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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