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Resistance to the macrocyclic lactone moxidectin is mediated in part by membrane transporter P-glycoproteins: Implications for control of drug resistant parasitic nematodes

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ABSTRACT

Our objective was to determine if the resistance mechanism to moxidectin (MOX) is similar of that to ivermectin (IVM) and involves P-glycoproteins (PGPs). Several *Caenorhabditis elegans* strains were used: an IVM and MOX sensitive strain, 13 PGP deletion strains and the IVM-R strain which shows synthetic resistance to IVM (by creation of three point mutations in genes coding for α-subunits of glutamate gated chloride channels [GluCls]) and cross-resistance to MOX. These strains were used to compare expression of PGP genes, measure motility and pharyngeal pumping phenotypes and evaluate the ability of compounds that inhibit PGP function to potentiate sensitivity or reverse resistance to MOX. The results suggest that *C. elegans* may use regulation of PGPs as a response mechanism to MOX. This was indicated by the over-expression of several PGPs in both drug sensitive and IVM-R strains and the significant changes in phenotype in the IVM-R strain in the presence of PGP inhibitors. However, as the inhibitors did not completely disrupt expression of the phenotypic traits in the IVM-R strain, this suggests that there likely are multiple avenues for MOX action that may include receptors other than GluCls. If MOX resistance was mediated solely by GluCls then exposure of the IVM-R strain to PGP inhibitors should not have affected sensitivity to MOX. Targeted gene deletions showed that protection of *C. elegans* against MOX involves complex mechanisms and depends on the PGP gene family, particularly PGP-6. While the results presented are similar to others using IVM, there were some important differences observed with respect to PGPs which may play a role in the disparities seen in the characteristics of resistance to IVM and MOX. The similarities are of concern as parasites resistant to IVM show some degree but not complete cross-resistance to MOX; this could impact nematodes that are resistant to IVM.

1. Introduction

The macrocyclic lactones which include the avermectins (e.g., ivermectin [IVM]) and milbemycins (e.g., moxidectin [MOX]) are natural fermentation products of soil-dwelling microorganisms which have been commercialized and are used to control nematode infections (Demain and Sanchez, 2009). The avermectins are produced by *Streptomyces avermilitis* and IVM is arguably the most widely used drug in this group. MOX is the most commonly used milbemycin due to its versatility, stability, high potency and safety (Prichard et al., 2012). It is a semisynthetic methoxime derivative of nemadectin, a fermentation product of *Streptomyces cyanogriseus* subsp. *noncyanogenus* (Shoop et al., 1995). This There are several differences in the chemical structure of the avermectins and milbemycins, however the bisoleandrosyloxy substituent located at the C-13 position on the macrolide ring of avermectins, which is unsubstituted in the milbemycins, is the most notable (Campbell, 1989). Other differences include several different alkyl substituents at C-25 in both groups (Shoop et al., 1995) and the C-23 methoxime in MOX (Prichard et al., 2012). IVM was the first macrocyclic lactone that was approved for use in both animals and humans and others (e.g., abamectin, emamectin and MOX) were subsequently commercialized for the veterinary market. IVM remains the sole macrocyclic lactone registered for use in humans to treat filarioid, strongyloides and mite infections (Omura and Crump, 2004). However, MOX has undergone Phase 1, 2 and 3 clinical trials against human onchocerciasis (Prichard et al., 2012).

Despite the structural differences between the avermectins and milbemycins, the primary mechanism of action is similar and results in paralysis and death of nematodes through activation of

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glutamate-gated chloride channels (GluCls) in muscle and nerve cells (Cully et al., 1994; Dent et al., 1997; Hibbs and Gouaux, 2011) and through effects on gamma-aminobutyric acid (GABA) receptors (Feng et al., 2002). Activation of these chloride channels results in paralysis of the pharynx and somatic muscle in nematodes (Prichard et al., 2012). In mammals, the macrocyclic lactones can induce neurotoxicity by acting on GABA receptors in the central nervous system (Ménez et al., 2012).

Although both IVM and MOX act on GluCl and GABA receptors there are significant differences in their effects at different chloride channels, in different invertebrate species. Some of these differences have been summarized in Prichard et al. (2012). For example, in IVM selected strains of *Haemonchus contortus*, pharyngeal pumping was altered by IVM, but remained unchanged in the presence of MOX (Paiement et al., 1999). In *Caenorhabditis elegans* exposed to a gradient (ranging from 0 to 5000 nM) of IVM and MOX, differences were observed in pharyngeal pumping, larval development and motility of wild type and IVM resistant worms. This study concluded that the product of the *glc-2* gene may play a role in sensitivity to MOX, but not to IVM, while the products of *avr-14*, *avr-15* and *glc-1* may be important for the effects of IVM, but less so for MOX (Ardelli et al., 2009). Similarly, there are differences in the action of IVM and MOX on mammalian GABA receptors (Ménez et al., 2012).

Parasites resistant to IVM show some degree but not complete cross-resistance to MOX (see review by Prichard et al., 2012). A recent study suggested a role for the ABC systems proteins P-glycoproteins (PGPs) (Ardelli and Prichard, 2013) in IVM resistance. In this study, significant changes in movement and pharyngeal pumping were noted in an IVM resistant strain of *C. elegans* (Dent et al., 2000) in the presence of compounds known to inhibit or disrupt PGP function. However, the compounds did not completely disrupt movement and pharyngeal pumping, indicating that there are probably multiple avenues for IVM action that include receptors other than the GluCls that had been silenced.

Because there is a degree of cross-resistance between IVM and MOX, but usually not complete cross-resistance (Prichard et al., 2012), a better understanding of mechanisms of cross-resistance is required, particularly as clinical trials are underway to assess the efficacy of MOX against *Onchocerca volvulus* (WHO, 2009), a parasite for which IVM resistance has been confirmed (Osei-Atweneboana et al., 2007, 2011). The objective of this study was to determine if the resistance mechanism of MOX is similar to that of IVM and involves PGPs. To assess this we used several strains of *C. elegans* including an IVM and MOX sensitive wild-type strain, 13 PGP deletion strains and a triple IVM receptor (*avr-14/avr-15/glc-1*) knock-out strain showing synthetic resistance to IVM (Dent et al., 2000) and cross-resistance to MOX (Ardelli et al., 2009). These strains were treated with MOX and used to (1) compare the gene expression signatures of 15 PGPs in the wild-type and resistant strains following treatment; (2) measure motility and pharyngeal pumping phenotypes in the wild-type, resistant and PGP deletion strains before and after treatment; and (3) evaluate the ability of compounds that are competitive inhibitors, or that block PGP function directly, to potentiate sensitivity or reverse resistance to MOX in the wild type, IVM resistant and PGP deletion strains.

