THE ROLE OF DRUG EFFLUX SYSTEMS IN ANTHELMINTIC RESISTANCE IN PARASITIC NEMATODES

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DVM, MPhil

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ABSTRACT

Trichostrongyloid nematodes have adapted various mechanisms to combat the toxic effects of anthelmintic drugs. These mechanisms can be classified as specific (e.g. modified drug receptors) or non-specific (altered drug metabolism or increased drug transport) mechanisms. Our knowledge of non-specific mechanisms is less advanced as most attention to date has been focused on specific drug target-based resistance mechanisms. ATP binding cassette (ABC) transporters are involved in active efflux of anthelmintics, and are considered to be important non-specific mechanisms of resistance. This PhD project aimed to explore the role of ABC transporters in anthelmintic resistance in *Haemonchus contortus* using a drug-susceptible and two drug-resistant isolates of this nematode.

For this purpose, two *in vitro* parasitological bioassays (larval development and migration assays) were used to explore the effects of multidrug-resistance inhibitors (MDRIs) on the susceptibility of the selected isolates to different anthelmintics. The effects of anthelmintics on expression patterns of ABC transporters were measured by qPCR following pre-exposure of worms to different anthelmintics. The phenotypic consequences of anthelmintic exposure were evaluated using migration assays, and by measuring the effects on efflux of the fluorescent dye Rhodamine-123 (R-123). Finally, a monepantel-resistant isolate (MPL-R) was also phenotypically characterised, and expression patterns of ABC transporters were determined using molecular assays.

Significant increases in sensitivity of larvae to anthelmintics were observed in the presence of third generation MDRIs in *in vitro* assays, particularly with ivermectin (IVM) (synergism ratios up to 6-fold). Several of the inhibitors increased the sensitivity of both a drug-resistant and -susceptible isolate, while others had significant effects on the resistant isolate only. This suggests that some of the inhibitors interact with P-glycoproteins (P-gps) representing intrinsic efflux pathways present across nematode populations with quite different drug sensitivities, while other inhibitors interact with P-gps of significance only to resistant nematodes; hence, most-likely representing an acquired resistance mechanism. Zosuquidar, tariquidar and crizotinib rendered the drug-resistant isolate equally sensitive, or more sensitive, to IVM than the drug-susceptible isolate in migration assays (Chapter 2).

Analysis of the data from gene transcription experiments showed that the drug-resistant isolate exhibited over-expression of three P-gp genes (*pgp*-1, *pgp*-9.1 and *pgp*-9.2) as compared to the susceptible isolate. In addition, pre-exposure to IVM and levamisole (LEV) for 3 h significantly increased the expression levels of multiple ABC transporters in the resistant isolate only. In
contrast, both isolates showed an increased R-123 efflux following exposure to the drugs, suggesting that the drug exposure stimulated the activity of existing transporter proteins (Chapter 3). In contrast, exposure to moxidectin (MPL) significantly increased transcription of multiple ABC transporter genes in both the drug-susceptible and -resistant isolates, not only at 3 h but also at longer drug exposure periods (6 and 24 h). These increases in transcription were consistent for pgp-11, pgp-12 and pgp-14 across all the time points in both isolates. In addition, pgp-11 maintained the elevated levels of upregulation 24 h after the end of 3 h MPL exposure of drug-resistant L3 (Chapter 4). Drug-exposed worms showed an increased ability in a proportion of the larval population to tolerate higher IVM concentrations in subsequent migration assays. This subsequent ability to tolerate higher IVM concentrations following pre-exposure to IVM, LEV and MPL suggests a protective role of some ABC transporters across different chemical entities.

The LDA was able to detect resistance to MPL in this isolate and the resistance was shown to exist in two distinct forms, with subpopulations showing resistance factors of 7-fold and 1000-fold compared to the susceptible isolate. This suggests that at least two separate moxidectin resistance mechanisms are acting within this isolate, with one or more mechanisms or combinations of mechanisms conferring a much higher level of resistance than the other(s). In the MPL-resistant isolate, the expression level of pgp-11 was significantly decreased as compared to the drug-susceptible isolate, whereas, transcriptions of four ABC transporter genes (pgp-2, pgp-9.2, pgp-11 and mrp-1) were at significantly lower levels in the MPL-resistant isolate as compared to the multi-drug resistant isolate (susceptible to MPL) (Chapter 5).

In conclusion, this thesis highlights the capacity of the third generation MDRIs to increase the sensitivity of nematodes to anthelmintics. In addition, the study also demonstrates an interaction between transcription of nematode ABC transporters and anthelmintic drugs. The study is the first published work to indicate an interaction between MPL and ABC transporters in nematodes. The results also show that ABC transport proteins are important in protecting parasitic worms against a variety of structurally unrelated compounds. Further research is needed to: (i) study the in vivo effects of anthelmintic/MDRI combination therapy on the efficacy of anthelmintics, (ii) explore the molecular mechanisms involved in the two MPL-resistant subpopulations, and (iii) discover the physiological functions of ABC transporters to expose their vulnerability as potential drug targets in nematodes.
DECLARATION BY AUTHOR

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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CONTRIBUTIONS BY OTHERS TO THE THESIS

My supervisory team, Dr Andrew Kotze, Dr Steven Kopp and Dr Abdul Jabbar provided guidance with presentation and structure of the literature review. My principal supervisor Dr Andrew Kotze as well as co-supervisors Dr Steven Kopp and Dr Abdul Jabbar provided guidance on the preparation and layout of the thesis chapters. All the experimental work, analysis and manuscript/chapter preparation was undertaken primarily by myself with the assistance of my principal supervisor, Dr Andrew Kotze. Proof-reading and editing of the articles and thesis as a whole was provided by my supervisors. The contributions of co-authors in publications are duly described in the above section.

STATEMENT OF PARTS OF THE THESIS SUBMITTED TO QUALIFY FOR THE AWARD OF ANOTHER DEGREE

None
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ABBREVIATIONS

ABC = ATP binding cassette transporters

ABCB1 = ATP binding cassette transporters B1 sub-family

ABCF = ATP binding cassette transporters F Family

ALB = Albendazole

ALK = Anaplastic lymphoma kinase

AR = Anthelmintic resistance

B. = Brugia

BZ = Benzimidazole

Ca. = Caenorhabditis

Co. = Cooperia

CI = Confidence interval

CSIRO = Commonwealth Scientific and Industrial Research Organisation

CYP450 = Cytochrome P450

DMSO = Dimethyl sulfoxide

EHA = Egg hatch assay

FECRT = Faecal egg count reduction test

FEN = Fenbendazole

GIN = Gastrointestinal nematode

H. = Haemonchus

HAF = Half transporters

IC50 = 50% inhibitory concentration

IVM = Ivermectin

L3 = Third-stage larva

LDA = Larval development assay

LEV = Levamisole

LFIA = Larval feeding inhibition assay

LMA = Larval migration assay
LMIA = Larval migration inhibition assay
LMIT = Larval migration inhibition test
MDR = Multidrug resistance
MDRI = Multidrug resistance inhibitor
MOX = Moxidectin
MPL = Monepantel
MPL-R = Monepantel resistant
MRP = Multidrug resistance protein
nAChR = Nicotinic acetylcholine receptor
NSCLC = Non-small cell lung cancer
NSW = New South Wales
O. = Ostertagia
OXF = Oxfendazole
PCR = Polymerase chain reaction
P-gp = P-glycoprotein
qPCR = Quantitative polymerase chain reaction
R-123 = Rhodamine-123
RAF = Rafoxanide
S. = Schistosoma
SE = Standard error
SNPs = Single nucleotide polymorphisms
SR = Synergism ratio
TBZ = Thiabendazole
Te. = Teladorsagia
TMDs = Transmembrane domains
Tr. = Trichostrongylus
WAL = Wallangra
CHAPTER 1

1. Review of literature

1.1 Introduction

Gastrointestinal helminthosis is one of the major constraints to the health and productivity of small ruminants globally. A number of gastrointestinal nematodes (GINs) can affect sheep and goats, leading to clinical illness ranging from anorexia, diarrhoea, anaemia, oedema of some body parts and even death of young, aged or immunocompromised animals (Fox, 1997; Perry and Randolph, 1999; Thompson, 1999). Thus, GINs in small ruminants have a significant effect on farm profitability (Roebert et al., 2013), and the economic importance of GINs can be appreciated from the fact that they cost the sheep and wool industry in Australia more than 430 million Australian dollars per annum (Meat and Livestock Australia, 2015). Among various GINs affecting goats and sheep, Haemonchus contortus is a highly pathogenic and economically important nematode distributed throughout the world, mainly in tropical and subtropical regions. It inhabits the abomasum and feeds on blood and it can also infect other ruminants such as cattle (Jacquet et al., 1998).

Haemonchus contortus (Nematoda: Strongylidia; Trichostrongyloidea) along with Trichostrongylus spp. and Teladorsagia circumcincta (Te. circumcincta) comprise a triad of the most pathogenic nematodes of small ruminants worldwide (Anderson, 2000; Besier and Love, 2003; Hoberg et al., 2004). The buccal cavity of H. contortus, an abomasal parasite, is equipped with a lancet that enables this nematode a haematophagous parasite. The body length of this nematode is up to 30 mm (female 18-30 mm; male 10-20 mm) (Bowman et al., 2009) and female worms have a distinctive feature of a white, egg-filled uterus which spirals around the blood-filled intestine giving a specific so-called barber pole appearance; hence, giving the name ‘barber’s pole worm’ (Sutherland and Scott, 2010). Infection with H. contortus produces a disease called haemonchosis.

Eggs of H. contortus are approximately 70-85 μm long by 44 μm wide, and the early stages of cleavage contain between 16 and 32 cells. The egg hatches within 1-2 days and the first-stage larva (L1) is released which feeds on bacteria and develops consecutively to second-stage larva (L2) and ensheathed third-stage larvae (L3) in the environment (faeces or soil). The sheath of the L3 protects it from harsh environmental conditions. Hosts get infected by ingesting infective larvae (L3) while grazing. Once the L3 reaches to abomasum, it develops to fourth-stage larva (L4) and finally an
adult (male or female worms) in about three weeks. When environmental conditions are less favourable (usually at the end of grazing season), the larvae can undergo a state of arrested development within the host, called hypobiosis (Miller et al., 1998; Sutherland and Scott, 2010). Hypobiotic larvae activate in the following spring and resume their development, which may result in increased faecal egg counts at the start of the spring season. The main pathogenic effects are caused by L4 and adult worms that feed on blood, leading to severe anaemia which is more apparent two weeks after infection (Baker et al., 1959). The impact of haemonchosis can be explained by the high fecundity rate of female worms that leads to an extensive pasture contamination, the blood sucking nature of the parasite that causes mortality in lambs and kids and its ability to withstand the unsuitable environmental conditions through hypobiosis (Waller et al., 2004). Severity of disease depends on intensity of infection, and acute disease is usually characterized by microcytic and hypochromic anaemia, pale mucous membranes, dark coloured faeces, oedema (especially in between lower jaws called bottle jaw), laboured breathing, weakness, reduced wool production and muscle mass, and sometimes sudden death. Chronic disease is associated with reduced feed intake, weight loss and anaemia (Jacobson et al., 2009; Roos, 2009).

1.2 Control of parasitic nematodes

Control of GINs in ruminants in an extensive grazing system is one of the most significant challenges in veterinary medicine (Craig, 2006). For decades, anthelmintics have been widely used worldwide to treat and control GINs. Benzimidazoles (BZ), imidothiazoles/tetrahydropyrimidines and macrocyclic lactones (MLs) are the major classes of anthelmintic drugs that are generally used in parasite control programs. The members of BZ class of anthelmintics act by inhibiting the functions of microtubules after binding to β-tubulin, which is essential for cell structure, resulting in death of the parasite (Kohler, 2001). The imidothiazole group including LEV mimics the action of acetylcholine. These drugs act upon the nicotinic acetylcholine receptors (nAChR) on the surface of somatic muscle cells in nematodes, leading to depolarization and spastic paralysis that facilitates parasite expulsion (Unwin, 1995; Evans and Martin, 1996). Macro cyclic lactones (avermectins and milbemycins) cause flaccid paralysis of the somatic musculature in the parasite body, thus disrupting food ingestion by inhibiting the pharyngeal pump (Kotze, 1998; Sangster and Gill, 1999; Rana and Misra-Bhattacharya, 2013).

The intensive use of these drugs in livestock has led to the development of progressive resistance to the available drugs (Taylor et al., 2002; Ihler, 2010). Anthelmintic resistance (AR) is generally defined as “when a previously effective drug is unable to kill the parasite population while exposed to therapeutic doses (Jabbar et al., 2006) or loss of sensitivity to a drug in parasitic
population that was sensitive to the same drug which is thought to be genetically transmitted (Kohler, 2001). Resistance to all the major classes of anthelmintics, including benzimidazoles (BZ), imidodthiazoles/tetrahydropyrimidines and macrocyclic lactones (MLs) has been reported worldwide (Kaplan, 2004). In addition, there have been reports describing the development of resistance to the most recently introduced anthelmintic, monepantel (MPL) within four years of its introduction (Scot et al., 2013; Love, 2014; Mederos et al., 2014; Van den Brom et al., 2015). Anthelmintic resistance has been documented in parasites of different animal species, including cattle (e.g., Eagleson and Bowie, 1986; Loveridge et al., 2003; Mejia et al., 2003; Rendell, 2010), sheep and goats (e.g., Barton, 1983; Overend et al., 1994; Eddi et al., 1996; Coles, 2005; Domke et al., 2012), horses (e.g., Kaplan, 2002; Traversa et al., 2009; Reinemeyer, 2012) and dogs (e.g., Jackson et al., 1987; Kopp et al., 2007). AR is a major issue with trichostrongyloid nematodes of small ruminants and reports of *H. contortus* resistant to multiple classes of anthelmintics are becoming more common; therefore, most research has been focused on this group of GINs (e.g., Sangster and Gill, 1999; Kaplan, 2004; Howell et al., 2008). Several factors, including inappropriate administration, under-dosing, inaccurate estimation of body weight and the use of low quality or expired drugs might result in treatment failure, and this tends to complicate detection of true AR in the field (Charleston, 1981). The emerging significance of AR demands an urgent need for the development of reliable, reproducible and standard methods/assays for its detection (Coles et al., 2006). Furthermore, it is important to understand the mechanism(s) involved in the development of AR. It has been estimated that the global market for antiparasitic drugs used in livestock and pet animals is 11 billion US dollars and the cost of developing a new anthelmintic product for livestock is around 40 million US dollars (Brown et al., 2006), highlighting the need to understand the mechanism(s) involved in AR so that it can be better managed without the need to constantly bring new drug families to market. Accurate and timely detection of AR and the knowledge of the mechanism(s) involved in its development might aid to adopt the measures to slow the development of resistance as well as informing the screening of new anthelmintic drugs as the control of GINs will remain dependent on anthelmintics in the foreseeable future (Taylor et al., 2002).

A number of studies have reported the possible specific mechanisms involved in AR based on changes and/or modifications of receptors or drug target sites in GINs (Dent et al., 2000; Sarai et al., 2013; Knapp-Lawitzke et al., 2015). In addition, non-specific mechanisms involving transmembrane transport channel proteins (ATP binding cassette transport proteins) have also been implicated in the development of AR (Schinkel et al., 1994a; Lespine et al., 2012). For example, the ATP binding cassette transport proteins (ABC transporters) are associated with non-specific
mechanisms of resistance, as they modulate the concentration of different drugs inside the cells irrespective of the class of drug (Lespine et al., 2012). These ABC transporters have also been reported in nematodes (Sheps et al., 2004) and have known capacity to act as efflux pumps, facilitating the ATP-dependent movement of xenobiotics including drugs. Therefore, ABC transporters might be involved in active efflux of anthelmintic drugs away from their target sites, resulting in decreased drug concentration and increased parasite survival. It has been suggested that commonly used anthelmintics such as ivermectin (IVM), levamisole (LEV) and thiabendazole are substrates of ABC transporters (Kerboeuf and Guégnard, 2011; Falasca and Linton, 2012), and there is inconsistent evidence that exposure to anthelmintics modulates the expression patterns of different ABC transporters in nematodes (James and Davey, 2009; Areskog et al., 2013; De Graef et al., 2013). Modulation of the abundance or activity of these transport proteins (particularly P-glycoprotein) has been suggested as a possible mechanism of AR in nematodes (Blackhall et al., 1998a). In addition, most of the previous studies focused on measuring the effects of macrocyclic lactones (MLs) on the expression patterns of ABC transporters in parasitic nematodes. However, the role of ABC transporters in AR is still unclear, and there is no evidence available on the effects of anthelmintics other than MLs on the expression patterns of ABC transporters. Therefore, further investigations are required to explore the interaction between ABC transporters with different anthelmintic drugs and their association with AR in GINs of livestock.

1.3 Historical hierarchy of anthelmintic resistance

Despite recent progress in the discovery of novel anthelmintics, AR has arisen as one of the major economic challenges throughout the world, and currently has the most impact upon small ruminant industries (Waller, 1997). For example, in Australia, the prevalence and extent of resistance to all major classes of anthelmintics is so widespread that it compromises parasite control and threatens the profitability of sheep farming (Besier and Love, 2003). This problem was initially highlighted in the mid-20th century when resistance to phenothiazine was reported in *H. contortus* (Drudge et al., 1957) and then later in small strongyles of horses (Gibson, 1960). Thiabendazole was introduced in 1961 as a broad spectrum anthelmintic with low toxicity, however, resistance to thiabendazole was reported in *H. contortus* within a few years of its discovery (Conway, 1964). The same problem occurred after the introduction of two new groups of anthelmintics, imidazothiazoles/tetrahydropyrimidines and avermectins/milbemycin in the 1970s and 1980s, respectively, and by the early 1980s, multiple AR was reported for the very first time (Taylor and Hunt, 1989; Beveridge et al., 1990; Prichard, 1990; Echevarria et al., 1991). Unfortunately, the development of resistance to a newly introduced anthelmintic, monepantel, has also been reported in trichostrongyloid nematodes of small ruminants in New Zealand, Australia, Uruguay and the
Netherlands, within just four years of its introduction (Scott et al., 2013; Love, 2014; Mederos et al., 2014; Van den Brom et al., 2015). These patterns of resistance development highlight the need to either adopt strategies to slow down the development of resistance or hasten the discovery of new anthelmintics.

### 1.4 Prevalence of anthelmintic resistance

There is a great deal of literature available on the prevalence of AR in livestock, horses, companion animals, and human parasitic nematodes globally. Resistance to all three broad spectrum anthelmintics has been reported and reviewed extensively in small and large ruminants (e.g., Drudge et al., 1957; Prichard, 1994; Echevarria et al., 1996; Eddi et al., 1996; Coles et al., 2006; Jabbar et al., 2006; Rendell, 2010).

Drug resistance in populations of *H. contortus* in small ruminants due to intensive use of benzimidazoles (BZ), LEV and IVM has already been reported throughout the world. In most instances, where resistance to various anthelmintics has been reported, closantel remained the only effective available drug, signalling the urgent need to develop new anthelmintics or alternate control strategies (Getachew et al., 2007). *H. contortus* has been widely used as a model organism to study the development and mechanism of AR, since this nematode has often been the first to show resistance and is also one of the most pathogenic sheep GINs (Roos, 2009). This shows that multi-drug resistant populations of *H. contortus* are prevalent, which suggests that strains of *H. contortus* resistant to one group of drugs also show resistance to other groups.

