and albendazole-mebendazole combinations behave as strongly synergistic (CI = 0.3–0.7). A good correlation between the *ex vivo* and *in vivo* data was observed. The three drug combinations showing a strongly synergistic effect *ex vivo* (albendazole-mebendazole, albendazole-ivermectin and mebendazole-ivermectin) also induced a synergism *in vivo* (Keiser et al., 2012). Furthermore, a synergistic effect was observed in both *ex vivo* and *in vivo* (*T. muris* infected-mice) experiments for the combination oxantel pamoate-mebendazole (Keiser et al., 2013). In contrast, combinations containing either pyrantel pamoate or levamisole combined with ivermectin (Keiser et al., 2012) or oxantel pamoate combined with either levamisole, albendazole or ivermectin (Keiser et al., 2013) were found to be antagonistic *in vivo*. The synergistic effect observed after *in vivo* experiments in the mouse model (Keiser et al., 2012) had some success to predict the real situation in humans naturally infected with *T. trichiura*. In a randomized controlled trial including children naturally infected with *T. trichiura*, higher egg reduction rates after the mebendazole-ivermectin (97%) or albendazole-ivermectin (91%) combinations were observed in comparison to mebendazole (67%) or albendazole (40%) given alone (Knopp et al., 2011). Unfortunately, this study did not include a group treated with ivermectin alone, in order to determine if a true synergistic/additive effect was occurring. Additionally, in children naturally infected with *T. trichiura*, a higher egg reduction rate was observed after the combined administration of albendazole-ivermectin (98%) compared to the albendazole (54%) or ivermectin (87%) alone (Belizario et al., 2003). However, a different situation was described in children infected with hookworms, where cure rates of 29.4% (ivermectin), 95.5% (albendazole) and 92.6% (ivermectin-albendazole) were observed, which indicates that the use of the combination did not improve the efficacy of the albendazole alone treatment (Ndyomugyenyi et al., 2008). Finally, although some improvement in the cure rate (defined as the proportion of parasitized individuals with a negative egg count test after treatment) was observed after albendazole-mebendazole combination (46.1%) compared to albendazole (6.0%) or mebendazole (11%) alone administration to school-aged children infected with *T. trichiura* (Namwanje et al., 2011), the success of the treatment (even the combination) was far from optimal.

Concerning veterinary nematode species, the *ex vivo* effects on *Toxocara canis* motility and tissue morphology was assessed after administration of pyrantel and fenbendazole, each drug alone or in combination (Mehlhorn et al., 2003). Although there was no significant difference observed between the effects of the single drugs

Table 1
Summary of *in vivo* performed trials assessing the efficacy of anthelmintic combined treatments against resistant nematodes.

Anthelmintic combination	Treatment	Animal species	Route of administration	Efficacy estimation method	Parasite infection	Efficacy (%)	Type of PD interaction	Reference
FBZ + LEV	FBZ alone LEV alone Combined	Sheep	Oral	FECRT	Natural[1]	83 16 97	Additive	Anderson et al., 1988
RBZ + LEV	RBZ alone LEV alone Combined	Sheep	Oral	Controlled	Artificial (*Teladorsagia*)	80 90 97	Additive	Anderson et al., 1991
RBZ + LEV	RBZ alone LEV alone Combined	Sheep	Oral	Controlled	Artificial (*Trichos.*)	86 36 95	Additive	Anderson et al., 1991
LEV + FBZ	LEV alone FBZ Combined	Goats	Oral	FECRT	Natural[2]	23 1 62	Synergism	Miller and Craig, 1996
ABZ + IVM	ABZ alone IVM alone Combined	Sheep	i.r. s.c. i.r./s.c.	FECRT	Natural[3]	44 80 71	Indifference	Entrocasso et al., 2008
ABZ + LEV + IVM	ABZ alone LEV alone IVM alone Combined	Sheep	Oral	FECRT	Natural[4]	52 72 80 87	Additive	Suarez et al., 2014
CLO + MXD	CLO alone MXD alone Combined	Sheep	Oral	FECRT	Natural[5]	83 98 100	Additive	Suarez et al., 2013

FBZ: fenbendazole; LEV: levamisole; RBZ: ricobendazole; ABZ: albendazole; IVM: ivermectin; CLO: closantel; MXD: moxidectin; PD: pharmacodynamic; FECRT: faecal egg count reduction test; i.r., intraruminal; s.c.: subcutaneous.[1]Faecal culture: *Teladorsagia* spp. and *Trichostrongylus* spp.; [1]*Haemonchus* spp.; [2]Faecal culture: Haemonchus spp.; [3]Faecal culture: *Trichostrongylus* spp., *Teladorsagia* spp. and *Haemonchus* spp.; [4]Faecal culture: *Haemonchus* spp. and *Trichostrongylus* spp.; [5]Not data available.

and the drug combination on worm motility, a synergistic effect of pyrantel and fenbendazole was manifested by an earlier onset of morphological alterations in worm tissues and organs observed by light and electron microscopy (Mehlhorn et al., 2003). Furthermore, a synergistic interaction between derquantel and abamectin occurs under *in vitro* laboratory conditions (Puttachary et al., 2013). In this study, the effects of derquantel, abamectin and their combination on somatic muscle nicotinic acetylcholine receptors and pharyngeal muscle glutamate gated chloride receptor channels of *Ascaris suum* was assessed. The study demonstrated that abamectin and derquantel interact at the nicotinic acetylcholine receptors on the somatic muscle. At this level, the effect of the combination was significantly greater than the predicted by an additive effect of both drugs, suggesting a synergistic effect of the combination (Puttachary et al., 2013).

