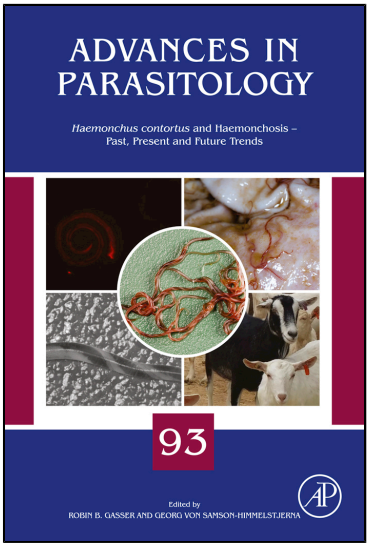

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CHAPTER ELEVEN

Gaining Insights Into the Pharmacology of Anthelmintics Using *Haemonchus contortus* as a Model Nematode

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Contents

Abstract

Progress made in understanding pharmacokinetic behaviour and pharmacodynamic mechanisms of drug action/resistance has allowed deep insights into the pharmacology of the main chemical classes, including some of the few recently discovered anthelmintics. The integration of pharmaco-parasitological research approaches has contributed considerably to the optimization of drug activity, which is relevant to

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preserve existing and novel active compounds for parasite control in livestock. A remarkable amount of pharmacology-based knowledge has been generated using the sheep abomasal nematode *Haemonchus contortus* as a model. Relevant fundamental information on the relationship among drug influx/efflux balance (accumulation), biotransformation/detoxification and pharmacological effects in parasitic nematodes for the most traditional anthelmintic chemical families has been obtained by exploiting the advantages of working with *H. contortus* under in vitro, ex vivo and in vivo experimental conditions. The scientific contributions to the pharmacology of anthelmintic drugs based on the use of *H. contortus* as a model nematode are summarized in the present chapter.

1. INTRODUCTION

Parasitic nematodes of ruminants represent one of the greatest infectious disease problems in grazing livestock systems worldwide. Despite promising research results, nonchemical parasite control strategies are not yet available for routine, commercial use. Thus, nematode control in livestock still relies on the use of antiparasitic drugs, which comprise the largest sector of the animal pharmaceutical industry. The integration of available information on the host-parasite-environment relationship with an understanding of the pharmacological properties of traditional antiparasitic molecules has contributed to more efficient parasite control. However, due to the magnitude of the investment in drug medications, it is necessary to acquire scientific information on how to improve the use of available molecules, and particularly, to avoid/delay the development of anthelmintic resistance. 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 to assess the potency of anthelmintics in vitro. This inconvenience has been a major limitation to estimating the active drug concentration required to achieve optimal in vivo activity and has delayed advances in drug development. However, progress made in understanding pharmacokinetic behaviour and pharmacodynamic mechanisms of drug action/resistance has allowed deep insights into the pharmacology of the main chemical classes including some of the few recently discovered anthelmintics. A remarkable amount of pharmacology-based knowledge has been acquired using the sheep abomasal nematode *Haemonchus contortus* as model. The scientific contributions to the pharmacology of anthelmintic drugs based on the use of *H. contortus* as a model nematode are summarized in the present chapter.

2. BACKGROUND ON THE PHARMACOLOGY OF ANTHELMINTIC DRUGS

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 when it reaches the predilection site of the parasite in the host. Knowledge of the processes of drug/metabolite 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. The activity of an anthelmintic drug depends not only on its binding to the specific receptor (pharmacodynamics), but also on its ability to reach high and sustained concentrations in the tissue(s) where the parasite is located, which allows the delivery of effective drug concentrations at the receptor within the parasite cells, in sufficient time, to cause the therapeutic effect (Thompson et al., 1993). There is a close relationship between pharmacokinetics (which determine drug exposure at the location site of the parasite) and pharmacodynamics (drug effect). The drug needs to pass through different 'barriers' to reach its specific target receptor within a target parasite (ie, *H. contortus*). Dissolution of drug particles in gastrointestinal (GI) fluids is a particularly important phenomenon for drugs administered as suspensions by the oral route (such as benzimidazole compounds and morantel/pyrantel). Dissolution is a crucial step because drug particles must dissolve in the enteric fluids, to allow absorption through the GI mucosa and/or penetration through the external surface of GI nematodes. The undissolved drug particles passing down the GI tract in the luminal content are excreted in the faeces without exerting anthelmintic action. Anthelmintic compounds formulated as drug solutions for parenteral injection in domestic animals (eg, macrocyclic lactones and levamisole) do not require dissolution before systemic absorption. In those cases, the GI secretion process (ie, abomasal secretion) is an important step to assure drug-nematode contact. Drug absorption is a main limiting factor that determines the amount of drug reaching the systemic circulation (systemic exposure). The reversible exchange between the bloodstream and tissues allows the drug to achieve concentrations that are anthelmintically active in the tissues where the parasite is located. The overall pharmacokinetic process, including drug absorption, tissue distribution and its biotransformation/elimination pattern, is crucial to allow the drug to reach the target parasites located in different tissues at sufficient concentrations/time to exert an anthelmintic

effect. Finally, the access of anthelmintic molecules to intracellular sites of action depends on their ability to penetrate the external cuticular (nematodes) or tegumental (cestodes and trematodes) structure of the parasite. Lipophilicity and concentration of the active drug, physicochemical features of the parasite-surrounding medium, the structure of parasite's external surface, are among the factors affecting the transfer (diffusion) and accumulation of the active drug into the target parasite(s). The particular mode of action of each compound will affect the onset and the characteristic of the anthelmintic effect. Altogether, these different factors will determine the final anthelmintic activity.

The time that a parasite is exposed to an active drug concentration 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, and its relationship with the mode of action of each particular molecule provides a basis for understanding the differences in efficacy observed for the different chemical families. Benzimidazoles (albendazole, fenbendazole, etc.), imidazothiazoles (levamisole), macrocyclic lactones (eg, ivermectin, doramectin and moxidectin), salicylanilides (closantel), tetrahydropyrimidines (eg, morantel and pyrantel), organophosphates (eg, coumaphos and naphthalophos) and the novel spiroindoles (derquantel) and aminoacetonitrile derivatives (AADs, monepantel) are the main chemical families used to control nematodes of ruminants, including *H. contortus*. The potency of most anthelmintics is dependent on their affinity for specific receptors, but also on the kinetic properties that facilitate the achievement of effective drug concentrations at the site of action (Thompson et al., 1993). The main pharmacological properties and the pharmacokinetic–pharmacodynamic relationships for the most common drugs used to control *H. contortus* and other nematodes of ruminants are described in the following.

Benzimidazoles: Benzimidazole and pro-benzimidazole anthelmintics are widely used in veterinary medicine. The pro-benzimidazoles (febantel and netobimin) are inactive prodrugs that are metabolically converted into anthelmintically active molecules in the host. The benzimidazole methylcarbamates are usually most commonly used. Albendazole, fenbendazole and their sulphoxide derivatives (albendazole sulphoxide or ricobendazole and oxfendazole, respectively) are currently among the most extensively used benzimidazole anthelmintics in ruminants. They are indicated for the removal and control of a broad spectrum of helminth parasites, including tapeworms, abomasal and intestinal nematodes (adults and L_4), lungworms

(adults and larval stages) and have ovicidal activity (~8 h after treatment). Additionally, albendazole is active against adult (>12 week-old) *Fasciola hepatica* (liver fluke). The pharmacological activity of benzimidazoles is based on the binding to parasite β-tubulin, which produces subsequent disruption of the tubulin-microtubule dynamic equilibrium (Lacey, 1988). Thus, all the functions ascribed to microtubules at the cellular level are altered (cell division, maintenance of cell shape, cell motility, cell secretion, nutrient absorption and intracellular transport). This particular mode of action requires a sufficient time of drug-parasite contact to assure that the biochemical changes following the disruption of the equilibrium of the tubulin-microtubule dynamic result in parasite expulsion. After in vivo administration of albendazole to artificially *H. contortus* infected lambs, parasites were recovered viable (with appreciable movement) up to 24 h following treatment (Alvarez et al., 2000). This and previous observations (Sangster and Prichard, 1985) demonstrate that expulsion from the predilection site is due to an inability of the parasite to maintain homeostasis. Adults of *H. contortus* may be able to survive for a short time after treatment, but if the impairment of essential functions is extended for a sufficiently long time, the ability of the parasite to remain in the abomasum is affected. Assisted by the flow of ingesta, adult parasites are readily expelled from the lumen of the gut, which helps explain why helminth parasites located outside of the luminal space (ie, mature *F. hepatica*, interstitial dwelling and/or arrested larval stages) are less susceptible to drug treatment compared with adult stages situated within the gut lumen.

As a chemical class, the benzimidazole methylcarbamates have a limited solubility in water and are prepared as suspensions for oral or intraruminal (IR) administration to ruminants. Drug particles must dissolve in the enteric fluids to facilitate absorption of the benzimidazoles through the GI mucosa. Benzimidazole dissolution is notably increased by extreme pH values. In ruminants, benzimidazole dissolution occurs mainly in the abomasum, favoured by a low pH. A drug that does not dissolve in the GI contents passes down the gut and is excreted in the faeces without exerting its action. Shortly after administration, benzimidazole compounds are almost completely adsorbed to particulate matter in the ingesta, reaching an equilibrium between particulate and fluid portions of the gut content (Hennessy, 1993). The rumen acts as a drug reservoir by slowing the transit time of ingesta, which results in improved systemic availability of benzimidazole compounds as a consequence of a greater dissolution of drug particles in the abomasum at a low pH (Lanusse and Prichard, 1993a). The plasma levels

of the parent sulphides (ie, albendazole, fenbendazole) and/or its active sulphoxide metabolites (albendazole sulphoxide and oxfendazole) reflect the amount of drug dissolved in the GI tract. The drug dissolved in the GI fluid is available for absorption and/or for diffusion through the cuticle of adult *H. contortus* located in the abomasum. Thus, *H. contortus* is largely exposed to "free" dissolved (not adsorbed to digest a material) drug molecules available in the GI content, and to drug present in gastric secretions, which recycles between plasma and the abomasum content. Since adult *H. contortus* is a blood-feeding parasite, it may also be exposed to drug available via the bloodstream (Alvarez et al., 2000).

Since benzimidazole anthelmintics are mainly administered by the oral route, "first pass" metabolism is relevant in the kinetic behaviour of these compounds. Aromatic benzimidazole derivatives, such as fenbendazole and oxfendazole, require more extensive hepatic oxidative metabolism than aliphatic derivatives (albendazole and albendazole sulphoxide) to achieve sufficient polarity for excretion (Hennessy, 1993). Consequently, while low fenbendazole concentrations are found in plasma following its oral/IR administration to sheep and cattle, albendazole is not detected in the bloodstream after its administration as a parent drug in either species. Biotransformation takes place predominantly in the liver, although metabolic activity is apparent in extrahepatic tissues such as lung parenchyma and small intestinal mucosa (Virkel et al., 2004). Therefore, albendazole sulphoxide and oxfendazole can be reduced back to their respective thioethers by ruminal and intestinal microflora and may act as a source of albendazole and fenbendazole, respectively, in the GI tract. The high efficacy of albendazole sulphoxide and oxfendazole against *H. contortus* depend, in part, on this bacterial reduction of the sulphoxide to the more pharmacologically active thioethers (Lanusse and Prichard, 1993b). In fact, although albendazole is not detected in the bloodstream, it has been detected in high concentrations in the abomasal mucosa as well as within *H. contortus* recovered from treated sheep (Alvarez et al., 2000). The extensive distribution of benzimidazole methylcarbamates from the bloodstream to the GI tract and to other tissues may contribute to achieve good anthelmintic efficacy against parasites located in peripheral tissues such as the GI mucosa and lungs.

