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Short Communication

Synergism between ivermectin and the tyrosine kinase / P-glycoprotein inhibitor crizotinib against *Haemonchus contortus* larvae *in vitro*

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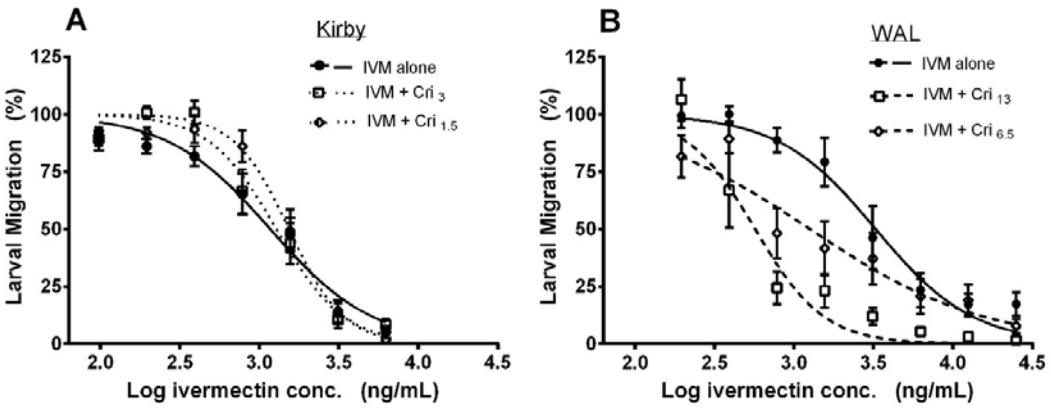
Graphical abstract



Highlights:

- we examined interaction of P-gp inhibitor crizotinib with ivermectin in *Haemonchus contortus* larvae *in vitro*

- crizotinib increased the toxicity of ivermectin towards a resistant isolate in migration assays

- less synergism observed in larval development assays

- assay differences suggest life-stage specific patterns of ivermectin / P-gp interaction.

- study highlights potential of P-gp inhibitors to reverse ivermectin resistance

Abstract

Anthelmintic resistance is a major problem in parasitic nematodes of livestock worldwide. One means to counter resistance is to use synergists that specifically inhibit resistance mechanisms in order to restore the toxicity, and hence preserve the usefulness, of currently available anthelmintics. P-glycoproteins (P-gps) eliminate a wide variety of structurally unrelated xenobiotics from cells, and have been implicated in anthelmintic resistance. Crizotinib is a tyrosine kinase inhibitor under development as a cancer therapeutic. The compound also inhibits P-gps, and has been shown to reverse multidrug resistance in cancer cells. We were therefore interested in determining if the compound was able to increase the sensitivity of *Haemonchus contortus* larvae to ivermectin, as measured by *in vitro* larval development and migration assays with a drug-resistant and a –susceptible isolate. In migration assays, co-administration of crizotinib increased the toxicity of ivermectin to resistant larvae (up to 5.7-fold decrease in ivermectin IC_{50}), and rendered the resistant larvae equally or more sensitive to ivermectin than the susceptible isolate. On the other hand, co-administration of crizotinib had no effect on ivermectin sensitivity in the

susceptible isolate. In development assays, significant increases in the sensitivity of both the resistant (up to 1.9-fold) and susceptible (up to 1.6-fold) larvae to ivermectin were observed , although the magnitude of the observed synergism was less than seen in migration assays, and the resistant larvae retained significant levels of ivermectin resistance. By highlighting the ability of the P-gp inhibitor crizotinib to increase the sensitivity of *H. contortus* larvae to ivermectin, this study provides further evidence that P-gp inhibitors are potential tools for modulating the efficacy of anthelmintics. In addition, the differences in the outcomes of the two assays, with 'resistance-breaking' effects being much more marked in migration assays, suggest that some life-stage-specific aspects may exist in the interaction of ivermectin with P-gps in the two worm isolates.

