P-glycoprotein interfering agents potentiate ivermectin susceptibility in ivermectin sensitive and resistant isolates of *Teladorsagia circumcincta* and *Haemonchus contortus*

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SUMMARY

P-glycoprotein (P-gp) homologues, belonging to the ATP Binding Cassette (ABC) transporter family, are thought to play an important role in the resistance of gastro-intestinal nematode parasites against macrocyclic lactones. The aim of this study was to investigate the influence of various P-gp interfering compounds on the efficacy of ivermectin (IVM) in sensitive and resistant nematode isolates. The feeding of IVM resistant and sensitive *Teladorsagia circumcincta* and *Haemonchus contortus* first-stage larvae (L_1) was assessed using a range of IVM concentrations (0·08–40 nM) with or without P-gp inhibitors: valspodar, verapamil, quercetin, ketoconazole and pluronic P85. The P-gp inhibitors were selected on the basis of their ability to interfere with P-gp transport activity in an epithelial cell line over-expressing murine P-gp. In the presence of P-gp interfering agents, the *in vitro* susceptibility to IVM of both sensitive and resistant isolates of *T. circumcincta* and *H. contortus* was increased. These results show that compounds interfering with P-gp transport activity could enhance IVM efficacy in sensitive isolates, and also restore IVM sensitivity in resistant nematodes. These results support the view that ABC transporters can play an important role in resistance to IVM, at least in the free-living stages of these economically important gastro-intestinal nematodes.

Key words: ABC transporters, P-glycoprotein, anthelmintic resistance, ivermectin, *Teladorsagia circumcincta*, *Haemonchus contortus*.

INTRODUCTION

In the 1980s ivermectin (IVM), which belongs to the macrocyclic lactone (ML) class of anthelmintics, was introduced onto the agricultural market. Its broad-spectrum activity and high safety profile soon made it the cornerstone of modern anthelmintic therapy for treating many endo- and ecto-parasites in livestock (Geary, 2005). However, widespread ML resistance has developed in some nematode parasites of sheep, goats and cattle (Jackson and Coop, 2000; Kaplan, 2004; Wolstenholme *et al*. 2004). The mechanisms of ML resistance are poorly understood at present, but may involve target-site mutations or non-specific mechanisms involved in the transport and/or metabolism of the anthelmintics. Previous studies have suggested that genetic variability within the glutamate-gated chloride channels (Prichard, 2005; von Samson-Himmelstjerna, 2006) and amphidial neurone genes (Freeman *et al*. 2003; Guerrero and Freeman, 2004; Yates *et al*. 2003) may affect the phenotypic expression of ML resistance. In the case of the non-specific mechanisms, decreased cuticular penetration of the drug (Scott, 1989), increased drug metabolism (Scott, 1989) and possible effects on the influx/efflux of xenobiotics by multidrug resistance transporters have been implicated in ML resistance. (Beugnet *et al*. 1997; Kerboeuf *et al*. 2002, 2003; Prichard and Roulet, 2007; Xu *et al*. 1998).

P-glycoprotein (P-gp) is a membrane-bound protein belonging to the ATP binding cassette (ABC) transporter family, whose main function is the active efflux of various structurally unrelated exogenous compounds, thus protecting both vertebrate and invertebrate organisms against potentially toxic molecules (Gottesman and Pastan, 1993). The over-expression of P-gp has been demonstrated in tumour cells in response to chemotherapy and severely restricts anti-cancer drug effectiveness (Borst *et al*. 1999). Interestingly, efflux pumps from the P-gp family of transporters have also been described in *C. elegans* (Broeks *et al*. 1995) and *H. contortus* (Blackhall *et al*. 1998; Le Jambre *et al*. 1999; Prichard and Roulet, 2007) and have been implicated in nematode resistance to all 3 broad-spectrum anthelmintics: benzimidazoles (Beugnet *et al*. 1997;

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Kerboeuf *et al*. 2002), imidazothiazoles/tetrahydropyrimidines (Rothwell and Sangster, 1997) and MLs (James and Davey, 2009; Sangster *et al*. 1999; Xu *et al*. 1998).