Anthelmintic resistance is a particular problem in small ruminants, which has resulted in the use of nematodicidal combinations of two or more anthelmintics in several countries such as Australia, New Zealand (Sutherland and Leathwick, 2011) and Uruguay (Suarez et al., 2014). The results collected from different *in vivo* trials to investigate the potential additive or synergistic PD effects of different nematodicidal drugs used in combination against resistant nematodes in sheep and goats are summarized in Table 1. The overall assessment of the field data appears to indicate that "additive" is the predominant PD effect observed after the combined use of nematodicidal anthelmintic molecules. In the presence of highly resistant nematode population, the combined treatments did not offer a clinically relevant increase in efficacy against highly multiple-resistant nematodes (Suarez et al., 2014).

In vivo data obtained under "real" field conditions, indicate that the use of anthelmintic combinations in sheep production systems (where anthelmintic resistance is common) may have a limited sustainability. However, a different situation could be observed in cattle production systems, where individual active ingredients still maintain relatively high efficacy. Recent work (Canton et al., 2014) evaluated the clinical efficacy (FECRT) observed after the subcutaneous administration of ivermectin and ricobendazole given either separately or co-administered to calves naturally parasitized with gastrointestinal nematodes resistant to ivermectin. The observed efficacies were 48% (ivermectin), 94% (ricobendazole) and 98% (ivermectin-ricobendazole). Since no significant differences in faecal egg counts were obtained between groups treated with ricobendazole alone and the combined treatment (Canton et al., 2014), no therapeutic advantage was observed for the combination. Additionally, evaluation of potential advantages derived from the subcutaneous administration of ricobendazole and levamisole given either separately or co-administered to naturally parasitized calves in two different seasons (winter and spring) with predominance of different nematode populations, was recently reported Canton et al. (2015). An ideal situation for the combined treatment was observed in winter (June/July in the southern hemisphere), with a FECR at 14 days post-treatment of 100% (combined treatment) compared to 96.1% (ricobendazole) or 99.8% (levamisole). In winter time, the *Ostertagia* spp. population observed in the faecal cultures (control group) represented only 10%. However, in spring (October) where *Ostertagia* spp. represented a 28% of the total third stage larvae recovered, the FECRs were 95.1 (ricobendazole), 93.1 (levamisole) and 96.1% (ricobendazole-levamisole). The increased presence of *Ostertagia* spp. in spring determined a tendency to reduced clinical efficacies compared to winter time, even for the combined nematodicidal treatment.

Preliminary results indicate that the combination of macrocyclic lactones (parenteral) and levamisole (oral) used in combination was highly effective in minimizing the survival of macrocyclic lactone resistant nematodes and the subsequent transport of those parasites between farms (Smith, 2014). Since a key factor for the "optimal results" of a combined nematodicidal treatment is that the individual molecules reached their highest efficacy (Geary et al., 2012), the use of anthelmintic combinations in cattle production systems may be an important tool to delay resistance (Lanusse et al., 2014).

The experimental work reviewed here indicates that most cases of PD interactions between nematodicidal drugs in the target animal species (sheep, cattle) and under practical conditions (natural infections, use of therapeutic doses, *etc.*) appear to be limited to an additive effect. When multiple resistance refers to the presence of different worm genera, each one resistant to a 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. There is some evidence of *in vivo* synergistic or antagonistic action of anthelmintics given in combination. Thus far, the possible mechanisms underlying the reported effects are not understood. Data extrapolation from *in vitro* assays to laboratory animals and from laboratory animals to domestic animals may be inadequate since differences in drug exposure (PK) and/or drug–receptor interaction (PD) among experimental models may occur. However, since combination chemotherapy is a common practice in different medical fields such as cancer, bacterial infections and malaria (Keiser and Utzinger, 2010; Geary et al., 2012), follow-up work on the assessment of the potential PD interactions between anthelmintics drugs is required. This type of pharmacology-based evaluation of drug interactions is becoming relevant since drug combinations is now widely used as an alternative to control resistant helminth parasites in livestock. This is enforced by the fact that the European Medicine Agency (EMEA) will only consider as acceptable, fixed combination products (combined drugs included in the same commercial product) based on valid therapeutic principles (EMEA, 2006).