Imidazothiazoles: Levamisole is readily available imidazothiazole compound for use in veterinary medicine. Levamisole (the L-isomer of tetramisole) is a nematodicidal drug with a broad activity in several host species against lung and GI nematodes but is inactive against parasitic cestode and trematodes. Levamisole is active against mature stages of the major GI

nematodes and both mature and larval stages of lungworms. However, levamisole shows little activity against arrested larval stages (Einstein et al., 1994). Levamisole is highly effective against adult stages of species of *Haemonchus*, *Ostertagia*, *Cooperia*, *Nematodirus*, *Bunostomum*, *Oesophagostomum*, *Chabertia* and *Dictyocaulus*. A major advantage is its formulation flexibility allowing various routes of administration (oral, parenteral and topical). Levamisole is a cholinergic receptor agonist and elicits spastic muscle paralysis due to prolonged activation of the excitatory nicotinic acetylcholine receptors (nAChR) on nematode body wall muscle. Since levamisole affects the neuromuscular coordination, it causes a spastic paralysis and rapid release of adult *H. contortus* from its location in the abomasum. The rate of levamisole absorption differs according to the route of administration. The systemic availability of levamisole in sheep is significantly lower after oral/IR administration (42–45%) compared with subcutaneous (SC) injection (Bogan et al., 1982). Levamisole is rapidly and extensively metabolized to a large number of metabolites in the liver. The main metabolizing pathways appear to be oxidation, hydrolysis and hydroxylation. *Haemonchus contortus* is able to metabolize levamisole into an unidentified compound (Sangster et al., 1988). Levamisole and metabolites (glucuronyl or S-cysteinyl-glycine conjugates) are excreted via urine (60%) and faeces (30%). Unchanged levamisole accounts for 5–10% of the dose in urine and faeces in cattle, sheep and swine (Nielsen and Rasmussen, 1982). Since levamisole is short-lived in plasma and GI contents, the peak concentration rather than the duration of exposure is important for its anthelmintic effect.

Tetrahydropyrimidines: Pyrantel and its methyl analogue morantel are the two members of this family available in the veterinary market. Pyrantel is formulated as tartrate, citrate or pamoate (also known as embonate) salts. Pyrantel pamoate is practically insoluble in water, while the tartrate salt is more water-soluble. Morantel is mainly formulated as a tartrate salt. Tetrahydropyrimidine compounds act selectively as agonists at synaptic and extrasynaptic nAChR on nematode muscle and produce contraction and spastic paralysis (Martin, 1997). These anthelmintics share biologic properties with acetylcholine and act essentially by mimicking the paralytic effects of excessive amounts of this natural neurotransmitter. The pyrantel pamoate salt is poorly absorbed from the GI tract, with the tartrate salt being more readily absorbed than the pamoate. Peak plasma levels occur at highly variable times in ruminants. Absorbed drug is rapidly metabolized to inactive metabolites and excreted into the faeces. Morantel is negligibly absorbed via the gut in ruminants and is thus largely excreted as the unmetabolized parent

compound in the faeces. Both pyrantel and morantel are highly efficacious against adult and immature stages of *H. contortus* in the abomasum and have minimal activity against arrested larval stages (Einstein et al., 1994). The low GI absorption and/or efficient metabolism to inactive metabolites explain the absence of systemic anthelmintic activity against lungworms as well as arrested tissue larvae.

Organophosphate compounds: Organophosphate compounds have insecticidal and nematodicidal activities. The main organophosphates used as anthelmintics in ruminants are haloxon, coumaphos, naphthalophos and crufomate. Organophosphates' mode of action related to an irreversible inhibition of acetylcholinesterase by phosphorylation, which results in acetylcholine accumulation at cholinergic receptors. The resultant accumulation of acetylcholine leads to a paralysis and death of the parasite. Overall, after its oral administration, organophosphates have satisfactory efficacy against nematode parasites of the abomasum (particularly *Haemonchus*) and small intestine, but lack satisfactory efficacy against nematodes of the large intestine (*Oesophagostomum* and *Chabertia*). Naphthalophos efficacy against adult *H. contortus* reaches 99–100% even at a reduced dose of 25 mg/kg (at 75 mg/kg is also active against *H. contortus* fifth stage) (Courtney and Roberson, 1995). As described for levamisole and pyrantel/morantel, the efficacy of the organophosphates against arrested stages of *H. contortus* and other abomasal and intestinal nematodes is rather low. This finding could be explained by the short time of drug-parasite contact and the reduced exposure of the larval/immature stages located at the GI mucosa.

Salicylanilides: The most used salicylanilide compound to control *H. contortus* is closantel. It is formulated for both, oral and intramuscular administration. Closantel is highly effective for the treatment of adult flukes and is also highly effective against *H. contortus* in sheep. It is used as an alternative drug for the treatment of ivermectin-, benzimidazole-, levamisole- and morantel-resistant isolate of this nematode. It is also effective against certain ectoparasites such as lice, ticks, mites and the nasal bot *Oestrus ovis* (see Lanusse et al., 2009). The mode of action of closantel and other salicylanilides is mainly based in the uncoupling of oxidative phosphorylation. Additionally, closantel has been identified as a potent and specific inhibitor of filarial chitinases (Gloeckner et al., 2010). Closantel pharmacokinetic behaviour is characterized by high plasma protein binding, low volume of distribution (in spite of its high lipophilicity) and long elimination half-life. Its broad-spectrum activity relies on its long persistence in the systemic

circulation (up to 90 days in sheep), which facilitates drug accumulation into target parasites, particularly haematophagous parasites. These characteristics explain the high efficacy against immature and adult *H. contortus* for up to 28 days after treatment (Lanusse et al., 2009). Following intramuscular administration of closantel to sheep, the expulsion of susceptible *H. contortus* begins at about 8 h following treatment and at 16 h most of the worms are removed (Rothwell et al., 1993). Additionally, it has been shown under in vivo conditions that *H. contortus* expulsion from its predilection site starts once motility is affected by closantel (Rothwell et al., 1993).

Macrocyclic lactones: The avermectins and milbemycins are closely related 16-membered macrocyclic lactones, and are active at extremely low (0.2 mg/kg) dosages against endo- and ectoparasites (Shoop et al., 1995). The avermectin family includes a series of natural and semisynthetic molecules, such as abamectin, ivermectin, doramectin, eprinomectin and selamectin. Ivermectin was the first marketed endectocide molecule. Nemadectin, moxidectin and milbemycin oxime belong to the milbemycin family. The macrocyclic lactones induce a reduction in motor activity and paralysis in both arthropods and nematodes. The paralytic effects are mediated mainly through glutamate-gated chloride channels (GluCl) (Martin, 1997). Furthermore, a study of *Caenorhabditis elegans* (see Hernando and Bouzat, 2014) revealed that the muscle levamisole-sensitive receptor (L-AChR), one of the main excitatory receptors involved in parasite locomotion is profoundly inhibited by ivermectin. Macrocyclic lactones are extremely effective against adult and larval stages of most GI and lung nematodes, exerting a prolonged 'protective' effect. The clinical efficacy of the macrocyclic lactones is closely related to their pharmacokinetic behaviour, being the time of parasite exposure to active drug concentrations, required to obtain optimal and persistent antiparasitic activity. The highly lipophilic macrocyclic lactones are extensively distributed via the bloodstream to different tissues. The persistence of the broad-spectrum anthelmintic activity against adult and immature GI parasites is facilitated by their advantageous pattern of distribution and prolonged residence in the mucosal tissues of the digestive tract. The long persistence of macrocyclic lactones is mainly explained by their wide tissue distribution, low metabolism/organic clearance and enterohepatic recycling. As pointed out by Hennessy (2000), the extent to which the biliary-secreted drug is presented to the gut lumen, reabsorbed as free compound and participates in the enterohepatic cycle is a major contributor to parasite exposure. The deposition of macrocyclic lactones in adipose tissue may represent a drug reservoir that contributes

to the long persistence of these compounds in the bloodstream and in different sites of parasite location. Following oral administration, ivermectin reaches a relatively high concentration in plasma (lower than that obtained after the parenteral treatment), but the concentrations attained in the GI tissue are relevant in terms of efficacy against resistant *H. contortus* (see specific comments in Section 3.2).

Monepantel: Monepantel is a novel AAD that is active against larval and adult stages of GI nematodes of sheep, including *H. contortus* which are highly resistant to all the other available anthelmintic chemical families. Genetic studies of the free-living nematode *C. elegans* and of *H. contortus* have shown that monepantel acts as an agonist on the nAChR, producing spastic paralysis and death of the worms (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). The systemic availability of the monepantel parent compound was significantly lower than that observed for its main metabolite, monepantel sulphone. Furthermore, after the oral administration of monepantel to sheep, the persistence of monepantel sulphone (9 days) in plasma was significantly longer than that of monepantel (2 days) (Lifschitz et al., 2014). An equivalent pharmacological potency of the monepantel parent drug and its sulphone metabolite against larvae of GI nematodes has been suggested using in vitro evaluation assays (Karadzovska et al., 2009). It seems unlikely that monepantel will adequately control lung nematodes at the dose used for GI 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 plasma concentrations of monepantel sulphone were detected until 9–12 days after administration, an efficacy study has confirmed that monepantel is a short-acting anthelmintic (Hosking, 2010). Thus, monepantel anthelmintic activity may be based on a substantial drug/metabolite accumulation in the GI tissues and fluid contents in the first 2–3 days following treatment. It is also likely that the level of drug concentration <0.1 μg/mL measured in plasma between days 4 and 9 after treatment may not be sufficient to obtain adequate activity against the different species of nematodes located in different segments of the digestive tract (Lifschitz et al., 2014).

Derquantel: Derquantel is a semisynthetic derivative of paraherquamide and belongs to the chemical family of spiroindoles. The anthelmintic activity of derquantel is based on interference with B-subtype nAChR, acting as an antagonist and leading to a flaccid paralysis in nematodes (Ruiz-Lancheros et al., 2011). This differential mode of action contributes to its activity against nematodes that are resistant to other currently available chemical groups. In an attempt to optimize its anthelmintic activity and to decrease the selection pressure on resistant nematode isolates, derquantel has been launched for use in combination with abamectin. Derquantel combined with abamectin has $\geq$98.9% efficacy against *H. contortus* (fourth stage and adult stages) (Little et al., 2011), and derquantel reaches an absolute bioavailability of 56% following the oral combined treatment (EMEA, 2010). There is no significant adverse kinetic interaction between derquantel and abamectin when they are co-administered (Friedlander et al., 2012). Derquantel undergoes biotransformation to a large number of metabolites over a short time period (EMEA, 2010).

Although comparative work showed that the derquantel–abamectin combination failed to reduce burdens of L4s of macrocyclic lactone-resistant *H. contortus* and *Teladorsagia* spp. (George et al., 2012; Kaminsky et al., 2011), there are no reports or known field cases of anthelmintic resistance to derquantel. There is pharmacology-based evidence of a synergistic interaction between derquantel and abamectin (Puttachary et al., 2013). Derquantel may interact additively with the macrocyclic lactone abamectin, and, at higher acetylcholine concentrations, the interaction is synergistic. This interaction is based on the noncompetitive antagonism of abamectin on the nicotinic receptor that potentiates the competitive antagonism produced by derquantel (Puttachary et al., 2013). However, this combination is not based on drugs with similar pharmacokinetic profiles. Concerns about matching half-lives of elimination to minimize exposure to suboptimal concentrations of single constituent actives or their bioactive metabolites at the tail of the elimination curve may be more relevant for synergistic combinations compared with combinations that produce additive effects (Geary et al., 2012). It is expected that the derquantel–abamectin combination achieves adequate parasite drug exposure to ensure the synergistic effect observed in vitro. However, the different plasma profile between derquantel and abamectin indicates that the combination will not prevent abamectin from selecting for resistance during a period of suboptimal concentrations at the tail of the elimination curve, as would occur if abamectin is used alone.

3. ASSESSMENT OF PARASITE EXPOSURE TO ANTHELMINTIC DRUGS USING *HAEMONCHUS CONTORTUS* AS A TARGET NEMATODE

3.1 *Haemonchus contortus* as a tool to explore ex vivo and in vivo drug accumulation, metabolism and activity in nematode parasites

The drug-parasite relationship can be characterized using in vitro, ex vivo and/or in vivo approaches. The transport of different substances has been investigated in vitro using isolated nematode cuticle, which offers some advantages over intact organisms, particularly for the interpretation of permeability data (Thompson et al., 1993). Ex vivo assays involve the use of intact, live parasites in a closed perfusion system, allowing the study of the relative contributions of the transcuticular and oral pathways as well as the influence that underlying tissues, such as somatic muscles and gut, may have on cuticular drug transport (Ho et al., 1992). Ex vivo characterization of drug transfer offers technical advantages and reliable results (Cross et al., 1998; Ho et al., 1992; Mottier et al., 2006). Additionally, ex vivo experiments have been performed, to deeply understand drug effect on parasites exposed to anthelmintics. However, it is clear that the drug-parasite interaction may differ under in vivo conditions, where worms are exposed to changing drug concentration over the time in a variable physiological environment.