Some selected studies reporting AR over the last two decades in GINs of small ruminants are summarized in Table 1.1.
Table 1.1. Selected reports of anthelmintic resistance in small ruminants from different parts of the world since 1990

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<td>GINs of goats</td>
<td>IVM, BZ, LEV, Doramectin, Morantel</td>
<td>FECRT, LDA</td>
<td>Georgia, United States of America</td>
<td>Terrill et al. (2001)</td>
</tr>
<tr>
<td><em>H. contortus</em></td>
<td>BZ, LEV, IVM, MOX</td>
<td>FECRT</td>
<td>Australia</td>
<td>Love et al. (2003)</td>
</tr>
<tr>
<td>GINs of sheep and goat</td>
<td>BZ</td>
<td>LDA</td>
<td>Philippines</td>
<td>Ancheta et al. (2004)</td>
</tr>
<tr>
<td><em>Trichostrongylus</em> spp.,</td>
<td>BZ, LEV, IVM, MOX</td>
<td>LDA</td>
<td>United States</td>
<td>Howell et al. (2008)</td>
</tr>
<tr>
<td><em>H. contortus</em></td>
<td>LEV, IVM</td>
<td>FECRT, PCR</td>
<td>Sweden</td>
<td>Hoglund et al. (2009)</td>
</tr>
<tr>
<td><em>H. contortus</em></td>
<td>Eprinomectin, BZ</td>
<td>FECRT</td>
<td>Switzerland and Northern Germany</td>
<td>Scheu erle et al. (2009)</td>
</tr>
<tr>
<td><em>H. contortus</em>, <em>Teladorsagia</em> spp., <em>Trichostrongylus</em> spp.,</td>
<td>LEV, MOX, ALB, IVM, Nitroxynil, Disophenol, Trichlorphon, Closantel</td>
<td>FECRT</td>
<td>Brazil</td>
<td>Cezar et al. (2010)</td>
</tr>
<tr>
<td>GINs of sheep</td>
<td>BZ, LEV</td>
<td>FECRT</td>
<td>West Indies</td>
<td>George et al. (2011)</td>
</tr>
<tr>
<td><em>Trichostrongylus</em> spp.</td>
<td>IVM, BZ, LEV</td>
<td>FECRT</td>
<td>Germany</td>
<td>Voigt et al. (2012)</td>
</tr>
<tr>
<td><em>Tr. colubriformis</em>, <em>Te. circumcincta</em></td>
<td>Monepantel</td>
<td>FECRT, Post-mortem worm count</td>
<td>New Zealand</td>
<td>Scott et al. (2013)</td>
</tr>
<tr>
<td><em>Tr. colubriformis</em>, <em>Teladorsagia</em> spp.</td>
<td>Monepantel</td>
<td>FECRT</td>
<td>Australia</td>
<td>Love (2014)</td>
</tr>
<tr>
<td><em>H. contortus</em></td>
<td>Monepantel</td>
<td>FECRT</td>
<td>Uruguay</td>
<td>Mederos et al. (2014)</td>
</tr>
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<td><em>H. contortus</em></td>
<td>Monepantel</td>
<td>FECRT</td>
<td>Netherlands</td>
<td>Van den Brom et al. (2015)</td>
</tr>
</tbody>
</table>

*H. = Haemonchus; Te. = Teladorsagia; Tr. = Trichostrongylus; BZ = Benzimidazole; ALB = Albendazole; OXF = Oxendazole; FEN = Fenbendazole; TBZ = Thiabendazole; LEV = Levamisole; IVM = Ivermectin; MOX = Moxidectin; RAF = Rafoxanide; GIN = Gastrointestinal nematode; EHA = Egg hatch assay; LDA = Larval development assay; FECRT = Faecal egg count reduction test; PCR = Polymerase chain reaction
1.5 Importance of anthelmintics and development of resistance

Despite the advancement of veterinary science and discovery of new anthelmintics to control helminths, nematodes are one of the major constraints to animal health and production worldwide. Broad spectrum anthelmintics are generally recommended for their prophylactic and therapeutic use to control the nematode infections in livestock (Barger, 1993; Thompson, 1999). Regular strategic use of oral, topical or injectable anthelmintic formulations remains a cornerstone of controlling the helminth infections (Prichard et al., 2007). According to the World Association for the Advancement of Veterinary Parasitology (WAAVP), resistance to anthelmintic drugs is suspected when the efficacy of the drug at a recommended dose is $<95\%$ with the lower limit of 95% confidence interval less than 90% (Coles et al., 2006). However, while applying this cut-off limit, factors such as inappropriate administration, under-dosing, inaccurate estimation of body weight and the use of low quality or expired drugs that may result in treatment failure should also be considered (Charleston, 1981). The regular use of anthelmintic drugs without a judicious strategy is likely a major factor in increasing selection pressure on GINs of livestock (Papadopoulos, 2008).

Drug resistance in parasites generally results from the selection of a sub-population of parasites that can withstand the toxic effects of drugs which were previously lethal to them. Drug pressure selects specific gene alleles that allow parasites to survive. An increase in the frequency of these alleles is responsible for the development of resistance. The rate of resistance development is defined by the frequency of alleles coding for resistance when the worms are exposed to the drug (Gillett and Beech, 2007; Ihler, 2010). Anthelmintic resistance is usually a multi-component phenomenon that involves more than a single genetic change and quite often non-receptor based mechanisms also contribute to resistance (Beech et al., 2011). Dosing rates and frequency of drug exposure also influence the development of AR. Suboptimal dosing can allow more resilient fractions of a worm population to survive and proliferate, while frequent use of anthelmintics exposes more generations to the drug, especially when prepatent periods are shorter as compared to the parasites with longer prepatent periods. This phenomenon is more likely associated with development of AR (Ihler, 2010).

Selection pressure can be reduced if treatment of selected animals with only higher number of faecal egg counts is practised, such that the impact of helminthosis can be minimised while maintaining a degree of refugia (Martin et al., 1981; Coles, 2003; Dobson et al., 2011). Refugia is a term used for the parasite population not exposed to the drug and it is well known that resistance is inversely proportional to the number of parasites in refugia as it lowers the selection pressure on the whole population (Shalaby, 2013). It has been suggested that maintenance of refugia is very
important to hold back the process of the development of AR in GINs of livestock species (Coles, 2005). Therefore, treatment of selected animals and adaption of alternate parasite control strategies would aid to slow the development of AR.

1.6 Mechanisms of anthelmintic resistance

Trichostrongyloid nematodes have developed various mechanisms to combat the toxic effects of anthelmintics either directly (e.g. modified drug receptors) or indirectly (altered drug metabolism or increased drug transport away from target sites) resulting in reduced drug efficacy. A summary of different mechanisms of AR is discussed in the following sections.

1.6.1 Specific mechanisms of anthelmintic resistance

Specific mechanisms associated with AR, also known as targeted resistance, are usually due to modification of the receptors that are target sites for the drugs, and therefore the mode of action of the drug is affected. Targeted resistance can mainly be due to: (i) single nucleotide polymorphisms (SNPs) or any other genetic modifications which alter the amino acid sequence of the drug receptors and the affinity of receptors to bind drugs, (ii) altered ancillary proteins or other substances which affect receptor functionality, and (iii) changes in regulatory components that modify the expression level of receptors or ancillary proteins (Lespine et al., 2012).

The mechanism associated with BZ resistance is a change in structure of β-tubulin isotypes as this class of anthelmintics acts by inhibiting the functions of microtubules after binding to β-tubulin, which is essential for cell structure, resulting in death of the parasite (Kohler, 2001). This association was previously suggested when a radioligand binding assay showed that phenotypically resistant *H. contortus* worms carry modified microtubule subunit proteins and high affinity binding sites for BZ were missing in these isolates (Kwa et al., 1993). Further investigations revealed that single point mutations at either codon 167 (F167Y) or 200 (F200Y) resulting in amino acid replacement (phenylalanine by tyrosine) are linked with BZ resistance (Kwa et al., 1994; Hodgkinson et al., 2008; Knapp-Lawitzke et al., 2015). Another study showed that glutamate (GAG) to alanine (GGC) substitution in codon 198 (E198A) is also responsible for BZ resistance in *H. contortus* isolates (Ghisi et al., 2007). Kotze et al. (2012) also reported that high level of thiabendazole resistance in *H. contortus* L3 was more associated with the SNP in codon 198 than the SNP at 200. In addition, Rufener et al. (2009a) also reported E198A substitutions of beta-tubulin protein but not at either position 167 or 200 following *in vitro* selection of *H. contortus* to BZ. These polymorphisms increase as a result of selection pressure and lead to treatment failure but still there are situations where exposure to BZs does not result in appearance of the polymorphisms.
usually associated with resistance (Schwenkenbecher et al., 2007). In spite of this, it is generally accepted that the mechanism involved in BZ resistance is well understood and the major genetic determinant of BZ resistance in most, if not all, trichostrongyloid nematodes is the possession of SNPs, in the parasite’s isotype-1 β-tubulin gene at codons 167, 198, and 200. In addition, mutations at β-tubulin isotype-2 have also been associated with BZ-resistance in ruminant parasites. The SNPs F167Y and F200Y have been reported in *H. contortus*, however the practical field importance of these mutations in nematodes is still unknown (reviewed by Wolstenholme et al., 2004).

The imidodiazole group including LEV mimics the action of acetylcholine. These drugs act upon the nicotinic acetylcholine receptors (nAChR) on the surface of somatic muscle cells in nematodes, leading to depolarization and spastic paralysis that facilitates parasite expulsion (Unwin, 1995; Evans and Martin, 1996). The laboratory-produced LEV resistant *Caenorhabditis elegans* worms showed that a large number of genes are involved in the observed resistance. These genes code for a LEV-sensitive muscle nAChR (Fleming et al., 1997), the contraction signalling pathway (Kagawa et al., 1997) and the processing and assembly of subunit ancillary proteins (Gottschalk et al., 2005). The mechanism of clinical levamisole resistance in parasitic nematodes is less well understood, and may incorporate different mechanisms and genes. The mechanism of resistance to levamisole is most likely due to a change in the ability of target sites to bind the drug. Studies conducted on the pig nematode, *Oesophagostomum dentatum*, involving different members of this class including levamisole and pyrantel showed that there was a lower percentage of opened ion-channels in resistant worms compared to the counterparts, suggesting an increased desensitization of drug receptors in resistant isolates (Robertson et al., 1999, 2000). Neveu et al. (2010) described the existence of abbreviated isoforms of the gene *unc-63*, referred to as *unc-63b*, in resistant worms of *H. contortus*, *Te. circumcincta* and *Tr. colubriformis*. The *Hco-unc-63b* gene encodes a truncated isoform that was subsequently shown to have a dominant negative effect on the LEV nAChR expression in Xenopus oocytes (Boulin et al., 2011). Further research showed that resistance to levamisole is associated with a spliced variant of the *acr-8* gene, referred to as *Hco-acr-8b* which showed elevated expression in resistant worms and was undetectable in susceptible worms (Williamson et al., 2011). Neveu et al. (2010) reported the presence of four paralogues of the gene *unc-29*: *Hco-unc-29.1, Hco-unc-29.2, Hco-unc-29.3* and *Hco-unc-29.4*. These observations suggest that the LEV receptor of some parasitic nematodes may not be the same as those found in *C. elegans*. The subunits that arise from these four paralogues may not function in the same way, with some rendering the receptor pentamer less sensitive to LEV (Kotze et al., 2014a). Sarai et al. (2014) showed that the highest level of LEV resistance was associated with decreased receptor subunit genes (*Hco-unc-63a* and *Hco-unc-29* paralogs) as well as genes involved in receptor assembly.
(Hco-unc-74, Hco-unc-50 and Hco-unc-3.1). These findings suggest that the higher level resistance to LEV is due to altered receptor subunit composition as a result of changes in both subunit composition and in the levels of proteins involved in receptor assembly. A more recent study showed that acr-21 and acr-25 showed increased expression alongside reduced expression of Ode-unc-63 in LEV-resistant adult male worms of *O. dentatum* (Romine et al., 2014). These modified nAChR genes were accompanied with four SNPs that were associated with the resistant worms. These studies suggest that resistance to cholinergic anthelmintics in parasitic nematodes is polygenic rather than a simple single-gene mechanism. Since LEV acts a substrate of P-gps (Kerboeuf and Guegnard, 2011), there might be a possible contribution from P-gps towards LEV resistance. However, further work is required to test this hypothesis.

Macrocyclic lactones (avermectins and milbemycins) cause flaccid paralysis of the somatic musculature in the parasite body, thus disrupting food ingestion by inhibiting the pharyngeal pump (Kotze, 1998; Sangster and Gill, 1999; Rana and Misra-Bhattacharya, 2013). Molecular studies have demonstrated that target sites for this class are invertebrate specific glutamate-gated chloride ion channels (GluCl), which hold alpha subunits. IVM acts as an agonist of glutamate that prolongs opening time of the targeted channels. The binding of IVM with receptors leads to irreversible chloride ion fluxes followed by hyperpolarisation of the cell membrane, which ultimately results in paralysis of the muscles (Martin, 1996; Kohler, 2001). The mechanism of IVM resistance is still unclear, as genetic analysis of a resistant isolate of *H. contortus* indicated that only one major gene is associated with resistance to IVM (Le Jambre et al., 2000), while other studies suggest that this mechanism seems to be influenced by more than one gene (Gill and Lacey, 1998; Ardelli and Prichard, 2004).

In the nematode *C. elegans*, high-level resistance to IVM was reported to be associated with simultaneous mutation of three genes (*avr-14, avr-15 and glc-1*) that encode GluCl channel alpha-type subunits (Dent et al., 2000). In parasitic nematodes, however, evidence describing the selection of specific alleles in GluCl responsible for IVM resistance is still limited. Some studies showed marked variation in response to IVM inhibition of pharyngeal activity between resistant and susceptible *H. contortus* isolates (Kotze, 1998; Sangster and Gill, 1999; Glendinning et al., 2011), which suggests that IVM resistance is more likely due to changes in target sites, the receptors on pharyngeal muscles (Kohler, 2001). There is still little evidence that resistance to IVM in *H. contortus* is associated with genetic changes to GluCl channels (Williamson et al., 2011). Some other studies also support the evidence that polymorphism of gamma-aminobutyric acid channel (Blackhall et al., 2003) and a putative amino-acid gated anion channel subunit HG1 (Prichard, 2001) are also involved in development of resistance to IVM. Another major mechanism implicated
for the development of ML resistance in nematodes is increased drug transport through transmembrane proteins because MLs, particularly IVM, are good substrates of these transporter proteins (Lespine et al., 2012). The role of these drug transporters in IVM resistance is outlined below.

### 1.6.2 Non-specific mechanisms of anthelmintic resistance

Generally, drug resistance is associated with more than one genetic changes and quite often non-receptor based mechanisms also contribute to the development of resistance (Beech et al., 2011). The major risk associated with this type of resistance is that several classes of drugs having different modes/targets of action may be equally impacted and their efficacies can be compromised by the modified pharmacokinetics (Lespine et al., 2012). Resistance to one anthelmintic due to non-receptor based mechanisms may lead to cross resistance to other anthelmintics to which the parasite has not been exposed, as indicated by reduced sensitivity of *C. elegans* to LEV due to selection for IVM resistance (Ardelli and Prichard, 2008). This type of resistance is associated either with altered levels of enzymes required for drug metabolism, defensive molecules required for survival of the cells, or modified transport mechanisms that control the concentration of drugs that reach the receptor sites (reviewed by Lespine et al., 2012). The continuous intrusion of environmental toxic substances has led organisms to develop special cellular mechanisms to combat the effects of such substances. These mechanisms include altered drug transport and modified drug metabolism (James et al., 2009). Modified drug metabolism may include (i) increased drug metabolism and inactivation and (ii) reduced activation in case of pro-drugs (reviewed by Lespine et al., 2012). Certain defensive molecules are also produced inside the cellular machinery of parasitic nematodes, which alter the drug metabolism and make these compounds less toxic. Glutathione and thioredoxin systems work in parallel to oxidize or reduce the toxins, scavenge the free radicals and protect the cells from oxidative damage (James et al., 2009). Some previous studies showed that increased expression of glutathione and/or thioredoxin is associated with AR in *H. contortus* isolates (Kerboeuf and Aycardi, 1999; Sotirchos et al., 2008).

The modulation of drug transport includes (i) increased efflux of the drugs from target cells and (ii) decreased uptake of the drugs (Lespine et al., 2012). P-gps are members of a large superfamily of trans-membrane transport proteins (ABC transport proteins) that have been associated with AR in nematodes (Prichard and Roulet, 2007; James and Davey, 2009). These proteins are proposed to be important players in the development of resistance to IVM because there have been reports describing an over-expression of multiple P-gps in different nematodes resistant to this class of anthelmintic (Sangster and Gill, 1999; Le Jambre et al., 2000; Dicker et al., 2011). The up-regulation of helminth P-gps gene expression enhances the parasite’s ability to
survive IVM exposure (Areskog et al., 2013) and also supports its role in the development of resistance to this drug. These efflux proteins are also called multi-drug resistance transporters and have been identified as a mechanism of resistance both in mammals and nematodes.

### 1.6.2.1 Multidrug-resistance transporters

ABC transporters are members of a well-conserved family of membrane proteins which mediate ATP dependent transport of a number of structurally unrelated endogenous and exogenous compounds, including drugs (Gottesman and Pastan, 1993). These transporters utilize energy generated by hydrolysis of ATP and export a wide variety of xenobiotics, lipids and metabolic products across the membrane (Falasca and Linton, 2012). The drug efflux through ABC transporters can represent a non-specific mechanism of AR because these modulate the concentration of different drugs at their target sites, which then limits the optimum efficacy of drugs (Lespine et al., 2012). Such an assertion is supported by the well documented association of multidrug resistance (MDR) transporters (P-glycoproteins, multi-drug resistance proteins and half transporters) with movement of various anticancer drugs across the cell membrane and development of resistance to various drugs in mammals (Lespine et al., 2008; Falasca and Linton, 2012). In mammals, certain ABC transporters that are associated with active efflux of anticancer drugs include: ABCB1 (P-gps, MDR1), ABCC1 (Multidrug-resistance-associated protein; MRP1), ABCC2 (MRP2), ABCC3 (MRP3), and ABCG2 (Breast cancer resistance protein; BRCP) subfamilies (Falasca and Linton, 2012). Similarly, P-gps and MRPs have been implicated in the development of AR against MLs in parasitic nematodes (Blackhall et al., 1998a; Xu et al., 1998; Ardelli and Prichard, 2008). The possible role of other members of the ABC transporter family requires further elucidation.