While some *in vitro* results may indicate that a synergistic action between nematodicidal drugs could be achieved, the PD advantage is not easily seen under real situations in the field. Drug chemical features, the type of target parasites and several host-related factors (*i.e.* immune response) should all concur to achieve an *in vivo* synergist interaction (see Fig. 2). In the presence of multi-resistance, the use of combined treatments may not significantly improve the observed clinical efficacy. To achieve the maximum benefit of the combined treatments in managing anthelmintic resistance, it is necessary to "design" the combination based on basic parasitological information of each individual farm, including degree of parasite pasture contamination, type of parasites/epidemiological behaviour, level of animal infection and the presence of drug resistant populations. Only upon integrated comprehension of all these aspects, an "adequate" anthelmintic combination could be designed and applied at the "correct" time to the "proper" animals. Under this context, sustainable anthelmintic control can only be successfully managed by veterinarians and the use of "fixed" anthelmintic combinations must be avoided.

3. Pharmacological assessment of combinations between anthelmintic and non-anthelmintic molecules

Anthelmintic drugs have often been initially marketed at the smallest dose which demonstrated a high (≥95%) efficacy. However, when anthelmintic resistance arises, the amount of drug that reaches resistant worms is not enough to eliminate them. There is a strong relationship between the amount of drug reaching the target parasite and the induced anthelmintic effect. Thus, even for resistant worms, increasing drug exposure (by increasing the concentration, the time of exposure or both) may help recover high anthelmintic efficacy (Várady et al., 1996; Moreno et al., 2004; Barrère et al., 2012; Lloberas et al., 2015; Alvarez et al., 2015). The enhancement of drug systemic exposure will facilitate that higher drug concentrations reach the parasite location for a sufficient time to improve the antiparasitic effect, particularly against worms carrying resistance genes. The combination of anthelmintic with non-anthelmintic molecules is intended to increase the parasite-drug exposure. Such a goal can be achieved by enhancing the active drug systemic exposure in the host or favouring drug accumulation within the target parasite. Interference/modulation on drug biotransformation or excretion/efflux mechanisms at either the level of the host, target parasite or both simultaneously, may achieve enhanced drug exposure and improve efficacy as is reported in the literature (see Lanusse et al., 2014).

Metabolic modulators have been studied for their potential to optimize the pharmacology of anthelmintics. It has been established that co-administration with methimazole, a metabolic inhibitor of flavin monooxigenase (FMO), potentiates the efficacy of netobimin and albendazole against gastrointestinal nematodes in cattle (Lanusse and Prichard, 1992). Furthermore, it has been demonstrated that co-administration of fenbendazole with piperonyl butoxide, a potent inhibitor of the P-450-mediated oxidation, markedly affected the pharmacokinetic disposition of fenbendazole/metabolites in sheep and goats, potentiating nematodicidal activity against benzimidazole-resistant strains of *Teladorsagia circumcincta* (Benchaoui and McKellar, 1996). Similarly, Sanchez Bruni et al. (2005) evaluated the effects of piperonyl butoxide on the relative plasma availability and efficacy of oxfendazole given orally to naturally parasitized horses. In the presence of piperonyl butoxide, enhanced pharmacokinetic profiles correlated with increased anthelmintic efficacy.

Biochemical studies have demonstrated that the FMO and cytochrome P-450 enzyme pathways are involved in the metabolism of triclabendazole by the fluke *Fasciola hepatica* and are up-regulated in triclabendazole-resistant flukes (Robinson et al., 2004; Alvarez et al., 2005), supporting the concept that altered drug metabolism contributes to the mechanism of resistance to triclabendazole. *Ex vivo* electron microscopic studies have shown that co-incubation of triclabendazole or its sulphoxide metabolite with metabolic inhibitors, such as methimazole (Devine et al., 2009), piperonyl butoxide (Devine et al., 2011) and ketoconazole (Devine et al., 2012), leads to greater surface disruption in triclabendazole-resistant flukes compared to that observed after adult fluke incubation with each anthelmintic alone. These results provide evidence that the condition of a triclabendazole-resistant fluke can be modified by inhibition of the parasite metabolic activity. However, a study performed in sheep artificially infected with a triclabendazole-resistant isolate of *F. hepatica* demonstrated that the presence of methimazole (as metabolic inhibitor) and ivermectin (as modulator of the P-glycoprotein [P-gp]-mediated drug efflux) did not affect the disposition kinetics of triclabendazole and its metabolites in the host (Ceballos et al., 2010). Furthermore, since the combined drug treatment did not reverse the poor efficacy of triclabendazole against triclabendazole-resistant *F. hepatica*, alternative mechanisms of resistance may play a critical role under *in vivo* conditions.