The helminth's external surfaces serve as a barrier which shields the organism from external conditions, and are vital for nutrient uptake, osmoregulation, immunoprotection and structural support. The tegument of flatworms is a membrane-bound syncytium, allowing the active transport of nutrients and drugs (Fetterer and Rhoads, 1993). In contrast, a nematode's cuticle has been considered to be a barrier limiting the entry of large molecules into the worm (Fetterer and Rhoads, 1993; Ho et al., 1990). *Haemonchus contortus* lives in the abomasum attached to the mucosa or free in the abomasal content and, because of its haematophageous nature, anaemia is often a clinical feature of the infection. Anthelmintic drugs can reach *H. contortus* either by oral ingestion of blood or by transcuticular uptake/diffusion (Geary et al., 1995). Therefore, the concentration of active drug in the bloodstream (oral ingestion) and/or abomasal fluid/mucosa (transcuticular diffusion) is relevant to the clinical efficacy against this parasite. Albendazole is not found in the bloodstream after enteral administration to sheep and cattle, and the active albendazole sulphoxide metabolite has been postulated to be responsible for

the activity against lungworms and tissue-dwelling parasites (Marriner and Bogan, 1980). However, higher concentrations of albendazole were measured in *H. contortus* recovered from infected treated sheep, compared with those of its sulphoxide metabolite (Alvarez et al., 2000). Albendazole and albendazole sulphoxide were rapidly taken up by *H. contortus* exposed in vivo to albendazole, being detected in the parasite between 0.5 and 12 h following administration (Alvarez et al., 2000). Interestingly, the concentration profiles of albendazole and albendazole sulphoxide during that period of time were greater in the *H. contortus* specimens than in abomasal mucosa and abomasal fluid. Since albendazole was not detected in peripheral plasma, only drug from the pool found in abomasal fluid and mucosa may be able to reach the nematode via transcuticular diffusion. The lipoidal hypocuticle tissue is the rate-determining barrier and only allows sufficiently small molecules to traverse the aqueous-filled, negatively charged collagen matrix of the cuticle (Ho et al., 1992). Lipophilicity facilitates drug diffusion through the nematode cuticle (Ho et al., 1992); thus, lipophilic drugs such as albendazole may have a greater capability to cross the external surface of the nematode than the more polar albendazole sulphoxide. The exposure of the nematode to high concentrations of albendazole sulphoxide in the abomasal lumen may compensate for its lower rate of diffusion across the cuticle. In spite of the lower concentrations of albendazole in abomasal fluid, its high lipophilicity may enhance penetration through the external parasite surface. It should also be pointed out that *H. contortus* may feed on portal blood and the relevance of the portal circulation as a source of albendazole might be considered. However, the low albendazole concentrations found in portal blood (Alvarez et al., unpublished observations) do not explain the high concentrations measured in *H. contortus* (see Alvarez et al., 2000). These findings confirm the relevance of the transcuticular diffusion process, even in a blood-feeding parasite, such as *H. contortus*, where the higher lipophilicity of albendazole (octanol–water partition coefficient: 3.83) may have accounted for its greater penetration compared with its sulphoxide derivative (partition coefficient: 1.24) (Mottier et al., 2003). The greater antiparasitic activity of albendazole compared with albendazole sulphoxide has been demonstrated in vitro by assessing binding to parasite tubulin (Lacey, 1990; Lubega and Prichard, 1990) as well as nematode motility (Petersen et al., 1997). The higher affinity to parasite tubulin and the greater capacity to diffuse into the parasite observed for albendazole suggest that the parent drug may exert greater activity than its metabolite albendazole sulphoxide against abomasal nematodes.

Available information (Cross et al., 1998; Geary et al., 1995; Ho et al., 1992; Sims et al., 1996) indicates that oral ingestion does not allow major drug accumulation in nematodes, particularly for lipophilic molecules present in large concentrations in tissues and in gut contents. A different situation might occur with anthelmintics that bind strongly to plasma proteins. The nematodicidal activity of closantel is almost restricted to *H. contortus*. It is likely that the anthelmintic effect of closantel against blood-feeding parasites depends on its intraparasite accumulation mediated by oral ingestion. Closantel is a highly lipophilic compound that is extensively bound (>99%) to plasma proteins and has a long half-life (14.5 days) (Michiels et al., 1987). Closantel-resistant *H. contortus* accumulated significantly less [^{14}C]closantel than did susceptible worms (Rothwell and Sangster, 1997). The reduced closantel accumulation in resistant worms was attributed to different factors including increased efflux from cells, reduced absorption of closantel across the worm intestine and/or a reduced feeding rate in the resistant isolate (Rothwell and Sangster, 1997). The measurement of reduced closantel levels in resistant *H. contortus* provided a clear in vivo association between drug resistance and decreased drug accumulation (closantel) in a target nematode. Although oral ingestion could be an important route of closantel entry into *H. contortus*, ex vivo data indicate some degree of closantel accumulation by transcuticular transport (Rothwell and Sangster, 1997).

Drug molecules move across cell membranes either by passive diffusion or specialized transport mechanisms. In the passive diffusion process, the membrane behaves as an inert lipid-pore boundary, and drug molecules traverse this barrier either by diffusion through the lipoprotein region or, alternatively, by filtering through aqueous pores (channels) without the expenditure of cellular energy if they are of a sufficiently small size (Ho et al., 1992). Specialized transport is another potential mechanism of drug entry into target parasites. This type of transport process is relatively selective towards the chemical nature of a substance and requires direct expenditure of energy (Baggot, 1982). If the entry of an anthelmintic into a target parasite is mediated by specialized transport (a saturable process), the exposure time is critical to determine the amount of drug accumulated inside the parasite. On the other hand, if passive diffusion is the main mechanism of entry of anthelmintics (over active transport), the concentration and the time will determine the drug concentration within the worm, and the restrictions imposed by cuticular lipid barriers will probably be similar to those of standard cellular membranes. The

knowledge of the mechanism of drug entry to parasites is a key to understand drug-parasite relationship.

There is previous evidence that would indicate that different chemical substances, including anthelmintic drugs, are mainly taken up through the external surfaces of flatworms (Alvarez et al., 1999, 2000, 2001, 2004; Bennett and Köhler, 1987; Mottier et al., 2003) and nematodes (Alvarez et al., 2001; Cross et al., 1998; Ho et al., 1990; Sims et al., 1992a) as opposed to oral ingestion by the parasite. In both cases (tegument/cuticular or intestinal entry), the anthelmintic molecules need to pass through cell membranes to reach the biophase around the specific receptor, which may be influenced by the pharmacokinetic behaviour of the compound, the concentration gradient and time of drug-parasite contact. In the case of passive diffusion, as the concentration gradient increases on one side of the membrane, drug concentrations on the other side will increase in favour of the concentration gradient. If transporters are involved in the mechanism of drug absorption, saturation may follow the increase of drug concentration on one side of a membrane. Therefore, the concentration achieved on the other side does not necessarily follow a linear relationship with the surrounding concentration in the medium.

Mottier et al. (2006) reported that under ex vivo conditions, benzimidazole anthelmintics accumulated inside *Moniezia benedeni* (fenbendazole), *F. hepatica* (triclabendazole sulphoxide) and *Ascaris suum* (fenbendazole and oxfendazole) in favour of the concentration gradient. Absorption of benzimidazole was linear for the range of concentrations assayed. Additionally, it is well known that lipid solubility facilitates drug diffusion through the external surfaces of *A. suum* (see Ho et al., 1992), *M. benedeni* (see Mottier et al., 2003) and *F. hepatica* (see Mottier et al., 2004). Oxfendazole, the sulphoxide metabolite of fenbendazole, is pharmacologically less active and it has a lower lipid solubility (octanol–water partition coefficient: 2.03) than the parent compound (octanol–water partition coefficient: 3.93) (Mottier et al., 2003). Fenbendazole concentrations inside ex vivo incubated *M. benedeni*, for the entire concentration gradient assayed (Mottier et al., 2006), were greater than those observed for its sulphoxide metabolite. The main way that a given drug molecule reaches the interior of a cestode parasite is by passing through its tegument and it is evident that concentration and lipophilicity are the major factors determining drug penetration (Mottier et al., 2006). Similarly, the higher lipophilicity of albendazole (octanol–water partition coefficient: 3.83) (Mottier et al., 2003) accounted for its greater penetration through the external parasite surface, compared with its sulphoxide derivative

(octanol–water partition coefficient: 1.24), observed in *M. benedeni* under ex vivo conditions (Mottier et al., 2003). Data reported by Bártíková et al. (2012) showed that flubendazole is able to effectively penetrate adult *H. contortus* under ex vivo conditions due to its high lipophilicity. Passive diffusion is probably the only mechanism involved in both flubendazole import and efflux from *H. contortus* (see Bártíková et al., 2012). Additionally, no differences in flubendazole ex vivo accumulation were found among four *H. contortus* isolates with distinct sensitivities to anthelmintics (Bártíková et al., 2012). These results relate to those described by Alvarez et al. (2004), who reported that physicochemical composition of the parasite's surrounding environment (in which it is immersed) plays a pivotal role in the process of drug access to *F. hepatica*. Since the concentration gradient, drug lipid solubility, physicochemical characteristics of the incubation medium as well as the structure and composition of the external surface of a helminth parasite are critical for the penetration of benzimidazole molecules through these surfaces; it is clear that passive diffusion is the main mechanism implicated in the entry of these anthelmintic drugs into nematodes and cestodes. The same is likely to be true for other anthelmintic drugs.

The small size of *H. contortus* limits the ex vivo experiments intended to evaluate the mechanism of drug entry into this nematode parasites. However, information has been obtained using other nematodes as models. The transcuticular transfer appeared to be the main route of passage of ivermectin into the filarial nematode *Onchocerca ochengi* (see Cross et al., 1998). The entry of ivermectin into adult *O. ochengi* occurs by the transcuticular route, where the marked foldings of its cuticle greatly increase the surface area, favouring ivermectin diffusion. Additionally, in the absence of pharyngeal pumping, first-stage larvae of *C. elegans* submerged in ivermectin became paralysed (Smith and Campbell, 1996), which reinforces the relevance of ivermectin transcuticular penetration. The in vitro accumulation of [^{14}C] closantel in adult *H. contortus* was measured both in the absence and presence of ivermectin (used to prevent closantel oral uptake) (Rothwell and Sangster, 1997). Closantel lipophilicity may explain its transcuticular entry into closantel-susceptible and closantel-resistant *H. contortus*, even in the presence of ovine serum albumin and when oral ingestion was abolished (Rothwell and Sangster, 1997). Nevertheless, the extensive binding to albumin may facilitate the oral ingestion of closantel by haematophagous parasites such as *H. contortus*.

The rate of penetration across the *H. contortus* cuticle depends mainly on lipophilicity, and, in the case of acidic or basic drugs, on the ionized and

unionized (lipid-permeable) fractions of the drug; this rate is determined by the relationship between drug pK and pH of the aqueous environment in the cuticle. The cuticle consists of (1) collagen-like proteins that form the medial and basal layers; (2) noncollagen proteins that form the epicuticular and external cortical regions and (3) nonstructural proteins associated with the external surface (Fetterer and Rhoads, 1993). The lipoidal hypocuticle tissue is the rate-determining barrier to molecules sufficiently small to traverse the aqueous-filled negatively charged collagen matrix of the cuticle (Ho et al., 1992). Lipophilicity facilitates drug diffusion through the nematode hypocuticle (Ho et al., 1992); thus lipophilic drugs, including benzimidazole methylcarbamates and macrocyclic lactones, may be better able to cross the external surface of the nematode than polar compounds. The water-filled and negatively charged collagenous matrix of the nematode cuticle permits the passage of molecules by molecular size restricted-diffusion (Ho et al., 1992). If the molecule is sufficiently small, it could traverse the aqueous-filled negatively charged collagen matrix of the cuticle (Ho et al., 1992). For example, the external surface of *A. suum* can be breached by drugs, and the rate-determining barrier for passive transport is the lipoidal hypocuticle tissue. The drug lipid—water partition coefficient, pH/pKa relationship and molecular size influencing the diffusion of uncharged molecules indicate that the transcuticular transport of weak acids and bases will be controlled largely by the pH at this surface, since, in the absence of facilitated transport, only unionized species can partition across a lipoidal surface (Sims et al., 1992b). *Ascaris* excretes a number of volatile fatty acids as well as lower levels of two nonvolatile organic acids (end-products of carbohydrate metabolism) via the transcuticular route at sufficient rates to establish and maintain a buffered microenvironmental pH of ~5.0 in the aqueous space of the pores in the cuticle (Sims et al., 1992b). Most drugs are weak organic bases or acids, and exist in solution as both nonionized and ionized forms. While the poor lipid solubility of ionized molecules excludes them from passive diffusion, lipophilic, nonionized moieties passively diffuse across cell membranes until an equilibrium is established. Accumulated data (Alvarez et al., 2001; Bennett and Köhler, 1987; Cross et al., 1998; Ho et al., 1990; Mottier et al., 2003, 2006; Sims et al., 1992a) would indicate that passive diffusion over specialized transport is implicated in drug entry into parasites. However, we cannot exclude the possible participation of an active entry mechanism in parasitic helminths.