Keywords: Crizotinib, P-glycoprotein, Inhibitor, *Haemonchus contortus*, Anthelmintic resistance

1. Introduction

The development of resistance to almost all available anthelmintics threatens our ability to control parasitic nematodes in livestock enterprises worldwide. Given the time and cost of developing new drugs (Woods and Williams, 2007), there is a need to manage the use of the existing drugs to preserve their usefulness for as long as possible. Means to achieve this include the elucidation of resistance mechanisms in order to develop resistance diagnostics (Kotze et al., 2014), as well as the use of compounds that can act to inhibit resistance mechanisms, and hence restore anthelmintic susceptibility to resistant worms (for example Lespine et al., 2012).

ATP binding cassette (ABC) transporters are a superfamily of transmembrane proteins which mediate the ATP-dependent efflux of a wide range of structurally and mechanistically unrelated compounds including various anticancer and anthelmintic drugs (Gottesman and

Pastan, 1993; Lespine et al., 2012). Multiple ABC transporter genes have been reported in free-living and parasitic nematodes (Sheps et al., 2004; Ardelli et al., 2010; Laing et al., 2013). The overexpression of some of these transporters has been observed in drug-resistant isolates of different nematode species compared to susceptible reference isolates (Dicker et al., 2011; Williamson et al., 2011; Sarai et al., 2013; Raza et al., 2016), suggesting a potential role for ABC transporters in anthelmintic resistance. Numerous *in vitro* and *in vivo* studies have shown that an anthelmintic/ MDRI combination therapy increases the toxicity of the anthelmintic to both drug-susceptible and –resistant isolates of different nematode species (Bartley et al., 2009; Pérez et al., 2010; Heckler et al., 2014). Recently, Raza et al. (2015) reported that zosuquidar and tariquidar, members of the so-called third generation of MDRIs (Falasca and Linton, 2012) significantly increased ivermectin (IVM) toxicity to *Haemonchus contortus* larvae *in vitro*.

Tyrosine kinase inhibitors are an important new class of targeted chemotherapeutic agents that represent a promising group of anticancer drugs in current clinical trials and clinical use. (Shawver et al., 2002). Crizotinib is a tyrosine kinase inhibitor that has been examined as a cancer therapeutic for the treatment of patients with anaplastic lymphoma kinase (ALK)-positive advanced non-small cell lung cancer (NSCLC). It works by inhibiting c-Met (a gene that encodes hepatocyte growth factor receptor) and ALK. In NSCLC, it also inhibits echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase (EML-ALK 4) translocation (reviewed by Sahu et al., 2013). As well as being an inhibitor of tyrosine kinase, crizotinib also acts as a competitive inhibitor of P-gps (Zhou et al., 2012). The compound significantly increased the sensitivity of ABCB1 over-expressing cells to doxorubicin and paclitaxel, and the combination of crizotinib with paclitaxel markedly increased anti-tumour activity of paclitaxel in the KBv200 tumour xenograft model.

Given that, i) crizotinib increases toxicity of anti-cancer drugs in mammalian cells and xenograft experimental models by acting as an MDRI in inhibiting the activity of P-gps, ii) that P-gps have been implicated in some anthelmintic resistances, and iii) that other MDRIs have been shown to partially reverse anthelmintic resistances, we hypothesized that crizotinib might be able to inhibit nematode P-gps, and hence restore the sensitivity of resistant worms to anthelmintics. The present study therefore aimed to investigate whether crizotinib was able to synergise the toxicity of IVM against drug-resistant and -susceptible isolates of *H. contortus* using larval development and migration assays.

2. Materials and methods

2.1. Parasites and chemicals

Two isolates of *H. contortus* were used for the present study: the drug-susceptible Kirby isolate (Albers and Burgess, 1988) and the multi-drug-resistant Wallangra (WAL) isolate (Love et al., 2003). Infections were maintained in sheep at the CSIRO Agriculture FD McMaster laboratory, Armidale, New South Wales (NSW), and faecal samples were collected and transported to the CSIRO laboratories in Brisbane, QLD. All animal procedures were approved by the FD McMaster Animal Ethics Committee, CSIRO Agriculture (Animal Ethics Approval Number AEC 13/23). Worm eggs and infective stage L3 larvae were prepared for use in larval development and migration assays, respectively, as described previously (Raza et al., 2015).