The influence of cell transporter systems in the pharmacokinetic behaviour of different drug compounds is considered as a new paradigm. This has been particularly relevant within the pharmacology of the macrocyclic lactone ecto-endoparasiticides. Among all the identified cell transporters, P-gp has been the most studied. 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). *In vivo* trials performed on different animal species provided information on the action of different P-gp modulators such as verapamil, loperamide, quercetin, itraconazole and ketoconazole, on the macrocyclic lactones PK disposition (Molento et al., 2004; Lifschitz et al., 2002; Dupuy et al., 2003; Ballent et al., 2007; Alvinerie et al., 2008). P-gp-mediated drug efflux has been proposed as a potential resistance

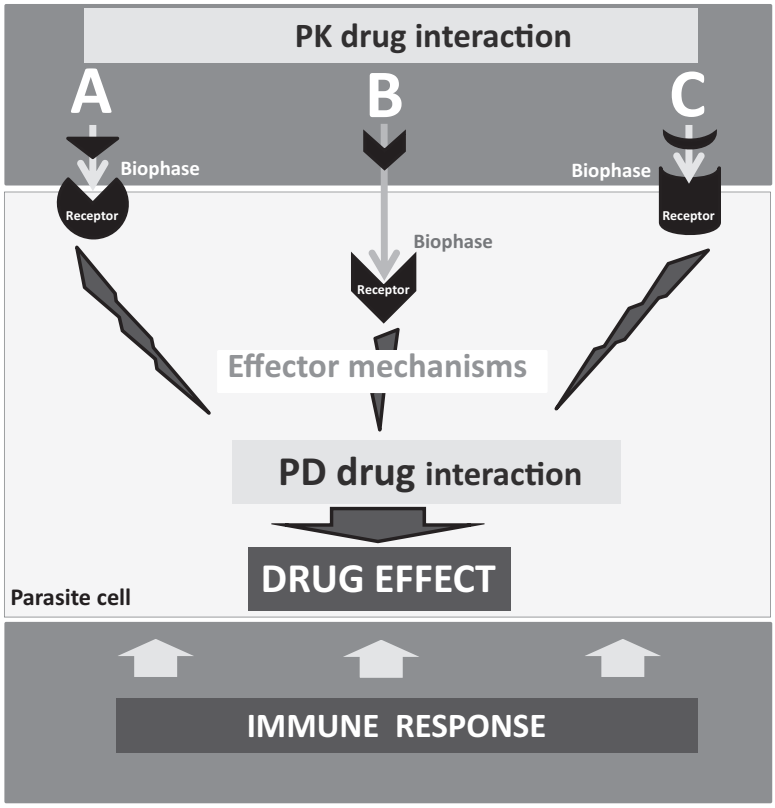


Fig. 2. Schematic representation of the events expected to occur after a combined treatment with anthelmintic drugs (named A, B and C) acting by different modes of action. The drug–receptor interaction will depend on different factors such as drug affinity, number of receptor expressed, drug concentration achieved at the biophase (ambient surrounding the receptor), *etc.* While working under *in vitro*/*ex vivo* conditions allows to accurately define the drug concentration, different pharmacokinetic (PK) interactions can alter the amount of drug reaching the biophase under *in vivo* conditions. The final pharmacological effect obtained after use of nematodicidal drug combinations, may either depend on the sum of individual drug effects or on the occurrence of pharmacodynamic (PD) drug–drug interactions among the co-administered anthelmintics. The host immune system could also modify the final clinical response (pharmacological effect) under *in vivo* conditions.

mechanism for macrocyclic lactones in different helminths (Xu et al., 1998; Kerboeuf et al., 2002). Modifications to the pattern of P-gp expression have been observed in resistant nematodes recovered from lambs treated with macrocyclic lactones (Prichard and Roulet, 2007). The modulation of P-gp activity has been assayed as a pharmacology-based strategy not only to increase systemic availability of macrocyclic lactones at host animal, but also to induce a drug–drug interaction at the parasite level, which would account for improved clinical efficacy (Lifschitz et al., 2012; Lespine et al., 2012).

Although ivermectin has no activity on *F. hepatica*, an interesting effect was observed after its co-incubation with triclabendazole (Mottier et al., 2006). The ivermectin-induced modulation of P-gp activity decreased triclabendazole efflux from resistant flukes and higher concentrations of triclabendazole and triclabendazole sulphoxide were recovered from a resistant *F. hepatica* isolate in the presence of ivermectin (Mottier et al., 2006). However, the *in vivo* 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 *ex vivo*/*in vivo* pharmacological interaction between triclabendazole and ivermectin into a change on the flukicidal efficacy in the host.

The modulation of P-gp increased the *in vitro* activity and the *in vivo* efficacy of ivermectin against ivermectin-sensitive and resistant parasites. The presence of P-gp modulators enhanced the ivermectin activity against larvae of *Teladorsagia circumcincta*, *H. contortus* and *Cooperia* spp. (Bartley et al., 2009; Demeler et al., 2013). While the presence of the P-gp modulator pluronic 85 did not improve the *in vivo* ivermectin efficacy against resistant *H. contortus* (Bartley et al., 2012), the efficacy of both ivermectin and moxidectin against resistant *Cooperia* spp. in naturally parasitized cattle tended to increase after their co-administration when loperamide was used as a P-gp modulator (Lifschitz et al., 2010a). Similarly, a significant increase in ivermectin efficacy against resistant nematodes in sheep, together with an enhancement on ivermectin systemic availability, was obtained after its combination with loperamide (Lifschitz et al., 2010b).