Resistance due to increased drug inactivation is one of the most common biochemical mechanism accounting for the antibiotic resistance that is

encountered in the clinical treatment of bacterial infections (Pratt, 1990). The worldwide development of resistance to insecticides is one of the most common examples of biochemical inactivation as a resistance mechanism. In helminths, mechanisms involved in the development of resistance to anthelmintics can result from changes in the target molecule, in drug uptake/efflux mechanisms and also in drug metabolism (Ouellette, 2001). Metabolism/biotransformation of active anthelmintic drugs by resistant helminth parasites could account for drug resistance. The sulphoxidation of triclabendazole to triclabendazole sulphoxide (Alvarez et al., 2005) and triclabendazole sulphoxide to the triclabendazole sulphone metabolite (Robinson et al., 2004) are both greater in resistant than in susceptible flukes. Indeed, triclabendazole resistant flukes have a 39% greater capacity to metabolize the parent drug (Alvarez et al., 2005).

Haemonchus contortus has also been used as a model to explore potential implications of drug metabolism as mechanisms of resistance. Rothwell and Sangster (1997) did not find differences between resistant and susceptible *H. contortus* isolates related to closantel metabolism, and concluded that the contribution of the increased metabolism to closantel resistance between isolates must be trivial. The metabolism of albendazole and the activities of selected biotransformation and antioxidant enzymes was investigated in vitro and ex vivo in three different isolates of *H. contortus*: susceptible and resistant to benzimidazoles, and multidrug resistant (Vokřál et al., 2013a,b). The in vitro data showed significant differences between the susceptible and both resistant isolates regarding the activities of peroxidases, catalase and UDP-glucosyltransferases. Albendazole sulphoxidation was significantly lower in benzimidazole resistant than in the susceptible isolate. Under ex vivo conditions, four metabolic products were identified in *H. contortus* specimens incubated with albendazole, including the sulphoxide and three glucosides derivatives. Interestingly, in both resistant isolates, the ex vivo formation of all albendazole glucosides was significantly higher than in the susceptible isolate (Vokřál et al., 2013a,b). This finding indicates that the altered activities of particular detoxifying enzymes may partly protect *H. contortus* against the toxic effect of albendazole contributing to resistance. Similar experiments were performed to investigate the biotransformation pattern of flubendazole in different *H. contortus* isolates (benzimidazole resistant and susceptible) (Bártíková et al., 2012). Similar to findings for albendazole (Vokřál et al., 2013a,b), the ex vivo formation of all flubendazole metabolites was significantly higher in the resistant compared with a susceptible isolate of *H. contortus* (see Bártíková et al., 2012). Altogether, these findings

are an indication that resistant nematode isolates may have an increased ability to deactivate anthelmintics via biotransformation as a mechanism contributing to drug resistance.

Haemonchus contortus appears to be able to metabolize monepantel under ex vivo conditions via oxidation and hydrolysis reactions (Stuchlíková et al., 2014). The study of monepantel biotransformation in *H. contortus* revealed four monepantel metabolites, including monepantel sulphoxide, monepantel sulphone and two monepantel metabolites derived from the hydrolysis of two different nitrile groups (Stuchlíková et al., 2014). Unlike sheep liver, *H. contortus* adult specimens failed to metabolize monepantel via phase II biotransformation. Results reported by Alvinerie et al. (2001) demonstrate that homogenates of adult *H. contortus* contain enzymes that were able to metabolize moxidectin. A 24-h incubation period was required for the production of detectable amounts of the unique metabolite observed, which did not correspond to the metabolites previously described in vertebrates (Alvinerie et al., 2001). By contrast, adults *H. contortus* were not able to deactivate ivermectin through biotransformation, which indicates that this mechanism does not contribute to the development of ivermectin resistance (Vokřál et al., 2013a,b).

Toxicity of rotenone towards the larvae of *H. contortus* and *Trichostrongylus colubriformis* was increased in the presence of piperonyl butoxide, a well-known cytochrome P450 inhibitor (Kotze et al., 2006). Furthermore, in the same ex vivo experiment, rotenone activity against adult *H. contortus* was also significantly enhanced following pretreatment with piperonyl butoxide. The synergistic effect observed between rotenone and piperonyl butoxide indicates that *H. contortus* and *T. colubriformis* are able to utilize a cytochrome P450 enzyme system to detoxify rotenone and indicates that a role may exist for cytochrome P450 inhibitors to act as synergists for other anthelmintics that are susceptible to oxidative metabolism within the nematode (Kotze et al., 2006). Using the ex vivo approach, *H. contortus* larvae were treated with phenobarbital, as an inductor of detoxification enzymes (Kotze et al., 2014). Co-treatment of larvae with phenobarbital and naphthalophos resulted in a significant increase in the naphthalophos 50% inhibitory concentration (IC50) compared with the treatment of larvae with the anthelmintic alone (Kotze et al., 2014). The phenobarbital-induced drug tolerance was reversed by co-treatment with the UDP-glucuronosyltransferases inhibitors, demonstrating that *H. contortus* larvae possess one or more UDP-glucuronosyltransferase enzymes able to detoxify naphthalophos. In insects, such as houseflies, the typical mechanism of resistance

against chlorinated hydrocarbons (DDT), cyclodienes (dieldrin), organophosphates and carbamates relates to an overproduction of drug-metabolizing enzymes (Pratt, 1990). It is likely that increased biotransformation activity could relate to a mechanism of drug resistance in helminth parasites. The in vitro and ex vivo data summarized here, using *H. contortus* as a nematode model, appear to indicate that at least for some anthelmintic drugs, metabolism/detoxification are associated with anthelmintic resistance mechanisms.

On the other hand, *H. contortus* has been the main nematode of veterinary interest used to interpret different aspects related to the understanding of drug action. It was established early that, under ex vivo conditions, ivermectin inhibits the contraction of pharyngeal muscles responsible for feeding in nematodes and causes paralysis of their body musculature (Geary et al., 1993). Inhibition of pharyngeal pumping was more potent than that of motility in *H. contortus* (see Geary et al., 1993). It was also established that the transcuticular uptake of glucose in *H. contortus* was not altered by ivermectin. Thus, it was hypothesized that if oral ingestion of other nutrients is essential for long-term survival in vivo, disruption of pharyngeal pumping might represent the primary mechanism of ivermectin action (Geary et al., 1993). The interruption of a vital function, such as pharyngeal pumping, which is implicated in nutrient ingestion, excretion, regulation of turgor pressure, etc., may be critical for worm survival. The effect of ivermectin on pharyngeal uptake of (^{3}H-inulin) was measured in larvae of both ivermectin-susceptible and ivermectin-resistant isolates of *H. contortus* (see Kotze, 1998). A higher ivermectin concentration was necessary to reduce by 50% the feeding by the resistant compared with the susceptible isolate (Kotze, 1998). This information indicates that susceptible and resistant isolates can be readily distinguished on the basis of the sensitivity of pharyngeal uptake to macrocyclic lactones. However, the in vivo assessment of ivermectin feeding inhibition in *H. contortus* fails to demonstrate the inhibition of pharyngeal pumping. Adult *H. contortus* recovered from sheep treated with ivermectin 4 h prior to the intravenous [^{3}H]inulin administration showed equivalent feeding levels (over a 1 h period) to those recovered from sheep not treated with ivermectin (Sheriff et al., 2005). There was no difference in the radioactivity in nematodes of an ivermectin-susceptible and ivermectin-resistant isolate recovered from individual sheep with concurrent infections after ivermectin treatment (Sheriff et al., 2005). These results indicate that ivermectin given orally at a dose sufficient to remove drug-susceptible worms by 8 h did not cause a significant reduction of

[^{3}H]inulin uptake in *H. contortus*, which questions the significance of feeding inhibition as in vivo toxic effect of ivermectin on *H. contortus* (see Sheriff et al., 2005). In addition, some more recent findings (Kotze et al., 2012) indicate that, when sensitive worm motility assessment methods are utilized under ex vivo conditions, worm motility is affected at lower abamectin concentrations than worm feeding, suggesting that somatic musculature is a more important target site for abamectin, and likely also for other macrocyclic lactones.

3.2 Influence of the route of drug administration on parasite exposure

The choice of the administration route for anthelmintic drugs in ruminants is based on either management factors or influenced by the technical marketing of the pharmaceutical companies. The selected administration route is relevant and relates to the potency of most anthelmintics and is dependent on their affinity for a specific receptor (site of action) but also on the kinetic properties that facilitate achieving effective drug concentrations at the site of action. A remarkable amount of work on the kinetic behaviour 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 behaviour need to be understood to optimize drug efficacy. The administration of the anthelmintic drug by different routes may account for significant differences in the final parasite drug exposure. The influence of the administration routes will depend on the physicochemical features of each drug. The lack of water solubility is an important limitation for the formulation of benzimidazole compounds, which results in their preparation as suspensions, pastes or granules for oral or IR administration. Thus, after the oral administration of benzimidazole compounds, the dissolution of drug particles in gut contents is a relevant step that has a direct influence on overall anthelmintic efficacy.

Different factors may affect the dissolution process and the pharmacokinetic behaviour of benzimidazoles. The effect of ruminant oesophageal groove closure was described as an important modification for the benzimidazole absorption process. Occasionally, reduced systemic availability and efficacy of benzimidazole methylcarbamates have been found after oral administration compared with IR administration (Hennessy and Prichard, 1981). A portion of the orally administered anthelmintic may bypass the rumen, rapidly entering the abomasum via the oesophageal groove. As a

consequence, the poor absorption due to insufficient time for dissolution of benzimidazole suspension's particles results in a reduced plasma bioavailability of active benzimidazole metabolites. Such an effect may indicate that the so-called 'reservoir' and 'slow delivery' effects of the rumen would be lost and the resultant efficacy significantly reduced. The GI transit time is another factor that may influence on the benzimidazole dissolution and, therefore, on its accumulation and efficacy against *H. contortus*. Feeding management has been recommended to restore the anthelmintic action of those benzimidazole compounds whose potency has been compromised by resistance (Ali and Hennessy, 1995). An enhanced plasma availability of oxfendazole induced by temporary feed restriction in sheep accounted for an increased efficacy of the drug against a benzimidazole-resistant nematode isolate (Ali and Hennessy, 1995). Fasting the animals prior to IR treatment resulted in pronounced modifications to the absorption and disposition kinetics of albendazole metabolites in sheep in which the administered drug appeared to be absorbed to a greater extent than in fed animal (Lifschitz et al., 1997). Starvation decreases the flow rate of ingesta. A delayed GI transit time that decreased the rate of passage of the anthelmintic drug down the GI tract may have accounted for the enhanced absorption observed in fasted animals compared with fed animals. The fasting-induced changes to the kinetic behaviour and quantitative tissue distribution of benzimidazole methylcarbamates may have particular relevance for designing strategies to increase activity against susceptible parasites. However, a fasting-induced improvement of benzimidazole dissolution/absorption may not be useful when a high level of drug resistance is already established in the treated nematode population (Alvarez et al., 2010).

In the case of levamisole, the available formulations for ruminants are prepared as solutions to be administered by oral, SC and topical routes. The rate of levamisole absorption differs with the route of administration. The drug is most rapidly absorbed following intramuscular or SC injection in cattle, and the highest plasma levels (>1 μg/mL) are observed at 0.5–2 h. Several oral formulations gave similar absorption rates (time to peak concentration [Tmax] = 3 h), and slower absorption was observed after dermal application (Bogan et al., 1982). Bioavailability differs depending on the route of administration. In sheep, the highest mean plasma concentrations were achieved after the SC (3.1 μg/mL) compared with oral (0.7 μg/mL) administration at a dose rate of 7.5 mg/kg (Bogan et al., 1982). Following pour-on administration in cattle, plasma and GI concentrations of levamisole were lower than those measured following parenteral and oral treatments

(Forsyth et al., 1983), which agrees with the limited anthelmintic efficacy reported for the topical preparation. In small ruminants, the systemic availability of levamisole has been shown to be 25–33% higher after parenteral administration compared with the oral route (Fernandez et al., 1998; Sahagun et al., 2000, 2001).