2. Materials and methods

2.1. Maintenance of C. elegans strains

C. elegans were grown on NGM agar plates seeded with *Escherichia coli* OP_{50} as described (Ardelli and Prichard, 2013). The Bristol N2 (wild-type), the synthetically resistant *avr-14/avr-15/glc-1* triple mutant (designated IVM-R) and the PGP deletion strains NL132 (*pgp*-1), GH378 (*pgp*-2), RB2349 (*pgp*-3), VC2159 (*pgp*-4), RB959 (*pgp*-5), RB104 (*pgp*-6 and *pgp*-7), RB1916 (*pgp*-8), RB1045 (*pgp*-10), VC26 (*pgp*-12), RB894 (*pgp*-13), RB2008 (*pgp*-14), and RB1041 (*pgp*-15) were used. The IVM-R strain contains a point mutation in each of the GluCl α-subunits *avr-14*, *avr-15* and *glc-1*. These point mutations make the IVM-R strain approximately 4000-fold less sensitive to IVM and it was considered resistant to IVM (Dent et al., 2000) and cross-resistant to MOX (Ardelli and Prichard, 2008; Ardelli et al., 2009). Bioinformatic and expression analysis of the PGP deletion strains indicated that they are functional nulls, homozygous, hermaphrodite stocks that are superficially wild type. The strains were out-crossed and cultures were synchronized prior to use.

2.2. Drug exposure

MOX and the PGP inhibitors R(+)-verapamil monohydrochloride monohydrate, vincristine sulfate, doxorubicin, etoposide, actinomycin D, colchicine, vinblastine, rhodamine 123, quinidine, quinine and forskolin were purchased from Sigma Life Science. All compounds were dissolved in a final concentration of 0.25 v/v of dimethylsulfoxide (DMSO). The concentrations of MOX and inhibitors are indicated within each experiment. In addition to being inhibitors of PGP proteins, these inhibitors have an additional mechanism of action including targeting of ion channels (i.e., verapamil, quinidine, quinine), tubulin (i.e., vincristine, vinblastine, colchicine), DNA replication (i.e., actinomycin, doxorubicin, etoposide) and enzymes (i.e., rhodamine, forskolin) (Palmiera et al., 2012).

2.3. Effects of MOX on PGP gene expression

The innate defense mechanism conferred by PGP against toxins in mammals is often observed as changes in expression levels (Chin et al., 1990). To determine if PGP expression responds in a similar manner in *C. elegans*, the wild-type and IVM-R strain were treated with MOX and the transcriptional profiles of the 15 PGP genes were measured to determine if treatment would result in changes in gene expression. Previous studies that evaluated GluCls, PGP and MRP expression *in C. elegans* after IVM treatment used a final concentration of 2.5 nM the same concentration was used in this study for comparative purposes (Ardelli and Prichard, 2008, 2013; Ardelli et al., 2009). The method used for expression analysis has been described (Ardelli et al., 2010). The primer sequences for the PGP genes and the control genes have been reported (Ardelli and Prichard, 2008, 2013; Ardelli et al., 2009).

To analyze gene expression, NGM agar plates were treated with either 2.5 nM MOX or no drug and inoculated with 250 adult hermaphrodite worms of each strain for each treatment (i.e., non-treated controls and MOX treated worms). Worms were removed from plates every 30 min (i.e., 0.5 h, 1 h, 1.5 h, 2 h and 2.5 h) and total RNA was extracted at each time point for use in real-time PCR analysis. The details of the real-time PCR protocol and gene expression analysis were as described (Ardelli et al., 2009; Ardelli and Prichard, 2013).

2.4. Microplate assay

Preliminary tests of motility and pharyngeal pumping were conducted in 48 well plates using adult hermaphrodites of all strains. A volume of 10 μL of worms ($n = 50$) in M9 Buffer, MOX (2.5 nM, 5.0 nM and 10.0 nM) and inhibitors (2.5 nM, 5.0 nM and 10.0 nM) were distributed to the appropriate well of the plate. Treatments included a control, MOX, an inhibitor, and MOX co-administered with an inhibitor. After three hours, worms were checked for pharyngeal pumping and movement using a Nikon

Eclipse Ti microscope. The results were considered positive if the majority of worms were moving or displayed pharyngeal pumping, and negative if there was no movement or pharyngeal pumping.

The results of this pilot study indicated that there were differences in the effects of MOX on pharyngeal pumping and motility in the wild-type, IVM-R and PGP deletion strains that are concentration dependent. MOX alone did not affect phenotype in the wild-type or IVM-R strain at any concentration tested for the three hour time period used in this study; this was similar to a previous study (Ardelli et al., 2009). However, incubation for longer periods resulted in a loss of motility and pharyngeal pumping in the Bristol N2. Based on these results further detailed analyses of motility (i.e., velocity and path length) and pharyngeal pumping were conducted at 5.0 nM using agar plates as described below. The results of the pilot study are reported in Supplementary Tables 1–3.

2.5. Macroplate assay

NGM agar plates were seeded with *E. coli* OP_{50} and controlled for temperature, touch effects, light and developmental stage as described (Ardelli et al., 2009; Ardelli and Prichard, 2013). To assess motility of worms, plates were inoculated with 15.0 µL of 5.0 nM MOX, 5.0 nM of an inhibitor, or MOX co-administered with an inhibitor (each at a concentration of 5.0 nM). Control plates were not inoculated with MOX or an inhibitor. Plates were incubated overnight at room temperature to allow bacteria to grow and then seeded with 10 adult hermaphrodite worms for each treatment. Motility and pharyngeal pumping were recorded after three hours. The macroplate assay was repeated three times (i.e., three independent trails) with 10 worms measured per trial. Motility was determined using a QImaging Qicam interfaced with a Nikon SMZ1500 microscope networked with a computer. Using the program NIS Elements version 4.0, the movement of each worm was recorded for one minute. Path velocity (px/s) and distance traveled (px) were determined. The number of pharyngeal pumps of each worm was recorded for a period of one minute. Full movement of the grinder to the back of the terminal bulb and return to the initial position within the terminal bulb was considered one pharyngeal pump.