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. This is an open field which must be addressed for the future of the macrocyclic lactones as antiparasitic agents, if the combination of anthelmintic molecules turns into an alternative for parasite control in resistant populations. Chemical modulation of the activity of drug transport proteins both in the host animal and target parasite could be a further step in the search for pharmacological tools that optimize drug therapy in veterinary medicine.

4. Novel ruminant anthelmintic molecules. Integrated understanding of their main pharmacological features

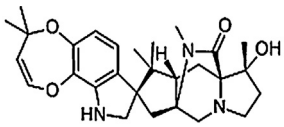
The need for new molecules that do not share mechanisms of resistance has driven the anthelmintic drug development process in animal health. Despite intensive efforts over many years, only a few promising molecules with potent and unique pharmacological activity have been identified. The diketopiperazines (*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 discovered. Among them, only two compounds, derquantel and monepantel, have reached the market for nematode control in sheep. Novel pharmacological data on derquantel and monepantel, as representatives of modern anthelmintics, is summarized here, along with pharmaco-parasitological knowledge considered critical to extend the lifespan of these molecules.

4.1. Kinetic-dynamic bases of derquantel anthelmintic activity

Derquantel (2-desoxoparaherquamide) is a semisynthetic derivative of paraherquamide belonging to the chemical family, spiroindoles. Derquantel has lower toxicity in mammalian species compared to the parent compound, paraherquamide (Lee et al., 2002). The anthelmintic activity of derquantel is based on interference with B-subtype nicotinic acetylcholine receptors, acting as an antagonist leading to a nematode flaccid paralysis (Ruiz-Lancheros et al., 2011). This distinct mode of action contributes to its activity against nematode strains resistant to other currently available chemical groups. Published information on the disposition kinetics of derquantel given alone in target animal species is scarce. However, the pharmacokinetic behaviour of paraherquamide has been described in sheep and could be useful in extrapolating overall kinetic features. After oral administration of paraherquamide to lambs, rapid absorption with a peak plasma concentration between 0.5 and 2 h post-treatment was observed (Aloysius et al., 2008). Paraherquamide undergoes extensive liver metabolism, showing a plasma elimination half-life of 8.5 h. Eighty % of the dose is excreted in faeces largely as metabolic products (Aloysius et al., 2008). In an attempt to optimize the spectrum of its anthelmintic activity, derquantel has been registered for distribution in combination with abamectin (Little et al., 2011). The approved dose is 2 mg/kg derquantel plus 0.2 mg/kg abamectin administered by the oral route to sheep. After oral administration, derquantel reached its peak plasma concentration at 4 h (T_{max}) post-treatment. The absolute bioavailability reached 56% following the oral combined treatment. Derquantel is a lipophilic substance, which contributes to its large tissue distribution volume (3.2 l/kg). The elimination half-life after the oral administration in sheep was 9.3 h (Friedlander et al., 2012). The potential pharmacokinetic interaction between derquantel and abamectin after oral combined administration has been evaluated: not significant adverse kinetic interaction was observed (Friedlander et al., 2012). *In vitro* metabolism studies of [^{14}C]-2-desoxoparaherquamide in rat, sheep, dog and human liver hepatocytes demonstrated that derquantel is subject to extensive metabolism (Friedlander et al., 2012) and derquantel undergoes biotransformation to a large number of metabolites over a short period (EMEA, 2010). The chemical structure and main pharmacological features of derquantel are shown in Fig. 3.

Several studies performed in different countries have shown that the derquantel-abamectin combination kills a broad range of gastrointestinal and lung nematodes of sheep, regardless of the resistant status of the parasites to other chemical groups (Little et al., 2011). Although comparative work showed that derquantel-abamectin failed to reduce fourth-stage larvae of macrocyclic lactone-resistant *H. contortus* and *Teladorsagia circumcincta* isolates (Kaminsky et al., 2011; George et al., 2012), there are no reports or known field cases of anthelmintic resistance to derquantel. The effect of several possible treatment scenarios using derquantel as a single active or combined with abamectin, was evaluated using a predictive sheep parasite model (Learmount et al., 2012). In this study, resistance to derquantel was predicted to develop more slowly when combined abamectin in comparison to administration



DERQUANTEL

- ✓ Paraherquamide derivative
- ✓ Highly lipophilic compound
- ✓ Large volume of tissue distribution
- ✓ High liver metabolism
- ✓ Faecal excretion
- ✓ Active against gastrointestinal nematodes
- ✓ Succesful combined use with abamectin

Fig. 3. Chemical structure and main pharmacological features of derquantel, a paraherquamide derivative anthelmintic compound.