### 1.6.2.2 ABC transporters in nematodes

In human medicine, P-gps were the first type of ABC transporter to be implicated in multidrug resistance in tumour cells (Dano, 1973). Nematodes possess a vast array of MDR transporter genes as compared to mammals, which have comparatively few MDR transporters (Prichard and Roulet, 2007). Active P-gp pumps were identified in *H. contortus* using UIC2 monoclonal antibody, which specifically interacts with the active form of the protein (Sangster, 1994; Kerboeuf et al., 2003b). Genes that are homologous to the mammalian ABCB1 subfamily are also known to encode P-gps in nematodes. Other MDR transporter genes include ABCC that encode MRPs, and half transporters encoding genes that are more closely related to ABCB sub-family in nematodes, than that of ABCG2 sub-family in mammals (Lespine et al., 2012).
Nematodes possess a greater diversity of MDR transporters. *C. elegans* is known to have 15 P-gp genes (*pgp*), eight MRP (*mrp*) and nine HAF genes (*haf*) (Sheps et al., 2004). In parasitic nematodes, eight P-gp, five MRPs and eight HAF transporter genes were reported in *Brugia malayi* (Ardelli et al., 2010), two P-gp genes in cyathostomins (Drogemuller et al., 2004), 11 partial sequences of P-gp genes in *Te. circumcincta* (Dicker et al., 2011) and four P-gp and three HAF encoding genes have been reported in *Onchocerca volvulus* (Huang and Prichard, 1999; Bourguinat et al., 2008). Whereas, in *H. contortus*, 11 P-gp genes, one HAF gene and two MRP genes have been reported (Williamson and Wolstenholme, 2012; Laing et al., 2013). The reason for this diversity of MDR transporters in nematodes is still not clear; however, it has been suggested that such diversity might be essential for protection of nematode neurons from a broad range of toxins (Prichard and Roulet, 2007). Additionally, all the stages of *C. elegans* do not express all ABC transporters and in adult worms, expression appears to be restricted to excretory cells, intestine, amphids, neurons, muscles, pharynx, hypodermis, and some other tissues including the vulva in female worms (Zhao et al., 2004). However, in the case of *H. contortus*, P-gp-A is expressed in the posterior pharynx and anterior intestine (Smith and Prichard, 2002), while anti-human *mdr-1* monoclonal antibody staining showed that P-gps are also present in egg shell (Kerboeuf et al., 2003b) and within the cuticle of adult and larval stages of *H. contortus* (Riou et al., 2005). However, antibodies also detected some lower molecular mass protein bands, which suggested that the anti-human *mdr1* antibodies are not 100% reactive with P-gps in nematodes and they can also cross-react with proteins other than P-gps. This suggests that anti-human *mdr-1* antibodies may not be suitable to detect the presence of nematode P-gps.

**1.6.2.3 ABC transporters and drug resistance in nematodes**

P-gps are associated with AR as elevated expression levels and allele frequencies of P-gps have been reported in anthelmintic-resistant populations of nematodes. Current evidence supports that the absorption, distribution and elimination of anthelmintics, especially MLs, in hosts and parasites are influenced by multidrug resistance transporters, including P-gps (Kerboeuf et al., 2003a; Lespine et al., 2008). IVM was the first ML reported to be a substrate for P-gps when a recommended antiparasitic dose resulted in toxicity and death of genetically engineered mice lacking the gene coding for P-gps. The concentration of IVM was found to be 100-fold higher in the brain of mutant mice than the wild type mice (Schinkel et al., 1994b). Apart from strong interaction with P-gps, MLs also interact with MRPs (MRP1, MRP2 and MRP3), suggesting that efflux of MLs is under the control of more than one transporter protein (Lespine et al., 2006). Likewise, the same mechanism might be inferred for other anthelmintic classes. The combined effects of these efflux proteins have a considerable impact on bioavailability and efficacy of the drugs by interfering
with the absorption, distribution and elimination of anthelmintics (Borst et al., 1999). The efficiency of drug elimination is higher with the increased affinity of anthelmintics for P-gps, therefore, anthelmintic drugs including MLs are eliminated from the organism more rapidly in relation to the relatively shorter resident time (Lespine et al., 2008), which ultimately reduces the drug’s efficacy.

There is accumulating evidence that MLs are a more effective substrate for P-gps than the other anthelmintic classes (Prichard and Roulet, 2007). Some evidence suggests that imidothiazoles are also a substrate of P-gps but LEV does not show any stimulatory effects; however, the BZ albendazole shows very slight stimulatory effects on the mammalian P-gps (Efferth and Volm, 1993; Naito et al., 1998). On the other hand, in eggs of a multi-drug resistant isolate of *H. contortus*, it has been shown that LEV and MLs other than IVM are highly stimulatory of P-gps (Kerboeuf and Guegnard, 2011). However, it was recently described that IVM markedly inhibited rhodamine-123 (R-123; a fluorescent substrate) transport through *pgp-2, pgp-9.1* and *pgp-16* from adult worms of *H. contortus* expressed in mammalian cells (Godoy et al., 2015a; Godoy et al., 2015b; Godoy et al., 2016), whereas moxidectin caused less inhibition of R-123 efflux as compared to IVM. The authors concluded that IVM is a better substrate of nematode P-gps than moxidectin and this may help to explain the slower rate of development of resistance in *H. contortus* to moxidectin compared with the avermectins. Their work also suggests that the transport mechanism mediated through MDR transporters may vary in different developmental stages of nematodes. The difference in interaction of anthelmintics across various life-stages in nematodes also supports the hypothesis that variation might exist in expression patterns of ABC transporters between developmental stages of nematodes. Sarai et al. (2013) described life-stage and isolate-specific differences in expression patterns for various P-gp genes within isolates of *H. contortus*.

Since anthelmintics are usually substrates of P-gps and therefore interact directly with these transporter proteins, they may have the capacity to regulate the expression levels of P-gps through transcriptional or post-transcriptional mechanisms (Schrenk et al., 2001). Macrocyclic lactones select for certain alleles of P-gp genes and also induce over-expression of different P-gps (Prichard and Roulet, 2007). Furthermore, P-gps have been implicated in resistance to anthelmintics, with a number of studies describing an increased transcription of specific transporter genes in drug-resistant nematodes (Dicker et al., 2011; Sarai et al., 2014). There is also accumulating evidence that exposure to anthelmintics increases gene transcription for ABC transporters in nematodes. Increased expression levels of several P-gps and *mrp-1* genes has been reported in *C. elegans* post-exposure to IVM (James et al., 2009). Recently, Ardelli and Prichard (2013) studied the effects of IVM on the expression patterns of P-gps and they found that IVM induces changes in the abundance of 15 P-gp gene products in *C. elegans* whereas inactivation of certain *pgp* genes (*pgp-2*,...
pgp-5, pgp-6, pgp-7, pgp-12 and pgp-13) resulted in increased sensitivity to IVM as compared to the wild-type *C. elegans*. Furthermore, another IVM-selected strain of *C. elegans* also showed an over-expression of multiple ABC transporters as compared to wild-type strain (Yan et al., 2012).

Some recent studies in parasitic nematodes reported overexpression of *pgp-11*, *pgp-16* and *mrp-1* in *Cooperia oncophora* recovered from animals treated with IVM (De Graef et al., 2013; Tydén et al., 2014). Furthermore, De Graef et al. (2013) also reported a significant increase (3-5 fold) in transcription levels of *pgp-11* in *C. oncophora* adult worms 14 days after treatment with IVM or moxidectin compared to non-exposed adults. The authors further described a 4-fold transcriptional up-regulation of *pgp-11* in L₃ of a resistant isolate compared to susceptible L₃ after an *in vitro* exposure for 24 h to different concentrations of IVM (8.7 and 87 ng/mL). Studies on *H. contortus* have also showed that multi-drug resistance is associated with increased P-gp mRNA expression levels in an IVM resistant strain (Smith and Prichard, 2002), while Lloberas et al. (2013) reported that treatment of infected lambs with IVM increased the transcription levels of *pgp-2* in resistant worms of *H. contortus* compared to the worms collected from untreated control animals. However, there were no significant differences observed between the expression patterns of P-gp genes before and after IVM treatment in *C. oncophora* and *H. contortus* (Williamson and Wolstenholme, 2012; Areskog et al., 2013). Therefore, the role of these trans-membrane proteins as a drug efflux mechanism in nematodes is still clouded and needs further investigation.

1.7 Methods of studying ABC transporters

The identification/localisation of P-gps in nematodes is very challenging as nematodes have different life stages including free-living and parasitic stages, which vary in structure and function. Moreover, being complex organisms, nematodes have highly differentiated organs and protective structures (sheath and cuticle); therefore, confirmation of P-gps in different body tissues of nematodes requires a laborious and careful approach. In addition to this, sometimes differentiation between P-gps and other proteins of the ABC transporters family is difficult due to the nature of methodologies employed, or the abundance of other ABC transporters that may be found in every selected location (Kerboeuf et al., 2003a). In addition, existence of P-gp isoforms that have not yet been characterised also creates problem in identifying P-gps (Sangster et al., 1999). Therefore, more investigations regarding identification of P-gps are required.

The methods that have been used to detect P-gps in mammals and nematodes are (i) detection of corresponding genes, (ii) quantification of mRNA, (iii) localisation of P-gps using monoclonal antibodies, (iv) *in vitro* functional assays, and (v) use of certain *in vivo* models to assess the presence of specific transport proteins.
There have been various reports describing the presence of genes that encode different ABC transporters in nematodes including *H. contortus* (Laing et al., 2013), *C. elegans* (Sheps et al., 2004), *Brugia malayi* (Ardelli et al., 2010), *Te. circumcincta* (Dicker et al., 2011) and *O. volvulus* (Huang and Prichard, 1999). Smith and Prichard (2002) examined the distribution of P-gp mRNA using *in situ* hybridisation on transverse cryosections of adult *H. contortus* using a digoxigenin-labelled cDNA encoding the ATP-binding domain of *H. contortus* P-gps. The probe sequence targeted a conserved ATP-binding region of P-gp A with 97.9% identity. They also demonstrated 49.7-71.1% identity with 11 other P-gp sequences identified earlier in *H. contortus* and suggested that it could be hybridisation of these sequences that gave an overall measure of the total P-gp mRNA distribution.

Several studies have reported the use of specific monoclonal antibodies for the detection of P-gps in nematodes. The use of C219 and UIC2 monoclonal antibodies for the detection of human and mouse *mdr-1* gene products has been documented, while in *H. contortus*, UIC2 also confirmed both the presence and activity of P-gps, as it was reported earlier in tumour cells (Georges et al., 1993; Kerboeuf et al., 2003b). Furthermore, Riou et al. (2005) also used mouse monoclonal anti-human *mdr-1* antibody UIC2 and electron microscopy to localise P-gps in the egg shells and cuticle of free-living and parasitic stages of *H. contortus*.

Certain *in vitro* methods are available to detect P-gps that are based on measurement or inhibition of the activity of trans-membrane transport proteins and classified as functional assays. Accumulation and/or efflux assays are well developed for mammalian tumour cell studies. Accumulation studies are based on uptake of a radiolabelled or fluorescent probe into the cell in the presence of ABC transporter inhibitors, which block the efflux proteins leading to increased accumulation of the probe and hence evidence of the presence of these transport proteins. Studies in mammalian cells have been conducted using transfected cell lines that over-express a specific transporter protein of interest in comparison to wild type cells (Zhang et al., 2003). The efflux studies involve measurement of a fluorescent dye eliminated through these proteins pumps. Rhodamine-123 (R-123) is one of the most commonly used P-gp probes that have been employed in mammalian tumour and transfected cells. Several agents identified as inhibitors of the transport proteins using efflux / accumulation assays have potential applications in chemotherapy of drug resistance in human cancers and livestock parasitic nematodes (Scala et al., 1997; Kerboeuf and Guegnard, 2011). In nematodes, R-123 accumulation/ efflux has been examined using eggs of *H. contortus* in the presence of P-gp inhibitors, especially verapamil. The results showed that P-gp inhibitors increase the accumulation of R-123 in the eggs of *H. contortus*, indicating partial or complete inhibition of drug efflux by these inhibitors (Beugnet et al., 1997; Kerboeuf et al., 1999).
ATPase assay is another functional tool capable of identifying the presence of specific transport protein channels by detecting specific ATPase activity. Binding of ATP at a nucleotide binding domain on the P-gps is crucial for substrate transport followed by hydrolysis of ATP by P-gp-specific ATPase (Rosenberg et al., 2001). This assay requires prepared cell membrane enriched with the efflux protein of interest, ATP, an analytical method to detect inorganic phosphate liberated from ATP hydrolysis, and a mechanism for discriminating between general and P-gp specific ATPase activity. Some of general ATPases including Ca-ATPases, Na+ ATPases, K-ATPases and mitochondrial ATPases are inhibited by using specific ATPase inhibitors (Zhang et al., 2003). At present, ATPase assay has been most widely used for the determination of P-gp-mediated drug efflux transport in mammals, while no information is available about its use in nematodes. Studies in mammalian cells showed that various compounds including verapamil, cyclosporine A, vinblastin and loperamide were able to stimulate P-gp ATPase activity (Adachi et al., 2001; Garrigues et al., 2002).

A couple of in vivo methods are currently being used in mammalian studies to detect the presence of specific transport proteins. Transgenic animal models are a well-established means of evaluating genes and their protein products. Animals can be genetically modified such that a specific protein can be over-expressed or blocked by addition or deletion of a gene or genes. The phenomenon of removal or silencing of a gene is called homologous recombination or more commonly gene knockout (Zhang et al., 2003). The role of P-gps in drug absorption and elimination has been studied by silencing single or both mdr genes (mdr-1 and mdr-2) that encode P-gps in mice. The results showed increased absorption/accumulation of various drugs in different body tissues of mice lacking P-gps as compared to a control group of mice (Schinkel et al., 1994a; Kim et al., 1998). CF-1 mouse (P-gp mutant) and TR (-) rat (MRP-2 mutant) are naturally deficient in the expression of specific drug efflux proteins. Merck, a pharmaceutical organisation, reported a CF-1 mouse strain that has a genetic defect and lacks the expression of P-gps (Umbenhauer et al., 1997). Various studies conducted on CF-1 mice have indicated that the drug concentration in blood was significantly increased as compared to wild type mice representing decreased elimination of the drugs. In a toxicological study in pregnant mice, a photoisomer of IVM (L-652,280) induces cleft palate (developmental toxicity) in 100% of foetuses with a deficit in P-gp (-/-), whereas the heterozygous (+/-) littermates were less sensitive and only 30% of them developed cleft palate. In contrast, the homozygous foetuses (+/+) with normal P-gp expression levels were totally insensitive to the developmental abnormality caused by the drug (Lankas et al., 1998). Later on, Kwei et al. (1999) also reported that CF-1 mice showed increased blood concentration of IVM and cyclosporine A with decreased intestinal excretion. In addition, brain concentration of both drugs
was also higher as compared to wild type mice after intravenous or oral administration. The use of gene silencing to study the role of P-gps in nematodes is still limited and no success has been reported with parasitic nematodes. In the free-living nematode *C. elegans*, genetic modification has also been used as a tool to study the role of P-gps in anthelmintic sensitivity. Deletion of some P-gp genes in *C. elegans* resulted in increased sensitivity of the worms to IVM compared to the wild-type worms (Ardelli and Prichard, 2013). However, this technique has not yet been successfully adapted for use with parasitic nematodes and needs further work.

1.8 Detection of anthelmintic resistance

Apart from developing new anthelmintics and adapting alternate strategies to slow down the development of resistance, detection of resistance at an early stage is also very important. Various *in vitro* and *in vivo* tests have been developed for the detection of AR in different nematodes, however each method has limitations regarding applicability in the field conditions, versatility, reproducibility or sensitivity. Moreover, most of the tests employed to detect AR are for GINs of livestock and horses (Coles et al., 2006; Jabbar et al., 2006), while relatively few *in vitro* tests have been developed and adapted for GINs of other species (Kotze et al., 2004; Kotze et al., 2005; Kopp et al., 2008). Therefore, there is an ongoing need to develop and refine methods for the detection of AR at an early stage in field conditions, particularly with respect to MLs.

1.8.1 *In vivo* tests for the detection of anthelmintic resistance

1.8.1.1 Faecal egg count reduction test

The faecal egg count reduction test (FECRT) is the most commonly used *in vivo* test in livestock and provides an estimation of anthelmintic efficacy based on the percentage of reduction in faecal egg counts after the administration of anthelmintics. Faecal egg counts are determined before and after a specific period of treatment (depending upon the drug used), and according to published standards, a single dose of an anthelmintic should reduce the egg burden by more than 95% with lower limit of 95% confidence interval (CI) >90% and upper CI limit >95%, where the efficacy of that drug is expected to be ≥99%. (Coles et al., 1992; Taylor et al., 2002; Coles et al., 2006). FECRT is not a reliable test for all nematodes, for instance it is less useful for monitoring drug response in the canine hookworm, *A. caninum*, due to the ability of female worms to modulate egg production at changes in worm burden in intestine (Krupp, 1961; Kopp et al., 2007). The FECRT can result in either false negative (Jackson, 1993) or false positive (Grimshaw et al., 1996) results, due to different developmental stages of parasite species. This test is only reliable if the resistance level is higher than 25% of the total worm population (Martin et al., 1989). Larval culture
is required to determine the species involved, although culture conditions may favour the development of one species over another. However, if more than one genus is found in pre-treatment larval culture then a post-treatment larval culture may still have utility, because it may provide some context as to which species fraction of the total population is most resistant (McKenna, 1997). Parasites with high biotic potential, for example H. contortus, may exert a disproportionate influence on the results and correction factors have been proposed by some authors (Webb et al., 1979; Edwards et al., 1986). Besides these limitations, FECRT is still the most commonly used test to detect AR in field conditions.

**1.8.1.2 Critical and controlled anthelmintic efficacy tests**

The critical test is based on estimation of number of worms recovered from animal faeces collected for at least four days following anthelmintic treatment. The animals are euthanized, worms still present in the gastrointestinal tract are counted and the percent efficacy is calculated by dividing the number of expelled worms by the residual number and multiplying by 100 (Gordon, 1950). The worm burdens, typically achieved via artificial infection using susceptible and resistant isolates, are compared after treatment (reviewed by Jabbar et al., 2006). The key benefit of this test is that fewer animals are required as each animal also acts as its own control. The critical anthelmintic test is unsatisfactory for estimating anthelmintic efficacy against abomasal parasites of sheep because they undergo digestion during their passage through the gut (Reinecke et al., 1962).

The controlled anthelmintic efficacy test is considered to be the most reliable method for assessing AR and has, therefore, been widely used to confirm the results of FECRT as well as for validating different *in vivo* tests (Boersema, 1983; Presidente, 1985). This test compares the worm burdens of animals following anthelmintic treatment which have been naturally or artificially infected with susceptible or suspected resistant isolates of nematodes (Reinecke et al., 1962). Guidelines for measuring anthelmintic efficacy using controlled test have been published previously (Powers et al., 1982; Wood et al., 1995). In the case of mixed infections, larvae of different species are identified by faecal cultures. Resistance is generally confirmed when the reduction in geometric mean worm counts is less than 90%, or greater than 100 worms surviving treatment (Presidente, 1985).

Critical and controlled tests are not desirable in companion animals as the requirement to euthanize the recruited animals at the endpoint is conflicting to animal welfare considerations. Furthermore, these tests are also laborious, cumbersome and expensive (Johansen, 1989; Kopp et al., 2008). Laboratory animal models have been used to reduce the cost and time for this experiment (Kelly et al., 1981).
1.8.2 *In vitro* tests for the detection of anthelmintic resistance

1.8.2.1 Egg hatch assay

The egg hatch assay (EHA) is used to measure the effects of anthelmintics on hatching of the nematode eggs. The ability of anthelmintics to prevent nematode egg hatching is measured, therefore, the assay is not suitable for anthelmintics lacking oxicidal effects, for example IVM. It was first reported by Le Jambre (1976) and later on modified by Coles et al. (1992). EHA is most frequently used to detect BZ resistance in ruminant nematodes. Later, a dose dependent relationship for BZs was reported for human hookworms (Kotze et al., 2005) but utilization of this test to detect AR in human and dog hookworms is still a work in progress. An *in vitro* EHA has also been described by Dobson et al. (1986) for detecting resistance of nematodes to LEV. Recently, the EHA has also been used to measure the oxicidal effects of MPL in *H. contortus, Tr. axei* and *Te. circumcincta* (Bartley et al., 2016). The authors concluded that the EHA may be a promising tool for the phenotypic characterisation of MPL sensitivity. The sensitivity of eggs to thiabendazole decreases with age; therefore, eggs should be used within 3 h of collection or stored anaerobically. BZ sensitivity also decreases as embryonation progresses, so unembryonated eggs are a prerequisite for this assay (Hunt and Taylor, 1989). Similar to the FECRT, the EHA is capable of detecting resistance when at least 25% of the worm population carry resistance genes as shown by artificial infection of animals with mixtures of helminth populations with a known level of AR (Martin et al., 1989). Despite the above shortcomings, EHA is still widely used in the field along with FECRT to determine AR.