2.6. Data analysis

We determined if overall differences existed between phenotypic responses of control and MOX treated worms with an analysis of variance (ANOVA). A Tukey's Honest Significant Difference (HSD) test with Bonferroni adjustment was used for pairwise comparisons of the mean response to the different treatments. This was to determine which of the treatments differed from the others and at what time point the difference was detected. We controlled for MOX exposure by testing treatments against negative controls (no MOX or inhibitor) using Spearman's Chi squares probability and found all test contrasts to be significant (results not shown). Thus we are confident that any effects due to the addition of inhibitor are real. Significance for all statistical tests was taken at $p < 0.05$.

3. Results

3.1. Transcriptional profiles of ABC systems genes following treatment with MOX

The transcriptional profiles of 15 PGPs in the wild-type and the IVM-R strain were determined after exposure to 2.5 nM MOX (Fig. 1). The transcriptional profile for each gene was determined over 2.5 h beginning 30 min after introduction of worms to agar plates, and for every 30 min thereafter, up to 2.5 h after drug exposure. Significant differences in the transcriptional profiles (indicated by an asterisk [∗]) for these two strains were noted for *pgp-1* (2.5 h), *pgp-5* (2.5 h), *pgp-6* (1.5 and 2.5 h), *pgp-12* (0.5, 1.5 and 2.5 h), *pgp-14* (1.5 and 2.5 h) and *pgp-15* (1.5 and 2.5 h). Significant differences were not detected for *pgp*-2, *pgp*-3, *pgp*-4, *pgp*-7, *pgp*-8, *pgp*-9, *pgp*-10, *pgp*-11 and *pgp*-13 following treatment with MOX (Fig. 1).

3.2. Pharyngeal pumping

Fig. 2(A–E) shows the significant results obtained for the pharyngeal pump rate (pumps/minute) for *C. elegans* following treatment with MOX alone, inhibitor alone or MOX administered in combination with an inhibitor. The complete data set is shown in Supplementary Fig. 1. Following treatment of the Bristol-N2 (MOX sensitive, wild-type) strain with MOX, the pharyngeal pump rate was significantly lower compared to the non-treated control, but it did not cease (Fig. 2A). However, pharyngeal pumping stopped following treatment of this strain with MOX in combination with an inhibitor (Fig. 2B–E).

3.2.1. IVM-R (cross-resistant to MOX)

The IVM-R strain was created by introducing point mutations into the alpha subunit genes *avr-14*, *avr-15* and *glc-1* (Dent et al., 2000). These mutations did not affect the pump rate in this strain as it was not significantly different from that of the wild-type (Fig. 2A). Treatment of the IVM-R strain with MOX either alone or in combination with inhibitors lowered the pump rate however it did not cease (Fig. 2B–E). For the majority of the inhibitors administered alone or in combination with MOX, there was an increased pump rate (e.g., verapamil, quinidine and colchicine) when compared to the non-treated IVM-R control (indicated by the short, red dotted line). These results are similar to those observed in the wild-type strain.

3.2.2. Pgp-2 and pgp-14 deletion strains

PGP-2 and PGP-14 are normally expressed in the pharynx (Zhao et al., 2004). Disruption of these genes affected the pump rate and it was significantly higher in these strains than that of the wild-type (Fig. 2A). Pumping ceased following administration of MOX for the *pgp-2* but not the *pgp-14* deletion strain. Co-treatment of the *pgp-14* deletion strain with MOX and an inhibitor caused pharyngeal pumping to cease, with the exception of quinidine, colchicine and forskolin (2B–E). These results are different to those observed for the wild-type strain.

3.2.3. Pgp-8 and pgp-15 deletion strains

PGP-8 and PGP-15 are normally expressed in the neurons (Zhao et al., 2004). Disruption of these genes affected the pump rate and it was significantly higher in these strains compared to the wild-type strain (Fig. 2A); however pumping ceased following MOX treatment.

3.2.4. Pgp-1, pgp-5 and pgp-10 deletion strains

PGP-1, PGP-5 and PGP-10 are normally expressed in the intestine (Zhao et al., 2004). Disruption of these genes resulted in a significantly higher pump rate in these strains when compared to that of the wild-type (Fig. 2A). Following treatment with MOX, pumping ceased in the *pgp-1* and *pgp-10* deletion strains, but not in the *pgp-5* strain, although it was significantly reduced (Fig. 2A). Similar to effects observed in the wild-type, treatment of the *pgp-5* deletion strain with MOX administered in combination with an inhibitor caused pharyngeal pumping to cease (Fig. 2B–E).