as a single active. This was corroborated for high refugia as well as low refugia scenarios. Even in low refugia scenario, the combination had a better performance than the single active used sequentially or rotated annually with abamectin in a high refugia situation (Learmount et al., 2012). As indicated above, there is pharmacology-based evidence of a synergistic interaction between derquantel and abamectin (Puttachary et al., 2013). Derquantel may interact additively with abamectin and at higher acetylcholine concentrations the interaction is synergistic. Abamectin acts as a non-competitive antagonist on the nicotinic receptor contributing to potentiate the antagonistic action of derquantel (Puttachary et al., 2013). Therefore, this combination approach may enhance the therapeutic effects and further parasitological studies are required to understand its contribution in parasite control. The complex scenario that includes the presence of multi-resistant nematode strains leads to the search of strategies to optimize the use of this new pharmacological tool. However, it seems reasonable from the accumulated experience with the traditional anthelmintic chemical families that avoiding derquantel overuse and taking advantage of all the available pharmacological knowledge will help optimize its anthelmintic activity and effective lifespan.

4.2. Disposition kinetics and pharmacodynamic basis supporting monepantel broad spectrum nematodicidal activity

The discovery of the amino-acetonitrile derivatives (AADs) as a new chemical class of synthetic anthelmintics and the development of monepantel, increase the available therapeutic options to control gastrointestinal nematodes (Kaminsky et al., 2008). From the many compounds evaluated within the chemical family, the racemic molecule AAD 96 was selected and the active s-enantiomer of this molecule, monepantel, was launched for oral administration to sheep in 2009 (Hosking et al., 2009). Monepantel is active against larval and adult stages of gastrointestinal nematodes of sheep and cattle (Kaminsky et al., 2009; Hosking et al., 2009). It is highly effective against nematode isolates resistant to all other available chemical families. Genetic studies conducted on the free-living nematode *Caenorhabditis 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). Monepantel exerts its action at a new target as a positive allosteric modulator of the nematode specific receptor MPTL-1, which belongs to the DEG-3 subfamily of acetylcholine receptors (Rufener et al., 2010). Monepantel binding to the receptor accounts for an alteration in ion flux and leads to

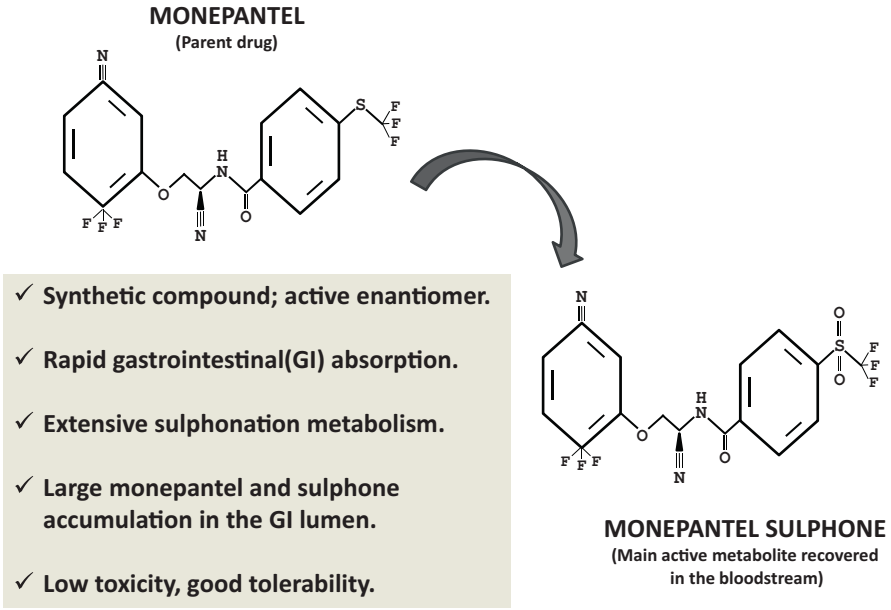


Fig. 4. Chemical structures and main pharmacological features of the novel nematodicidal amino-acetonitrile derivative monepantel and its sulphone metabolite. *Data taken from* Kaminsky et al. (2008), Karadzovska et al. (2009), Lifschitz et al. (2014).

nematode paralysis (Epe and Kaminsky, 2013). This novel mechanism of action explains the high efficacy of monepantel against nematodes resistant to other anthelmintic classes (Baker et al., 2012).