Technical grade IVM was purchased from Sigma Chemical Co. and a stock solution was prepared at 10 mg/mL in dimethyl sulfoxide (DMSO). Crizotinib was purchased from

SelleckChem, and a stock solution was prepared in DMSO at a concentration of 5 mg/mL. The stock solutions for both chemicals were further diluted by two-fold serial dilutions in DMSO to produce multiple separate drug solutions. The drug solutions were stored at -20 ^{0}C.

2.2. Worm bioassays

The ability of ivermectin, alone or in combination with crizotinib, to inhibit the migration of L3 stage larvae through an agar/mesh system was measured using a larval migration assay (LMA) in 96-well microtitre plates (Kotze et al., 2006), as described previously (Raza et al., (2015). Final concentration ranges for IVM were 25000-195.30 ng/mL for WAL, while the ranges used for Kirby were 6250-48.8 ng/mL (final DMSO concentration was 1% v/v). The plates were incubated for 48 h, and the drug-exposed worms were then transferred using a multichannel pipette to the agar / filter mesh / receiver plates. After 24 h, the agar / filter plates were removed, and the worms that had migrated into the receiver plate wells were killed by adding Lugol's iodine (10 µL), and counted.

A larval development assay (LDA) was used to study the effects of IVM, alone or in combination with crizotinib, on the development of *H. contortus* larvae from the egg to the L3 stage following the method described by Kotze et al. (2009). The assay was performed in 96-well plates, with drugs impregnated into agar. Final IVM concentration ranges were 39-0.076 ng/mL for the WAL isolate, and 2.44-0.0048 ng/mL for the Kirby isolate (final DMSO concentration was 1 % v/v). Eggs were added to each well, the larvae were fed the next day (with a growth medium prepared as described by Kotze et al. (2009)), and finally larvae were killed after 7 days using Lugol's iodine, and the number of fully grown infective L3 in each well was counted.

For both the LDAs and LMAs, each experiment consisted of triplicate wells at a range of IVM concentrations, either alone or combined with crizotinib, as well as at least 12 control

wells (DMSO or crizotinib only) per plate. Three separate experiments were performed for IVM alone and IVM in combination with crizotinib with each worm isolate.

2.3. Data Analysis

For each experiment, the number of L3 in each well was converted to a percentage of the mean number of L3 in multiple control wells. The data were then analysed using non-linear regression with GraphPad Prism® software (GraphPad Software Inc., USA, version 5.03).

The effects of crizotinib on the sensitivity of larvae to IVM were described using synergism ratios, calculated as: IC_{50} IVM alone / IC_{50} IVM in combination with crizotinib. The ratios were considered to indicate a significant degree of synergism if they were derived from IC_{50} values which showed non-overlapping 95% confidence intervals (CIs).

3. Results and Discussion

Dose-response curves from a number of preliminary LMA and LDA experiments with crizotinib-alone were used to select two concentrations of crizotinib for use in subsequent assays in combination with IVM (Fig. 1A, B). Crizotinib concentrations that resulted in less than 20% inhibition of larval migration or development were chosen: LMA, Kirby 3 and 1.5 µg/mL, WAL 13 and 6.5 µg/mL; LDA, Kirby 10 and 5 µg/mL, WAL 20 and 10 µg/ mL (Table 1). The Kirby larvae showed increased sensitivity to crizotinib in both the LMA and LDA compared to WAL, with dose-response curves shifted to the left for Kirby compared to WAL, and hence lower combination treatment crizotinib concentrations were selected for subsequent combination assays with Kirby compared to WAL. It has previously been