It has been clearly established that the administration of P-gp inhibitors *in vivo* to animals increases the bioavailability of ML (Lespine *et al*. 2008) providing a possible strategy to increase drug efficacy. Since all these data point to the importance of the role played by P-gp in the modulation of ML pharmacokinetics, it seems reasonable to assume that they have some potential as bio- or molecular targets for ML resistance.

The aim of the present study was to investigate the potential of various P-gp interfering agents to enhance the *in vitro* susceptibility of resistant and sensitive nematode isolates to IVM. Although mammalian and nematode P-gps have a low homology, and since no cell cultures over-expressing nematode efflux pumps are available, cells over-expressing mammalian P-gp were used to test the ability of various compounds to inhibit P-gp transport activity and to provide a concentration range of these compounds for use in the larval feeding inhibition test (LFIT) (Alvarez-Sanchez *et al*. 2005). Compounds identified in this way were used to determine changes in sensitivity to IVM using both sensitive and resistant *Teladorsagia circumcincta* and *Haemonchus contortus* isolates.

MATERIALS AND METHODS

Chemicals

Ivermectin, rhodamine 123 (Rho123), verapamil hydrochloride (VER), quercetin (QUER) and pluronic P85 (P85) were obtained from Sigma-Aldrich (St Louis, MO, USA). Valspodar (VAL) was a gift from Novartis (Basel, Switzerland). Ketoconazole (KET) was from ICN Biochemicals (CA, USA). All the compounds were dissolved in dimethylsulphoxide (DMSO) with the exception of P85 which was dissolved in water.

Cell culture and P-gp transport activity

In order to determine the concentration of P-gp inhibitors to be used for larval feeding inhibition tests (LFIT), P-gp transport activity was assayed by following Rho123 accumulation in an *in vitro* model of a recombinant pig kidney epithelial cell line LLC-PK1 over-expressing murine P-gp as previously described (Lespine *et al*. 2007). The effect of 5 P-gp interfering compounds on P-gp activity transport was explored. Prior to the experiments, the cells were plated into 24-well cluster plates and incubated for 2 h in Hank's Buffered Salt Solution containing 10 μM Rho123 with or without IVM (0·05–15 μM), VAL (0·01–10 μM), VER (0·1–10 μM), KET (1–100 μM), QUER (1–100 μM) or P85 (1–110 μM). The final DMSO concentration in the medium never exceeded 0·2% (v/v). The intracellular fluorescence was then measured in cell lysates and the values were normalized to the protein content per well. Valspodar was used as the reference compound for maximal inhibition of P-gp transport activity. The results obtained were expressed as percent of total valspodar inhibition.

Nematode isolates

Two *T. circumcincta* (MTci3, MTci4) and 2 *H. contortus* isolates (MHco3 and MHco4) were characterized using the LFIT. MTci3 and MHco3 are phenotypically IVM sensitive (unpublished data) whilst MTci4 (Jackson *et al*. 1992) and MHco4 (van Wyk *et al*. 1987) are phenotypically IVM resistant. The isolates were passaged through parasite naïve lambs that were housed under conditions that precluded contamination with other nematode species prior to use.

Larval feeding inhibition test (LFIT)

Nematode ova from the faeces of monospecifically infected animals were extracted as described previously (Bartley *et al*. 2003). The harvested eggs were washed, resuspended in water in a 10 cm Petri dish prior to being incubated for 16 h at 22 °C. Following this incubation period, the embryonated eggs were placed in a mini-Baermann apparatus (mesh aperture 25 μm) which was submerged in water in a 6-well cluster plate and incubated at 22 °C until the eggs hatched and the emerging first-stage larvae (L_1) migrated through the mesh. The L_1 concentration was adjusted so that 1498 μl of water in a 2 ml microcentrifuge tube contained 100 larvae.