The plasma disposition kinetics of monepantel has been assessed in sheep after intravenous and oral administration (Karadzovska et al., 2009; Hosking et al., 2010; Lifschitz et al., 2014). Monepantel is rapidly converted in the liver into different metabolites (Karadzovska et al., 2009). The systemic availability of the monepantel parent compound was significantly lower than that observed for its main metabolite monepantel sulphone (Karadzovska et al., 2009; Lifschitz et al., 2014) The monepantel sulphone to monepantel plasma concentration profiles ratio (values expressed in AUC) reached a value of 12 (Lifschitz et al., 2014). As the persistence of monepantel sulphone is significantly longer than that of monepantel, this metabolite is the marker for tissue residue studies. Although the sulphone was the main metabolite detected in the bloodstream of sheep after monepantel administration, nine phase I and phase II metabolites were recovered after *in vitro* monepantel incubation in primary culture of ovine hepatocytes (Stuchlíková et al., 2013). Recently, ten monepantel metabolites were found in urine and eight monepantel metabolites were recovered in faeces (Stuchlíková et al., 2014). Sheep are able to metabolize monepantel *via* several metabolic reactions of phases I and II (Stuchlíková et al., 2014). It seems that monepantel may be metabolized in extrahepatic tissues such us kidney and intestine tissues (Stuchlíková et al., 2014). However, the exact amount of each metabolite produced *in vivo* is unknown and therefore the *in vivo* importance of the different metabolites should be evaluated in future. An equivalent pharmacological potency between monepantel parent drug and its sulphone metabolite against larvae of gastrointestinal nematodes has been suggested using *in vitro* evaluation assays (Karadzovska et al., 2009). Thus, as the sulphone seems to be the only active monepantel metabolite, its pharmacokinetic behaviour is relevant for the overall interpretation of the data collected from efficacy studies. The main pharmacological features of monepantel are summarized in Fig. 4. Although the evaluation of drug concentration profiles in the bloodstream contribute useful information, monepantel and monepantel sulphone exert their anthelmintic effect in non-vascular target tissues such as the gastrointestinal tract where the nematode parasites are located (Kaminsky et al., 2009). The characterization of monepantel and monepantel sulphone concentration profiles attained at specific gastrointestinal sites and the establishment of the relationship between their plasma and gastrointestinal content/tissues availabilities are relevant to understand its antiparasitic action (Lifschitz et al., 2014).

In the abomasal content of sheep orally treated with monepantel, high concentrations of monepantel were measured (2 and 4 μg/g) during the first 48 h post-treatment. Interestingly, monepantel sulphone was also detected in the abomasal contents but the concentrations were significantly lower compared to those of parent compound. Gastric secretions may be involved in the appearance of monepantel sulphone in abomasal contents as it has been demonstrated for benzimidazole compounds in sheep (Hennessy, 1993). Such a kinetic pattern may support the high efficacy of monepantel against the abomasal nematode *H. contortus* (Kaminsky et al., 2009). Both monepantel and its sulphone metabolite may reach the target parasite from plasma by oral ingestion. However, considering that they are highly lipophilic compounds (Karadzovska et al., 2009), the high availability of monepantel and monepantel sulphone in abomasal contents could facilitate accumulation of both active molecules within the parasite through a transcuticular diffusion process. Fig. 5 illustrates the comparative plasma and abomasal contents concentration profiles of monepantel and its sulphone metabolite measured in treated sheep (adapted from Lifschitz et al., 2014).

Monepantel and the sulphone metabolite have also been recovered in different segments of the sheep intestine (Lifschitz et al., 2014). The partitioning of both compounds between the intestinal contents and mucosal tissue was different for monepantel and the sulphone metabolite. Whereas the significantly high accumulation of monepantel observed along the intestine (fluid content) may be mainly related to non-absorbed orally administered drug, the high concentrations of monepantel sulphone recovered from the mucosal tissues may be due to its blood-mucosa transfer in the different intestinal segments (Lifschitz et al., 2014). The active intestinal secretion of lipophilic anthelmintics such as ivermectin was previously demonstrated (Laffont et al., 2002). The involvement of P-gp on the intestinal secretion of ivermectin

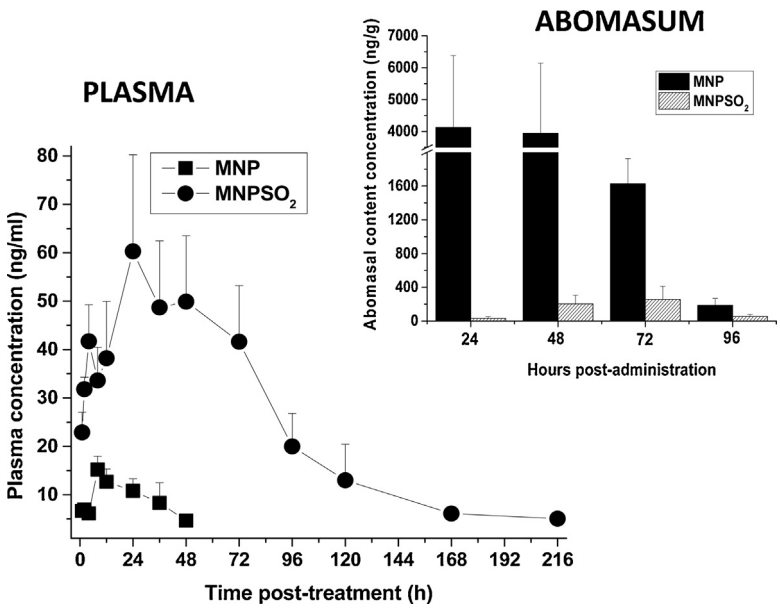


Fig. 5. Mean concentration profiles of monepantel (MNP) and monepantel sulphone ($MNPSO_2$) ($n = 6$) in plasma and abomasal content obtained after the oral administration of MNP to sheep (2.5 mg/kg).
Adapted from Lifschitz et al. (2014).

was corroborated by *in vivo* and *ex vivo* trials (Laffont et al., 2002; Ballent et al., 2006). While parent drugs such as albendazole and fenbendazole did not show interaction with the breast resistance cancer protein (BCRP) transporter, their sulphoxide metabolites showed a highly efficient transport by BCRP in culture cells (Merino et al., 2005). Therefore, the involvement of ABC transporters in the intestinal secretion of monepantel and its sulphone derivative should be examined. This may be relevant to understand its pharmacological behaviour and to identify potential drug–drug interactions if monepantel is co-administered with other anthelmintics.

