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Research paper

Response of drug-susceptible and -resistant *Haemonchus contortus* larvae to monepantel and abamectin alone or in combination *in vitro*

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ABSTRACT

There is an increasing interest in the use of combination anthelmintic products for the control of intestinal nematode parasites of livestock. These products are seen as attractive options for parasite control in the face of increasing levels of resistance to the different anthelmintic drug classes, as well as a means to slow the rate at which resistance develops to the individual components of the combination. With the recent introduction of an anthelmintic combination product containing abamectin and monepantel (at 1:12.5), we were interested in measuring the response of drug-susceptible and drug-resistant isolates of *Haemonchus contortus* to these two drugs alone and in combination, using larval development assays. The GWBII isolate showed resistance to abamectin (12-fold) alongside susceptibility to monepantel. The resistance ratio was reduced from 12- to 3.2-fold when the two drugs were combined. The MPL-R isolate was resistant to both drugs, with resistance factors of 6-fold towards abamectin, and 10.6- and 1008-fold towards monepantel in two sub-populations present in the isolate. This isolate showed 6.4-fold resistance to the drug combination. Hence, for both GWBII and MPL-R, the level of resistance towards the combination was reduced compared to the resistance towards abamectin or monepantel alone, respectively, but was not abolished. However, for GWBII, this *in vitro* resistance to the drug combination would be expected to have no impact on the *in vivo* efficacy of the combination drench product as the isolate is resistant to only the abamectin component of the drench, with monepantel remaining effective. On the other hand, the observed *in vitro* resistance to the combination shown by the MPL-R isolate is derived from significant levels of resistance towards both components separately, and hence may impact on *in vivo* efficacy of the combination. Isobologram analysis did not find any evidence for a synergistic interaction between the two drugs in larval development assays. We examined the predicted effects of varying the abamectin:monepantel ratio in drug combinations, assuming that the two drugs acted in an additive fashion. For GWBII, resistance to the drug combination was reduced to almost zero as the abamectin:monepantel ratio increased from 1:12.5 to 1:100, reflecting its resistance to only the abamectin component of the combination. For MPL-R, on the other hand, the resistance increased as the relative proportion of monepantel in the combination was increased, reflecting the extreme level of *in vitro* resistance shown by this isolate to monepantel.

1. Introduction

Anthelmintic resistance in gastrointestinal nematode parasites of livestock impacts on the efficacy of almost all of the currently-available anthelmintic drug classes. Resistance in nematodes of small ruminants towards benzimidazoles, imidothiazoles and macrocyclic lactones has been reported for many years (Kaplan, 2004; Wolstenholme et al., 2004), while a number of recent reports have described resistance to the amino-acetonitrile derivative (AAD), monepantel (Scott et al., 2013; Mederos et al., 2014; Van den Brom et al., 2015; Cintra et al., 2016; Sales and Love, 2016). There are many reports of multi-drug resistance occurring in different regions of the world (for example, Love et al., 2003; Cezar et al., 2010; Veríssimo et al., 2012; Papadopoulos et al., 2012; Playford et al., 2014; Lamb et al., 2017). The prevalence and degree of resistance has been viewed as a threat to the sustainability of sheep production systems worldwide for a number of years (Kaplan and Vidyashankar, 2012), while the impact on cattle production systems is also increasing (Geurden et al., 2015; Ramos et al., 2016; Waghorn et al., 2016).

One strategy to deal with the increasing levels of resistance to anthelmintics, and the increased prevalence of multi-resistant isolates, has been to utilise drug combination products consisting of two or more

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drugs from different chemical classes. For example, in Australia presently there are eleven dual-active, six triple-active and one quadruple-active combinations available for the control of intestinal nematode parasites of sheep (Wormboss, 2017). The ability of drug combinations to provide protection against parasites in the face of existing resistances to at least one of the separate components of the mixture, as well as to slow the further development of resistance, has been recognised for many years (Anderson et al., 1988; Barnes et al., 1995). A number of more recent studies have also shown that the use of drug mixtures can have significant effects in slowing the development of resistance (Leathwick, 2012; Bartram et al., 2012).

A combination product released onto the market recently, Zolvix® Plus, contains abamectin and monepantel at a ratio of 1:12.5 (2 mg/mL abamectin and 25 mg/mL monepantel). The use of the combination product at the recommended dose of 0.5 mL/5 kg results in the administration of 0.2 mg/kg and 2.5 mg/kg of abamectin and monepantel, respectively. This is equivalent to the dose of monepantel when administered as the single-active drench product (Zolvix®), and the normal dose of abamectin when using this drug as a single-active product (for example, Virbamec® Oral) or as part of double-, triple- or quadruple-active combinations (for example, Sequel®, Hat-Trick® and Q-Drench®, respectively). Given that resistance to macrocyclic lactones, including abamectin, is widespread in field isolates of some livestock nematodes, and that monepantel resistance has been reported on a number of occasions, we were interested in how resistance to these two chemicals may impact on the efficacy of the Zolvix® Plus drug combination. We used *in vitro* larval development assays to measure the toxicity of abamectin and monepantel, alone or in combination, towards larvae of susceptible and drug-resistant isolates of *Haemonchus contortus*. One of the resistant isolates showed resistance to macrocyclic lactones and monepantel, while the other was resistant to the former drug class only. Given that the two drugs in Zolvix® Plus have been documented as acting in a synergistic manner *in vivo* (Rolfe et al., 2013) we examined whether their toxicity in *in vitro* assays differed from that expected for an additve interaction, and used isobologram analysis to further examine the nature of any interaction between the two drugs.