For the LFIT, IVM concentrations ranged from 0·07 to 35 ng/ml (0·08–40 nM) whilst maintaining a single concentration of P-gp interfering compound: VAL 5 μM; VER and QUER 50 μM; KET 10 μM and P85 22 μM. Control assays were performed with only DMSO or P-gp interfering agents alone at the concentration described above. All tests were run in duplicate. The microcentrifuge tubes were incubated horizontally at 25 °C for 2 h after which time 10 μl of fluorescein isothiocyanate (FITC)-labelled *Escherichia coli* (Geary *et al*. 1993) were added. The tubes were again incubated horizontally for a minimum of 18 h at 25 °C. Following this incubation, the tubes were centrifuged at 3000 ***g*** for 20 sec and 750 μl of supernatant was then removed. Larvae were transferred onto a glass slide for counting and were examined at a magnification of ×100 using an inverted fluorescence microscope fitted with a UV blue range filter (495 nm). Larvae with FITC-labelled *E. coli* visible throughout the gastrointestinal tract were considered to be feeding.

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Table 1. Influence of several selected compounds on P-gp transport activity in LLC-PK1-mdr1a cells

(LLC-PK1-mdr1a cells were incubated in the presence of rhodamine123 (rho123) with increasing concentrations of the compounds of interest. Maximal effect (E_{max}) was calculated relatively to the maximal effect obtained in the presence of valspodar (100%). IC_{50} was the concentration needed to reach 50% of rho123 efflux inhibition. Values are mean ± S.D. of 3 experiments.)

Compound	E_{max} (% of valspodar effect)	Concentration to reach E_{max} (μM)	IC_{50} (μM)
Valspodar	100	5	0·11 ± 0·03
Ivermectin	86·1 ± 2·1	2	0·44 ± 0·04
Verapamil	50·0 ± 1·4	50	3·2 ± 1·0
Ketoconazole	46·1 ± 4·9	10	5·0 ± 1·2
Quercetin	30·3 ± 8·9	50	10·0 ± 2·1
Pluronic 85	740 ± 18	22	11·1 ± 1·5

Statistical analysis

The LFI_{99} estimates i.e. the concentration of IVM at which 99% of the L_1 did not feed was performed using a probit model on uncorrected raw data. The analyses were carried out using Genstat 6.0. Sensitivity factors were determined for each of the isolates using the standard formula: (LFI_{99} estimate of IVM alone) ÷ (LFI_{99} estimate of IVM + inhibitor). Resistance factors were determined using the equation (LFI_{99} estimate of resistant isolate) ÷ (LFI_{99} estimate of IVM sensitive isolate).

RESULTS

Characterization of P-gp interfering agents

As shown in Table 1, IVM was a potent P-gp inhibitor, with a maximum effect (E_{max}) of 86% obtained at 2 μM, and a half-maximal inhibition (IC_{50}) of 0·4 μM, compared with the reference inhibitor VAL (E_{max} of 100% obtained at 5 μM and IC_{50} = 0·11 μM). KET and VER induced Rho123 accumulation with respective E_{max} values that were 46% and 50% of those seen with VAL, obtained at 10 and 50 μM with IC_{50} values of 5 and 3·2 μM, respectively. P85 produced the greatest effects on Rho123 accumulation greater than that seen with VAL (740%) at a concentration for E_{max} of 22 μM and an IC_{50} value of 11 μM. QUER was the least potent inhibitor with an E_{max} of 30% of that recorded with VAL, at a concentration of 50 μM and an IC_{50} value of 10 μM. The concentration giving the E_{max} for each inhibitor was selected for use in the LFIT.

Isolate sensitivity to IVM and effects of P-gp interfering agents on the LFIT

Table 2 contains details of the isolate nomenclature, their IVM resistance status, the effects of P-gp inhibitor on feeding behaviour, together with estimates of the LFI_{99} and changes in sensitivity resulting from exposure to the inhibitors in combination with IVM.