The concentrations of monepantel and its active sulphone metabolite required at the site of parasite location to inhibit parasite establishment and/or larval development have not been determined. However, the assessment of the pattern of drug accumulation at target tissues may provide useful information to predict the level of drug concentration below which the effectiveness against larval and adult parasites begins to decrease. The AADs act on a nematode specific acetylcholine receptor and produce marked effects on the movement, growth and viability of nematodes (Kaminsky et al., 2008). *In vitro* experiments have shown that the phenotypic effects of these compounds on free-living nematodes and adult *H. contortus* are observed at 50–100 ng/mL, but full lethality occurs at drug concentrations above 1000 ng/mL (Kaminsky et al., 2008). The speed at which a reduction of nematode eggs in the faeces of sheep occurs after monepantel treatment was also evaluated (Sager et al., 2010). A significant reduction on egg production was obtained 36 h post-treatment and faecal egg counts were reduced to 0 at 72 h post-treatment. This time-course of pharmacological activity correlates with the highest monepantel concentrations measured at the abomasum during the first 48 h post-treatment (Lifschitz et al., 2014).

The low to moderate action of monepantel against nematodes located in different systemic tissues may be explained by disposition kinetic data. It seems unlikely that monepantel will adequately control lung nematodes at the dose used for gastrointestinal nematodes (Hosking, 2010). The systemic drug availability is relevant for the exposure of lung nematodes to the active drug/metabolites. Thus, the levels of monepantel/sulphone systemically available after its oral administration at 2.5 mg/kg to sheep may be below the critical amount required to reach an optimal efficacy against lung nematodes (Lifschitz et al., 2014). Although monepantel sulphone plasma concentrations were detected until 9–12 days post-administration, efficacy studies confirmed that monepantel is a short-acting anthelmintic (Hosking, 2010). Thus, monepantel anthelmintic activity may be based on the great drug/metabolite accumulation in the gastrointestinal tissues and fluid contents during the first 2–3 days post-treatment. It is also likely that the level of drug concentration below 0.1 μg/mL measured in plasma between 4 and 9 days post-treatment may not be sufficient to obtain a good activity against the different species of nematodes located in different segments of the digestive tract (Lifschitz et al., 2014).

The development of resistance to the traditional anthelmintic families in sheep and goats is a seriously increasing problem worldwide. There is a real need to include new pharmacological tools in nematode control programmes in livestock, but it is also necessary to further optimize the use of traditional and modern anthelmintics (Kaminsky et al., 2013). Inappropriate use of the new anthelmintics may rapidly lead to the development and spread of resistance. Unfortunately, field cases of monepantel resistance have already been reported in goats and sheep (Scott et al., 2013; Mederos et al., 2014; Van den Brom et al., 2015). Resistance to monepantel was corroborated in *Teladorsagia circumcincta*, *Thichostrongylus colubriformis* (New Zealand) and *H. contortus* (Uruguay and Netherlands). Resistance to monepantel was established in a short period (between 2 and 4 years) after a limited number of generation cycles of nematodes (Scott et al., 2013; Van den Brom et al., 2015). Whereas, the reported resistance cases in New Zealand and Netherlands showed a history of highly frequent monepantel treatments (between 13 and 17 treatments in a 2 years period), the administration frequency of monepantel in Uruguay was much lower (Mederos et al., 2014). Previous research has demonstrated that mutant *C. elegans* and *H. contortus* had a reduced susceptibility to monepantel (Kaminsky et al., 2008; Rufener et al., 2009). Several mutations were found in the *acr-23* and Hco-mptl-1 genes in these parasites which may account for a resistance mechanism to monepantel activity (Kaminsky et al., 2008; Rufener et al., 2009). However, as monepantel sig-

nificantly increases cytochrome P-450-related activities in sheep (Stuchlíková et al., 2015), and also interacts with the ABC transporter, BCRP (Halwachs et al., 2014), the involvement of other mechanisms of resistance should be evaluated.

The emergence of new anthelmintic compounds into the veterinary pharmaceutical market reinforces the need for deeper understanding of their pharmacological properties to avoid their misuse and therefore, delay the appearance and spread of resistance. It is also necessary to develop molecular methods for the early detection of resistance to the new compounds in order to inform treatment strategies in the field and, thus, prolong the lifespan of these molecules for controlling parasitic diseases in livestock.

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