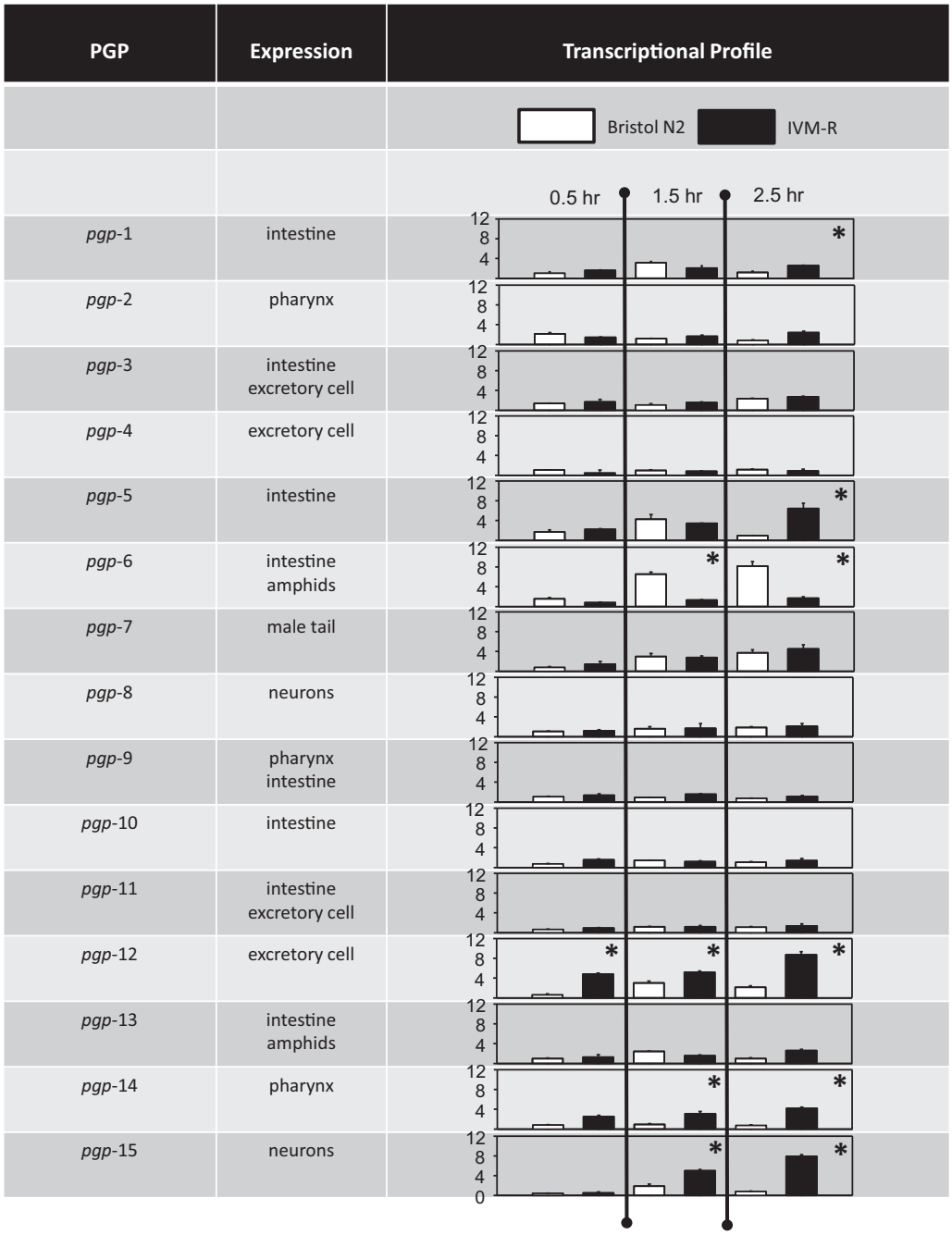

Fig. 1. Transcriptional profile of 15 PGP genes in *C. elegans* wild-type and deletion strains following treatment with MOX. The figure shows the strain, expression site (Zhao et al., 2004) and transcriptional profile of each gene at 0.5 h, 1.5 h and 2.5 h after treatment with 2.5 nM MOX. The profile of the wild-type strain is represented by a white bar (□) and that of the IVM-R strain by a black bar (■). The *Y*-axis shows the fold over-expression compared with expression in the absence of MOX. Significant differences in the profiles between the two strains are indicated by an asterisk (∗).

3.2.5. Pgp-6, pgp-13, pgp-3, pgp-4, pgp-12 and pgp-7 deletion strains

Disruption of PGP-13 (expressed in intestine and amphids), PGP-3 (expressed in the intestine and excretory cell) and PGP-4 and PGP-12 (both expressed in the excretory cell) had a significant effects on the pump rate (Fig. 2A), and it ceased following MOX treatment. PGP-6 and PGP-7 are found in a four gene cluster with PGP-5 and PGP-8 and are expressed in the intestines and amphids (PGP-6) and the tail (PGP-7; Zhao et al., 2004). Disruption of PGP-6 and PGP-7 genes did not affect the normal non-treated pharyngeal pump rate; however it ceased following treatment with MOX.

3.3. Motility: path length and velocity

For motility, the parameters path length (px) and velocity (px/s) were measured, however only results for velocity (Fig. 3A–E) are reported as the patterns produced for path length and velocity were similar (i.e., a significant increase or decrease in velocity was correlated with a significant increase or decrease in path length). Gene disruption did not affect velocity in *pgp-14* and *pgp-15* deletion strains as it was not significantly different from the non-treated wild-type. For all other strains, the gene mutations