Isolate sensitivity. As expected, the concentration of IVM required to inhibit 99% of larval feeding (LFI_{99}) was lower in IVM sensitive isolates than in IVM resistant ones (Table 2). The LFI_{99} estimates were 56 and 24 ng/ml for the 2 IVM sensitive isolates, MTci3 and MHco3 respectively compared with 137 and 33 ng/ml for the 2 IVM resistant isolates MTci4 and MHco4 respectively. Resistance factors were 2·5 and 1·4 for *T. circumcincta* and *H. contortus* respectively. Based on the LFI_{99} estimates the resistant and sensitive *H. contortus* isolates were 2·3- and 4·1-fold more sensitive to the effects of IVM when compared with the corresponding *T. circumcincta* isolates.

Effects of P-gp interfering agents. The effects of the P-gp interfering agents on larval feeding behaviour varied between species, isolates and drug sensitivity, as shown in Table 2. Figures 1, 2 and 3 show the dose-response curves for MTci3, MTci4 and MHco4 and clearly show in all cases a curve shift to the left in the presence of P-gp inhibitors, reflecting a decrease in larval feeding. The feeding of both IVM resistant and sensitive isolates of *T. circumcincta* (Figs 1 and 2) and *H. contortus* (Fig. 3) was influenced by the co-administration of interfering agents with IVM compared with IVM alone. For the *T. circumcincta* isolates the IVM was between 3- and 77-fold more potent, as determined by changes in sensitivity factors, whilst with the *H. contortus* isolates the increase in potency ranged between 19- and 69-fold (Table 2). The effect obtained was more pronounced with P85, VAL and VER than with QUER.

LFI_{99} estimates with IVM + P85 tended to be lower than those with the other inhibitors and ranged between 0·3 and 2·4 ng/ml (Table 2). In the 3 isolates (MTci3, MTci4 and MHco4) that were tested with IVM + VAL the LFI_{99} estimates were also low, ranging from 0·6 to 2·9 ng/ml. In the *T. circumcincta* isolates quercetin had the least effect upon LFI_{99} estimates, ranging from 36·4 (MTci3) to 48·6 ng/ml (MTci4). As a consequence of their low LFI_{99} estimates both P85 and VAL gave the highest sensitivity values which produced an average 46-fold increase in sensitivity in the *T. circumcincta* isolates compared with a 63-fold increase in *H. contortus* isolates.

DISCUSSION

The aim of this study was to investigate the ability of various P-gp interfering compounds to enhance IVM efficacy in both sensitive and resistant isolates of *Teladorsagia circumcincta* and *Haemonchus*

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Table 2. Probit analysis estimates of *in vitro* larval feeding of two *Teladorsagia circumcincta* and two *Haemonchus contortus* isolates

(Larvae feeding inhibition was performed by treating larvae with IVM from 0·07 to 35 ng/ml (0·08–40 nM) in the presence or not of interfering agents: valspodar (VAL, 5 μM), verapamil hydrochloride (VER, 50 μM), quercetin (QUER, 50 μM), pluronic P85 (P85, 22 μM) or Ketoconazole (KET, 10 μM). LFI_{99} values are mean ± S.E.M. of 3 experiments, except for MHco3 for VAL and KET that were done only in duplicate. For technical reasons no results were obtained for VER for MHco3.)

Isolate	IVM resistance status[a]	Treatment	Estimated LFI_{99} (ng/ml ± S.E.M.)	Sensitivity factor[b]
MTci3	Sensitive	IVM	55·6 (±6·1)	—
		IVM+P85	1·6 (±0·1)	36
		IVM+QUER	36·4 (±9·8)	2
		IVM+VAL	2·9 (±0·3)	19
		IVM+VER	5·6 (±1·0)	10
MTci4	Resistant	IVM	136·7 (±27·4)	—
		IVM+P85	2·4 (±0·2)	56
		IVM+QUER	48·6 (±4·6)	3
		IVM+VAL	1·8 (±0·1)	77
		IVM+VER	2·1 (±0·3)	67
MHco3	Sensitive	IVM	24·2 (±2·3)	—
		IVM+P85	0·3 (±0·0)	69
		IVM+VAL	0·3 (±0·0)	69
		IVM+KET	0·9 (±0·2)	23
		IVM+VER	n.d.	—
MHco4	Resistant	IVM	33·0 (±3·2)	—
		IVM+P85	0·6 (±0·1)	57
		IVM+VAL	0·6 (±0·1)	57
		IVM+KET	2·2 (±0·2)	15
		IVM+VER	1·8 (±0·3)	19