1.8.2.2 Larval development assay

The effects of anthelmintics on the development of parasites provides a chance to develop techniques useful for detection of AR (Gill et al., 1995). In the LDA, the eggs or L1 larvae are exposed to different concentrations of anthelmintics incorporated into agar wells in a 96-well plates or in a small test tube containing growth medium (Hubert and Kerboeuf, 1992; Kotze et al., 2009). Methods based upon development inhibition are more laborious and time consuming than for the EHA but are useful to detect resistance to all the major groups of anthelmintics including MLs (Jabbar et al., 2006). The larval development assay (LDA) is more sensitive than the FECRT as it identifies resistance when it is present in a worm population at levels down to 10% (Dobson et al., 1996). The suitability of the LDA for detection of resistance to pyrantel in livestock nematodes has also been established (Kotze et al., 1999). On the other hand, Amarante et al. (1997) reported that the LDA is not suitable for detecting ML resistance in *T. circumcincta* while the assay was able to detect resistance to BZ and LEV anthelmintics. This test is considered as reliable, inexpensive and
suitable for use in the field investigations of AR. The test can also utilize L1, therefore, there is no prerequisite for undeveloped eggs or fresh faecal samples (Coles et al., 1988).

1.8.2.3 Larval paralysis test

This test was established to detect the resistance against LEV and morantel. After exposure of larvae to different drug gradients for 24 h, the proportion of paralyzed larvae for each concentration is calculated and the dose dependent response is referenced to a control replicate (Martin and Jambre, 1979). The repeatability of the test is questionable, as in some studies, the same response could not be reproduced (Boersema, 1983). Geerts et al. (1989), however, reported fairly good reproducibility of this test and attributed any differences in repeatability to the age of larvae. Sutherland and Lee (1990) described a modification of the larval paralysis assay by incubating L3 of trichostrongyle nematodes in an acetylcholinesterase inhibitor (esrine), and found it to be suitable for detecting resistance to thiabendazole due to the presence of higher levels of acetylcholinesterase in resistant isolates. The test is not widely used as compared to other available tests, most likely due to difficulty in interpretation and lack of repeatability.

1.8.2.4 Larval motility assay

The larval motility assay is used to identify resistance to BZs, MLs and LEV or morantel, however for morantel, a definitive discrimination among resistant and susceptible strains is not always possible (Geerts et al., 1989; Conder and Campbell, 1995). A micromotility meter was developed for measuring the motility of larval and adult nematodes after exposure to anthelmintics (Bennett and Pax, 1986). The instrument uses microprocessor technology to measure light refraction at the meniscal interface. Movement of larvae in solution is claimed to change the angle of light refraction entering the photodiode. This light deviation is measured and information passed to a computer to give a motility index. An in vitro assay using dog hookworms (A. caninum and A. ceylanicum), human hookworm (N. americanus) and Strongyloides species demonstrating the effects of BZ and IVM has been established (Kotze et al., 2004). Each parasite species showed distinct differences in dose dependent motility against anthelmintics. The usefulness of the assay in resistance detection requires correlation with clinical responses among individuals infected with the same strains of parasite that show different drug sensitivities. This assay is not in wide use for the detection of AR. The larval motility assay has also been suggested as a potential method for detection of resistance to pyrantel in dog hookworms (Kopp et al., 2008).
1.8.2.5 Larval arrested morphology assay

The larval arrested morphology assay is performed by exposing hookworm larvae to different concentrations of anthelmintics, the presence of which alters the posture of the larvae into a dormant state. This assay is validated for dog hookworm using isolates of intermediate susceptibility to pyrantel (Kopp et al., 2008). Larvae in control (drug free) environments assume straight conformation and are not deviated or twisted. Increased severity of kinking (deviations) occurs with increased concentrations of pyrantel and at very high concentrations of drug, certain larvae are coiled. The sensitivity of this assay was demonstrated to be significantly higher than larval motility and migration assays, which makes it likely that the larval arrested morphology assay is a more appropriate tool for detecting resistance to pyrantel in dog hookworms (Kopp et al., 2008).

1.8.2.6 Larval migration assay

The larval migration assay (LMA) was developed as a modification of the previously discussed motility assay (Gill et al., 1991) to detect the sheep nematodes resistant to IVM (Kotze et al., 2006). The test is useful for investigating the action of a range of paralysing agents, for example, IVM. Infective stage larvae (L3) are exposed to various dilutions of IVM for 48 h and then allowed to migrate through an agar/ filter mesh system fitted over a receiver plate, for the next 24h. The assay is able to detect a 10% IVM-resistant fraction in a population of *H. contortus* but it proved to be ineffective for two other nematodes, including *Tr. colubriformis* and *O. circumcincta*. On the other hand, Demeler et al. (2013) reported the usefulness of the LMA for measuring the effects of MLs in three species of sheep trichostrongylid nematodes. The migration assay can also be used in detecting anthelmintic activity of agents other than drugs. For instance, detection of anthelmintic effects of enzyme systems generating oxygen radicals, and in detection of inhibitory effects of double stranded RNA (dsRNA) with L3 *H. contortus* (Kotze and Bagnall, 2006). When used to detect resistance to pyrantel in the dog hookworm (*A. caninum*), the LMA did not produce satisfactory results, most likely due to the adaption of a quiescent state by *A. caninum* larvae in contrast to constant movement of *H. contortus* larvae (Kopp et al., 2008).

1.8.3 Molecular techniques

Different molecular techniques have been developed for the detection of specific mutations that are associated with AR, which include restriction enzyme digestion, direct sequencing, pyrosequencing and diagnostic PCR. These techniques have been used to reveal a pattern of substitutions associated with BZ resistance. DNA polymorphisms were investigated in the genome of resistant and susceptible larvae and adult *H. contortus* using restriction fragment length
polymorphisms (RFLPs) preceded by southern blotting (Roos et al., 1990). It was suggested that BZ resistant worms possess a transformed, perhaps decreased, pair of β-tubulin genes in contrast to susceptible worms. Later on, an allele specific PCR was introduced capable of identifying transformation of a single amino acid (Phenylalanine to Tyrosine) at codon 200 in β-tubulin isotype I gene in *H. contortus*, which was implicated for the development of BZ resistance (Kwa et al., 1994). A similar substitution at P167 has also been reported in *H. contortus* (Prichard, 2001).

There is currently little evidence to support a definite molecular mechanism involved in resistance to IVM and LEV. In the case of LEV, previous studies suggest that the development of genetic mutations alter target sites, resulting in an inability of drugs to bind to the receptors (Williamson et al., 2011; Sarai et al., 2013) It has been reported earlier that the L subtype channel was absent in LEV resistant *Oesophagostomum dentatum* (Robertson et al., 1999). A number of genes have been implicated in IVM resistance in *H. contortus*, either via a reduction in polymorphism or selection of specific alleles in resistant populations. These include the glutamate-gated chloride channels α-subunit (GluClα) (Blackhall et al., 1998b), gamma-aminobutyric acid channel (GABA) (Blackhall et al., 2003) and a putative amino-acid gated anion channel subunit HG1 (Prichard, 2001). Analysis of a replicated portion of GluClα from *H. contortus* using single strand polymorphism suggested that resistance to IVM was related to altered frequencies of two alleles of α-subunit gene (Blackhall et al., 1998b). Moreover, research on ABC transporters suggests that these may also play a vital role in the development of resistance to MLs (Xu et al., 1998; Ardelli et al., 2006; James and Davey, 2009; Ardelli and Prichard, 2013). However, further research is required to determine the molecular mechanisms involved in the development of resistance to IVM and LEV.

Polymerase chain reaction (PCR) is capable of detecting resistance even when 1% of the worms are resistant in a population, which makes it more sensitive than any other available techniques (Roos et al., 1995). Although more high-throughput and rapid techniques are now available as direct real-time PCR, sequencing and pyro-sequencing are being used to detect the proportion of susceptible to resistant genes in worm populations (Beech et al., 2011), these techniques are expensive compared to RFLPs. All the polymorphisms within a defined region can be determined by direct sequencing of the PCR amplified DNA containing the region of interest from individual diploid parasites (de Lourdes Mottier and Prichard, 2008; Palcy et al., 2010). Variations in the sequence of interest are identified by comparing the sequence chromatograms with a reference sequence. A limitation of direct sequencing is the inability to identify specific haplotypes, although they can be inferred if certain assumptions are made about the population sampled (Beech et al., 2011). Pyrosequencing (Ronaghi et al., 1998), using PCR primers designed
to detect specific polymorphisms can be used to screen relatively large numbers of individual parasites and also quantify the presence of different alleles in bulk DNA samples (Hodgkinson et al., 2008; von Samson-Himmelstjerna et al., 2009). Initially, the technique was expensive and intended to identify SNPs within only a short stretch of DNA, but recent advances in technology are increasing this range along with reducing the cost. The use of diagnostic PCR is another simple and cost effective approach to identify polymorphisms in a sequence of interest. The diagnostic PCR primers bind only to specific sequence variants with the 3’ end of the primer overlapping the SNP of interest (Schwenkenbecher et al., 2007; Rufener et al., 2009a; Palcy et al., 2010). Standardization of the primers for which the most 3’ nucleotide lies on the SNP of interest is often difficult, but the introduction of a deliberate mismatch in the penultimate nucleotide of the primer has been shown to achieve specific detection (Rufener et al., 2009a; Beech et al., 2011). Besides higher sensitivity, molecular techniques do not represent functional assays for detecting resistance. These molecular techniques only detect the presence of alleles that are correlated with resistance, therefore, these techniques should be concurrently used with some other functional assays used for phenotypic detection of resistance, for example FECRT.

In recent years, there has been rapid development and application of high-throughput sequencing technologies for the identification of different microbes and parasites. Loop-mediated isothermal amplification (LAMP) is a powerful innovative gene amplification technique which has been recently developed for simple rapid identification and early detection of microbial disease agents (Martinez-Valladares and Rojo-Vazquez, 2016; Melville et al., 2014). The full LAMP procedure can be completed in less than 1 h under isothermal conditions (Parida et al., 2008). In addition, the development of microsatellite markers, based on genomic data, is another approach to identify parasite isolates (Pajuelo et al., 2015). Microsatellites or simple sequence repeats, are repetitive DNA sequences consisting of nucleotide blocks, with each block containing 1-6 nucleotides. These blocks are repeated up to 60 times and are highly polymorphic with respect to number of repeated units (Barker, 2002). Apart from identification of microorganisms, these techniques could also be useful for detecting AR at an early stage. Although these high-throughput techniques are expensive, researchers are exploiting such techniques for their potential application in exploring the development and spread of AR worldwide (e.g. Chaudhry et al., 2016; Rashwan et al., 2016), and efforts are underway for modifying such techniques to make them cost effective.

Genetics and functional genomics have been playing a vital role in discovering the possible molecular mechanisms of insecticide resistance. Research on insecticide resistance provides support for the potential utility of these approaches both in identification of resistance genes and assessing their relative importance to the resistance phenotype (Gilleard, 2006). For example, identification of
the molecular mechanisms involved in resistance to cyclodiene (*Rdl* a GABA receptor) (Ffrench-Constant et al., 1993) and dichloro-diphenyl-trichloro-ethane (DDT) (*Cyp6g-I* gene) (Daborn et al., 2002) provides illuminating insights into the power of an integrated genetic and genomic approach to study drug resistance. However, it is quite clear that both the availability of an annotated, largely complete genome sequence and a system of forward genetics were essential for the identification of *Rdl* and *Cyp6g-I* as major determinants of resistance to DTT and cyclodiene (Gilleard, 2006). On the other hand, the situation of AR in parasitic nematodes is somewhat different since *C. elegans* is the only nematode with significant genomic resources and for which genetic tools are widely available. This free-living nematode is a useful model for research on AR; however, it does not naturally live under selection pressure to anthelmintics as parasitic nematodes do, and does not face the hostile environment of a host and its immune system. Consequently, to apply approaches like forward genetics and functional genomics for the identification of resistance-conferring mutations under field conditions, genomic and genetic resources for some of the important parasitic nematode species need to be developed (Gilleard, 2006). Therefore, parasitology researchers have been working on exploring the genome sequences for many parasites, and draft genome sequences are now available for a number of animal, plant and human parasitic nematode species. Draft genome sequences have been published for *B. malayi* (Ghedin et al., 2007), *Trichinella spiralis* (Mitreva et al., 2011), *Ascaris suum* (Jex et al., 2011), *D. immitis* (Godel et al., 2012) and *H. contortus* (Laing et al., 2013; Schwarz, et al., 2013). In addition, a large number of genome studies, including projects on important ruminant strongyles for which AR has already been reported, are currently underway. These genome sequences, once fully available along with their transcriptomic data, will provide major insights into the biology of parasitic nematodes and mechanisms involved in resistance, and thereby aid in the identification of specific AR markers (reviewed by Kotze et al., 2014a).

1.9 Modulation of P-glycoproteins and anthelmintic resistance

Studies reporting non-specific mechanisms of AR in nematodes reveal that P-gps are the only member of the ABC transporter family to be well understood with respect to a role in AR. Research to overcome the MDR in nematodes is still less advanced and further work is required to understand this mechanism. Development of drugs that are known not to be the substrates of ABC transport proteins is a good option to overcome the problem of MDR, however this is a challenging requirement and may not be practical. It may be more appropriate to seek increased efficacy of anthelmintics against parasites by modifying the pharmacokinetics in the host or by blocking the resistance-conferring transport mechanism in parasites (Lespine et al., 2012). This is highlighted by the fact that emphasis is currently placed upon development of effective MDR inhibitors (MDRI),
or reversal agents (Friedenberg et al., 2006; Falasca and Linton, 2012; Abdallah et al., 2015). The concept of P-gp modulation in nematodes to partially or completely reverse the drug resistance is based on the principal mechanism of action of these transporters. These transport proteins reduce the drug toxicity by transporting the drug away from its target site. Therefore, reducing the activity of transporters by using such compounds would increase drug toxicity.

Ideally, an effective MDRI would be non-toxic to the host, and a potent and specific inhibitor of the relevant ABC transporters, with no adverse effects on the pharmacokinetics of anthelmintic agents (Falasca and Linton, 2012). Several compounds have been evaluated in vitro and in vivo in mammals while some of them have also been studied in nematodes. These compounds are classified into three different generations of ABCB1 inhibitors. Previous studies that reported the effects of MDR inhibitors on sensitivity of parasites to anthelmintics and on plasma availability of MLs are summarised in Table 1.2.
Table 1.2. Effects of multidrug resistance inhibitors on sensitivity of parasites to anthelmintics and plasma availability of macrocyclic lactones

<table>
<thead>
<tr>
<th>Inhibitors used</th>
<th>Nematode spp.</th>
<th>Study design</th>
<th>Effect on anthelmintic efficacy/availability</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td><em>H. contortus</em></td>
<td>EHA</td>
<td>Increased BZ sensitivity of resistant and susceptible isolates</td>
<td>Beugnet et al. (1997)</td>
</tr>
<tr>
<td>Verapamil</td>
<td><em>H. contortus</em></td>
<td>EHA</td>
<td>Increased sensitivity of resistant isolate to thiabendazole</td>
<td>Kerboeuf et al. (1999)</td>
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<tr>
<td>pluronic acid P85</td>
<td><em>T. circumcincta</em>,</td>
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<tr>
<td>Verapamil and valspodar</td>
<td><em>C. elegans</em></td>
<td>In vitro</td>
<td>Significantly restored IVM sensitivity</td>
<td>James and Davey (2009)</td>
</tr>
<tr>
<td>Verapamil, cyclosporin A, vinblastine, and</td>
<td><em>B. malayi</em></td>
<td>In vitro motility assay</td>
<td>Increased susceptibility of adult and microfilariae to IVM</td>
<td>Tompkins et al. (2011)</td>
</tr>
<tr>
<td>daunorubicin</td>
<td><em>C. elegans</em></td>
<td>Microplate assay</td>
<td>Worm motility: All inhibitors reduced motility of worms in wild-type worms, only verapamil reduced</td>
<td>Ardelli and Prichard (2013)</td>
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<tr>
<td>R(+) - Verapamil monohydrochloride monohydrate,</td>
<td><em>C. oncophora</em></td>
<td>In vitro (LDA, LMIA)</td>
<td>Completely restored sensitivity of IVM resistant isolate</td>
<td>Demeler et al. (2013)</td>
</tr>
<tr>
<td>vincristine sulfate, doxorubicin, etoposide,</td>
<td>Cattle nematode</td>
<td>EHA, LDA, LMIA</td>
<td>Increased IVM sensitivity</td>
<td>AlGusbi et al. (2014)</td>
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<td>actinomycin D, colichicine, vinblastine,</td>
<td><em>C. elegans</em></td>
<td>In vitro</td>
<td>Inhibit motility and pharyngeal pumping in different P-gp deletion strains and IVM resistant strain in</td>
<td>Bygarski et al. (2014)</td>
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<td>rhodamine-123, quinidine, quinine, forskolin</td>
<td><em>H. placei</em></td>
<td>In vitro (LMIT)</td>
<td>All inhibitors increased IVM sensitivity of resistant isolate, except diminazine aceturate</td>
<td>Heckler et al. (2014)</td>
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<tr>
<td>Cyclosporin A, ceftriaxone, dexamethasone,</td>
<td><em>S. mansoni</em></td>
<td>In vitro (worm motility)</td>
<td>Significantly increased sensitivity of adult worms to praziquantel</td>
<td>Kasinathan et al. (2014)</td>
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<tr>
<td>diminazine aceturate, quercetin, trifluoperazine,</td>
<td><em>C. elegans</em>, <em>H. contortus</em></td>
<td>In vitro</td>
<td>Increased the susceptibility of wild-type and ML-selected isolates to IVM and MOX</td>
<td>Menez et al. (2016)</td>
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<td>verapamil, vinblastin</td>
<td></td>
<td>LDA</td>
<td></td>
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<tr>
<td>Inhibitors used</td>
<td>Nematode spp.</td>
<td>Study design</td>
<td>Effect on anthelmintic efficacy/availability</td>
<td>Reference(s)</td>
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<tr>
<td>----------------</td>
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</tr>
<tr>
<td>Loperamide</td>
<td>Cattle nematodes</td>
<td><em>In vivo</em> in cattle</td>
<td>Increased IVM and MOX efficacy in terms of reduced FECR</td>
<td>Lifschitz et al. (2010b)</td>
</tr>
<tr>
<td>Loperamide</td>
<td>Sheep nematode</td>
<td><em>In vivo</em> in sheep</td>
<td>Increased IVM efficacy in terms of reduced FECR, increased plasma availability and longer half life</td>
<td>Lifschitz et al. (2010a)</td>
</tr>
<tr>
<td>Verapamil</td>
<td>-</td>
<td>Sheep</td>
<td>Significantly higher IVM availability in maternal and foetal plasma</td>
<td>Pérez et al. (2010)</td>
</tr>
<tr>
<td>Cyclosporin A, dexteverapamil, curcumin derivative (C-4), tariquidar, MK-571</td>
<td><em>S. mansoni</em></td>
<td><em>In vitro</em> and <em>in vivo</em> in mice</td>
<td><em>In vitro and in vivo</em> disruption of egg production in resistant isolate</td>
<td>Kasinathan et al. (2011)</td>
</tr>
</tbody>
</table>

EHA = egg hatch assay; LFIA = larval feeding inhibition assay; IVM = ivermectin; MOX = moxidectin; ML = Macrocyclic lactones; BZ = Benzimidazole; LMIT = larval migration inhibition test; FECR = faecal egg count reduction; LMIA = larval migration inhibition assay; LDA = larval development assay; B. = Brugia; Ca. = Caenorhabditis; Co. = Cooperia; H. = Haemonchus; S. = Schistosoma; T = Teladorsagia
1.9.1 First generation ABCB1 inhibitors

The first-generation inhibitors include a number of agents that were developed for some other use such as verapamil (anti-hypertensive), cyclosporine A (immunosuppressant) and quinine (antimalarial). Verapamil is a well-studied multidrug resistance inhibitor and has been shown to inhibit mainly the functions of P-gps both in mammalian tumour cells and nematodes (Kerboeuf et al., 2003a; Falasca and Linton, 2012). However, in mammals, these inhibitors were found to be ineffective in clinical studies, despite their in vitro effects. These agents were also reported to be toxic and some were observed to induce undesirable pharmacokinetic complications (cardiac toxicity in case of verapamil) in mammals (Darby et al., 2011).