reported that WAL larvae tolerate higher levels of some MDRIs than Kirby larvae, for example verapamil and zosuquidar (Raza et al., 2015). The present study and our earlier data therefore suggest that WAL larvae may have defensive systems that allow them to survive in the presence of higher concentrations of crizotinib and other MDRIs. One possible mechanism may be the use of P-gps to efflux the compounds, as WAL larvae have been reported to show higher expression of several P-gp genes compared to Kirby (Sarai et al., 2013; Raza et al., 2016). In addition, both the isolates also showed higher tolerance to crizotinib in LMAs as compared to LDAs. The variation in toxicity of the compound in the two bioassays may be due to life-stage-specific differences in expression patterns of different P-gps genes, as previously observed in comparisons of P-gp gene expression patterns between different life stages of *H. contortus* (Sarai et al., 2013).

Dose responses in LMAs with IVM alone, or in combination with crizotinib, are shown in Fig. 2, with IC_{50} values in Table 1. Crizotinib had no significant effect on the IVM dose response for Kirby larvae (Fig. 2A). On the other hand, crizotinib shifted the WAL IVM dose-response significantly to the left, amounting to a 2.6-fold decrease in IVM IC_{50} at 6.25 µg/mL crizotinib, and a 5.7-fold decrease in IVM IC_{50} at 12.5 µg/mL crizotinib (Fig. 2B). The IC_{50} for IVM against WAL larvae in combination with 12.5 µg/mL crizotinib (570 ng/mL) was significantly less than for Kirby larvae with IVM alone (1171 ng/mL), indicating that the presence of crizotinib resulted in an increase in the sensitivity of WAL larvae to IVM to levels greater than that observed with IVM alone and the drug-susceptible Kirby isolate.

Co-administration of crizotinib with IVM shifted the LDA dose response curves towards the left and significantly decreased IVM IC_{50} values for both WAL and Kirby isolates, as indicated by non-overlap of 95% CIs (Figure 3, Table 1). Synergism ratios were 1.4 and 1.6 for the Kirby isolate (at 10 and 5 µg/mL crizotinib, respectively) and 1.9 and 1.5 for the WAL isolate (at 20 and 10 µg/mL crizotinib, respectively).

There were several points of difference between the LMA and LDA results. Firstly, crizotinib acted synergistically with WAL larvae only in the LMA, while showing equivalent levels of synergism with both isolates in the LDA. Secondly, the effects of crizotinib with WAL larvae in the LMA were much more marked than those seen with either isolate in the LDA (SRs of 2.6 and 5.7 in the LMA with WAL, compared to 1.4 – 1.9 in the LDA with both isolates). Thirdly, leading directly on from the last point, co-administration of crizotinib and IVM to WAL larvae in the LMA rendered the larvae more sensitive to IVM than the Kirby isolate (Kirby IVM IC_{50} = 1171 ng/mL, compared to WAL IVM IC_{50} in presence of crizotinib = 570 ng/mL). That is, the IVM resistance displayed by the WAL larvae was removed by crizotinib in the LMA. In contrast, in the LDA, the WAL IVM IC_{50} in the presence of crizotinib remained 11-fold higher than IVM-alone IC_{50} value for Kirby larvae (2.09 ng/mL compared to 0.19 ng/mL). These differences may reflect differences in the nature of the two assays. The assays measure the effects of IVM on quite different phenotypic traits (larval development and larval migration), focused on different life stages, with the LDA focusing on the early larval stages, while the LMA assesses migration ability in infective L3 stage larvae.

The minor levels of synergism observed with both isolates in the LDA, alongside the more significant synergism with WAL larvae only in the LMA, suggests that the nature of the IVM resistance mechanism(s) that distinguishes Kirby from WAL larvae, and specifically the contribution of P-gps towards the resistance, varies between the larval life stages. As mentioned above, Sarai et al. (2013) reported significant variation in the life-stage expression patterns of the various P-gp genes in L1 and L3 stages of *H. contortus* larvae within as well as between different isolates. The low level of synergism in both isolates as measured by LDA in the present study suggest that P-gps may play a minor role in the ability of early larval stages of both isolates to tolerate IVM. On the other hand, the significant synergism