[a] IVM resistance status as determined by faecal egg count reduction or controlled efficacy test (references in text).
[b] Sensitivity factor was determined using the standard formula [(estimated LFI_{99} of IVM alone) ÷ (estimated LFI_{99} of IVM+inhibitor)].
n.d., Not determined.

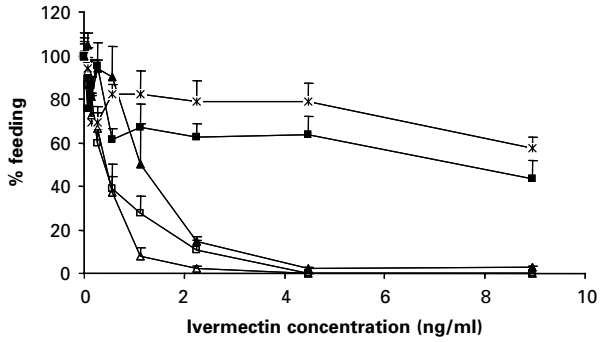

Fig. 1. Larval feeding inhibition dose-response curves generated for the *Teladorsagia circumcincta* IVM-sensitive isolate (MTci3) using IVM from 0·07 to 35 ng/ml (0·08–40 nM) (x) with or without pluronic P85, 22 μM (△);valspodar, 5 μM (□);verapamil 50 μM (▲) or quercetin, 50 μM (■). The IVM concentration range shown in this figure is from 0·07 to 10 ng/ml.

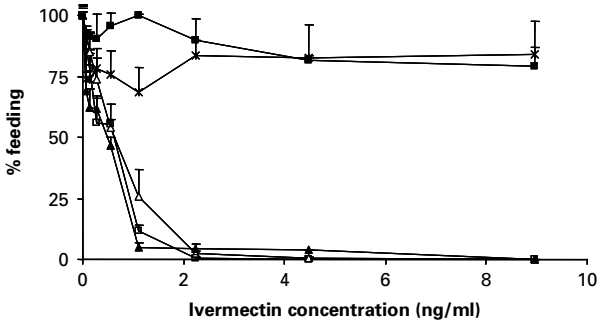

Fig. 2. Larval feeding inhibition dose-response curves generated for the *Teladorsagia circumcincta* IVM-resistant isolate (MTci4) using IVM from 0·07 to 35 ng/ml (0·08–40 nM) (x) with or without pluronic P85, 22 μM (△); valspodar, 5 μM (□); verapamil 50 μM (▲) or quercetin, 50 μM (■).The IVM concentration range shown in this figure is from 0·07 to 10 ng/ml.

contortus. This drug combination could be useful for preventing the emergence of drug resistance, increasing efficacy, or shortening the course of treatment of gastro-intestinal nematodes in livestock.