In nematodes, it has been shown that verapamil can reverse AR either partially or completely when co-administered with anthelmintics (Beugnet et al., 1997; Kerboeuf et al., 1999; Demeler et al., 2013). Verapamil has been studied in various in vitro assays such as EHA, LDA and LMIA using different developmental stages (eggs and L3) of C. elegans and trichostongylid nematodes (Beugnet et al., 1997; Ardelli and Prichard, 2013; Demeler et al., 2013). The results revealed that co-administration of verapamil increases the sensitivity of both the susceptible and resistant isolates of H. contortus and C. oncophora to anthelmintic agents by decreasing the IC$_{50}$ values as compared to anthelmintics alone. Verapamil also increased the thiabendazole toxicity in EHA and showed partial reversal of resistance (Beugnet et al., 1997). Furthermore, some in vivo studies showed that verapamil, when co-administered with anthelmintic agents, increased the bioavailability of the anthelmintic in jirds and sheep (Molento and Prichard, 1999; Molento et al., 2004). Investigations around host toxicity of verapamil are still in early stages in livestock and Pérez et al. (2010) reported that co-administration of verapamil/IVM in pregnant sheep increases the bioavailability of IVM not only in maternal blood but also in foetal blood, which may lead to IVM toxicity in the foetus.

1.9.2 Second generation ABCB1 inhibitors

This group of ABCB1 inhibitors were designed to counter the major drawbacks of first generation inhibitors (reduced specificity and increased toxicity). Valspodar, a derivative of cyclosporine A, is characterised by higher specificity and potency than its precursor along with no immunosuppressive effects in vitro, but it failed to improve the outcome of phase II clinical trials when administered with anticancer agents. Valspodar inhibits cytochrome P450 (CYP450), resulting in higher systemic concentrations of both the inhibitor and the therapeutic drug (Boesch et al., 1991; Friedenberg et al., 2006). Biricodar, derived from piperidine, was also a more potent ABCB1 inhibitor than the first-generation compounds and showed the ability to inhibit the ABCC1
transporter family as well. However, the use of biricodar showed no efficacy in phase II clinical trials when co-administered with doxorubicin or vincristine. In addition, this trial showed the additional undesirable clinical complication of neutropenia (Gandhi et al., 2007).

Information on the use of second-generation inhibitors against nematodes is limited. Bartley et al. (2009) reported that use of valsposdar in combination with IVM significantly increases the in vitro sensitivity of drug-susceptible and -resistant isolates of *H. contortus* and *Te. circumcincta* in a larval feeding inhibition assay. The authors also suggested that the combination of P-gp inhibitors with drugs could be useful to counter the emergence of AR, either by increasing the drug’s efficacy or by reducing the required dose of the drug. Valspodar has also been reported to reverse resistance in an IVM-selected *C. elegans* isolate. This isolate also showed increased expression of P-gps and MRPs following IVM selection, and reversal of resistance on addition of valsposdar clearly suggests that it interferes with the functions of ABC transporters thus ultimately reversing the resistance (James and Davey, 2009).

### 1.9.3 Third generation ABCB1 inhibitors

The third generation ABCB1 inhibitors were specifically designed to counter the limitations of first two generation inhibitors. Therefore, inhibitor development was focused on the compounds that avoided the inhibition of CYP450 and do not alter the pharmacokinetics of co-administered drugs. The members of third generation inhibitors include tariquidar (an anthranilamide, XR9576), elacridar (an acridone caroxamide), zosuquidar (LY 335979) and CBT-1 (both quinolone derivatives) and laniquidar (a piperidine), which are currently in various stages of clinical trials in mammals (Falasca and Linton, 2012). They have higher potency, selectivity and lower toxicity than the agents of the previous two generations. Although these ABCB1 inhibitors are in phase II / III clinical trials, tariquidar has been disappointing and therefore zosuquidar is perhaps the most specific of the third-generation inhibitors and with the least effects on CYP450 (Dantzig et al., 1999; Shepard et al., 2003). However, a potentially dose-limiting neurotoxicity has been observed (Rubin et al., 2002).

The only evidence on the use of third generation inhibitors in pathogenic parasites is reported in *Schistosoma mansoni* (*S. mansoni*). Kasinathan et al. (2011) showed that tariquidar is capable of reducing the egg production in *S. mansoni* both in vitro and in vivo. It eliminated the egg production in vitro at a concentration of 12.5 µM. The disruption of parasite egg deposition in worms due to *SMDR2* and *SmMRP1* genetic knockdown suggests that MDR transporters play an important physiological role in egg production, hence it could be ventured that the absence of egg production was due to the effects of tariquidar on MDR transporters. Later on, Kasinathan et al. (2014)
reported that tariquidar, zosuquidar and elacridar enhanced the susceptibility of adult *S. mansoni* to praziquantel *in vitro*. However, there are no reports on the effects of these third-generation inhibitors on any life stage of GINs.

### 1.9.4 Circumvention of multi-drug resistance by alternative strategies

Some alternative strategies to counter the multi-drug resistance have been used in mammalian cancer research. Some have been employed at the level of gene expression of ABC transporters using anti-sense oligonucleotides or double-stranded small interference RNAs (siRNA) to regulate mRNA levels or targeting the signalling pathways that induce ABC transporters expression (Yague et al., 2004; Huang et al., 2007). There are also reports describing the use of genetic manipulations in nematodes, and some recent studies have demonstrated that silencing of some P-gp genes in *C. elegans* increases the sensitivity of genetically modified worms to MLs as compared to wild-type worms (Ardelli and Prichard, 2013; Bygarski et al., 2014). The use of RNAi to knock-down some specific genes has seen some success with *H. contortus*, as described by some previous studies (Samarasinghe et al., 2011; Zawadzki et al., 2012), however, it has also been reported to be ineffective with many genes in this species (Geldhof et al., 2006). In addition, there is no information available as to whether the technique can silence transporter genes in this species.

Interestingly, tyrosine kinase inhibitors such as crizotinib, sunitinib and laptinib, that are used to target malignant cells growth and tumour spread, have also been shown to interfere with ABC transporters activity in tumour cells (Zhou et al., 2012). Some natural compounds such as curcumin have also been associated with modulation of ABC transporter functions and improved efficacy of co-administered anticancer drugs (Nabekura, 2010). These strategies might also be fruitful when applied to parasites of veterinary importance.

### 1.10 Conclusion

In conclusion, this review of literature has highlighted evidence that the ABC transporters play a role in AR. A number of studies have described the effects of first and second generation MDRIs in increasing the toxicity/ availability of anthelmintics *in vitro* and *in vivo*. The use of first and second generation MDRIs have been discontinued in mammalian studies due to their side effects and poor response in combination therapies *in vivo*. Therefore, members of third generation inhibitors are the most interesting with respect to their potential usefulness in combination therapy in increasing the sensitivity of resistant worms. Secondly, most of the previous studies have reported the effects of MDRIs on MLs sensitivity, therefore, exploring the effects of MDRIs on anthelmintic classes other than MLs should also be considered in future studies. Similarly, previous
studies reporting the effects of anthelmintics on the expression patterns of ABC transporters in nematodes have focused on MLs. Although, MLs are well-known for their role as substrates of ABC transporters in nematodes and mammals, ABC transporters provide protection against different chemical entities, which suggests that anthelmintics other than MLs, for example LEV and MPL, may also be the substrates of ABC transporters, and exposure to these anthelmintics may also result in modified expression patterns of ABC transporters. In addition, it is important to examine the use of the various in vitro assays to detect resistance to MPL recently reported in *H. contortus*. MPL resistance in a laboratory selected isolate has been associated with various truncated forms of the target protein due to a number of mutations in the coding gene. However, the molecular mechanism involved in MPL-resistant field isolate is still unknown. It may be possible that MPL acts as a substrate for ABC transporters and these transporters are involved in resistance to this compound in a field isolate.

1.11 Aims of the study

This research project is intended to address the following objectives:

1. Since ABC transporters play an important role in AR, it is hypothesised that modification of the efflux proteins would increase the drug toxicity and reverse the AR either partially or completely. Previous studies have reported the use of MDRIs in *H. contortus* but most of them used the first-generation inhibitors *i.e.* Verapamil. Therefore, this study aims to **examine the effects of some recent MDRIs (second and third generations) in increasing the in vitro sensitivity of selected isolates of *H. contortus* to IVM, LEV, MPL and thiabendazole.**

2. The association of ABC transporters with AR suggests that there should be variation in ABC transporters expression patterns between drug-susceptible and -resistant nematodes. The expression patterns of P-gps in nematodes is quite variable, with some reports linking them to AR, and other studies finding no association. **This thesis aims to measure the variation in expression levels of different ABC transporter genes (if any) in selected isolates of *H. contortus*.**

3. There is accumulating evidence that anthelmintics are substrates of P-gps and exposure to anthelmintics increased gene transcriptions for some P-gps. However, the major focus has been the free living nematode model and IVM, whereas, in parasitic nematodes, these changes are quite inconsistent and only IVM has been studied for its effects on ABC transporter gene transcription. Therefore, **the present study aims to measure the effects of three anthelmintics (IVM, LEV and MPL) on the expression patterns of ABC transporters in a drug-sensitive and -resistant isolates of *H. contortus*.**
4. Resistance to monepantel has recently been reported in trichostrongyloid nematodes and there are no reports available on the possible mechanism of resistance to MPL from the field isolates. In order to understand the possible mechanism involved in resistance to this drug and ability of worm bioassays to detect MPL resistance, the ability of larval development and migration assays to measure the dose-response relationships against MPL-resistant and two susceptible isolates of *H. contortus* will be examined. In addition, the expression patterns of ABC transporters will also be compared with two other MPL-susceptible isolates to find a possible role of ABC transporters in resistance to MPL.
CHAPTER 2

2. Effects of multidrug resistance inhibitors on sensitivity of *Haemonchus contortus* to anthelmintics

2.1 General introduction

ABC transporters eliminate a wide range of drugs and other chemicals from the cells, therefore acting as protective pathways. These proteins have been studied intensively for their role in resistance to chemotherapeutic agents and anthelmintics in mammals and nematodes, respectively. There is a great deal of literature on the potential use of multidrug resistance inhibitors (MDRIs) to provide more effective control of gastrointestinal nematodes (GINs) of livestock (reviewed by Lespine et al., 2012). The use of inhibitors prevents the activity of efflux pumps and maintains the drug at higher concentrations in cells. This would overcome resistance if it is due to the increased efflux activity. Most of the previous studies showed that the use of MDRIs in nematodes was limited to the members of first and second generation of inhibitors. There have been no reports on the effects of third generation inhibitors on any life stage of GINs. Therefore, the present study was designed to extend the previous studies on the interaction of MDRIs with parasitic nematodes by comparing the ability of first, second and third generation MDRIs to increase drug sensitivity through inhibition of drug efflux pathways.

Additional figures AF-2.1 and AF-2.2 representing the dose-response curves of different multidrug resistance inhibitor-alone on the development and migration of *H. contortus* Kirby and WAL larvae are given at the end of section 2.3. In addition, the origin and drug susceptibility profiles of the parasite isolates used for this study are described at the end of this Chapter (Additional Table: Table 2.6).

This chapter contains three sections as follows:

2.2 Effects of third generation P-glycoprotein inhibitors on sensitivity of drug-resistant and -susceptible isolates of *Haemonchus contortus* to anthelmintics *in vitro*. Presented as published.

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2.3. Effects of P-glycoprotein inhibitors on the sensitivity of a drug-resistant and -susceptible isolate of *Haemonchus contortus* to monepantel. This section is presented as unpublished data.

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

**Citation:** Raza A, Kopp SR, Kotze AC., 2016. Synergism between ivermectin and the tyrosine kinase/ P-glycoprotein inhibitor crizotinib against *Haemonchus contortus* larvae *in vitro*. Veterinary Parasitology, 227: 64-68.
2.2 Effects of third generation P-glycoprotein inhibitors on sensitivity of drug-resistant and -susceptible isolates of *Haemonchus contortus* to anthelmintics *in vitro*

2.2.1 Abstract

P-glycoproteins (P gps) play an important role in the sensitivity of nematodes to anthelmintic drugs. They have been implicated in a number of anthelmintic resistances, particularly for macrocyclic lactone drugs. Hence, inhibition of nematode P-gps has been suggested as a means of reversing some types of anthelmintic resistance. The present study aimed to investigate the ability of the most-recently developed group of P-gp inhibitors (the so-called ‘third generation’ of inhibitors) including tariquidar, zosuquidar and elacridar, to increase the sensitivity of *Haemonchus contortus* larvae to various anthelmintics (ivermectin, levamisole and thiabendazole) *in vitro*. We compared these compounds to some older P-gp inhibitors (e.g. verapamil and valsapar). Larval migration and development assays were used to measure the sensitivity of larvae to anthelmintics alone, or in combination with P-gp inhibitors. Significant increases in sensitivity to ivermectin were observed with zosuquidar and tariquidar in larval migration assays (synergism ratios up to 6-fold). Several of the inhibitors increased the sensitivity of both the drug-resistant and -susceptible isolates (e.g. tariquidar with ivermectin in migration assays, zosuquidar with ivermectin in larval development assays), while others had significant effects on the resistant isolate only (e.g. zosuquidar with ivermectin in migration assays, verapamil with ivermectin in development assays). This suggests that some of the inhibitors interact with P-gps representing intrinsic pathways present across nematode populations with quite different drug sensitivities, while other inhibitors interact with P-gps of significance only to resistant nematodes, and hence most likely representing an acquired resistance mechanism. The study highlights the potential of the third generation of P-gp inhibitors for increasing the sensitivity of nematodes to anthelmintics.
2.2.2 Introduction

Gastrointestinal nematodes (GINs) are a major health concern for livestock production systems worldwide. Losses due to decreased production and increased treatment costs have a significant impact on farm profitability (Roeber et al., 2013). Control of parasitic nematodes in livestock relies heavily on the use of broad spectrum anthelmintics in the absence of vaccines, however, the intensive use of anthelmintic drugs has led to the development of resistance in common livestock GINs to all the major classes of anthelmintics including benzimidazoles, imidazothiazoles-tetrahydropyrimidines and macrocyclic lactones (Kaplan, 2004; Sutherland and Leathwick, 2011). Anthelmintic resistance has also been reported in companion animals, including horses (Reinemeyer, 2012) and dogs (Kopp et al., 2007; Bourguinat et al., 2011). There are also reports of the emergence of resistance in human filarial parasites (Osei-Atweneboana et al., 2007; Osei-Atweneboana et al., 2011), while the possibility that resistance may emerge in human soil-transmitted helminths is of concern (Vercruysse et al., 2011).

Anthelmintic resistance mechanisms can broadly be divided into two types: (i) specific mechanisms involving a change and/or modification of drug receptors, and (ii) non-specific mechanisms that generally apply to more than one chemical group, and are mediated by drug efflux pathways or detoxification enzymes. Among the non-specific mechanisms, multi-drug resistance proteins (ATP binding cassette transport proteins, including P-glycoproteins (P-gps), multi-drug resistance associated proteins, HAF transporters and others) are cellular efflux transport channels with wide substrate ranges. Some of these transport proteins, particularly the P-gps, have been implicated in a number of instances of anthelmintic resistance in nematodes (reviewed by Lespine et al., 2008; 2012). P-gp genes are significantly over-expressed in some resistant nematodes (Dicker et al., 2011; Williamson et al., 2011; Sarai et al., 2014), while De Graef et al. (2013) recently showed that P-gps are up-regulated in Cooperia oncophora in response to exposure to macrocyclic lactone anthelmintics in vitro and in vivo. Lloberas et al. (2013) reported an increased expression of P-gp 2 in resistant Haemonchus contortus after treatment of host animals with ivermectin (IVM).

In human medicine, drug efflux pathways are very important as they act to reduce the amount of drug reaching its target site within cells. The use of inhibitors (multi-drug-resistance inhibitors, MDRIs) to reduce the activity of the efflux pumps, and hence increase the concentration of drug retained in the cell, has been studied extensively. This is particularly the case for the use of such inhibitors as an adjunct to anti-cancer therapy, since ABC transporters are known to be over-expressed in several types of tumour cell, and can therefore act directly to reduce the effectiveness of anti-cancer drugs (Falasca and Linton, 2012). There is increasing interest in the potential use of MDRIs to provide more effective control of GINs in two ways: (i) reducing the efflux of drug from
nematodes, and hence increasing the amount of drug reaching its target site within the nematode, and (ii) reducing the excretion of drugs by host animals, and hence increasing their bioavailability (reviewed by Lespine et al., 2008; 2012). MDRIs have been identified over the years, and their development has been described as consisting of sequential steps in terms of first, second and third generation inhibitors (Darby et al., 2011; Falasca and Linton, 2012). A number of studies have reported on the effects of first and second generation MDRIs in increasing the toxicity of anthelmintics in vitro, and increasing bioavailability in vivo. For example, Heckler et al. (2014), showed that some MDRIs including verapamil and cyclosporine A potentiated IVM efficacy against an IVM- resistant field isolate of Haemonchus placei resulting in higher efficacy and lower IVM EC₅₀. Bartley et al. (2009) reported that co-administration of valspodar increased the sensitivity of resistant isolates of H. contortus and Teladorsagia circumcincta to IVM in vitro. James and Davey (2009) showed that valspodar was able to completely reverse IVM resistance in Caenorhabditis elegans. In vivo studies have shown that verapamil increases the toxicity of IVM and moxidectin towards H. contortus in jirds (Molento and Prichard, 1999), while also increasing the bioavailability of these two drugs in sheep (Molento et al., 2004) (the effects on drug efficacy were not measured in this study). Lifschitz et al. (2010a, b) showed that P-gp inhibitors could increase the efficacy of macrocyclic lactones against several species of GIN in sheep and cattle. With regard to the third generation MDRIs, Kasinathan et al. (2014) recently reported that tariquidar, zosuquidar and elacridar enhanced the susceptibility of adult Schistosoma mansoni to praziquantel in vitro. However, to our knowledge, there have no reports on the effects of these third generation inhibitors on any life stage of GINs.

The aim of the present study was to extend the previous studies on the interaction of MDRIs with parasitic nematodes by comparing the ability of first, second and third generation MDRIs to increase drug sensitivity through inhibition of drug efflux pathways. We used larval development and migration assays with drug-susceptible and drug-resistant isolates of H. contortus to determine the effects of MDRIs on their sensitivity to anthelmintics.