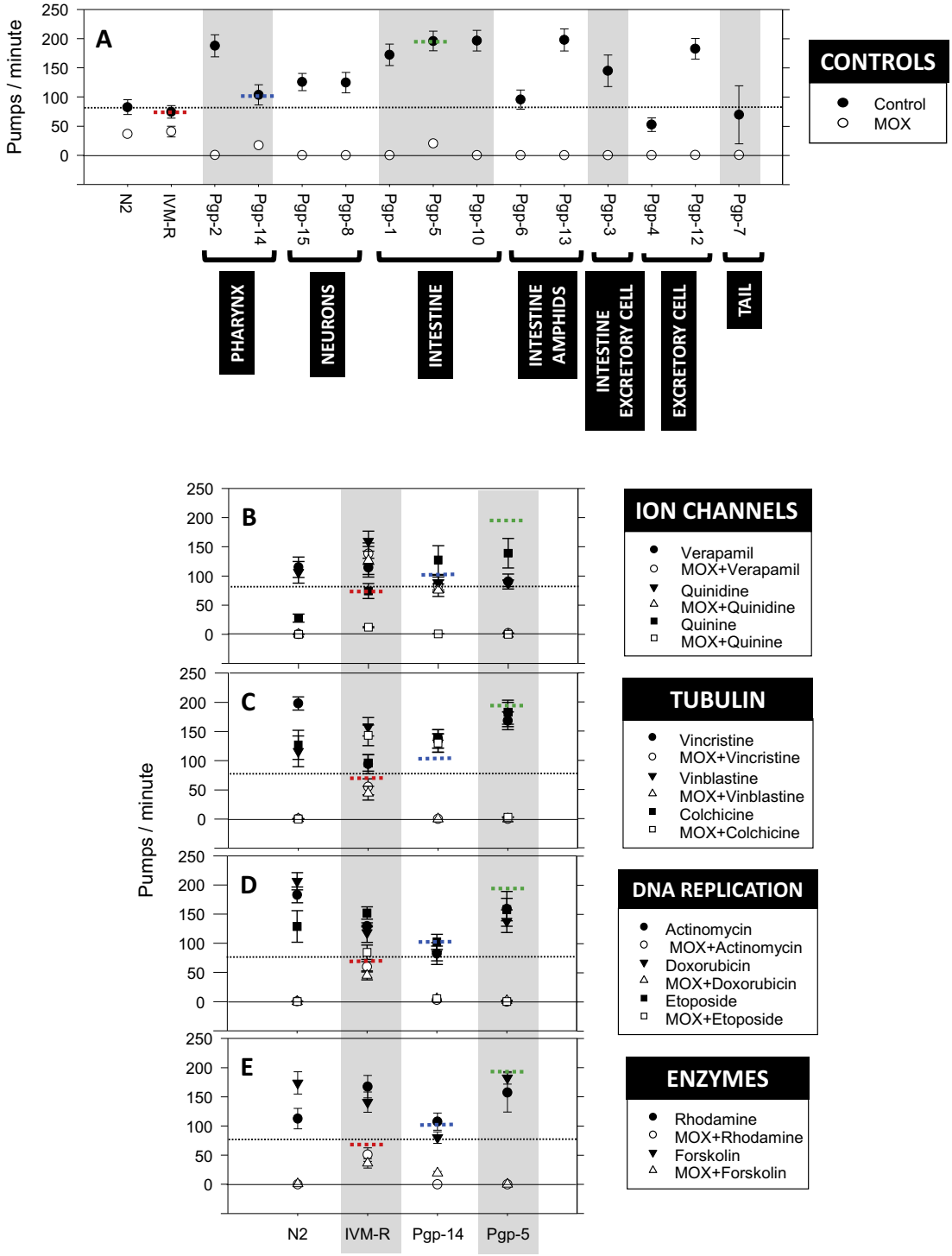

Fig. 2. (A–E) The pharyngeal pump rate (pumps/minute) following treatment with MOX (5 nM), inhibitor (5 nM) or MOX administered in combination with an inhibitor (both at 5 nM). (A) The *x*-axis shows the *C. elegans* strains which are subdivided according to the expression site (Zhao et al., 2004). Figures (B–E) are arranged according to the inhibitors tested and are subdivided based on their additional mechanism of action into ion channels (i.e., verapamil, quinidine and quinine), tubulin (i.e., vincristine, vinblastine and colchicine), DNA replication (i.e., actinomycin, doxorubicin and etoposide) and enzymes (rhodamine and forskolin). For each inhibitor, the results are represented by symbol pairs such that the closed symbols represent the mean ± standard deviation of inhibitor alone and the open symbols represent the mean ± standard deviation of the inhibitor administered in combination with MOX. Two lines were drawn across the entire graph for each treatment: the top dotted line indicates the mean pharyngeal pump rate of the wild-type sensitive strain without treatment and the bottom solid line was drawn from zero. As the Bristol N2 was the control (i.e., MOX sensitive wild-type strain), effects on pharyngeal pumping were compared against this strain. As the top dotted line is the mean response of the non-treated wild-type, any data point that falls above or below, and does not intersect, the line is statistically significant with respect to this strain. The shorter colored dotted lines represent the mean ± standard deviation of that mutant strain without treatment. Figures (B–E) depict the pharyngeal pump rate for the N2, IVM-R, *pgp*-14 and *pgp*-15 deletion strains as pumping in all other strains ceased following treatment with MOX. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

resulted in a significantly lower velocity. For all strains, including the wild-type, the velocity was significantly reduced following treatment with MOX, but it did not cease (Fig. 3A). The exception was the IVM-R strain in which there was no change in the velocity following MOX treatment.

As the Bristol N2 (MOX sensitive, wild-type) strain was still motile following treatment with MOX, a number of additional experiments were performed where the strain was treated with MOX in combination with a number of PGP inhibitors. For the 11 different treatment combinations, velocity was significantly reduced in this strain but it did not cease (Fig. 3B–E). Similar results were obtained for the 14 mutant strains examined.

4. Discussion

In this study we report on two phenotypes, pharyngeal pumping and velocity, in a MOX/IVM sensitive wild-type strain, an IVM/MOX resistant strain and 13 PGP deletion strains in an effort to understand the potential role of PGP in MOX resistance. Since some nematodes resistant to IVM are also cross-resistant to MOX, an understanding of the mechanism of resistance and cross-resistance in different species of nematodes is important, particularly as clinical trials have progressed to Phase 3 for MOX to assess its efficacy against *O. volvulus* (WHO, 2009), a parasite for which IVM resistance has been identified (Osei-Atweneboana