seen in the LMA only with WAL suggests that P-gps may play a significant role in the observed IVM resistance displayed by L3 of this isolate, while being of little consequence in the interaction of Kirby L3 with IVM. That is, the data suggest a significant role in IVM sensitivity for P-gps in L3 stage larvae, compared to a minor role in protection of early larval stages against IVM in both isolates, but no role in resistance in these early larval stages. This suggestion of no role in resistance in the early larval stages needs to be tempered by the fact that crizotinib may only interact with a sub-set of the ABC transporters present in nematode larvae, or at least its inhibitory effects may vary across the population of ABC transporters, and hence it is only the lack of a role for the specific ABC transporters inhibited by this compound that is indicated by the data for early stage larvae. Raza et al. (2015) also found that the synergistic effects of a number of MDRI were more marked in larval migration assays compared to LDAs; for example co-administration of zosuquidar and tariquidar rendered the WAL larvae more sensitive to IVM than Kirby larvae (synergism ratios 4.7-6.0), whereas these compounds had less effect on the toxicity of IVM towards WAL larvae in LDAs (SRs 1.6-2.4).

In examining the interaction of crizotinib with IVM sensitivity in *H. contortus* larvae, the present study aimed to explore two aspects of P-gp inhibition and anthelmintic sensitivity. The first of these was to use crizotinib as a test compound for exploring the IVM resistance mechanism in WAL larvae. The data described above add to the body of literature indicating that P-gps play a role in the sensitivity of nematode larvae to anthelmintics, particularly MLs (reviewed by Lespine et al., 2012), while the life-stage-dependant patterns of synergism across the two assays has allowed us to make suggestions as to the relative role of P-gps in IVM resistance in WAL larvae at different larval life stages. The second aspect of this study was to assess the potential for the use of crizotinib to reverse IVM resistance. A number of studies have reported on the use of MDRI compounds in combination with anthelmintics,

especially MLs, to increase the toxicity of these drugs *in vivo* (Molento and Prichard, 1999; Lifschitz et al., 2010a; 2010b). Importantly, for crizotinib, only our LMA data is supportive of such a potential use. Studies with adult parasites would be required to assess the practical applicability of the ML-resistance reversing ability of this compound, since this is the target life stage of most chemotherapeutic approaches to worm control. Initially, though, *in vitro* studies using sensitive assays may be informative as to compound concentrations (ratios) required in order to observe synergistic effects with the adult life stage. There are, however, significant barriers to such an approach. Most importantly, there is potential for toxic side-effects in the host as a result of inhibiting host animal ABC transporters alongside the nematode transporters (Lespine et al., 2012) Secondly, the cost of such drugs is currently prohibitive. Such combination therapies will not be a cost-effective option while cheaper drugs remain effective, however, such cost constraints may diminish in the future as multi-drug resistance becomes more intense and widespread.

Acknowledgments

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Figure captions:

Fig. 1. Effects of crizotinib alone on the migration (**A**) and development (**B**) of *H. contortus* Kirby and WAL larvae. Kirby dose-response curve shown with solid lines and closed symbols, WAL dose-response curve shown as dashed lines and open symbols. Arrows indicate crizotinib concentrations chosen for subsequent assays in combination with IVM. Each data point represents mean ± SE, n= 9 (pooled data from three experiments, each with assays in triplicate).

Fig. 2. Effects of IVM alone, or in combination with crizotinib, on the migration of L3 stage *H. contortus* Kirby (**A**) and WAL (**B**) larvae; IVM alone shown with solid lines and closed symbols, IVM plus crizotinib shown as dashed (WAL) or dotted (Kirby) lines, and open symbols. The concentration of crizotinib in µg/mL is shown as a subscript after the inhibitor name; Cri: crizotinib. Each data point represents mean ± SE, n= 9 (pooled data from three experiments, each with assays in triplicate).