2.2.3 Materials and methods

2.2.3.1 Parasites

Two isolates of H. contortus were used for the present study:

(i) Kirby: isolated from the field at the University of New England Kirby Research Farm in 1986; susceptible to all commercially available anthelmintics (Albers and Burgess, 1988).
(ii) Wallangra (WAL): isolated from the New England region of Northern New South Wales in 2003; at the time of isolation from the field it was resistant to benzimidazoles, closantel, levamisole (LEV) and IVM (Love et al., 2003). The isolate has been further selected using moxidectin (Cydectin®) over at least five generations. The current isolate is unaffected by a full registered dose of moxidectin. For the current study, sheep were infected with this isolate and were subsequently treated with a full dose of Cydectin 14 days after infection.

Infected animals were housed at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Agriculture Flagship FD McMaster laboratory at Armidale, NSW. All animal procedures were approved by the FD McMaster Animal Ethics Committee, CSIRO Agriculture Flagship (Animal Ethics Approval Number AEC 13/23).

Faeces was collected from infected sheep and processed in two ways depending on the final use of the eggs: (i) to provide eggs for LDAs, the faeces was placed into 50 ml centrifuge tubes, which were then filled with water and shaken in order to establish anaerobic conditions, as described by Coles et al. (2006); (ii) to provide third stage larvae (L3) for LMAs, the faeces was placed into large ziplock bags. The tube or bag samples were then sent by courier to the CSIRO Agriculture Flagship laboratories at the Queensland Bioscience Precinct, Brisbane, Queensland. Worm eggs were recovered from the tube samples following standard protocols as described by Kotze et al. (2009). Briefly, the faeces was passed through a series of fine filters (250 µm followed by 75 µm), and then centrifuged on a 2-step sucrose gradient (10% and 25%). The eggs were recovered from the interface of the two sucrose layers and washed over a 25 µm sieve with water to remove the sugar. Finally, the eggs were treated with a solution of sodium hypochlorite (8.4 mg/L) for 12 min, followed by thorough washing with water. The eggs were diluted in distilled water at a concentration of 38 eggs/ 10 µL after the addition of amphotericin B (final concentration 25.0 µg/mL) and tylosin (final concentration 800 µg/mL) and used in LDAs. To provide L3 larvae for LMAs, the bagged faecal samples were placed into 2L glass jars and most of the faecal pellets were broken up by hand. A small amount of water was added, and the jars were placed into an incubator at 27°C. After approximately one week, the L3 larvae moving up the sides of the jar were rinsed out with water, placed onto a 20 micron cloth suspended in water, and allowed to migrate into a collection vessel overnight. The larvae were collected and stored at 15°C for use in migration assays within 3 weeks.
2.2.3.2 Anthelmintics and MDRIs

Technical grade IVM, LEV and thiabendazole (TBZ) were purchased from Sigma-Aldrich. For each anthelmintic, a stock solution was prepared at 10 mg/mL in dimethyl sulfoxide (DMSO) followed by two-fold serial dilutions in DMSO to produce multiple separate anthelmintic solutions.

The MDRIs were purchased from different sources: verapamil, valspodar, ascorbic acid from Sigma-Aldrich; elcaridar from Santa Cruz; zosuquidar and tariquidar from SelleckChem. Stock solutions of verapamil and ascorbic acid were prepared in water at 50 mg/mL, while the other MDRIs were prepared in DMSO at a concentration of 5 mg/mL. The stock solutions were further diluted by two-fold serial dilutions in either water or DMSO to produce multiple separate drug solutions.

2.2.3.3 Larval development assay (LDA)

The ability of anthelmintics and MDRIs, alone and in combination, to inhibit the growth of *H. contortus* larvae (eggs to the L3 stage) was determined using LDAs following the protocol described by Kotze et al. (2009). Anthelmintics alone, or in combination with different MDRIs, were added to the wells of 96-well plates, and overlayed with 200 µL of 2% (w/v) molten agar (Devis Gelatin Co), and the agar allowed to set. The volumes of the various solutions added to the wells varied depending on whether the MDRI was dissolved in water or DMSO. For water-soluble MDRIs, 2 µL of anthelmintic and 10 µL of MDRI were added to wells, followed by agar. For DMSO-soluble MDRIs, 1 µL of anthelmintic and 1 µL of each MDRI were dispensed into each well, followed by agar. In this way, all wells received 2 µL of DMSO (= 1% v/v). Control wells received DMSO alone, or 10 µL of water-soluble MDRIs, or 1 µL of DMSO-soluble MDRIs and DMSO (2 or 1 µL), to give a final DMSO concentration of 1% v/v. Final drug concentration ranges were 39-0.076 ng/mL for IVM, 10000-4.88 ng/mL for LEV and 10000-19.50 ng/mL for TBZ for the WAL isolate, and 2.44-0.0048 ng/mL for IVM, 625-1.22 ng/mL for LEV and 78-0.15 ng/mL for TBZ for the Kirby isolate.

Egg suspension (30 µL) was dispensed into each well, and the plates incubated overnight at 27 °C. The next day, 10 µL of growth medium (live culture of *Escherichia coli* in a nutrient solution, as described by Kotze et al. (2009)) was added to the wells. The plates were incubated for a further 6 days. On day 7, the larvae were killed by addition of 10 µL of Lugol’s iodine, and the number of fully grown L3 was counted in each well.

Each experiment consisted of triplicate wells at a range of anthelmintic concentrations, either alone or combined with an MDRI, as well as at least 12 control wells (DMSO & MDRIs only) per
plate. Three separate experiments were performed for each anthelmintic and anthelmintic/MDRI combination with each worm isolate.

2.2.3.4 Larval migration assay (LMA)

The ability of anthelmintics and MDRIs, alone and in combination, to inhibit the migration of L3 stage larvae through an agar/mesh system was measured using LMAs, modified slightly from Kotze et al. (2006). The assay was not used with TBZ as this anthelmintic has very little effect on the migration of L3 stage *H. contortus* larvae (Kotze, unpublished data). L3 stage larvae of resistant and susceptible isolates of *H. contortus* were recovered from faecal cultures as described above, and then exposed to anthelmintics and MDRIs in 96-wells microtitre plates. The volumes of the various solutions added to the wells varied depending on whether the MDRI was soluble in DMSO or water. For water-soluble MDRIs, aliquots of each anthelmintic dilution (1 µL) were added to assay plate wells, followed by water (60 µL) and 10 µL of MDRI. For DMSO-soluble MDRIs, 0.5 µL of each anthelmintic dilution, water (70 µL) and 0.5 µL of MDRI were added to assay wells. In this way, both DMSO and water soluble MDRIs were examined in assays containing 1% DMSO (v/v). Control assays also contained 1% DMSO. Larvae were diluted in water to a concentration of 3-3.5/µL, and amphotericin B (250 µg/mL) was added at a rate of 100 µL/mL, and Penicillin/Streptomycin (P 10,000 U and Streptomycin 10,000 µg/mL) added at 10 µL/mL. Aliquots of this larval suspension (30 µL, approximately 90-105 larvae) were added to the assay plate wells. Plates were placed into zip-lock plastic bags and incubated at 27 °C for 48 h. Final concentration ranges for IVM & LEV were 25000-195.30 ng/mL for WAL, while the ranges used for Kirby were 6250-48.8 ng/mL for IVM and 25000-195.30 ng/mL for LEV.

During the final six hours or so of this drug incubation period, receiver plates (Millipore, Australia) were prepared as follows: for water-soluble MDRIs, each well of the receiver plate received 4µL of anthelmintic, 40 µL of MDRI and 260 µL of water, while for DMSO-soluble MDRIs each well received 2 µL of anthelmintic, 2 µL MDRI and 300 µL of water. The control wells received only DMSO (2µL or 4µL) and / water-soluble MDRI (40 µL) or DMSO-soluble MDRI (2 µL) to give a final DMSO content of 1% (v/v). These plates were placed at room temperature until required. The filter/agar plates were prepared as follows: 0.1% agar (w/v) was dissolved in water and allowed to cool down to 45 °C. After cooling, 75 µL of agar was poured to each well of a multiscreen mesh filter plate (20 µm filter) (Millipore, Australia). The plates were covered and allowed to sit at room temperature for a couple of hours. The agar plates were then lowered into the receiver plates and left for an hour on the bench to allow the drugs to equilibrate through the agar.
After 48 h of exposure of the worm larvae to anthelmintic/MDRI combinations in the initial treatment plates, the drug exposed worms were transferred using a multichannel pipette to the filter/agar/receiver plates. These plates were then placed into an illuminated incubator at 27 °C. After 24 h, the filter plates were removed, and the worms that had migrated into the receiver plate wells were killed by the addition of Lugol’s iodine (10 µL), and counted.

Each experiment consisted of triplicate wells at a range of anthelmintic concentrations, either alone or combined with an MDRI, as well as at least 12 control wells (DMSO & MDRI only) per plate. Three separate experiments were performed for each anthelmintic and anthelmintic/MDRI combination with each worm isolate.

2.2.3.5 Data analyses

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 model used to fit the data was based on a normalised response (dose response curve from 100% to 0%) and a variable slope. Data from each set of nine assays (3 experiments, each with triplicate assay wells) were pooled and used to calculate IC$_{50}$ values and 95% confidence intervals (CI) for each drug alone and in combination with MDRIs. Significant differences between IC$_{50}$ values were determined based on overlap of 95% CIs.

The effects of MDRIs on the sensitivity of larvae to anthelmintics were described using synergism and antagonism ratios in cases where the IC$_{50}$ was significantly decreased or increased, respectively, in the presence of the MDRI. The synergism/antagonism ratios were calculated as: IC$_{50}$ drug alone/IC$_{50}$ drug in-combination with MDRI. The ratios were considered to indicate a significant change in the IC$_{50}$ if they were derived from IC$_{50}$ values which showed non-overlapping 95% CIs.

2.2.4 Results

2.2.4.1 Effects of MDRIs alone

A number of preliminary dose-response experiments were undertaken with MDRIs alone in LDAs and LMAs (data not shown). These dose responses were used to select two concentrations of each MDRI which caused less than 20% inhibition of larval development or migration. These concentrations (shown in Table 2.1) were subsequently used in combination with anthelmintics as described in sections 2.2.3.2, 2.2.3.3 and 2.3.3.4. The two isolates showed equivalent sensitivities to many of the MDRIs (as indicated by equivalent concentrations shown in Table 2.1). However, there
were a number of instances of reduced sensitivity to MDRIs in WAL larvae compared to Kirby. In these cases, higher concentrations of MDRI were used in subsequent MDRI/anthelmintic assays for the WAL isolate, for example, the WAL isolate was more tolerant of verapamil, valsodar and zosuquidar than Kirby in LDAs (Table 2.1).

**Table 2.1.** Concentrations of MDRIs used in larval development assays (LDAs) and migration assays (LMAs) in combination with anthelmintics

<table>
<thead>
<tr>
<th>MDRI</th>
<th>LDA (µg/mL)</th>
<th></th>
<th></th>
<th>LMA (µg/mL)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kirby isolate</td>
<td>WAL isolate</td>
<td>Kirby isolate</td>
<td>WAL isolate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verapamil</td>
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<td>31.3</td>
<td>15.6</td>
<td>78</td>
<td>313</td>
<td>156.3</td>
</tr>
<tr>
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<td>40</td>
<td>20</td>
<td>50</td>
<td>50</td>
<td>25</td>
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<td>0.8</td>
<td>0.4</td>
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<tr>
<td>Zosuquidar</td>
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<td>40</td>
<td>20</td>
<td>3</td>
<td>13</td>
<td>6.5</td>
</tr>
<tr>
<td>Tariquidar</td>
<td>40</td>
<td>40</td>
<td>20</td>
<td>3</td>
<td>13</td>
<td>6.5</td>
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<td>125</td>
<td>125</td>
<td>62.5</td>
<td>1250</td>
<td>1250</td>
<td>625</td>
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</table>
2.2.4.2 MDRIs and ivermectin

The results of LDAs with IVM alone and in-combination with different MDRIs are described in Table 2.2, with some dose-response curves shown in Figure 2.1. The WAL isolate showed 18-fold resistance to IVM with an IC$_{50}$ of 3.94 ng/mL compared to 0.19 ng/mL for Kirby. The WAL IC$_{50}$ values towards IVM were significantly reduced by the co-administration of all the MDRI compounds as indicated by non-overlap of 95% CIs for assays performed with IVM alone compared to those in the presence of the MDRIIs. There was one instance where no synergism was observed (valspodar at 10 µg/mL), however, there was significant synergism measured at the alternate MDRI concentration tested for this compound (5 µg/mL). Synergism ratio (SR) values ranged from 1.6 to 5.2, with the highest value being for verapamil. Importantly, the presence of the MDRIs did not reduce the IVM IC$_{50}$ for the WAL isolate to the level measured for Kirby larvae. This is illustrated in Figure 1 where the response of WAL larvae to IVM plus verapamil lay to the right of the response of Kirby larvae to IVM alone. The IC$_{50}$ for WAL larvae co-treated with verapamil remained almost 4-fold higher than for Kirby treated with IVM alone (0.76 ng/mL compared to 0.19 ng/mL), while the WAL IC$_{50}$ in the presence of the other MDRIs remained at least 9-fold higher than for Kirby larvae treated with IVM alone. The sensitivity of the Kirby isolate to IVM was significantly increased in the presence of two inhibitors: valspodar and zosuquidar (Table 2.2 and Fig 2.1). The effects of these two compounds were approximately equivalent to their effects on WAL larvae in terms of the magnitude of the SRs. The greatest effect was with valspodar, resulting in a SR of 2.7.

The results of LMA dose-response experiments with IVM alone and in-combination with different MDRIs are described in Table 2.3, with some dose-response curves shown in Figure 2.2. The WAL isolate showed 3.3-fold resistance to IVM (IC$_{50}$ of 3826 ng/mL compared to 1158 ng/mL for Kirby). The WAL IC$_{50}$ values towards IVM were significantly reduced by the co-administration of all the MDRI compounds (SRs of 1.9 to 6.6) other than verapamil. Some of the MDRIs had greater effects in reducing the WAL IVM IC$_{50}$ in the LMA compared to the effects observed in the LDA (Table 2.3 compared to Table 2.2). The most pronounced effects were observed with tariquidar (SRs of 5.8 and 5.6) and zosuquidar (6.0 and 4.7). Both zosuquidar and tariquidar reduced the WAL IVM IC$_{50}$ to below that measured for Kirby with IVM alone (544 - 688 compared to 1158). Hence, in these cases the MDRIs rendered the WAL larvae more sensitive to IVM than Kirby larvae. Interestingly though, while tariquidar had a significant effect on the IVM IC$_{50}$ for both Kirby and WAL, zosuquidar only reduced the IC$_{50}$ for WAL larvae. Several MDRIs had no effect on the Kirby IVM IC$_{50}$, while others showed significant synergism (ascorbic acid, valspodar and
tariquidar). The effects seen with ascorbic acid were greater with Kirby than for WAL (SRs of 4.6 and 5.7 compared to 2.4 and 2.2, respectively).

**Fig. 2.1.** Effects of IVM alone, or in combination with MDRI s, 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 MDRI s shown as dashed (WAL) or dotted (Kirby) lines, and open symbols. The concentration of each MDRI in µg/ml is shown as subscript after the MDRI name. Each data point represents mean ± SE, n= 9 (pooled data from three experiments, each with assays in triplicate); Ver: verapamil, Val: valspodar, Zq: zosuquidar.
Table 2.2. Larval Development Assay: IC\textsubscript{50} and Synergism ratios (SRs) for ivermectin, levamisole and thiabendazole either alone or in the presence of different concentrations of MDRIs, with Kirby and Wallangra isolates

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<th>Anthelmintic</th>
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<th>Hc Kirby Conc. of MDRI (µg/mL)</th>
<th>IC\textsubscript{50}\textsuperscript{ab} (ng/mL)</th>
<th>SR\textsuperscript{cd}</th>
<th>Hc Wallangra Conc. of MDRI (µg/mL)</th>
<th>IC\textsubscript{50}\textsuperscript{ab} (ng/mL)</th>
<th>SR\textsuperscript{cd}</th>
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<tr>
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</table>

\(a^{*}\) Within an isolate, and within an anthelmintic, * denotes that the IC\textsubscript{50} in the presence of the MDRI was significantly less than the IC\textsubscript{50} for the anthelmintic alone, as determined by non-overlap of 95 % Confidence Intervals.

\(b^{*}\) Within an isolate, and within an anthelmintic, # denotes that the IC\textsubscript{50} in the presence of the MDRI was significantly greater than the IC\textsubscript{50} for the anthelmintic alone, as determined by non-overlap of 95 % Confidence Intervals.

\(c^{*}\) Synergism ratio = IC\textsubscript{50} for anthelmintic in the absence of MDRI / IC\textsubscript{50} for anthelmintic in the presence of MDRI

\(d^{*}\) SR values denoted by * or # are derived from IC\textsubscript{50} values significantly decreased or increased, respectively, by the presence of the MDRI
Table 2.3. Larval Migration Assay: IC$_{50}$ and Synergism Ratios (SRs) for ivermectin, and levamisole either alone or in the presence of different concentrations of MDRIs, with Kirby and Wallangra isolates

<table>
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<tr>
<th>Anthelmintic</th>
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<th>He Kirby</th>
<th>He Wallangra</th>
</tr>
</thead>
<tbody>
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<td>Conc. of MDRIs (µg/mL)</td>
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<tr>
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<td></td>
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</table>

a Within an isolate, and within an anthelmintic, * denotes that the IC$_{50}$ in the presence of the MDRI was significantly less than the IC$_{50}$ for the anthelmintic alone, as determined by non-overlap of 95 % Confidence Intervals.

b Within an isolate, and within an anthelmintic, # denotes that the IC$_{50}$ in the presence of the MDRI was significantly greater than the IC$_{50}$ for the anthelmintic alone, as determined by non-overlap of 95 % Confidence Intervals.

c Synergism ratio = IC$_{50}$ for anthelmintic in the absence of MDRI / IC$_{50}$ for anthelmintic in the presence of MDRI

d SR values denoted by * or # are derived from IC$_{50}$ values significantly decreased or increased, respectively, by the presence of the MDRI.
Fig. 2.2. Effects of IVM alone, or in combination with MDRIs, 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 MDRIs shown as dotted lines and open symbols. The concentration of each MDRI in µg/ml is shown as subscript after the MDRI name. Each data point represents mean ± SEM, n = 9 (pooled data from three experiments, each with assays in triplicate); Asc: ascorbic acid, Zq: zosuquidar, Tq: tariquidar.
2.2.4.3 MDRIs and levamisole

In LDAs, the WAL isolate showed 2.5-fold resistance to LEV at the IC$_{50}$ (326 ng/mL compared to 125.4 ng/mL) (Table 2.2). At least one concentration of most of the MDRIs resulted in significant synergism for both the WAL and Kirby isolates (Table 2.2, Figure 2.3). The exceptions to this were the absence of any observed effect with ascorbic acid and Kirby worms, as well as an absence of any synergism with elacridar and the WAL isolate. The extent of the observed synergism with the two isolates was similar with verapamil, valspodar and tariquidar. On the other hand, there were several instances of greater SRs for WAL compared to Kirby: this was most pronounced for 125 µg/mL ascorbic acid (SR of 5.6 compared to 1.1), and also with both concentrations of zosuquidar (SRs of 4.2 and 5.6 compared to 2.3 and 1.6).