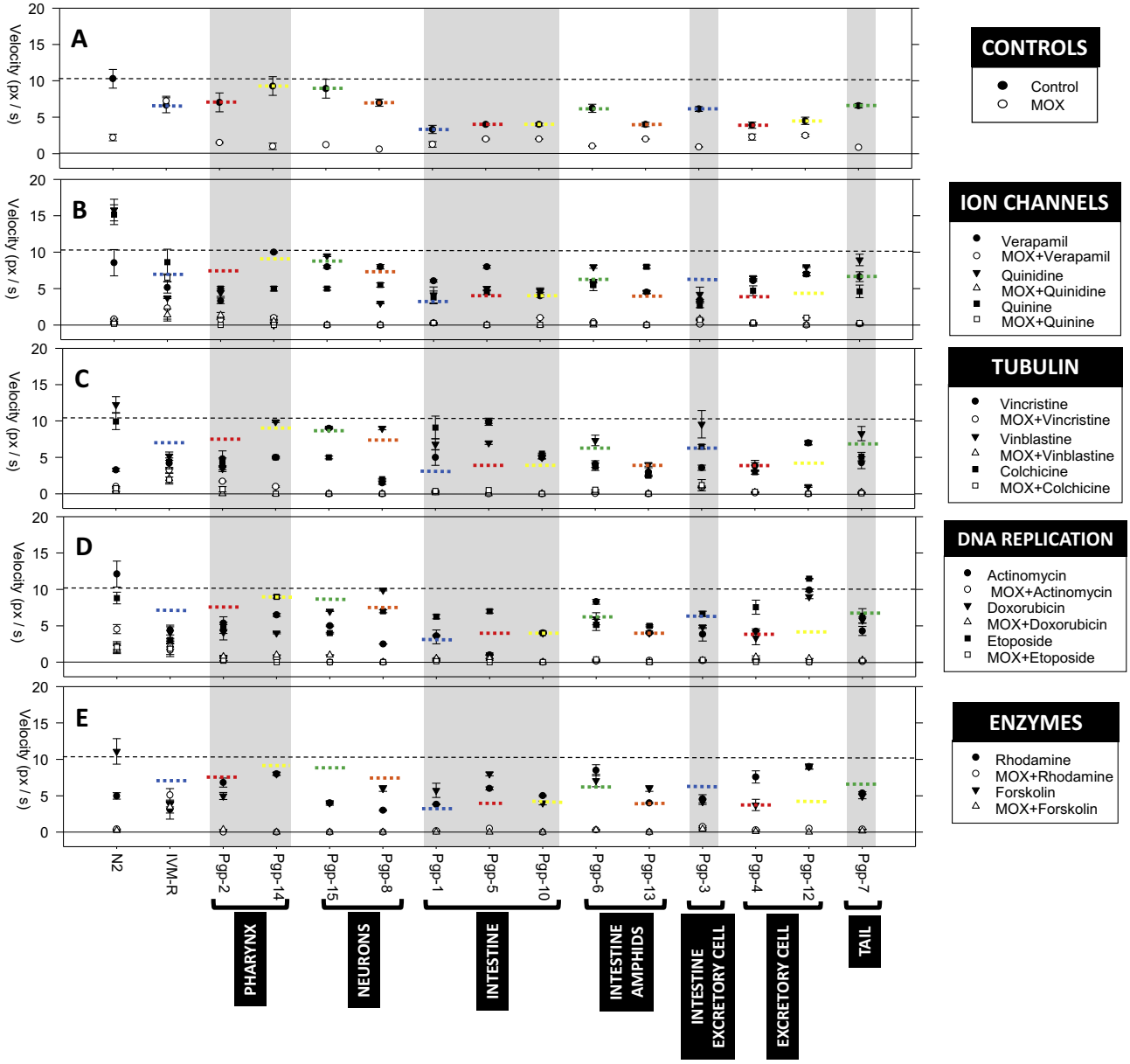

Fig. 3. (A–E) Velocity (px/second) following treatment with MOX (5 nM), inhibitor (5 nM) or MOX administered in combination with an inhibitor (both at 5 nM). The *x*-axis shows the *C. elegans* strains which are subdivided according to the expression site (Zhao et al., 2004). Figures (A–E) are arranged according to the inhibitors tested and are subdivided based on their additional mechanism of action into ion channels (i.e., verapamil, quinidine and quinine), tubulin (i.e., vincristine, vinblastine and colchicine), DNA replication (i.e., actinomycin, doxorubicin and etoposide) and enzymes (rhodamine and forskolin). For each inhibitor, the results are represented by symbol pairs such that the closed symbols represent the mean ± standard deviation of inhibitor alone and the open symbols represent the mean ± standard deviation of the inhibitor administered in combination with MOX. Two lines were drawn across the entire graph for each treatment: the top dotted line indicates the mean pharyngeal pump rate of the wild-type sensitive strain without treatment and the bottom solid line was drawn from zero. As the Bristol N2 was the control (i.e., MOX sensitive wild-type strain), effects on velocity were compared against this strain. As the top dotted line is the mean response of the non-treated wild-type, any data point that falls above or below, and does not intersect, the line is statistically significant with respect to this strain. The shorter colored dotted lines represent the mean ± standard deviation of the mutant strain without treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

et al., 2007, 2011). As a number of studies on nematodes have suggested efflux of IVM by PGP as a protective or resistance conferring mechanism (Ardelli and Prichard, 2008; James and Davey, 2009; Dicker et al., 2011; Williamson et al., 2011; Lespine et al., 2012), the aim of this study was to determine the contribution of PGPs to the effects of MOX, using *C. elegans* as a model organism. The majority of studies on PGPs and their role in drug resistance in nematodes have focused on IVM with limited studies on role of these transporters and MOX effects. While a number of the deletion strains appear to be superficially wild-type, the observations on pharyngeal pumping and motility suggest that deletion of some of the PGP genes has a measurable effect on phenotype. For example, the normal non-treated pharyngeal pump rate for the deletion strains *pgp*-1, *pgp*-2, *pgp*-3, *pgp*-5, *pgp*-10, *pgp*-12 and *pgp*-13 were significantly higher than that of the wild-type strain, suggesting that deletion of these genes does affect feeding in these strains. In contrast, where gene deletion increased the pharyngeal pump rate, the opposite was observed with motility in that velocity was significantly lower in the deletion strains in comparison with the wild-type. A study by Janssen et al. (2013) of the effects of PGP gene deletion on *C. elegans* demonstrated an effect on development in *pgp-8*, *pgp-14* and *pgp-11* deletion strains.

A study of GluCl genes in *C. elegans* suggested that the product of the *glc-2* gene may play a role in sensitivity to MOX, but not to IVM, while the products of *avr-14*, *avr-15* and *glc-1* genes may be important for the effects of IVM, but less so for MOX (Ardelli et al., 2009). A recent study of the IVM-R strain used in this study demonstrated increased sensitivity to IVM (i.e., paralysis or reduced pharyngeal pumping) following co-administration of IVM with verapamil, or actinomycin D, or daunorubicin, or colchicine (Ardelli and Prichard, 2013). As these drugs are known inhibitors of PGP, it was suggested that the action of IVM is mediated by receptors in addition to GluCls and likely includes members of the ABC systems family, particularly PGPs (Ardelli and Prichard, 2013). In the current study, co-administration of MOX with any inhibitor caused pharyngeal pumping to cease while velocity was significantly reduced by some inhibitors in the wild-type strain (in the three hour period used in this study). In the IVM-R strain, while exposure to some inhibitors in combination with MOX reduced motility and pharyngeal pumping, they did not cease. This suggests that the product of the *glc-2* gene, which is a β subunit (Laughton et al., 1997), is more important for the effects of MOX than are the products of the *avr-14*, *avr-15* and *glc-1* genes. In addition, PGPs may play a role in MOX resistance, as sensitivity to MOX increased following addition of a PGP inhibitor, however the effects were less pronounced than for IVM. This is not surprising as MOX has a weaker affinity for PGP than the avermectins (Prichard et al., 2012). However, the PGP inhibitors used can all have effects on non-PGP targets, and the potentiation of the combination of the inhibitor to MOX could be due to non-PGP effects caused by the inhibitor.