Fig. 3. Effects of IVM alone, or in combination with crizotinib, on the development of *H. contortus* Kirby and WAL larvae; Kirby set of dose responses lie to the left of the WAL set; IVM alone shown with solid lines and closed symbols, IVM plus crizotinib shown as dashed (WAL) or dotted (Kirby) lines, and open symbols. The concentration of crizotinib in µg/mL is shown as a subscript after the inhibitor name; Cri: crizotinib. Each data point represents mean ± SE, n= 9 (pooled data from three experiments, each with assays in triplicate).

Figure 1

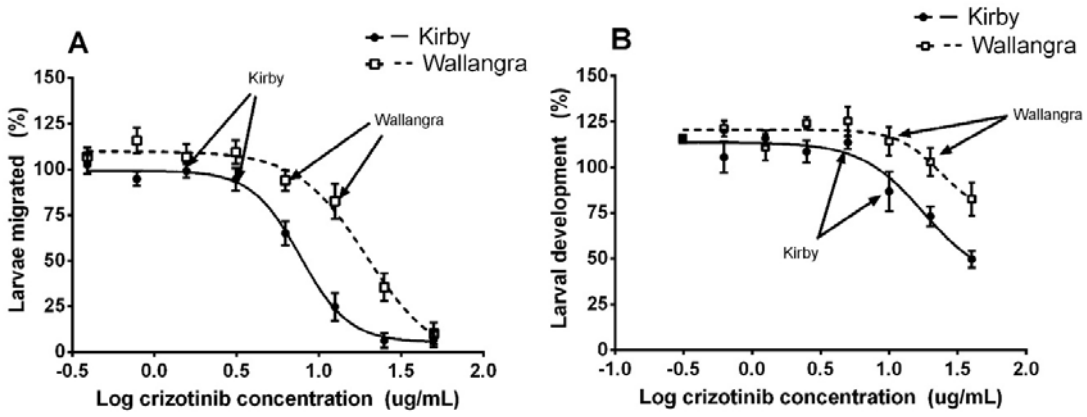

Figure 2

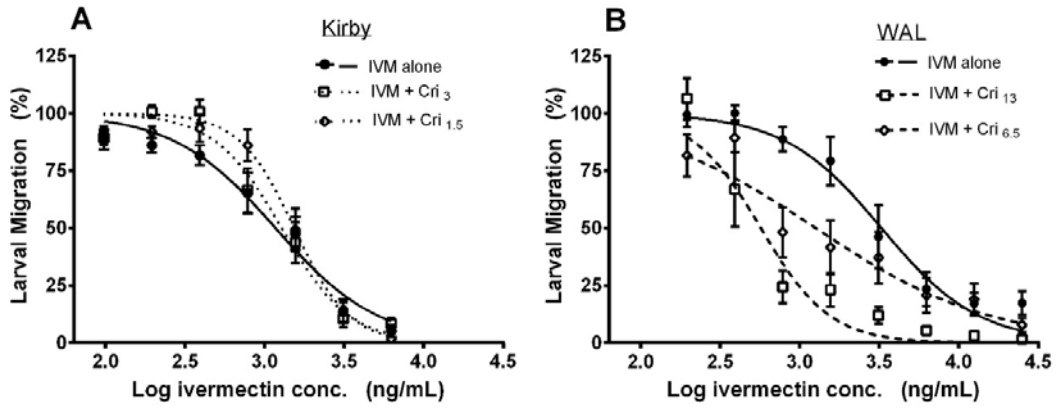

Figure 3

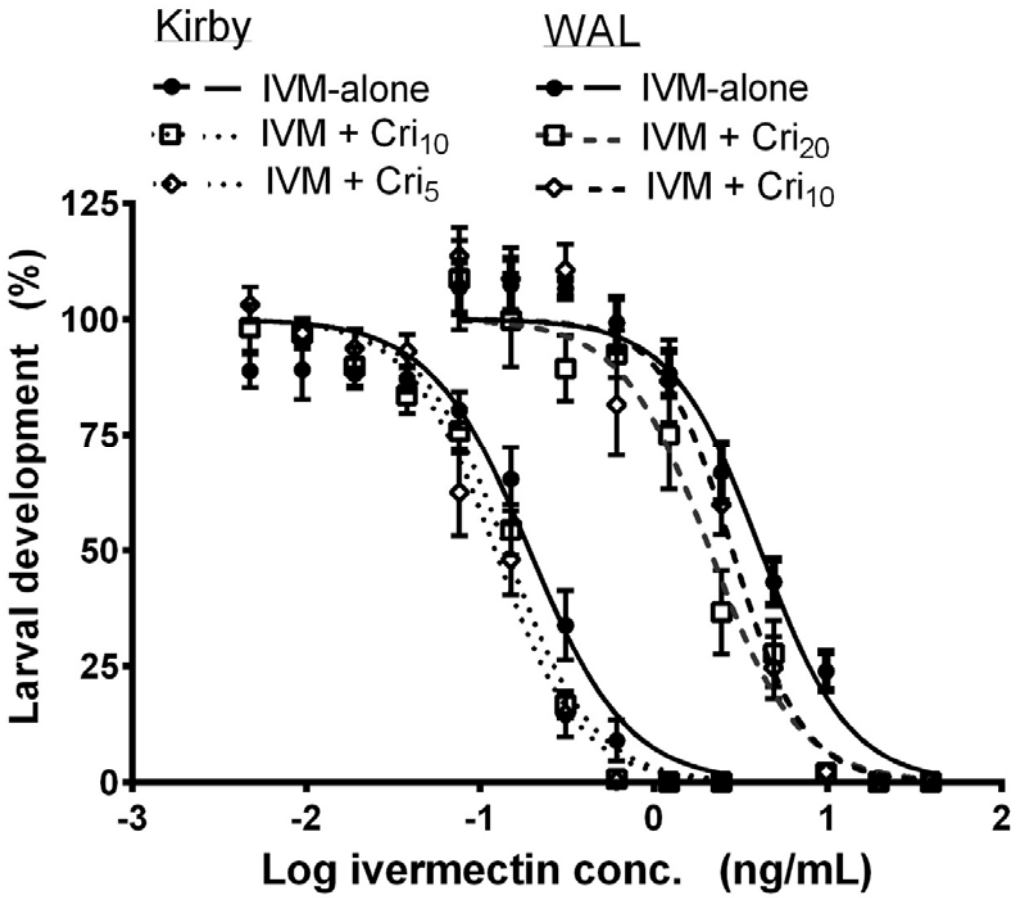

Table 1. Responses of Kirby and WAL larvae to ivermectin alone, or in combination with crizotinib, in larval migration assays (LMAs) and larval development assays (LDAs)