WAL showed no resistance to LEV at the IC$_{50}$ in the LMA (Table 2.3). Two of the MDRIs showed statistically significant effects in increasing the sensitivity of WAL larvae to LEV (zosuquidar and tariquidar; SRs 2.2-3.9), while only tariquidar had any effect in reducing the Kirby LEV IC$_{50}$ (SR 1.8) (Table 2.3, Figure 2.4). A number of the MDRIs increased the LEV IC$_{50}$ values with both isolates compared to treatment with LEV alone that is, resulted in reduced sensitivity of larvae to the effects of LEV in inhibiting larval migration (Table 2.3, Figure 2.4). This increase in IC$_{50}$ occurred with verapamil and ascorbic acid with WAL larvae, and with at least one concentration of all the MDRIs except tariquidar with Kirby. The increase in IC$_{50}$ was most marked with ascorbic acid, which resulted in increases of 5-6-fold in the LEV IC$_{50}$ with Kirby and 4-fold with WAL.

2.2.4.4 MDRIs and thiabendazole

The WAL isolate showed a high level of resistance to TBZ compared to the Kirby isolate in the LDA (IC$_{50}$ 252.2 ng/mL compared to 13.1 ng/mL; resistance ratio = 19) (Table 2.2). The effects of MDRIs on TBZ sensitivity were not as pronounced as observed for IVM and LEV. Several MDRIs had significant effects on TBZ toxicity to WAL larvae, particularly ascorbic acid which showed SRs of 2.5 and 4.6 at the two concentrations tested. Verapamil and tariquidar also significantly reduced the WAL TBZ IC$_{50}$ at one of the two concentrations tested. Only ascorbic acid showed any effect on TBZ sensitivity with the Kirby isolate, however the SR observed with this isolate was much less than for WAL (1.3 compared to 4.6). Importantly, as observed above for IVM, none of the MDRIs reduced the TBZ IC$_{50}$ for WAL larvae to the level shown by the Kirby isolate; the WAL IC$_{50}$ for TBZ in combination with ascorbic acid remained over 4-fold higher than for Kirby larvae exposed to TBZ alone.
Fig. 2.3. Effects of LEV alone, or in combination with MDRIs, on the development of *H. contortus* Kirby (A) and WAL (B) larvae; LEV alone shown with solid lines and closed symbols, LEV plus MDRIs shown as dashed lines and open symbols. The concentration of each MDRI in μg/mL is shown as subscript after the MDRI name. Each data point represents mean ± SE, n = 9 (pooled data from three experiments, each with assays in triplicate); Asc: ascorbic acid, Val: valspodar, Zq: zosuquidar.
Fig. 2.4. Effects of LEV alone, or in combination with MDRIs, on the migration of L3 stage *H. contortus* Kirby (A) and WAL (B) larvae; LEV alone shown with solid lines and closed symbols, LEV plus MDRIs shown as dashed lines and open symbols. The concentration of each MDRI in µg/ml is shown as subscript after the MDRI name. Each data point represents mean ± SEM, n = 9 (pooled data from three experiments, each with assays in triplicate); Asc: ascorbic acid, Zq: zosuquidar, Tq: tariquidar.
2.2.5 Discussion

The present study has shown that various MDRIs are able to increase the sensitivity of *H. contortus* larvae to anthelmintics in two *in vitro* assays. The effects of MDRIs on the sensitivity of *H. contortus* larvae varied considerably with the identity of the MDRI, the drug resistance status of the worm isolate, the anthelmintic examined, and the bioassays used to measure the interaction between the MDRI and the anthelmintic. The study has highlighted two aspects of the relationship between P-gp activity and drug sensitivity in these *H. contortus* larvae. Firstly, the equivalent impact of some MDRI on the sensitivity of both the drug-susceptible and drug-resistant isolates suggests that the efflux pathways inhibited by those specific MDRIs are acting to equivalent levels in the resistant and susceptible larvae, and hence are not responsible for the observed differences in drug sensitivity between the two isolates. Demeler et al. (2013) also demonstrated the effects of verapamil in increasing the efficacy of IVM in resistant and susceptible isolates of *C. oncophora*, while Bartley et al. (2009) reported similar effects on first stage larvae (L1) of both resistant and susceptible isolates of *H. contortus* in the presence of different P-gp inhibitors including valspodar and verapamil. Secondly, the effect of some MDRIs in increasing the sensitivity of only the resistant larvae in the present study suggests that the efflux pathways which interact with those specific MDRIs are more active in the resistant larvae compared to the susceptible larvae, and may therefore be contributing to the observed differences in drug sensitivity. Hence, the study has highlighted the presence of P-gs that may be considered as intrinsic pathways present in both drug-susceptible and-resistant isolates, along with other P-gps which are more active in resistant larvae and may therefore be a factor contributing to the observed resistance.

Some of the MDRIs had significant effects on the toxicity of anthelmintics in both LDA and LMA, for example zosuquidar and tariquidar with IVM. On the other hand, some MDRIs acted as synergists only in one assay type, for example verapamil with IVM in the LDA only. These patterns may be due to differences in life-stage expression levels of specific P-gp genes between early larval life-stages (examined in LDAs) compared to later infective-stage larvae (as examined in LMAs). A further layer of complexity was also apparent in cases where specific MDRIs acted as synergists for both the susceptible and resistant isolates in one assay type, while synergising only with one isolate in the other assay, for example, zosuquidar with IVM. This suggests that different patterns of life-stage expression of specific P-gp genes exist in the susceptible and resistant populations. Sarai et al. (2013) described life-stage differences in expression patterns for various P-gp genes within isolates of *H. contortus*, as well as difference between isolates.

One difficulty in interpreting the synergism data in the present study is that while some of the MDRIs have been characterised with respect to interactions with mammalian drug transporters,
very little is known about their interactions with nematode drug transporters. Nematodes possess many more P-gp genes than mammals (Ardelli, 2013). The properties of nematode efflux pumps may be quite different to those in mammals. An example of the difference between the two organisms is provided by Kerboeuf and Guegnard (2011) who described the effect of macrocyclic lactones in inhibiting P-gp-mediated efflux in mammals while activating the transport activity of nematode P-gps. The quite different patterns of interaction of the MDRI and anthelmintics observed in the present study may be due to specific interactions of some MDRI and anthelmintics as substrates and/or inhibitors of only a subset of the P-gps present in the nematode. The third generation MDRIIs are classified as non-competitive inhibitors and therefore do not act as substrates of mammalian P-gps, however, further work is needed to define the interactions of these inhibitors with specific nematode drug transporters.

Several studies have shown that nematode P-gps show quite different specificities with respect to their interactions with anthelmintics and other toxins. In *C. elegans*, deletion of P-gp 3 caused an increase in sensitivity to colchicine and chloroquine, while deletion of P-gp 1 had no effect (Broeks et al., 1995). *C. elegans* also showed hypersensitivity to heavy metals following targeted inactivation of *mrp-1* and P-gp 1 genes (Broeks et al., 1996). It has been recently shown that deletion of P-gp 6 in *C. elegans* results in higher sensitivity to moxidectin (Bygarski et al., 2014). These studies suggest that different nematode P-gps are responsible for protection against specific toxins and chemicals. This may partly explain the different patterns of interaction between specific MDRIIs and anthelmintics observed in the present study.

A surprising result was the effect of ascorbic acid in significantly decreasing the sensitivity of both WAL and Kirby larvae to LEV in LMAs. The LEV IC\textsubscript{50} was increased approximately 4-fold for WAL and 5-fold for Kirby larvae in the presence of ascorbic acid. A possible explanation for this lies in the known ability of ascorbic acid to increase the levels of the tri-peptide glutathione (GSH) in nematodes (Hartwig et al., 2009) and the activity of certain ABC transporters in the efflux of glutathione conjugates (James et al., 2009). Increased GSH levels following exposure to ascorbic acid may lead to greater levels of conjugation of LEV with GSH (mediated by glutathione transferase enzymes) and hence a greater rate of elimination of the LEV/GSH conjugate by specific GSH-conjugate pumps. This is, however, speculative, and requires confirmation with experiments focusing on the relationship between cellular GSH levels and LEV toxicity. In LDAs, ascorbic acid did not show this antagonistic effect, but rather, the combination of this compound with LEV resulted in increased sensitivity to the anthelmintic. These differing patterns in the interaction of the two compounds in the different life-stage assays may be a result of specific life-stage patterns in expression of individual P-gps, particularly those involved in the transport of GSH conjugates.
There were a number of additional instances in which the sensitivity to LEV was reduced in the presence of MDRIs other than ascorbic acid in LMAs, particularly with the Kirby isolate. These cannot be linked to GSH as described above with respect to ascorbic acid. Further studies of the pharmaco-dynamics and pharmaco-kinetics of anthelmintics/inhibitors in nematodes are required to explain these effects.

The MDRIs had little effect on the sensitivity of the larvae to TBZ in the present study. This suggests that they play only a very limited role in the high-level resistance to the compound shown by WAL larvae in LDAs (resistance rate for TBZ was 19.3-fold; Table 2.2). This is to be expected as it is well known that the major determinants of resistance to this drug are changes in beta-tubulin genes. The WAL isolate is known to show high frequencies of both the F200Y and E198A SNPs associated with resistance to benzimidazole drugs (Kotze et al., 2012). The contribution of P-gps to TBZ resistance in the WAL isolate may be less significant than has been reported for other TBZ-resistant worm isolates in which MDRIs (particularly verapamil) have been reported to increase the sensitivity to this anthelmintic (Beugnet et al., 1997). The exception to this general lack of synergism with TBZ was the significant effects of ascorbic acid in increasing the sensitivity of WAL larvae to TBZ. The mechanism for this is unknown, however it may be due to an action of ascorbic acid other than direct inhibition of P-gps, as described above for the antagonistic interaction of ascorbic acid and LEV in LMAs.

While understanding the role of drug efflux pumps as intrinsic or acquired pathways is important as part of efforts to define resistance mechanisms, it is less important when considering the implications of our data on potential therapeutic approaches to worm control. Both intrinsic and acquired pathways would be valid targets for chemotherapeutic approaches to worm control. The interaction of some MDRIs with the drug-resistant isolate only (for example, zosuquidar and IVM in LMAs) highlights the potential usefulness of a combination therapy (MDRI and anthelmintic) to restore the sensitivity of resistant worms to levels observed with susceptible worms that is, as a “resistance breaking” strategy. On the other hand, the ability of some of the MDRIs to increase the sensitivity of an anthelmintic to the susceptible isolate (for example, tariquidar and IVM in LMAs) raises the possibility of reducing the recommended dose of an anthelmintic while maintaining 100% efficacy against susceptible worms. Such combination therapies could be considered as a means to reduce dose rates of new anthelmintics currently under development.

In conclusion, the potential usefulness of first and second generation P-gp inhibitors to increase drug efficacy in vivo when co-administered with anthelmintics has been recognised for many years (reviewed by Lespine et al., 2008; 2012). The present study suggests that some third
generation MDRIs may also be considered as candidates for use in combination with anthelmintics to overcome drug efflux pathways that act to reduce the amount of anthelmintic within parasitic nematodes. However, the cost of the new MDRIs will be important in determining their suitability for livestock applications. In addition, it will be important to consider the potential effects that the action of MDRIs on host animal ABC transport proteins may have on the interaction of anthelmintics with the host animal; for example, inhibition of host transporter proteins at the blood brain barrier could induce neurotoxic effects by IVM (Menez et al., 2012). These factors may impact on the practical use of MDRI/anthelmintic combination therapies.
2.3 Effects of P-glycoprotein inhibitors on the sensitivity of drug-resistant and -susceptible isolate of *Haemonchus contortus* to monepantel

2.3.1 Introduction

Monepantel (MPL), an amino-acetonitrile derivative was introduced in New Zealand for the control of GINs in 2009. This class of anthelmintics was the first to appear on the livestock medicine market 25 years after the discovery of ivermectin (Kaminsky et al., 2008). However, there have been reports already describing the emergence of resistance to MPL in different parasitic nematodes (Scott et al., 2013; Mederos et al., 2014; Van den Brom et al., 2015). At present, the molecular basis of monepantel resistance in field isolates is unknown, and the interaction of MPL with drug efflux pathways has not been reported. Therefore, the present study was designed to explore the interaction of MPL with P-gps of *H. contortus* by measuring the effects of MDRIs (first, second and third generation inhibitors) on the sensitivity to this anthelmintic in two MPL-susceptible isolates using larval development assays.

2.3.2 Materials and methods

2.3.2.1 Parasites

The drug susceptible (Kirby) and multidrug-resistant isolate (WAL) isolates were used for the present study. Both isolates are susceptible to MPL. The details on drug-susceptibility status and origin of these isolates and the protocol used to recover eggs from the faeces are given in section 2.2.3.1.

2.3.2.2 Anthelmintics and MDRIs

The commercially available drench product Zolvix® (Novartis Animal Health, Australia) was used as a source of MPL in the study. The product was used as a stock solution (25 mg/mL) and multiple separate anthelmintic solutions were produced by two-fold serial dilutions in dimethyl-sulfoxide (DMSO). The details on MDRIs sources and dilutions are described in section 2.2.3.2.

2.3.2.3 Larval development assay (LDA)

The ability of MPL-alone, or in the presence of MDRIs, to inhibit the growth of *H. contortus* eggs to the L3 stage was determined using the LDA. The assay protocol is described in first part of this chapter (section 2.2.3.3). The data were analysed using Graph-Pad Prism software (GraphPad Software Inc., USA, version 5.03) as described in section 2.2.3.5.
2.3.3 Results and discussion

The concentrations of MDRIs that were used in combination with MPL in LDAs are shown in Table 2.1. The results of LDAs with MPL-alone and in the presence of different MDRIs are described in Table 2.4, with some dose-response curves shown in Fig. 2.5. Both the isolates showed equivalent sensitivities to MPL with IC$_{50}$ values of 3.54 µg/mL and 2.98 µg/mL for the Kirby and WAL isolates, respectively.

The co-administration of verapamil at 15.60 µg/mL and ascorbic acid at 125 µg/mL significantly reduced the Kirby IC$_{50}$ values for MPL, as indicated by non-overlap of 95% CIs for assays performed with MPL-alone compared to those in the presence of these MDRIs (Table 2.4). The dose-response curves were shifted slightly to the left (Fig. 2.5A). Synergism ratio (SR) values were 1.8 for verapamil and 1.7 for ascorbic acid (Table 2.4). There were no significant differences in MPL-IC$_{50}$ values with Kirby isolate in the presence of lower concentrations of these two compounds, as well as both concentrations tested for all other inhibitors.

As seen above for Kirby, the sensitivity of WAL isolate was significantly increased in the presence of the higher concentrations of verapamil and ascorbic acid (Table 2.4, Fig. 2.5B). The effects of these two compounds were approximately equivalent to their effects on Kirby larvae in terms of the magnitude of SRs (1.3 for verapamil and 1.9 for ascorbic acid). In contrast, co-administration of all other MDRIs resulted in decreased sensitivity of WAL larvae to MPL, with the dose-response curves shifted to the right (Table 2.4; Fig. 2.5C). This indicated that WAL larvae were able to tolerate higher concentrations of MPL following inhibition of efflux proteins by MDRIs. The greatest increase in IC$_{50}$ value was observed with zosuquidar (14.35 and 7.06 µg/mL compared to 2.98 µg/mL for MPL-alone). The synergism ratios for zosuquidar ranged from 0.2 to 0.4, representing 2.5-5-fold antagonism of MPL toxicity.

There is no evidence in the scientific literature that MPL acts as a substrate for ABC transporters. However, it was suggested that being hydrophilic in nature, MPL might be a substrate for the drug transport proteins in nematodes (James and Davey, 2009). Although, we do not have any direct evidence that MPL acts as a substrate for ABC transporters, the significant increases in tolerance of the WAL isolate to MPL in the presence of MDRIs suggests that drug transporters play a role in the pharmacodynamics and pharmacokinetics of MPL in nematodes.

The presence of inhibitors of drug efflux pathways would be expected to result in an increased presence of drug in the cells, and hence, increased toxicity (as seen with IVM and LEV in section
However, our data for WAL and MPL show that the presence of the drug efflux inhibitors results in a decreased sensitivity to MPL.

A possible explanation for the observed antagonism may lie in an interaction between drug efflux pathways and drug metabolism pathways. Stuchlíková et al. (2014) reported on the metabolic pathways of MPL in *H. contortus*, as measured *ex vivo* (Fig. 2.6). They detected metabolites produced by oxidation and hydrolysis reactions. The antagonism of MPL by MDRIs may result from an interaction between these detoxification pathways and P-gps. It is possible that inhibition of P-gps by MDRIs reduces the rate of efflux of the drug from cells, and hence increases the length of time that the drug is retained in the cells. This in turn may provide an increased opportunity for detoxification enzymes to metabolise the MPL into non-toxic metabolites. Hence, the levels of the toxic MPL parent molecule would be reduced and the sensitivity to MPL in LDAs would be decreased, as seen in Fig. 2.5 C and Table 2.4.
Fig. 2.5. Effects of MPL-alone, or in combination with MDRI s, on the development of *H. contortus* Kirby (A) and WAL larvae (B and C). MPL-alone shown with solid lines and closed symbols, MPL plus MDRI s shown as dotted lines, and open symbols. The concentration of each MDRI in µg/mL is shown as subscript after the MDRI name. Each data point represents mean ± SEM, n = 9 (pooled data from three experiments, each with assays in triplicate); Ver: verapamil, Asc: ascorbic acid, Zq: zosuquidar, Tq: tariquidar.
Table 2.4. Larval Development Assay: IC₅₀, 95% CI and SR values for MPL-alone (ng/mL) and in the presence of different concentrations of MDRI with Kirby and Wallangra isolates

<table>
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<th>MDRI</th>
<th>Conc. of MDRI (µg/mL)</th>
<th>MPL IC₅₀ᵃᵇ (ng/mL)</th>
<th>95% C.I</th>
<th>S.R  c,d</th>
<th>Conc. of MDRI (µg/mL)</th>
<th>MPL IC₅₀ᵃᵇ (ng/mL)</th>
<th>95% C.I</th>
<th>S.R  c,d</th>
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<td></td>
<td></td>
<td>3.0</td>
<td>2.7-3.3</td>
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<td>2.6-3.0</td>
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<td>40</td>
<td>5.5*</td>
<td>5.0-5.9</td>
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<td>20</td>
<td>4.7*</td>
<td>4.4-5.1</td>
<td>0.6*</td>
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</table>

a Within an isolate, * denotes that the IC₅₀ in the presence of the MDRI was significantly less than the IC₅₀ for the anthelmintic alone, as determined by non-overlap of 95 % Confidence Intervals.

b Within an isolate, # denotes that the IC₅₀ in the presence of the MDRI was significantly greater than the IC₅₀ for the anthelmintic alone, as determined by non-overlap of 95 % Confidence Intervals.

c Synergism ratio = IC₅₀ for anthelmintic in the absence of MDRI / IC₅₀ for anthelmintic in the presence of MDRI

d SR values denoted by * or # are derived from IC₅₀ values significantly decreased or increased, respectively, by the presence of the MDRI.
There are several examples of this type of interaction between mammalian drug efflux and drug detoxification pathways in the scientific literature. Chang et al. (2006) reported that raloxifene metabolism was increased in hepatocytes after inhibition of P-gps by the addition of verapamil. In this case, metabolism by cytochrome P450 resulted in the bioactivation of the raloxifene. Nickel et al. (1996) reported that the inhibition of drug transporters by forskolin was the most likely explanation for the effects of this drug in reducing the toxicity of brefeldin A (BFA) towards mammalian cells. In the presence of forskolin, the detoxification of BFA by conjugation with cysteine was increased. Stuchlíková et al. (2014) found that a resistant strain of *H. contortus* produced more MPL metabolites than worms of sensitive strain which suggests that resistant worms may have more active biotransformation enzymes. The results of section 2.1 showed that the effects of MDRIs in increasing the efficacy of anthelmintics were much greater in WAL larvae as compared to Kirby. It may be possible that WAL worms have more active MPL metabolic biotransformation enzymes than Kirby, and possess a higher ability to deactivate this drug. This greater detoxification capacity does not make MPL less toxic to the WAL larvae when the larvae are exposed to this compound only (as shown by the equivalent IC$_{50}$ values, 3.54 µg/mL and 2.98 µg/mL for Kirby and WAL, respectively). However, in the presence of P-gp inhibitors, with MPL retained in the cells for longer, the detoxification systems in the multi-gp inhibitors, with MPL may be able to respond more effectively than in the susceptible Kirby larvae.