The macrocyclic lactones are available to be administered by the parenteral, oral and topical routes to ruminants. In the early days, shortly after the introduction of ivermectin into the market, nematode susceptibility was high and equivalent efficacy patterns were observed against abomasal parasites after parenteral or oral treatment in sheep/goats. A similar pattern was described later on for other macrocyclic lactones from both the avermectin (abamectin) and milbemycin (moxidectin) families. A slightly improved ivermectin efficacy against sheep intestinal nematodes was observed after oral compared with parenteral treatment (Borgsteede, 1993). However, when the efficacy was assessed against ivermectin-resistant nematodes, a significant greater pharmacological activity was observed after oral administration of both abamectin and moxidectin compared with their SC administration to lambs (Alka et al., 2004; Gopal et al., 2001). Thus, the pharmacological basis underlying the observed differential efficacy patterns against *H. contortus* after the macrocyclic lactones administration by both routes was recently investigated (Lloberas et al., 2012). The simultaneous measurement of drug concentrations in blood, GI tissues of parasite location and within resistant target worms was performed on *H. contortus*-infected lambs. Enhanced ivermectin plasma concentrations were obtained after SC treatment compared with oral administration (Lloberas et al., 2012), which supports the use of the parenteral route to control ectoparasites. The higher ivermectin plasma profiles observed in the SC-treated ivermectin group related to an enhanced systemic availability. The mean ivermectin systemic availabilities (measured as AUC) were 129 (SC) and 58.4 ng d/mL (oral treatment). In addition, the described longer mean residence time and elimination half-life observed for ivermectin after its SC administration accounted for the persistent antiparasitic activity (over 10 days) against *H. contortus* in sheep (Borgsteede, 1993). Markedly lower ivermectin concentrations were recorded in the abomasal contents after the SC administration of the drug to infected lambs (Lloberas et al., 2012). The mean ivermectin concentrations achieved (3 days posttreatment) in the abomasal content were 143 ng/g (oral ivermectin) and 2.53 ng/g (SC ivermectin). The active secretion of ivermectin (and other macrocyclic lactones, such as doramectin) from the bloodstream to the abomasal lumen is of

little relevance (Hennessy et al., 2000), as opposed to that observed at the small intestine level. Consistently, early studies with ivermectin showed that its SC administration at ten times (2 mg/kg) the therapeutic dose to sheep resulted in very low concentrations in the abomasal content (Bogan and McKellar, 1988). This phenomenon may indicate that ivermectin concentrations achieved in the adult *H. contortus* after the parenteral treatment may relate mainly to the drug coming from the bloodstream. The amount of drug reaching the target parasite is influenced by the drug concentrations in the tissues where the parasite is located (Lifschitz et al., 2000). The higher concentrations measured in the abomasal content after the oral administration of ivermectin accounted for a greater amount of drug being measured within adult *H. contortus* recovered from treated sheep. The ivermectin concentrations in *H. contortus* were 74.4 ng/g (oral) and 5.19 (SC). These enhanced ivermectin concentrations explain the lower number of adult *H. contortus* specimens recovered after treatment and the enhanced efficacy obtained after the oral treatment, despite the high level of resistance observed. The high concentrations of drug detected in the GI tract during the first 2–3 days after the oral treatment may have a relevant effect on the resistant nematodes, being of great importance to induce the pharmacological action at the target site.

It is also likely that the concentrations of the dissolved drug attained in the gut lumen after oral administration of an anthelmintic preparation may be critical to the pharmacological activity against worms in the abomasum and small intestine, particularly if they have a reduced susceptibility to the drug. Lipophilic drugs, such as ivermectin, may reach the target parasite from the GI contents (transcuticular route) or from plasma (oral ingestion) if the nematode (*H. contortus*) feeds on host blood. Increasing drug exposure may be a useful strategy for killing heterozygous resistant parasites present in the earliest phases of development of resistance. Interestingly, a similar concept was applied to cattle against resistant *Cooperia* spp. A recent trial in cattle confirmed these findings (Leathwick and Miller, 2013). The activity of the drug against *Cooperia oncophora* was significantly higher after the oral treatment with moxidectin. Furthermore, the clinical efficacy against ivermectin-resistant nematodes in cattle was significantly greater after the oral (74%) compared with SC (54%) ivermectin treatment (Canton et al., 2015). Altogether, these findings show that the administration of macrocyclic lactones by the SC injection may achieve lower efficacy against nematodes located at the GI tract compared with oral treatment. These differences in 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 oral administration of macrocyclic lactones may be based on an enhanced drug exposure of the worms located at the lumen of the abomasum and/or small intestine. Such a finding may have direct impact on the practical use of the macrocyclic lactones in ruminant species accounting for a marked improvement in the control of macrocyclic lactone–resistant nematodes in the field, at least in the early stages of resistance development. The influence of the route of administration on the systemic exposure and efficacy of macrocyclic lactones against resistant *H. contortus* is shown in Fig. 1.

The AADs represent one of the newest anthelmintic classes (Kaminsky et al., 2008) introduced to veterinary medicine. From many compounds evaluated, the racemic molecule AAD 96 was selected and the active s-enantiomer of this molecule, named monepantel, was launched into the pharmaceutical market for oral administration to sheep in 2009 (Hosking et al., 2010). The monepantel plasma concentration profiles were determined after its intravenous and oral administration to sheep at 1, 3 and 10 mg/kg (Karadzovska et al., 2009). The monepantel and monepantel sulphone concentrations at the sites of parasites location were also shown to improve the understanding of its anthelmintic efficacy (Lifschitz et al.,

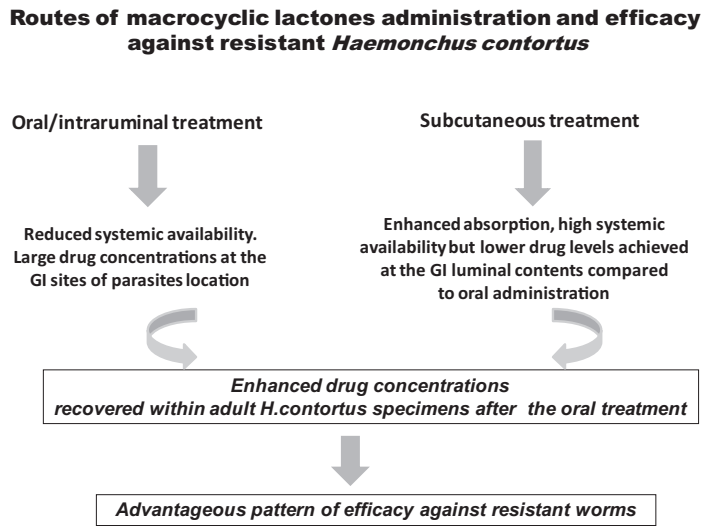

Figure 1 Impact of the route of administration on the systemic exposure and efficacy of the macrocyclic lactones against resistant *Haemonchus contortus*. *GI*, gastrointestinal tract.

2014). Higher concentration profiles of the metabolite monepantel sulphone compared with the parent drug were measured in the sheep bloodstream (Hosking et al., 2010; Karadzovska et al., 2009; Lifschitz et al., 2014). The peak concentration (Cmax) of monepantel sulphone was fourfold higher compared with that measured for the parent compound. Monepantel is rapidly converted in the liver into different metabolites (Karadzovska et al., 2009). Under the described circumstances, it is important to understand the fate of the drug within the GI tissues and fluid contents. In the abomasum, drug concentrations of the monepantel parent were much higher than those measured in plasma, but monepantel sulphone was also recovered. In the abomasal content, monepantel concentrations were recovered in a range between 2000 and 4000 ng/g during the first 48 h after treatment. Interestingly, monepantel sulphone was also detected in the abomasal content, but the concentrations were significantly lower compared with those of monepantel ($P < 0.05$) (gastric secretions may be involved in the appearance of monepantel sulphone in abomasal content, as was demonstrated for benzimidazole compounds in sheep (Hennessy, 1993)). The characterization of monepantel and monepantel sulphone accumulation in target digestive tissues provides relevant information on drug exposure for GI nematode parasites. Such a kinetic pattern may support the well-established high efficacy of monepantel against *H. contortus* (see Kaminsky et al., 2009). Both monepantel and its sulphone metabolite may reach the target parasite from plasma after oral ingestion. However, considering that they are highly lipophilic compounds (Karadzovska et al., 2009), the great availability of monepantel and monepantel sulphone in the abomasal content could facilitate an accumulation of both active molecules within the parasite through a transcuticular diffusion process. There is no published information that correlates the pharmacokinetics of monepantel with clinical efficacy. The concentrations of monepantel and monepantel sulphone at the site of the parasite required to inhibit parasite establishment and/or development have not been determined. However, the characterization of drug accumulation in target tissues may provide information to predict the drug concentration below which the effectiveness against larval and adult parasites begins to decrease. 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 lethality occurs at drug concentrations of >1000 ng/mL.

The study by Sager et al. (2010) might also provide useful information on the pharmacokinetic–pharmacodynamic relationship for this novel drug. These authors studied the speed at which a reduction of nematode eggs in the faeces of sheep occurs after monepantel treatment. A significant reduction of eggs in faeces was obtained 36 h after treatment, and the faecal egg counts reduced to zero 72 h following treatment. This time-course of monepantel pharmacological activity correlates to the highest monepantel concentrations in the abomasum during the first 48 h following treatment.

3.3 Dosage and relationship between enhanced drug exposure and anthelmintic efficacy

Haemonchus contortus has been used as a nematode model to assess the impact of increasing the dose rates on the pharmacokinetics and subsequent drug efficacy against resistant isolates. The therapeutic response to an increased dosage may depend on the genetic status of the resistant nematode population being exposed to the drug. The impact of large increases of dose on systemic concentrations of benzimidazole and on the subsequent efficacy against benzimidazole-resistant nematodes in ruminants remains unclear. In the case of benzimidazoles, it has also been demonstrated that their efficacy relies on the time the parasite is exposed to "toxic" concentrations and that anthelmintic activity is influenced by the residence time of the drug in the animal's body (Lanusse and Prichard, 1993b). The oral absorption of most drugs follows first-order kinetics, whereby a constant fraction of the total drug present is absorbed in each equal interval of time (Neubig, 1990). This statement, true for most of the drugs commonly used in veterinary therapeutics, remains unclear for the benzimidazole compounds in ruminant species. Moreno et al. (2004) evaluated the clinical efficacy of albendazole, given at two different dose rates, 3.8 mg/kg (a therapeutic dose recommended against GI nematodes in some countries) and 7.5 mg/kg against benzimidazole-resistant nematodes in naturally infected sheep. Significantly higher Cmax and AUC values were observed for both albendazole metabolites following the treatment at the highest dose rate (7.5 mg/kg) compared with 3.8 mg/kg. Also the time of residence (MRT) of albendazole sulphoxide was significantly longer after the albendazole treatment at 7.5 mg/kg. Although parasites were highly resistant to the treatment with albendazole orally administered at both dose rates, the clinical efficacy against *H. contortus* tended to be higher after the administration of albendazole at 7.5 mg/kg compared with that observed at the lowest investigated dose rate. For instance, clinical efficacy against *H. contortus*

increased from 5.9% (3.8 mg/kg treatment) up to 46.1% (7.5 mg/kg). Albendazole molecules adsorbed to ruminal particulate material gradually reached the abomasum and small intestine, the main sites of dissolution and absorption, respectively (Hennessy, 1993). Within a therapeutic dose range, when a higher oral dose is administered, a greater amount of the drug suspension will gradually pass to the abomasum being available to be absorbed in the duodenum over time following treatment. The lower plasma profiles of both albendazole metabolites observed at the 3.8 mg/kg treatment may be due to a lower total amount of albendazole being available to be absorbed, which explains the positive relationship between the amount of drug administered and the systemically available concentrations of both metabolites in the treated animals. Furthermore, a high correlation between the concentration profiles of albendazole metabolites measured in the bloodstream and those in tissues of parasite location and within target parasites collected from treated sheep has been demonstrated (Alvarez et al., 1999, 2000). These previous pharmacokinetic findings clearly indicate that the enhanced drug concentrations and the prolonged plasma detection of the drug observed for a higher dose may account for greater drug exposure of the target nematodes, which would also explain the tendency observed in the anthelmintic efficacy against resistant parasites. Further studies evaluated the pharmacokinetic and efficacy of albendazole against resistant *H. contortus* administered at 5, 15 and 45 mg/kg (Alvarez et al., 2012). The dose level affected the plasma disposition kinetics of albendazole sulphoxide in treated lambs. The time of albendazole sulphoxide detection in plasma increased from 1–48 h ($ABZ_{5\ mg/kg}$) to 1–96 h ($ABZ_{15\ mg/kg}$) and 1–120 h ($ABZ_{45\ mg/kg}$). The AUC of the active albendazole sulphoxide metabolite increased from 21.0 ± 6.4 μg h/mL ($ABZ_{5\ mg/kg}$) up to 158.6 ± 19.4 μg h/mL ($ABZ_{15\ mg/kg}$) and 389.7 ± 98.2 μg h/mL ($ABZ_{45\ mg/kg}$). Furthermore, when the mean AUC values were adjusted by the dosages, the albendazole sulphoxide plasma exposure after the administration of 15 and 45 mg/kg doses was 78% and 43% higher, compared with the therapeutic dose (5 mg/kg). These AUC values show a lack of proportionality in the relationship between dose and systemic availability. Additionally, a significantly longer MRT value was observed for albendazole sulphoxide in both $ABZ_{15\ mg/kg}$ and $ABZ_{45\ mg/kg}$ treated groups. Thus, the higher dose-normalized AUC ($ABZ_{15\ mg/kg}$) and the longer MRT values ($ABZ_{15\ mg/kg}$ and $ABZ_{45\ mg/kg}$) obtained for albendazole sulphoxide reflect some type of nonproportionality in the dose–plasma concentrations relationship. The lack of dose proportionality observed for albendazole