PGP homologues are found across a number of species, from microorganisms to humans (Higgins, 2001). Despite being found in varying numbers in many species, the structure and function of a typical PGP are similar and they confer an "innate resistance" against toxins. The activation of this defense mechanism in mammals and nematodes is often observed as changes in expression levels (Chin et al., 1990). There were differences in the expression profiles of 15 PGP genes in the Bristol N2 and the IVM-R strains following treatment with MOX (present study) and IVM (Ardelli and Prichard, 2013). Following 0.5 h of IVM treatment, there was a significant increase in the expression profiles of *pgp-1*, *pgp-9*, *pgp-11*, *pgp-12*, *pgp-13*, *pgp-14* and *pgp-15* in the IVM-R strain while significant differences were only noted for *pgp-12* after treatment with MOX. Similarly, after 1.5 h of treatment, the IVM-R strain showed increased expression of *pgp-4*, *pgp-7*, *pgp-11*, *pgp-13*, *pgp-14* and *pgp-15* with IVM, and of *pgp-6*, *pgp-12*, *pgp-14* and *pgp-15* with MOX treatment. After 2.5 h, treatment with IVM induced increased expression of *pgp-4*, *pgp-7*, *pgp-11*, *pgp-14* and *pgp-15* in the IVM-R strain, and of *pgp-1*, *pgp-5*, *pgp-6*, *pgp-12*, *pgp-14* and *pgp-15* after treatment with MOX. A study examining larval development in the *C. elegans* wild-type and the 15 PGP deletion strains following treatment with IVM suggested that *pgp-11* and *pgp-14* were important (Janssen et al., 2013). While there were a number of similar PGP expression patterns induced in the IVM-R strain after treatment with both MOX and IVM, there were also some unique expression profiles noted. For example, a significant increase in expression was noted for *pgp-6* (expressed in intestine [Zhao et al., 2004]) following MOX treatment that was not induced by IVM at any of the time points examined, while a significant increase in expression was noted for *pgp-4* (expressed in excretory cell), *pgp-7* (expressed in tail), *pgp-9* (expressed in pharynx and intestine), *pgp-11* (expressed in intestine and excretory cell) and *pgp-13* (expressed in intestine and amphids) following IVM treatment that were not noted for MOX. A change in transporter activity, induced by changes in gene expression, can have an impact on drug efficacy and resistance Pajic et al., 2009; Gao et al., 2010; Menez et al., 2012). The differences in PGP expression induced by MOX and IVM suggest some differences in the mechanism of action and efflux mechanisms that may contribute to resistance, as both studies were conducted using the same IVM resistant strain of *C. elegans*. As the main function of PGP is detoxification, the results of this study suggest that different detoxification mechanisms may be involved, as there was increased expression in some PGPs that were unique to MOX and some that were unique to IVM.

Studies of mammals with PGPs that was either deleted or disrupted showed increased sensitivity to IVM (Schinkel et al., 1994). It is of interest that in Mdrab (−/−) double knock-out mice there was an approximately 5-fold lower sensitivity to MOX toxicity than to IVM toxicity (Ménez et al., 2012). A study examining *C. elegans* strains that had all or portions of the multidrug resistance associated protein (MRP) genes deleted demonstrated that disruption of the MRP genes caused less change in the motility phenotype following MOX exposure compared with IVM exposure, suggesting that the MRPs may play less of a role in protecting *C. elegans* from MOX toxicity than they do in protecting the nematode from IVM toxicity. In this study, the effect of deleting part of or the entire PGP gene on sensitivity to MOX was examined in 13 PGP deletion strains by measuring changes in velocity and pharyngeal pumping. In terms of pharyngeal pumping, exposure to MOX caused pumping to cease in all PGP deletion strains except for the *pgp-5* and *pgp-14* deletion strains. Velocity was significantly reduced but did not cease in all of the PGP deletion strains following MOX treatment. The characteristic phenotype associated with motility in IVM exposed wild-type worms is a "hyperactivity" which peaks at 1.5 h (Ardelli et al., 2009). Inactivation of *pgp-2*, *pgp-5*, *pgp-6*, *pgp-7*, *pgp-12* and *pgp-13* resulted in increased sensitivity to IVM and the magnitude of this increased sensitivity varied (Ardelli and Prichard, 2013). Deletion of these genes resulted in an earlier onset of paralysis in treated worms than was observed in treated wild-type worms; pharyngeal pumping was not measured following exposure to IVM in the PGP deletion strains (Ardelli and Prichard, 2013). While the effects on velocity in a number of the PGP deletion strains were similar after treatment with both MOX (present study) and IVM (Ardelli and Prichard, 2013), there were also some unique patterns (with the exception of deletion strains *pgp*-1, *pgp*-3, *pgp*-8, *pgp*-10, *pgp*-14 and *pgp*-15 which were not examined by Ardelli and Prichard (2013)). For example, disruption of the *pgp*-2, *pgp*-5, *pgp*-12 and *pgp*-13 genes resulted in paralysis following 2.5 h of IVM exposure, but worms were still motile following exposure to MOX (although significantly reduced). As discussed previously (Ardelli and Prichard, 2013), *pgp-12* is

strongly expressed in the excretory cell and forms a four-gene cluster of duplicated genes in tandem with *pgp-13*, *pgp-14* and *pgp-15* (Zhao et al., 2004). As the excretory cell of *C. elegans* performs functions analogous to the mammalian kidney, PGPs associated with this cell would be expected to assist in toxin elimination. Targeted disruption of *pgp-12* could result in an inability to remove IVM and perhaps metabolic waste products, resulting in an increased disturbance to homeostasis, leading to a more pronounced paralysis. It has been suggested that IVM is excreted from the mammalian intestine through a PGP-dependent pathway, while MOX is mostly excreted via a PGP-independent pathway (Kiki-Mvouaka et al., 2010). The results of the current study are consistent with this hypothesis as *pgp*-12 and *pgp*-13 deletion *C. elegans* strains were more sensitive to IVM than to MOX.