2. Materials and methods

2.1. Parasites

Three isolates of *H. contortus* were used in this study:

i) Kirby: isolated from the field at the University of New England Kirby Research Farm in 1986; susceptible to all commercial anthelmintics (Albers and Burgess, 1988). The isolate has been continually passaged for at least the last 15 years.

ii) GWBII: a passaged variant of a multi-resistant field isolate (isolated in 2003 from Wallangra, NSW; Love et al., 2003) with demonstrated resistance to macrocyclic lactones, levamisole, benzimidazoles and closantel: treatment efficacies of 0% ivermectin, 67% moxidectin, 32% closantel, 16% albendazole and 67% levamisole were observed following a Faecal Egg Count Reduction Test (FECRT) when the original strain (GWB1) was first isolated in 2003 This strain has since been maintained in naive donor sheep by Invetus and further passaged with abamectin, albendazole and levamisole treatments The efficacy of abamectin was measured as 17.7% in 2015 (based on total worm counts, n = 8 animals per group).

iii) MPL-R: isolated from a property in southwest Queensland, Australia, in early 2014. *H. contortus* larvae were cultured from faeces collected from sheep that had shown clinical signs of scouring after a drench treatment (drench efficacy measured as 99.2%). These larvae were subsequently used to establish infections in a housed animal. This animal was treated with a full dose of Zolvix®, and larvae were collected and used to infect additional housed animals. This isolate therefore represents a field-derived isolate in which only the survivors of a drench treatment have been propagated further. This isolate showed a zero efficacy with Zolvix® when tested in 2014 (based on total worm counts, n = 4 animals per group). The isolate is also resistant to macrocyclic lactones (ivermectin efficacy 8.8%, moxidectin 79.3%) (based on total worm counts, n = 8 animals per group; tested in 2015).

Infected sheep were housed at the Invetus animal house facility in Armidale, NSW. Faeces was collected and sent by overnight courier to the CSIRO laboratory in Brisbane, Queensland, for recovery of eggs using filtration and sucrose gradient centrifugation as described by Kotze et al. (2009).

2.2. Chemicals

The commercial drench product Zolvix® was used as a source of monepantel. The drench solution (25 mg/mL monepantel) was serially diluted 2-fold in dimethyl sulfoxide (DMSO) to generate a series of working solutions. Technical grade abamectin was purchased from ChemService Inc. (West Chester, PA, USA). A stock solution was prepared in DMSO at 1 mg/mL followed by 2-fold serial dilutions in DMSO to generate multiple working solutions. Equal amounts of a 12.5 mg/mL solution of monepantel and the 1 mg/mL stock solution of abamectin were mixed together to make a stock solution of an abamectin:monepantel 1:12.5 mixture. This solution was then serially diluted 2-fold to generate a series of working solutions of the drug combination.

2.3. Larval development assay

A larval development assay (LDA) was used to measure the effects of abamectin or monepantel alone, or in combination at 1:12.5, on the development of *H. contortus* larvae from eggs to third-stage (L3) larvae following the method described previously (Kotze et al., 2009). The anthelmintics were impregnated into 200 µL of 2% agar in 96-well plates. Control assays received DMSO alone (final DMSO concentration in test chemical and control wells was 1% v/v). Nematode eggs were dispensed into each well, and plates were incubated overnight at 27 °C. The larvae were fed the next day with growth medium (prepared as described by Kotze et al., 2009). The plates were incubated for another six days, and finally larvae were killed using Lugol's iodine and the number of fully grown infective L3 were counted in each well.

Each dose response experiment consisted of triplicate assay wells at a range of chemical concentrations, as well as 12 control wells per plate (containing DMSO only). Each isolate was examined in three separate experiments.

2.4. Dose response analysis

The number of fully grown L3 larvae in each well was expressed as a percentage of the mean number of L3 in multiple control (DMSO only) wells. The data were then analysed using non-linear regression with GraphPad Prism® software (GraphPad Software Inc., USA, version 6.01). For abamectin alone, and for the abamectin:monepantel combination, dose- responses showed a sigmoidal shape, ranging from approximately 100%–0% development, and hence a normalised response model with a variable slope was used to fit the data. For monepantel alone, the MPL-R isolate showed a two-phase response (as described previously by Raza et al., 2016) and hence the dose response was examined in two stages (separately over low and high concentration ranges) using a non-normalised model with variable slope. IC_{50} values and 95% confidence intervals (CI) were calculated for each drug or drug combination for each isolate. Significant differences in IC_{50} values between isolates were judged based on overlap of 95% CI. Resistance ratios were calculated for each isolate and drug as: IC_{50} resistant isolate (GWBII or MPL-R)/IC_{50} Kirby isolate.

We utilised the dose response data at each drug concentration from the single-drug assays to calculate the expected response to a combination of the two drugs if they were acting in an additive fashion, as