(estimated as the albendazole sulphoxide systemic exposure) may be associated with a saturation of the enzymatic pathways involved in biotransformation. It has been demonstrated that the FMO-mediated sulphoxidation accounted for up to 60% of the albendazole sulphoxide production from albendazole, while CYP contributed the remainder (40%) in sheep liver microsomes (Virkel et al., 2004). Thus, a decreased metabolic rate for the conversion of albendazole sulphoxide into albendazole sulphone may have accounted for the marked changes observed in the albendazole sulphoxide disposition kinetics after the administration of three times ($ABZ_{15\ mg/kg}$) the therapeutic dose. As expected, there was a highly negative correlation between the albendazole sulphoxide AUC and Cmax values, and the number of adult *H. contortus* recovered from treated lambs. The enhanced systemic exposure achieved after albendazole treatments at the highest dosages correlated with a significant increment in drug efficacy against a resistant *H. contortus* isolate. In fact, the efficacies against resistant *H. contortus* were 16% ($ABZ_{5\ mg/kg}$), 59% ($ABZ_{15\ mg/kg}$) and 94% ($ABZ_{45\ mg/kg}$) (Barrere et al., 2012). Benzimidazole resistance has been correlated with genetic changes associated with the β-tubulin gene (Lubega and Prichard, 1990). These changes, mainly at positions 200 and 167 of the β-tubulin gene, determine a reduced binding affinity, which explains the development of anthelmintic resistance to benzimidazole compounds (Beech et al., 1994; Kwa et al., 1994). A loss of receptor affinity increases the concentration of drug needed for a given degree of response. These findings indicate that higher active drug/metabolite concentrations associated with the highest albendazole dosages resulted in significant increased drug efficacy. As benzimidazole metabolites are reversibly exchanged between the bloodstream and the GI tract (Lanusse et al., 1993), the enhanced drug concentrations associated with the increasing doses administered may account for GI nematodes being exposed to toxic drug concentrations for an extended period of time. This finding helps to explain the reversion of the drug resistance phenomenon observed after administration of albendazole at a high dose level. In addition, Barrere et al. (2012) evaluated the presence of single nucleotide polymorphisms (SNPs) in the β-tubulin gene of *H. contortus* after the albendazole treatment at three dose rates (5, 15 and 45 mg/kg). This investigation corroborated a strong association between the presence of SNPs at codons 167 and 200 in isotype 1 of β-tubulin and the survival of *H. contortus* individuals at a high dose rate of albendazole. The genotype (Phe/Phe)167-(Tyr/Tyr)200 was consistently observed in worms exhibiting high levels of resistance. Heterozygosity at both codons

167 and 200 conferred resistance with treatments of up to three times the recommended albendazole dose rate (Barrere et al., 2012). Routine tests for the evaluation of benzimidazole resistance, using analysis of the β-tubulin gene, should include the assessment of both the 167 and 200 codons to assess heterozygosity at both of these codons.

From a pharmacological point of view, anthelmintic drugs need to have the best opportunity to act on the specific target nematode site of action (Hennessy, 1997). This concept applies to the different strategies addressed to increase parasite drug exposure. For macrocyclic lactones, the impact of increasing dosage levels was evaluated in a high-resistance scenario, allowing the prediction of the consequences of rational dose adjustments. The comparison of the pharmacokinetics, distribution and efficacy against resistant *H. contortus* of single and double doses of ivermectin and moxidectin was recently evaluated in lambs (Lloberas et al., 2015). The plasma concentration profiles were related to the dose-rate administered for both drugs. For both ivermectin and moxidectin, the mean Cmax and AUC were higher for the 0.4 compared with 0.2 mg/kg treatments ($P < 0.05$), but there were no differences for other parameters. Ivermectin and moxidectin concentrations in the GI target tissues and in *H. contortus* were much higher compared with those measured in plasma. Mean drug concentration levels in abomasal contents were 26–47 (ivermectin) and 26–36 (moxidectin) fold higher than those achieved in plasma at day 1 following administration of both compounds at 0.2 and 0.4 mg/kg, respectively. Ivermectin and moxidectin concentrations in the GI target tissues were significantly higher after their administration at 0.4 mg/kg. The exposure of *H. contortus* to ivermectin and moxidectin was related to the level of the administered dose. Concentrations of the macrocyclic lactones measured in *H. contortus* correlated to those recovered from abomasal content. The increment of drug concentrations at the tissue sites of parasite location accounted for an enhancement on drug levels measured within the worm. Based on pharmacological principles, all the strategies that maximize drug availability (exposure) at the host–parasite interface may increase the nematodicidal effect. As drug concentrations at the GI target tissues/contents during the first 2–3 days after treatment are relevant for the effectiveness of the macrocyclic lactones against resident worms in sheep (Lloberas et al., 2013), the higher drug accumulation observed within the nematode after the administration of ivermectin and moxidectin at double doses (0.4 mg/kg) may be useful to increase the efficacy against resistant worms. Despite the high concentrations recovered from the abomasal content and within the worm, ivermectin

failed to control a highly resistant isolate of *H. contortus* (see Lloberas et al., 2013). Moxidectin showed a higher performance at both doses assayed. Ivermectin had no efficacy at both dose rates. In contrast, the efficacy of moxidectin against this ivermectin-resistant *H. contortus* isolate was 85.1% (0.2 mg/kg) and 98.1% (0.4 mg/kg). Interestingly, whereas the double dose of ivermectin remained ineffective against *H. contortus*, high efficacy (98.2%) was achieved after the administration of moxidectin at 0.4 mg/kg. Particular pharmacodynamic features for each macrocyclic lactone may play a relevant role on the activity against resistant nematodes. A differential pattern of interaction at the glutamated-gated chloride channel may support the higher efficacy of moxidectin (Hibbs and Gouaux, 2011; Prichard et al., 2012). The way a drug enters into the nematodes is relevant to the efficacy of the anthelmintics compounds. To corroborate the in vivo results, the accumulation of both compounds was also evaluated ex vivo. The incubation of adult nematodes in 0.5 μM of ivermectin and moxidectin reflects the in vivo concentrations of both drugs measured in the abomasal contents after their administration at 0.2 mg/kg to sheep. There was a concentration and time influence in the accumulation of ivermectin and moxidectin in *H. contortus*. A significantly higher amount of both drugs was measured within *H. contortus* after the incubation with 5 μM of ivermectin and moxidectin. In addition, the incubation for 3 h resulted in a higher accumulation of both drugs (five- to sevenfold) compared with the accumulation assay performed over 15 min. The ex vivo uptake of benzimidazole drugs by nematodes, trematodes and cestodes has been extensively studied (Alvarez et al., 1999, 2001; Mottier et al., 2006). Drug lipophilicity and the permeability of helminth external surfaces determine the effective concentrations that reach the site of action. These findings confirm that the increment on drug exposure for both ivermectin and moxidectin accounted for an enhanced amount of drug being recovered from the target parasite, which was observed in both, the ex vivo and in vivo experiments. The degree of susceptibility of a nematode population is a relevant topic to determine the impact of the increment of the local drug exposure. If the response of a drug reaches 80% of its maximum, the final effect will be insensitive to further changes in drug concentrations (Holford and Sheiner, 1981). If a clinical study is conducted with animals infected with a susceptible parasite isolate, a dose-dependant increased therapeutic effect will not be detected. However, if animals are infected with nematodes displaying reduced susceptibility, the clinical response to the increment of the drug exposure at the site of action may be increased (Martinez, 2014). The administration of

ivermectin and moxidectin at 0.4 mg/kg accounted for enhanced drug exposure in the target tissues as well as for higher drug concentrations within the resistant nematodes. Given the extremely high degree of resistance of the *H. contortus* isolate being tested, the administration of a double-dose treatment was only effective for moxidectin.

Alvarez et al. (2015) assessed the disposition kinetics and efficacy of ivermectin against resistant *H. contortus* after SC and IR administrations at one, five and ten times the therapeutic dose to lambs. The ivermectin systemic exposure increased from 41.9 ± 20.1 ng d/mL (IVM_{SCx1}) up to 221 ± 55.9 ng d/mL (IVM_{SCx5}) and 287 ± 100.4 ng d/mL (IVM_{SCx10}). The higher dose levels (five and ten times) were also correlated to a significant enhancement of the ivermectin peak plasma concentrations, which were obtained at the same time after treatment. Furthermore, when the mean AUC and Cmax values were normalized based on dose, differences among groups did not reach statistical significance to show a dose-proportional relationship. The plasma concentrations of ivermectin obtained after IR administration were also related to the level of the different doses. A higher AUC value was measured for the IVM_{IRx10} group (AUC = 323 ng d/mL) compared with that obtained after the IVM_{IRx1} treatment (AUC = 20.8 ng d/mL). As observed following the SC treatment, when the AUC and Cmax values were normalized by the dose, similar absorption rates were obtained for the treatments at different doses. The low efficacy level (42−50%) obtained at the therapeutic dose (0.2 mg/kg) after both routes of administration confirmed the high ivermectin-resistant status of the worms. The efficacy of ivermectin against adult *H. contortus* increased with the increment of the dose both, after the SC and IR treatment. After the SC treatment, an anthelmintic efficacy of 75% was observed for the administration of the five and ten times the doses. After the IR administration of ivermectin, these higher doses resulted in a significant ($P < 0.05$) reduction in adult *H. contortus* counts, compared with that observed both in the group treated at the therapeutic dose and the untreated control. A high efficacy was observed in both the IVM_{IRx5} (96%) and IVM_{IRx10} (98%) groups. Since drug accumulation in GI nematodes appears to be mainly related to drug diffusion from the surrounding medium (GI fluids), the enhanced ivermectin concentration in abomasal content after the IR administration may have accounted for an increased drug accumulation in *H. contortus*, explaining the improved efficacy observed after IR treatment at the highest doses. Within a population of *H. contortus*, individual parasites do not respond uniformly to treatment. Some were killed by a

therapeutic ivermectin dose, other tolerated this dose, but were eliminated by treatment at the higher doses (five and ten times), and some particular individuals survived even ten times the therapeutic dose. This 'dose-related behaviour' may be explained by genetic diversity in the parasite population (Prichard, 2001). High genetic diversity has been described for different *H. contortus* populations, also relating to genes encoding β-tubulins (Beech et al., 1994; Kwa et al., 1994), P-gp (Blackhall et al., 1998a; Sangster and Gill, 1999) and glutamate-gated chloride channel (GluClR) subunits (Blackhall et al., 1998b). Macrocyclic lactones resistance is quite complex, with mechanisms varying both within and between species (Gill and Lacey, 1998). The development of resistance to ivermectin requires of the simultaneous mutation of several genes to develop a high level of resistance (Martin et al., 2002). In this context, the variation in response according to the dosage administered may be explained by genetic diversity within the isolate. In conclusion, both the genetic variability and the potential differences on drug accumulation, according to the location of *H. contortus* within the abomasum, may have accounted for the observed differences in efficacy related to the dose level of ivermectin. These data shown the ivermectin resistance may be overcome by increasing the amount of the active drug at the biophase. Indeed, under experimental conditions, an IR ivermectin dose as high as 5- to 10-fold the therapeutic dosage was necessary to reach an acceptable efficacy level against resistant *H. contortus*. From a clinical point of view, the inconvenience of recommending high dose rates may be associated with the selection of highly resistant nematodes, in addition to the impact on drug residues, withdrawal times, etc., which would preclude its use as a 'practical' strategy when resistant parasite populations are present.