In this study, we showed, as expected, that the resistant *T. circumcincta* and *H. contortus* larvae required more IVM than sensitive larvae (1·4- and 2·5-fold, respectively) for full feeding inhibition. These results are in full agreement with previous studies (Sangster, 1996; Kotze, 1998; Sheriff *et al.* 2002) which have shown inter-specific and inter-isolate differences in IVM sensitivity. The difference

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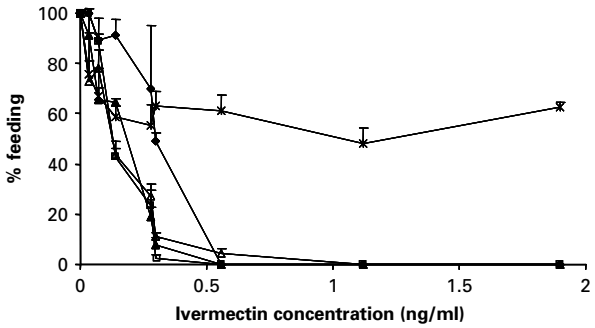

Fig. 3. Larval feeding inhibition dose-response curves generated for the *Haemonchus contortus* IVM-resistant isolate (MHco4) using IVM from 0·07 to 35 ng/ml (0·08–40 nM) (x) with or without pluronic P85, 22 μM (△); valspodar, 5 μM (□);verapamil, 50 μM (▲) or ketoconazole, 10 μM (◆). The IVM concentration range shown in this figure is from 0·07 to 1·9 ng/ml.

in resistance factors may reflect either differences in the ways that the two species handle IVM exposure or may be related to differences in the way that resistance was selected in these particular isolates, since the treatment regime has been shown to be an important factor in determining the phenotypic responses to anthelmintic treatment (Le Jambre *et al*. 1999; Sutherland *et al*. 2003). The small differences in resistance factors observed within these tests are similar to those seen in previous *in vitro* characterization studies using larval development and larval migration tests (Gill *et al*. 1995, 1998; Gill and Lacey, 1998; Le Jambre *et al*. 1995). Gill and Lacey, (1998) reported 2 to 3-fold reductions in the *in vitro* sensitivity of pre-parasitic stages to IVM in a number of *H. contortus* and *T. circumcincta* isolates while the *in vivo* efficacy of IVM against the adult stages of the same isolates ranged between 33 and 100%. Larger differences in resistance factors have been reported in controlled efficacy tests, where IVM treatments were directed against adult parasites (23 and 6 for *T. circumcincta* and *Trichostrongylus colubriformis*, respectively) (Shoop *et al*. 1993). The data from the various trials suggest that there are inter and intra (Gill and Lacey, 1998; Shoop *et al*. 1993) as well as stage (Bartley *et al*. 2005) and age of infection (Borgsteede and Couwenberg, 1987; Kerboeuf *et al*. 1989; Scott *et al*. 1989) specific differences in the way that nematodes handle anthelmintics. In the present *in vitro* studies, it is unclear why such a relatively small shift in sensitivity is sufficient to overcome IVM resistance, but it might be related to the low drug concentrations that are required to induce marked effects on the key biological processes used in the *in vitro* tests.

The mechanisms of anthelmintic resistance in nematodes are poorly understood and defined (Jabbar *et al*. 2006; Sangster *et al*. 2005; Wolstenholme *et al*. 2004) but changes in the distribution of the drug in the organism brought about by ABC transport proteins such as P-gp homologues have been proposed as one mechanism that nematodes might use in handling a range of different parasiticides (Prichard, 2007). For this reason we investigated the effect of various P-gp inhibitors on IVM efficacy. The compounds used in this study: valspodar, verapamil (Didier and Loor, 1996) and ketoconazole (Ward *et al*. 2004), the natural flavonoid quercetin (Hsiu *et al*. 2002) and a poloxamer, pluronic P85 (Kabanov *et al*. 2005), are all known to interfere with P-gp function. We showed that the presence of P-gp inhibitors increased the sensitivity to IVM in both IVM- sensitive and resistant isolates of the two parasite species. These results support the previous view that P-gp analogues play an important role in both the overall distribution of IVM and the mechanisms of IVM resistance of the free-living stages of parasitic nematodes (James and Davey, 2009; Kerboeuf *et al*. 2003; Prichard, 2007; Xu *et al*. 1998). The findings of this current study are in complete agreement with a previous study (Molento and Prichard, 1999) which demonstrated that verapamil potentiated the efficacy of IVM and moxidectin against unselected and IVM-selected strains of *H. contortus*. In the same vein, recent studies have demonstrated that exposure to verapamil or valspodar completely restores sensitivity to IVM in IVM resistant *C. elegans* (James and Davey, 2009). However, no interactive effect of P-gp inhibitors and IVM aglycone was found on the feeding of *H. contortus* L_1 (Kotze, 1998). There are no simple explanations for these different findings but they may be due to target specificity, differences in inhibitor concentrations or lower affinity for P-gp of the avermectin analogues used in the trials. Also one cannot exclude the possibility that this is solely a stage-specific phenomenon and that distinct drug-transport mechanisms may be involved at different stages of the parasite life cycle (Kotze *et al*. 2002). However, there is some evidence from *in vivo* studies using P-gp inhibitors that some mechanisms may be common to different life-cycle stages (Lanusse and Prichard, 1993; Lifschitz *et al*. 2007; Bartley *et al*. 2009).