The function of PGPs can be inhibited by substrates that are competitive inhibitors (e.g., verapamil) or that block their function directly. Treatment of MDR organisms with inhibitors restores drug sensitivity (Molento and Prichard, 1999; Summers et al., 2004). We explored the potential for PGP inhibition with substrates by exposing *C. elegans* wild-type, IVM-R and PGP deletion strains to a number of compounds that are known to interfere with PGP function. The results are depicted in Figs. 2 (pharyngeal pumping), 3 (velocity) and Supplementary Fig. 1 and are arranged based on the additional mechanism of action of the compound (i.e., ion channels, tubulin, DNA replication and enzymes). With pharyngeal pumping, the majority of compounds administered in combination with MOX inhibited the pharyngeal pump rate in the wild-type and PGP deletion strains, but not the IVM-R. Verapamil is a calcium channel blocker that is well documented as a viable PGP inhibitor in humans and other mammals (Summers et al., 2004; Syvanen and Hammarlund-Undenaes, 2010). When administered alone, verapamil reduced the pump rate in all PGP deletion strains, and it ceased in the *pgp*-2, *pgp*-4 and *pgp*-6 deletion strains. Following co-administration of verapamil with MOX pharyngeal pumping ceased in all strains with the exception of the *pgp*-14 (expressed in pharynx) and *pgp*-15 (expressed in neurons) deletion strains and the IVM-R strain. This is in contrast to a similar study examining the effects of IVM and verapamil on the IVM-R and wild-type strains (Ardelli and Prichard, 2013). In that study, verapamil was the only inhibitor that induced paralysis in both the wild-type and IVM-R strains when the worms were exposed to IVM, though as expected, the wild-type strain was more sensitive than the IVM-R strain. The observations of this study are consistent with verapamil being a potent PGP inhibitor in *C. elegans*. Similar to the results obtained following co-administration of IVM with verapamil, there were concentrations of MOX at which verapamil was able to induce paralysis of movement and pharyngeal pumping in the wild-type and IVM-R strains (see Supplementary Tables 1–3). A study examining the kinetics of IVM and MOX in sheep, following co-administration of the drugs with verapamil demonstrated an impact on IVM kinetics but not on MOX (Molento et al., 2004).

Of interest were the *pgp*-6 and *pgp*-7 deletion strains. For the *pgp*-7 deletion strain, all inhibitors, with the exception of verapamil, had a stimulatory effect in that the pharyngeal pump rate significantly increased in comparison to the non-treated control, however all inhibitors administered in combination with MOX caused pharyngeal pumping to cease. The opposite effect was observed in the *pgp*-6 deletion strain. With the exception of quinine and actinomycin, all inhibitors, administered alone or in combination, caused pharyngeal pumping to cease in this strain, pumping ceased with quinine and actinomycin following co-administration with MOX. Movement was less sensitive to the effects of MOX, administered alone or in combination, for these two strains. Many of the 60 *C. elegans* ABC transporter genes are found in clusters and pairs of sister genes (Zhao et al., 2004). Sixteen *C. elegans* ABC genes on the X chromosome are arranged in tandem, forming two four-gene clusters and four two-gene clusters. PGPs 6 and 7 are found in a four-gene cluster along with PGPs 5 and 8. We did find a similar pattern of induction of expression for these genes in both the wild-type and the IVM-R strain of *C. elegans* when exposed to MOX. PGP-6 showed the greatest change in expression in the N2 strain while PGP-7 showed the greatest change in the IVM-R strain following exposure to MOX. PGP-6 is expressed in the larval and adult intestine as well as the lateral sensilla at the tip of the adult head (amphids), while PGP-7 is expressed in the tail (Beuchner et al., 1999). The sensitivity of the *pgp*-6 deletion strain to MOX (either administered alone or in combination with inhibitors), as well as the significantly increased expression of the gene in the N2 strain following treatment with MOX, suggests that PGP-6 may be important in protecting *C. elegans* from MOX toxicity. In a study of PGP-6 expression in *C. elegans* following exposure to IVM, a significant over-expression of PGP-6 was demonstrated (Ardelli and Prichard, 2013). It was suggested that the significant over-expression could help protect the nematodes from the effects of IVM because the sensory endings of the extra pharyngeal neurons are in the amphids and drug uptake with food or via the chemosensory amphids are important means by which nematode cells become exposed to drugs (Bargmann and Horvitz, 1991). Thus, the over-expression of PGPs in these tissues could be expected if the worms use these pumps to remove the drug from the intestinal cells and amphidial neurons. It is plausible that a similar mechanism is operating in *C. elegans* with respect to MOX and PGP-6.

We have shown that *C. elegans* may use PGP regulation as a response mechanism to MOX. This response was measured in the over-expression of several PGPs, in both susceptible wild-type and the synthetically resistant IVM-R strains exposed to MOX. The significant changes in movement and pharyngeal pumping in the IVM-R strain in the presence of compounds known to inhibit or disrupt PGP function is consistent with the hypothesis that MOX action is mediated in part by PGP transporter proteins. However, the compounds did not completely disrupt expression of these phenotypic traits (within our 2.5 h study period), which indicates that there are likely to be multiple avenues for MOX action that may include receptors other than GluCls. If MOX resistance was mediated solely by GluCls, then exposure to PGP inhibitors should not have affected sensitivity to MOX. Targeted gene deletion has shown that protection of *C. elegans* against MOX involves complex mechanisms and depends in part on members of the PGP gene family, particularly PGP-6. The results obtained in this study are similar to those obtained using IVM (Ardelli and Prichard, 2013) however there were some important differences. The differences observed with respect to PGP may play a role in the differences seen in the expression of resistance to IVM and MOX. However, the similarities observed are of concern as parasites resistant to IVM show some degree but not complete cross-resistance to MOX (see review by Prichard et al., 2012); this could impact nematodes that are resistant to IVM, such as *O. volvulus*. Further research is needed to determine the extent to which these PGP transporters play a role in drug cross-resistance to IVM and MOX in parasitic nematodes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpddr.2014.06.002.

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