4. MODULATION OF DRUG EFFLUX TRANSPORT: IMPACT ON CLINICAL RESPONSE AGAINST RESISTANT *HAEMONCHUS CONTORTUS*

4.1 Relevance of cellular efflux transport on systemic exposure and drug action

The influence of cell transporter systems on the pharmacokinetic behaviour of different drug compounds has been profoundly studied, and the interaction between drug compounds at the transport proteins levels is now considered as a key pharmacological issue with a variety of potential therapeutic implications. For instance, the interactions of macrocyclic lactones with different ATP-binding cassette (ABC) transporters have been

thoroughly investigated. Of all identified cell transporters, P-gp has been the most studied. P-gp was initially described due to its capacity of preventing the intracellular accumulation and cytotoxic effects of antineoplastic drugs by actively removing them from the cell membrane before they reach their intracellular target. Besides tumour cells, P-gp has also been identified in several healthy tissues and particularly in organs actively involved in drug pharmacokinetics (Schinkel, 1997). P-gp is located in tissues and particularly in organs involved in the processes of drug absorption (eg, mucosa of the small and large intestine), distribution (eg, brain–blood barrier) and elimination (luminal surface of hepatocytes and ducts cells, kidney tubules and enterocytes) (Lin, 2003). The interaction of macrocyclic lactones with different P-gp has been well demonstrated (Lespine et al., 2007), and different aspects of this interaction recently reviewed in the literature (Lespine et al., 2012; Lifschitz et al., 2012). Considering the wide use of the macrocyclic lactones 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 induction of the activity of this transport system, for example, at the intestinal level, will lead to the reduction of the bioavailability of orally administered P-gp substrates, while an increment of the bioavailability would be observed when an inhibitor is co-administered with a P-gp substrate. The interaction between macrocyclic lactones and cell transporters was characterized by in vitro and in vivo trails. Ivermectin is actively secreted by cells transfected with gene encoding P-gp in mice (Schinkel et al., 1995). The interaction of moxidectin with the ABC transporters was demonstrated in cultured rat hepatocytes. Ketoconazole, quercetin and fumagillin increased the quantity of ^{14}C-moxidectin in hepatocytes (Dupuy et al., 2003, 2006). Recently, it has been shown that affinity by P-gp may differ among different macrocyclic lactone molecules (Lespine et al., 2007). The different macrocyclic lactones were tested for their ability to inhibit the P-gp mediated rhodamine 123 (Rho^{123}) transport function in recombinant cell lines over-expressing P-gp. The different avermectins (ivermectin, eprinomectin, abamectin, doramectin and selamectin) increased the intracellular Rho^{123} accumulation with a similar potency. It is interesting to note that moxidectin appears to have different P-gp efflux potential, with a half-maximal inhibitory effect (IC_{50}) approximately ten times higher than that reported for ivermectin (Griffin et al., 2005; Lespine et al., 2007). Using the everted sac technique, the ivermectin accumulation rate in 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-gps was demonstrated employing an Ussing chamber system (Ballent et al., 2012). The in vivo involvement of multiple transporters suggests a complex interplay between macrocyclic lactones and the different ABC transporter proteins, which could affect their systemic disposition.

In vivo trials performed on different animal species provided information on the action of different P-gp modulators on the macrocyclic lactones pharmacokinetic disposition. Important changes to the plasma disposition of the macrocyclic lactones 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 demonstrated. Significantly higher moxidectin plasma concentrations were observed after its co-administration with loperamide (an opioid derivative acting as P-gp substrate) compared with those measured after moxidectin given 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.

The differential affinities of macrocyclic lactones by P-gp were recently assayed in vivo using the knockout mouse model (Kiki-Mvouaka et al., 2010). P-gp deficiency led to a significant increase in the systemic availability of ivermectin (1.5-fold) and eprinomectin (3.3-fold), whereas the moxidectin availability remained unchanged. Ivermectin and to a greater extent eprinomectin were both excreted by the intestine via a P-gp-dependent pathway, whereas moxidectin excretion was less compared to the avermectin-type macrocyclic lactones (Kiki-Mvouaka et al., 2010).

Most studies of drug interactions mediated by cell transporters have been conducted 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 behaviour of macrocyclic lactones is not fully understood. 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 in relation to P-gp induction in vivo is not entirely clear. The experimental effect of the inducer agent phenobarbital on both plasma and GI disposition of ivermectin was

examined in our laboratory (Ballent et al., 2010). The ivermectin AUC values measured were significantly lower in the plasma, intestine and liver tissue of rats pretreated with phenobarbital. On the other hand, the chronic administration of dexamethasone in sheep produced a decrease in P-gp expression along the small intestine compared with 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 pharmacotherapy in livestock animals. More specifically, this is an open field for the future of the antiparasitic drugs that needs to be addressed if the combination of anthelmintic molecules turns into an alternative for parasite control in resistant populations.

4.2 Modulation on drug transport and efficacy against resistant *Haemonchus contortus*

Currently, resistance to antiparasitic drugs is recognized as a problem in small ruminant and bovine production systems (Kaplan and Vidyashankar, 2012; Prichard, 1994). The drug–drug interactions at the efflux transporter protein level are not only important in the host but also at the target nematodes. P-gp has been described not only in mammals but also in parasites, such as *Onchocerca volvulus* (see Kwa et al., 1998) and *H. contortus* (see Prichard and Roulet, 2007). Drug efflux mediated by P-gps in different parasites has been proposed as a potential resistance mechanism for different drugs (Xu et al., 1998). Increased scientific evidence supporting this concept has been reported during the last few years. Modifications of the pattern of P-gp expression have been observed in resistant nematodes recovered from lambs treated with macrocyclic lactones (Prichard and Roulet, 2007). An upregulation of P-gp in *H. contortus* recovered one day after ivermectin treatment has been reported, and 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 0.5 and 1 day after treatment compared with 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). The chemical structure differences between moxidectin and ivermectin may account for their differential pharmacokinetic, pharmacodynamic and P-gp interaction patterns (Prichard et al., 2012).

To evaluate the impact of P-gp in the parasites, the inhibition of the activity of P-gp has been assayed as a pharmacology-based strategy not only to increase the systemic availability of the macrocyclic lactones in the host animal, but also to improve their clinical efficacy. The combinations of VRP and CL347099 with either ivermectin or moxidectin significantly reduced worm counts of resistant isolates of *H. contortus* (see Molento and Prichard, 1999). In addition, the modulation of P-gp increased the in vitro activity of ivermectin against ivermectin-sensitive and resistant larvae of *H. contortus*. The presence of the P-gp modulators PSC833, VRP, ketoconazole and pluronic 85 in the larval feeding inhibition test enhanced the sensitivity of larvae (*H. contortus* resistant isolates) to ivermectin by between 15- and 57-fold (Bartley et al., 2009). Another study has shown that macrocyclic lactones anthelmintics, which inhibit P-gp-mediated efflux in mammals, activate transport activity in nematodes, suggesting a complex interaction of macrocyclic lactones with P-gps in the parasites (Kerboeuf and Guégnard, 2011).

Although a modification of macrocyclic lactone activity after P-gp modulation was confirmed in vitro, in vivo trials performed under field conditions are necessary to evaluate the clinical impact of the P-gp inhibition. The enhanced sensitivity of resistant larvae to ivermectin obtained after its co-incubation with pluronic 85 did not correlate with their in vivo co-administration to sheep (Bartley et al., 2009). In the in vivo trial, the presence of pluronic 85 did not improve the efficacy against resistant *H. contortus* (see Bartley et al., 2012). However, significant increment on ivermectin efficacy against resistant nematodes of sheep together with an enhancement on ivermectin systemic availability was obtained in the presence of loperamide (Lifschitz et al., 2010). The faecal egg count reduction increased from 78.6% (ivermectin alone treatment) to 96% after the co-administration with loperamide. A nematode population highly resistant to ivermectin was identified. The efficacy of ivermectin against *H. contortus* was 0%, and the percentage of reduction against intestinal nematodes, such as *T. colubriformis* and *Nematodirus* spp. was 77.9% and 85.5%, respectively. The clinical efficacy against the resistant nematodes was enhanced in the presence of loperamide, with percentages of reduction of 72.5% (*H. contortus*), 96.3% (*T. colubriformis*) and 93.0% (*Nematodirus* spp.) (Lifschitz et al., 2010). Thus, there is evidence that the P-gp-mediated drug—drug interaction increases the ivermectin systemic exposure in the host, and it may also decrease the P-gp-mediated efflux transport over-expressed in target resistant nematodes. The interaction at the parasite tissue-level was specifically investigated using *C. elegans*

as a model system. It was confirmed that different 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). Recently, the localization of P-gp2 was studied in *H. contortus*. P-gp2 was expressed in the pharynx, the first portion of the worm's intestine and perhaps in adjacent nervous tissue, suggesting a role for this gene in regulating the uptake of avermectins and in protecting nematode tissues from the effects of macrocyclic lactone anthelmintic drugs (Godoy et al., 2015). The impact of P-gp modulation on drug pharmacokinetics in the host and the efficacy against resistant *H. contortus* is shown in Fig. 2.

It is evident that a P-gp-mediated drug–drug interaction increases the systemic exposure of macrocyclic lactones in the host. However, such an interaction might also occur in the target worm, which would decrease the P-gp-mediated efflux transport over-expressed in target resistant nematodes. Different pharmacological approaches to delay the bile/intestinal secretions and to extend the plasma-intestine recycling time of macrocyclic lactones 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

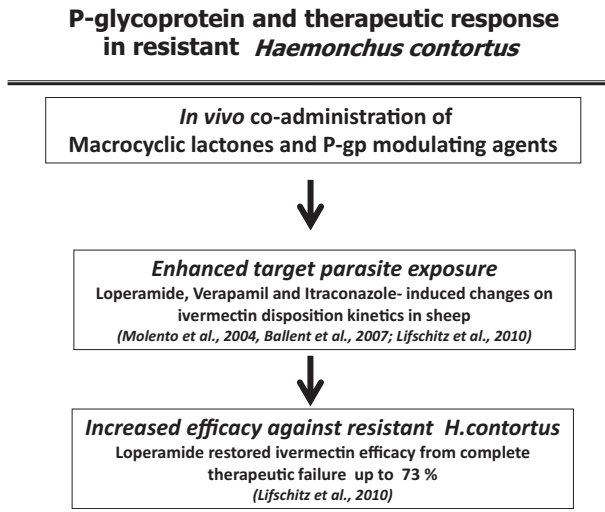

Figure 2 Influence of the modulation of the efflux transport protein P-glycoprotein (P-gp) on both drug pharmacokinetics in the host and efficacy against resistant *Haemonchus contortus*.

(target parasites) to different anthelmintic chemical groups has been outlined. 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 for specific P-gp modulators with high affinity to parasite transport proteins may identify useful pharmacological tools to extend the life span of the macrocyclic lactones in veterinary medicine. The effect of the most-recently developed group of P-gp inhibitors (the so-called 'third generation' of inhibitors, such as tariquidar, zosuquidar and elacridar) was evaluated (Raza et al., 2015). Zosuquidar restored the sensitivity of resistant worms to levels observed in susceptible worms. On the other hand, the ability of tariquidar to increase the sensitivity of ivermectin to the susceptible isolate raised the possibility of reducing the recommended dose of an anthelmintic, while maintaining 100% efficacy against susceptible worms (Raza et al., 2015). Further work is required to assess the practical pharmaco-parasitological implications of the chemical modulation of these cell efflux pump systems on antiparasitic therapy.