Since it is unclear to what extent activity *in vitro* can be correlated with activity *in vivo*, any extrapolation of results from laboratory studies to the field situation needs to be approached with caution. Nevertheless, studies of the interactions of P-gp and/or detoxification enzyme modulators administered to non-parasitized sheep have demonstrated that the co-administration of ML anthelmintics with compounds such as loperamide (Lifschitz *et al*. 2002), quercetin (Dupuy *et al*. 2003), verapamil (Molento *et al*. 2004), ketoconazole, piperonyl butoxide (Virkel *et al*. 2009) or itraconazole (Ballent *et al*. 2006) can significantly increase plasma drug concentrations. Work in parasitized animals is less readily available,

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but concomitant administration of verapamil (Xu *et al*. 1998), methimazole (Lanusse and Prichard, 1993), piperonyl butoxide (Benchaoui and McKellar, 1996), loperamide (Lifschitz *et al*. 2007), ketoconazole or pluronic P85 (Bartley *et al*. 2009) with an anthelmintic has generally resulted in an enhancement of drug efficacy.

Most of our current understanding on the structure and function of P-gp and substrate/inhibitor interaction derives from mammalian studies. Since some of the inhibitors of mammalian P-gp used in this study, such as P85 and valspodar, were able to partially restore IVM sensitivity in IVM-resistant nematodes, this suggests that they may also inhibit nematode P-gp homologues. If this is the case then it seems likely that the substrate binding regions of nematode P-gps share some characteristics with mammalian P-gp and may contain overlapping substrate specificities. Furthermore, it is interesting to note that the differences in the efficiency of the P-gp inhibitors identified using mammalian cells are similar when used to inhibit nematode P-gps. However, given the low amino acid identity (around 40%) between mammalian and nematode P-gps (Kerboeuf *et al*. 2003), we may expect some differences in some other drug interactions between them.

IVM also interacts with ABC-transporters other than P-gp such as the multidrug resistance associated proteins (MRPs) (Lespine *et al*. 2006) which are also involved in multidrug resistance (Lautier *et al*. 1996). Homologues of MRPs have been described in nematode parasites (Prichard, 2007) and MRP inhibitors have been shown to reverse IVM resistance in *C. elegans* (James and Davey, 2009). Since most P-gp inhibitors tested here also bind with mammalian MRP transporters (Seelig *et al*. 2000), we cannot exclude the possibility that MRPs may also play some role in IVM resistance mechanisms.

In conclusion, this study demonstrates that P-gp inhibitors are able to increase and restore sensitivity to IVM in sensitive and resistant nematode isolates of *T. circumcincta* and *H. contortus*. These findings support the view that ABC transport proteins, in particular P-gp homologues, play an important role in the mechanisms of resistance of the free-living stages of parasitic nematodes against IVM. Even though further investigation is required to test other P-gp inhibitors and concentrations and to clarify the role played by ABC transporters in nematodes, these observations open new perspectives for using compounds which selectively target nematode P-gps and thus improve the efficacy of anthelmintic treatment.

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