Worm bioassay	MDRI	Hc Kirby				Hc Wallangra			
		Crizotinib (μg/mL)	**IVM IC_{50} [a] (ng/mL)**	**95% CI**	**SR[b]**	**Crizotinib** (μg/mL)	**IVM IC_{50} [a] (ng/mL)**	**95% CI**	**SR[b]**
LMA	None	-	1171	965-1422	-	-	3268	2520-4238	-
	Crizotinib	3.0	1280	1081-1760	0.9	12.5	570*	459-708	5.7*
		1.5	1535	1340-1760	0.8	6.25	1262*	815-1954	2.6*
LDA	None	-	0.19	0.16-0.23	-	-	4.01	3.55-4.73	-
	Crizotinib	10	0.14*	0.12-0.15	1.4*	20	2.09*	1.66-2.64	1.9*
		5	0.12*	0.10-0.14	1.6*	10	2.81*	2.32-3.42	1.5*

[a] Within an isolate, and within an assay type, * denotes that the IC_{50} in the presence of crizotinib was significantly less than the IC_{50} for IVM alone, as determined by non-overlap of 95 % Confidence Intervals.

[b] SR = Synergism ratio = IC_{50} for IVM in the absence of crizotinib / IC_{50} for anthelmintic in the presence of crizotinib