5. PHARMACOLOGICAL EVALUATION OF DRUG-COMBINED THERAPY AGAINST RESISTANT *HAEMONCHUS CONTORTUS*

There is a long history of the use of drug combination for treating the most dreadful diseases. For instance, the use of drug combination in cancer chemotherapy was reviewed early (Goldin and Mantel, 1957). In 1959, it was shown that the administration of both streptomycin and isoniazid to tuberculosis patients markedly reduced the emergence of a drug-resistant strain of the tubercle bacillus (Conn et al., 1959). Concerning parasite control, a drug combination (ie, ivermectin-clorsulon) has been successfully used as a strategy to expand the efficacy spectrum (Geary et al., 2012). Additionally, in an attempt to overcome/manage anthelmintic resistance in ruminants, combinations of two or more anthelmintics are primarily being used (Geary et al., 2012). *Haemonchus contortus* has been extensively used as a model nematode to test different strategies/approaches related to drug combinations. The combination of nematodicidal drugs could be achieved using two or more pharmaceutical formulations containing a different active principle each, or, alternatively, through 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 of the use of two or more drugs with different modes of action is to increase the treatment

efficacy. Other possible favourable outcomes after the use of drug combinations include (1) the use of lower doses to avoid toxicity (reaching equivalent or even higher efficacy) and (2) minimizing or slowing down the development of drug resistance. The probability of selecting resistant parasites is decreased if two drugs with different mechanism of action and different biochemical pathways of resistance are administered together in combination chemotherapy. Thus, individual worms may have a lower degree of resistance to a multiple component formulation (each chemical with different mode of action) compared with 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 have been introduced into the veterinary pharmaceutical market in many countries. However, potential pharmacokinetic and/or pharmacodynamic interactions between drug components may occur.

A potential drug interaction refers to the possibility that one drug may alter the intensity of the pharmacological effects of another drug when given concurrently (Nies and Spielberg, 1996). The modified effect may result from a change in 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). Pharmacodynamic drug-to-drug interactions may occur at three different levels: (1) at the receptor site, (2) at the signalling (ie, second messenger) or (3) at the effector level. They can lead to both enhanced (additive or synergic effect) and diminished (antagonism) drug responses. Overall, an additive effect is present when the combined activity of two drugs equals 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 is greater than that of additive effect. The effect achieved in the presence of antagonism is less than additive (Chou, 2010). Synergism normally occurs between drugs that exert the same effect (ie, anthelmintic) via different modes of action. Since levamisole, albendazole, monepantel, derquantel, organophosphates, closantel and ivermectin are chemical entities that differ in their intrinsic anthelmintic mode of action, their co-administration may potentially induce a synergistic effect. On the other hand, when multiple resistance relates to 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.

In an effort to improve the control of highly resistant nematode (ie, *H. contortus*) different drug combinations have been assessed. Pharmacodynamic 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 appear to be potentially useful in veterinary medicine, particularly in delaying the emergence and spread of resistance, and/or controlling parasite populations with existing resistance (Geary et al., 2012). In an ideal situation, if an anthelmintic treatment reaches 100% efficacy, selection of anthelmintic resistance will not 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 an alternative to improving chemical control.

The development of anthelmintic resistance in *H. contortus* is a major global problem, which has motivated the development and 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). Early work investigated the potential additive or synergistic effect of different nematodicidal drugs used in combination against multiple resistant nematodes, particularly *H. contortus*. Evidence of synergism between fenbendazole and levamisole against *H. contortus* has been reported (Miller and Craig, 1996). In a goats flock naturally infected with a resistant isolate of *H. contortus*, the efficacy of fenbendazole, levamisole, fenbendazole–levamisole, ivermectin, albendazole and albendazole–ivermectin was evaluated using a faecal egg count reduction test (FECRT). The results showed no significant reduction for either fenbendazole (1%) or levamisole (23%), whereas the combination reduced egg counts up to 62%. When the animals were treated with albendazole or ivermectin alone, faecal egg count reductions of 72% (albendazole) and 0% (ivermectin) were observed. An enhanced efficacy (97%) was observed following the combined treatment

(Miller and Craig, 1996). These reductions in egg counts indicate an additive action, which was not enough to be clinically effective in the case of the fenbendazole–ricobendazole combination. 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 nAChR and pharyngeal muscle glutamate-gated chloride receptor channels of *A. suum* was assessed. The study demonstrated that abamectin and derquantel interact at the nAChR 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).

A faecal egg count reduction of 92% was reported in naturally infected lambs after the intravenous co-administration of albendazole and ivermectin, in comparison to 73% (albendazole) and 79% (ivermectin) (Entrocasso et al., 2008). The enhanced efficacy value observed for the albendazole–ivermectin treatment, in comparison to each drug administered alone, was related to an additive effect of both anthelmintic molecules acting via different modes on different parasite genus/species. Albendazole and ivermectin each administered alone by the intravenous route demonstrated high efficacies against *Haemonchus* spp. (95.1% and 99.3%, respectively). Furthermore, the highest reduction in *Haemonchus* spp. counts was observed with the albendazole–ivermectin combination (99.9%) (Entrocasso et al., 2008). In the same study, the combination of both chemicals administered by the IR (albendazole) or SC (ivermectin) route did not have a positive effect on eggs count reduction. In fact, the albendazole–ivermectin co-administration reached an efficacy of 71% in comparison to egg reductions of 44% (albendazole) and 80% (ivermectin). The faecal cultures showed *Haemonchus* spp. as the main parasite resistant to albendazole and ivermectin (Entrocasso et al., 2008). The combined action of albendazole and ivermectin on GI nematodes did not improve the efficacy of ivermectin administered alone by the SC route. Similar to these results, a recent field study undertaken in Uruguay (Suarez et al., 2014a) showed an equivalent efficacy against multiple resistant *H. contortus*, following the use of either a triple-combined treatment (levamisole–albendazole–ivermectin) or ivermectin alone. The observed anthelmintic efficacies were 87% (combined treatment), 80% (ivermectin treatment), 72% (albendazole treatment) and 52% (levamisole treatment), indicating no advantageous effect of the triple-combined preparation. However, the situation appears to be different when a combined treatment is designed to control *H. contortus*

with low/moderate pre-existing levels of resistance against the drugs included in the combination. The clinical efficacy of closantel and moxidectin administered each drug alone or in combination by the SC or the oral route was assessed in lambs naturally parasitized with resistant nematodes (mainly *H. contortus*) (Suarez et al., 2013). The results obtained showed that the administration of closantel and moxidectin as a single active principle reached efficacy levels (estimated as the FECRT) ranging from 80% (moxidectin oral), 84% (closantel oral) up to 85% (closantel SC) and 92% (moxidectin SC). However, the combined treatments given both orally and subcutaneously reached 100% efficacy. The combination permitted a restoration to maximum efficacy levels, which were not reached by the individual active ingredients. The above situation illustrates the performance of drug combinations when the efficacy of the individual ingredients is relatively high. However, when the individual components included in the combined product demonstrate a low efficacy, a different situation may occur. Suarez et al. (2014b) evaluated the efficacy of levamisole, albendazole, ivermectin and their combinations (levamisole–albendazole, levamisole–ivermectin, albendazole–ivermectin, levamisole–albendazole–ivermectin) against multiple resistant *H. contortus* in naturally infected lambs. The observed efficacies of the single drug treatments were 45% (levamisole), 68% (albendazole) and 0% (ivermectin). For the combined treatments, efficacies of 73% (levamisole–albendazole), 35% (levamisole–ivermectin), 63% (albendazole–ivermectin) and 71% (levamisole–albendazole–ivermectin) were observed. Thus, albendazole was the active compound with the highest individual efficacy, and only the combinations containing albendazole reached efficacies $\geq$60%. A 'worse' situation was described in a Brazilian sheep flock in which low efficacy against the nematode genera *Haemonchus* spp., *Trichostrongylus* spp. and *Ostertagia* spp. was observed for most of the anthelmintic groups commonly used to control nematodes (Cezar et al., 2010). The observed efficacies, evaluated by the FECRT, were 54% (moxidectin), 32% (nitroxynil), 26% (disophenol), 23% (levamisole) and 0% (albendazole sulphoxide, ivermectin, trichlorphon and closantel). The combination of levamisole, albendazole and ivermectin reached an efficacy of 68%, showing that, in this scenario of compromised chemical control, the combination (with its limitations) demonstrated some utility to control GI nematodes. The enhanced anthelmintic efficacy obtained after the use of nematodicidal combinations against resistant *H. contortus* derived from the occurrence of positive pharmacokinetic and/or pharmacodynamic interactions between combined molecules is illustrated in Fig. 3. The sustainability

of drug combinations in this scenario is unlikely. In conclusion, when a high resistance status is observed, the combined treatments can offer a slight increase in efficacy against multiple-resistant nematodes (including *H. contortus*), but it seems likely that it will become ineffective with long-term use.

In cattle production systems, where individual active ingredients still maintain their highest efficacy, the use of anthelmintic combinations could be a useful tool to delay the development of resistance. Recent work (Canton et al., 2014) evaluated the clinical efficacy (FECRT) observed after the SC administration of ivermectin and ricobendazole given either separately or co-administered to calves naturally parasitized with GI nematodes resistant to ivermectin (*H. contortus* L_3 represents 85% in faecal cultures). The observed efficacies were 48% (ivermectin), 94% (ricobendazole) and 98% (ivermectin–ricobendazole). Since no significant differences in the egg

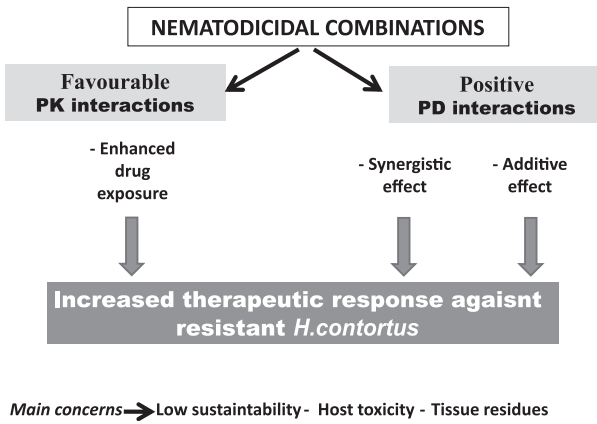

Figure 3 Increased anthelmintic efficacy may be obtained after the use of nematodicidal combinations against resistant *Haemonchus contortus* upon the occurrence of positive pharmacokinetic (PK) and/or pharmacodynamic (PD) interactions between combined molecules. The increased drug exposure (Alvarez et al., 2008; Suarez et al., 2014), additive (Anderson et al., 1988; Entrocasso et al., 2008) or synergistic PD effects (Miller and Craig, 1996) observed after the administration of a drug-combined product may help to control resistant *H. contortus*. The main concerns related to the use of 'potentially favourable' combinations are associated with a limited sustainability if the efficacy of each active included in the combined product is low (presence of highly multiple-resistant parasites) and modifications of the safety profile and/or the pattern of tissue residues depletion of a drug and/or its metabolites if large positive changes on drug exposure occurs. *Further details on the pharmacological basis of nematodicidal combinations can be obtained from Lanusse, C., Alvarez, L., Lifschitz, A., 2014. Pharmacological knowledge and sustainable anthelmintic therapy in ruminants. Vet. Parasitol. 204, 18–33.*

counts were observed between groups treated with ricobendazole alone and the combined treatment (Canton et al., 2014), no therapeutic advantage was observed for the combination. 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 parasites between farms (Smith, 2014). Since a key factor for an 'optimal result' 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). However, in vivo data obtained from sheep production systems indicate that the use of anthelmintic combinations may have limited success and sustainability, particularly in situations where *H. contortus* populations with multiple drug resistance patterns are extensively disseminated.

6. CONCLUDING REMARKS

Anthelmintic resistance in human and animal pathogenic helminths has been spreading in prevalence and severity. Multidrug resistance is becoming a widespread problem in livestock animals. The use of pharmacology-based information for existing and novel molecules is critical to the design of successful strategies for parasite control in the future. Modern technologies will likely contribute to some leading products in the field of diagnostic or drug discovery. Meanwhile, further pharmaco-parasitological integrated work, supported by the substantial progress achieved in parasite genomics, is required to generate the basic scientific knowledge necessary to optimize drug action and to preserve available and novel active ingredients as useful tools for parasite control in livestock animals. The remarkable amount of knowledge acquired using *H. contortus* as a target nematode has markedly contributed to the overall understanding of the pharmacology of anthelmintic drugs as well as of the comprehension of the mechanisms of drug resistance. Overall, this knowledge has had a favourable impact on parasite control both in veterinary and human medicine. Relevant, fundamental knowledge of the relationship among drug influx/efflux, biotransformation, accumulation and pharmacological effect in parasitic nematodes has been obtained exploiting the advantages of working with *H. contortus* under in vitro, ex vivo and in vivo experimental conditions.

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