Another possible explanation for the antagonism between MDRIs and MPL may be that efflux from cells in some locations within the nematode’s body results in movement of the drug towards other locations within the body in which the MPL-receptors are located. However, this is speculative and there are no reports of such a role of P-gps in drug movement towards receptors in the scientific literature.

Further studies are required to explore the pharmacodynamics and pharmacokinetics of MPL in relation to drug-transport proteins, and to explain why the antagonism of MDRIs to MPL occurred only in WAL larvae.
Fig. 2.6. Scheme of metabolic pathways of MPL in *H. contortus ex vivo* (Stuchlíková et al., 2014), MOP = Monepantel.
Additional figures

**Fig 2.7.** Effects of different multidrug resistance inhibitors-alone on the development of *H. contortus* Kirby and WAL larvae. Kirby dose-response curves shown with solid lines and closed symbols, WAL dose-response curves shown as dashed lines and open symbols.

**Fig 2.8.** Effects of different multidrug resistance inhibitors-alone on the migration of *H. contortus* Kirby and WAL larvae. Kirby dose-response curves shown with solid lines and closed symbols, WAL dose-response curves shown as dashed lines and open symbols.
2.4 Synergism between ivermectin and the tyrosine kinase/ P-glycoprotein inhibitor crizotinib against *Haemonchus contortus* larvae *in vitro*

2.4.1 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.
2.4.2 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., 2014a), 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., 2016a), suggesting a potential role for ABC transporters in anthelmintic resistance. Numerous in vitro and in vivo studies have shown that an anthelmintic/multi-drug-resistance inhibitor (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) P-gps have been implicated in some anthelmintic resistances, and iii) other MDRIIs 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.

### 2.4.3 Materials and methods

#### 2.4.3.1 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 °C.

#### 2.4.3.2 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.

### 2.4.3.3 Data analyses

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).

### 2.4.4 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. 2.9 A, 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 2.5). 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., 2016a). In addition, both the isolates also showed greater sensitivity 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. 2.8, with IC$_{50}$ values in Table 2.5. Crizotinib had no significant effect on the IVM dose response for Kirby larvae (Fig. 2.10A). 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 6.5 µg/mL crizotinib, and a 5.7-fold decrease in IVM IC$_{50}$ at 13 µg/mL crizotinib (Fig. 2.10B). The IC$_{50}$ for IVM against WAL larvae in combination with 13 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 2.11, Table 2.5). 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).
Fig. 2.9. 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 ± SEM, n = 9 (pooled data from three experiments, each with assays in triplicate).
Fig. 2.10. 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 ± SEM, n = 9 (pooled data from three experiments, each with assays in triplicate).
Fig. 2.11. 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 ± SEM, n = 9 (pooled data from three experiments, each with assays in triplicate).
Table 2.5. 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 | He Kirby | | | | He Wallangra | | | |
|---------------|------|----------|---|---|---|---|---|---|---|---|
|               |      | Crizotinib | ICl<sup>a</sup> | 95% CI | SR<sup>b</sup> | Crizotinib | ICl<sup>a</sup> | 95% CI | SR<sup>b</sup> |
| LMA           | None | -         | 1171 | 965-1422 | - | - | 3268 | 2520-4238 | - |
|               | Crizotinib | 3.0 | 1280 | 1081-1760 | 0.9 | 13 | 570* | 459-708 | 5.7* |
|               |      | 1.5 | 1535 | 1340-1760 | 0.8 | 6.5 | 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* |

<sup>a</sup> Within an isolate, and within an assay type, * denotes that the IC<sub>50</sub> in the presence of crizotinib was significantly less than the IC<sub>50</sub> for IVM alone, as determined by non-overlap of 95% Confidence Intervals.

<sup>b</sup> SR = Synergism ratio = IC<sub>50</sub> for IVM in the absence of crizotinib/ IC<sub>50</sub> for anthelmintic in the presence of crizotinib
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 reversed 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 suggests 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.
**Additional Table: Table 2.6.** Drug sensitivity profiles and origin of the *Haemonchus contortus* isolates used in the current study

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>Origin</th>
<th>Drug sensitivity profiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kirby</td>
<td>University of New England Kirby Research Farm in 1986</td>
<td>Susceptible to all commercially available anthelmintics</td>
</tr>
<tr>
<td>Wallangra</td>
<td>Northern New South Wales in 2003</td>
<td><strong>Efficacies:</strong> ivermectin 0%, abamectin 19%, moxidectin 67-84%, levamisole 79%</td>
</tr>
<tr>
<td>Monepantel-resistant</td>
<td>South East Queensland in 2010</td>
<td><strong>Efficacies:</strong> moxidectin 66%, levamisole 65 %, closantel 92%, and albendazole 40%</td>
</tr>
</tbody>
</table>
CHAPTER 3

This chapter is presented as a published article.


3. Effects of \textit{in vitro} exposure to ivermectin and levamisole on the expression patterns of ABC transporters in \textit{Haemonchus contortus} larvae

3.1 Abstract

This study investigated the interaction of ATP binding cassette (ABC) transport proteins with ivermectin (IVM) and levamisole (LEV) in larvae of susceptible and resistant isolates of \textit{Haemonchus contortus} \textit{in vitro} by measuring transcription patterns following exposure to these anthelmintics. Furthermore, we studied the consequences of drug exposure by measuring the sensitivity of L$_3$ to subsequent exposure to higher drug concentrations using larval migration assays. The most highly transcribed transporter genes in both susceptible and resistant L$_3$ were \textit{pgp-9.3}, \textit{abcf-1}, \textit{mrp-5}, \textit{abcf-2}, \textit{pgp-3}, and \textit{pgp-10}. The resistant isolate showed significantly higher transcription of \textit{pgp-1}, \textit{pgp-9.1} and \textit{pgp-9.2} compared to the susceptible isolate. Five P-gp genes and the \textit{haf-6} gene showed significantly higher transcription (up to 12.6-fold) after 3 h exposure to IVM in the resistant isolate. Similarly, five P-gp genes, \textit{haf-6} and \textit{abcf-1} were transcribed at significantly higher levels (up to 10.3-fold) following 3 h exposure to LEV in this isolate. On the other hand, there were no significant changes in transcriptional patterns of all transporter genes in the susceptible isolate following 3 and 6 h exposure to IVM or LEV. In contrast to these isolate-specific transcription changes, both isolates showed an increase in R-123 efflux following exposure to the drugs, suggesting that the drugs stimulated activity of existing transporter proteins in both isolates. Exposure of resistant larvae to IVM or LEV resulted, in some instances, in an increase in the proportion of the population able to migrate at the highest IVM concentrations in subsequent migration assays. The significant increase in transcription of some ABC transporter genes following 3 h exposure to both IVM and LEV in the resistant isolate only, suggests that an ability to rapidly upregulate protective pathways in response to drugs may be a component of the resistance displayed by this isolate.
3.2 Introduction

*Haemonchus contortus* is one of the most pathogenic gastrointestinal nematodes (GINs) of small ruminants, causing substantial economic losses to livestock industries worldwide. Owing to the unavailability of effective vaccines and/or other alternate control methods for GINs, anthelmintics are the cornerstone of control programs (Kaplan and Vidyashankar, 2012). However, resistance has developed to all the major classes of anthelmintics and this situation threatens the sustainability of many livestock enterprises (Kaplan, 2004; Sutherland and Leathwick, 2011).

A number of studies have shown that nematode ATP binding cassette (ABC) transport proteins (including P-glycoproteins (P-gps), multi-drug resistance proteins (MRPs) and Half (HAF) transporters) have a protective function through their role in the efflux of anthelmintics (Kerboeuf and Guegnard, 2011; Lespine et al., 2012; Janssen et al., 2013; Janssen et al., 2015; Kaschny et al., 2015). Increased expression of P-gps in nematodes after *in vitro* as well as *in vivo* exposure to anthelmintics suggests a role for P-gps in the efflux of anthelmintics (Dicker et al., 2011; Williamson et al., 2011; De Graef et al., 2013). The reported increase in sensitivity to some anthelmintics, particularly IVM, in different P-gp knock-out strains of *Caenorhabditis elegans*, also provides evidence for their role in protection from anthelmintics (Ardelli and Prichard, 2013; Bygarski et al., 2014). Furthermore, P-gps have been implicated in resistance to anthelmintics, with a number of studies describing an increased transcription of specific transporter genes in drug-resistant nematodes (Dicker et al., 2011; Williamson et al., 2011; Sarai et al., 2014). Increased expression levels of several P-gps and MRP genes have been reported in *C. elegans* post-exposure to IVM (James and Davey, 2009; Ardelli and Prichard, 2013). Similarly, in parasitic nematodes, overexpression of *pgp-11, pgp-16* and *mrp-1* was observed in Cooperia oncophora recovered from animals treated with IVM (De Graef et al., 2013; Tydén et al., 2014), while Lloberas et al. (2013) reported that treatment of infected lambs with IVM increased the transcription levels of *pgp-2* in resistant worms of *H. contortus* compared to worms collected from untreated control animals. The use of multi-drug resistance inhibitors (MDRIs) to increase the toxicity of anthelmintics to nematodes further indicates a role for drug transporters in resistance (Bartley et al., 2009; Heckler et al., 2014; Raza et al., 2015).

Nematodes possess a greater diversity of MDR transporters compared to mammals. *C. elegans* is known to possess 15 P-gp genes, eight MRP and nine Haf genes (Sheps et al., 2004). In parasitic nematodes, 11 P-gp genes, one HAF gene and two MRP genes have been reported in *H. contortus* (Williamson and Wolstenholme, 2012; Laing et al., 2013). Furthermore, eight P-gp genes, five MRP genes and eight HAF genes were identified in *Brugia malayi* (Ardelli et al., 2010), two P-
gp genes in cyathostominis (Drogemuller et al., 2004) and 11 partial sequences of P-gp genes have been reported in *Teladorsagia circumcincta* (Dicker et al., 2011). The diversity of nematode P-gps suggests that they may play a protective role in the efflux of a wide range of environmental toxins and internal metabolites (Prichard and Roulet, 2007).

The aim of the present study was to examine transcription patterns of ABC transporters in the third-stage larvae (L3) of *H. contortus*. Firstly, we examined the relative transcription levels of the various transporter genes in L3 of susceptible and -resistant isolates of the parasite, and identified differences between transcription levels of the various genes within each isolate, and between the two isolates. We then measured gene transcription patterns in both susceptible and -resistant L3 following exposure to IVM and LEV *in vitro*. Finally, we examined the functional consequences of this drug exposure by measuring efflux of the dye rhodamine-123 (R-123) from drug-exposed and control worms, and also by using migration assays to measure the sensitivity of larvae to IVM and LEV following pre-treatment with lower concentrations of these drugs.

### 3.3 Materials and methods

#### 3.3.1 Parasites

Two isolates of *H. contortus* were used for the present study:

(i) **Kirby**: isolated from the field at Kirby Research Farm, University of New England in 1986; susceptible to all commercially available anthelmintics (Albers and Burgess, 1988).

(ii) **Wallangra (WAL)**: isolated in 2003 from the New England region of Northern New South Wales (NSW). At the time of isolation from the field, WAL was resistant to benzimidazoles, closantel, LEV and IVM (Love et al., 2003). The isolate has further been selected against moxidectin (Cydectin®) for at least five generations.

Infected animals were housed at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Agriculture FD McMaster laboratory at Armidale, NSW. All animal procedures were approved by the FD McMaster Animal Ethics Committee, CSIRO Agriculture Flagship (Animal Ethics Approval Number AEC 13/23).

Faeces was collected from infected sheep, placed into large ziplock bags, and sent by courier to the CSIRO Agriculture laboratories at the Queensland Bioscience Precinct, Brisbane, Queensland. For recovery of L3, the faecal samples were placed into 2L glass jars and most of the faecal pellets were broken up by hand. The faecal mixture was slightly moistened with tap water,
and the jars were then placed in an incubator at 27°C. After approximately one week, the L3 moving up the sides of the jar were flushed out with tap water, placed onto a cloth (20 µm) suspended in water, and allowed to migrate into a collection jar overnight. Collected L3 were stored at 15°C for the later use in migration and molecular assays within three to four weeks.

3.3.2 RNA extraction and generation of complementary (c)DNA

Approximately 30,000 L3 were used for each RNA preparation. Total RNA was extracted using RNeasy mini kit (Qiagen®, Germany) following the manufacturer’s protocol. Briefly, the samples were homogenised in buffer RLT using a bead-based homogenizer (Power-lyzer® 24, Mo-Bio Laboratories, USA) followed by washing, binding and elution of total RNA. The extracted RNA was treated with Turbo-DNase (Ambion®, USA) to remove genomic DNA. RNA samples were quantified using a spectrophotometer (Nanodrop 8000, Thermo Scientific®, USA) and stored at -80°C for further use. cDNA was synthesised using DNase-treated RNA with Superscript III™ reverse transcriptase (Invitrogen®, USA) according to the manufacturer’s instructions. The final volume of cDNA was diluted to a concentration of 4 ng/µL for downstream applications. For each of the isolate and/or treatment, cDNA was generated in three distinct replicates using separate worm samples for subsequent gene expression analysis.

3.3.3 Quantitative PCR (qPCR)

Primer sequences for some of the ABC-transporters used herein were previously reported by Sarai et al. (2013) (GAPDH, actin, pgp-1, pgp-2, pgp-3, pgp-10, pgp-12, and pgp-14) and Issouf et al. (2014) (pgp-9.2 and pgp-16). Primers for β-tubulin, pgp-9.1, pgp-9.3, pgp-11, mrp-1, mrp-5, haf-6, and ABCF transporters were designed using Primer-3 from the sequence information available on Nembase-4 (see Supplementary Table 3.3). Three housekeeping genes, GAPDH, actin and β-tubulin, were used as reference genes for the qPCR analyses. Approximately 18 ng of cDNA was added to other standard PCR reagents in a total reaction volume of 25 µL and then split into four replicates, each of 5 µL. A Vii A7 thermocycler® (Applied Biosystems, USA) was used with SYBR® Green dye system (Applied Biosystems, USA) under the following PCR cycling conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, followed by a melt curve stage (95°C for 15 s, 60°C for 1 min and 95°C for 15 s). Each sample was represented by three biological replicates and run in four technical replicates in the real-time PCR. Amplification efficiency for each primer set was determined by performing PCR using either 2-fold or 1.5-fold cDNA dilutions. Standard curves for all primers showed an efficiency range between 80 and 99%. Melt-curves were analysed for each primer sequence to ensure the specificity of the primers. Furthermore, the PCR products were run on an electrophoresis gel and viewed under
UV illumination after SYBR safe staining to ensure that a single band without any non-specific product was visible. In order to confirm the identity of the PCR products generated using primers designed for the present study (pgp-9.1, pgp-9.3, mrp-1, mrp-5, abcf-1 and abcf-2), the products were cloned into Top-10 cells (Invitrogen®, USA) and sequenced (Big-Dye terminator, V3.1; Applied Biosystems, USA) using M13 forward/reverse primers. In each case, the PCR product corresponded to the specific targeted gene.

Expression values for all genes in each sample were normalised to the three housekeeping genes using REST 2009 (version v2.0.13) to: (1) determine the transcription levels of each gene within an isolate using the transcription level of pgp-1 as control; (2) compare the transcription profiles of ABC transporter genes between resistant and susceptible isolates, and (3) measure the transcriptional patterns of ABC transporters post-exposure to IVM and LEV (drug exposure regimen described below in section 2.4) using DMSO-treated samples as controls for each isolate. In order to compare the transcription profiles of control and test samples, the triplicate expression values were log_{10}-transformed and analysed using repeated measures ANOVA with Fischer’s LSD as multiple comparison test in GraphPad Prism® software (GraphPad Prism, USA version 6.01).

3.3.4 Drug exposure assays

Technical grade IVM and LEV were purchased from Sigma-Aldrich. For each anthelmintic, a stock solution was prepared at 10 mg/mL in dimethyl sulfoxide (DMSO) followed by two-fold serial dilutions in DMSO to produce multiple separate anthelmintic solutions.

To measure the effects of drug exposure on the expression profiles of ABC transporters, groups of approximately 30,000 L3 were exposed to IVM (0.2 and 0.8 µg/mL), LEV (0.4 µg/mL) or DMSO (vehicle control) for two different time periods (3 and 6 h). These selected concentrations were based on the dose-response curves described previously (Raza et al., 2015). The lower (0.2 µg/mL) concentration of IVM was non-toxic to resistant isolate, but inhibited about 12% of the larval migration for the susceptible isolate, whereas, the higher concentration of IVM (0.8 µg/mL) showed about 12% and 25% inhibition of larval migration in the resistant and susceptible isolates, respectively. The LEV concentration (0.4 µg/mL) showed about 10% inhibition of larval migration with both the isolates (Raza et al., 2015). The concentration of DMSO across all the treatments was 1% (v/v).

The worms were kept on a roller-mixer (BTR-5, Ratek®, Australia) for the entire duration of the drug-exposure period. Both the susceptible and resistant isolates were examined in three separate experiments. After exposure, the worms were snap frozen in liquid nitrogen and kept at -
80°C for further use. The transcriptional profiles of 11 P-gps, two MRPs, one HAF and two ABCF genes were measured by qPCR as described above (section 3.3.3).

3.3.5 Larval migration assay (LMA)

An LMA was used to determine the sensitivity of Kirby and WAL L3 larvae to a range of concentrations of IVM and LEV following 3 h drug-exposure for Kirby and 3 and 6 h drug-exposure for WAL as described in section 3.3.4. The LMA was modified from Kotze et al. (2006) as follows: (1) the assays used short incubation/migration periods (30 min each) compared to 48 and 24 h periods in the earlier paper, (2) filter-mesh plates were used without any agar, and (3) receiver plate well received only 300 µL water and no drug. Final concentration ranges used for IVM were 100-0.195 µg/mL and 12.5-0.024 µg/mL for LEV. Three separate experiments (each with assays in triplicate) were run for each combination of pre-exposure treatment (IVM at 0.2 µg/mL and 0.8 µg/mL or LEV at 0.4 µg/mL) and subsequent dose response (range of IVM or LEV concentrations) with the resistant Wallangra isolate. Assays with the Kirby isolate were only performed using L3 pre-exposed to the lower IVM concentration (0.2 µg/mL), as well as LEV (0.4 µg/mL), as a previous study had shown that the migration of Kirby larvae was reduced by 25% at the higher IVM concentration compared to controls (Raza et al. 2015), hence making it difficult to accurately measure the specific effects of subsequent drug exposure.

The data were analysed using non-linear regression in GraphPad Prism® software (GraphPad Prism, USA version 6.01). IC₅₀ values and 95% confidence intervals were calculated based on the pooled data from each set of nine assays, and significant differences were determined by the overlap of 95% confidence intervals. For assays with LEV, the larvae showed % migration ranging from approximately 100 % (equivalent to controls) down to 0% migration, and hence we used a normalised dose-response model in GraphPad (dose-response from 100% to 0%), and a variable slope. On the other hand, the dose response to IVM in the migration assay showed the presence of a plateau in response at the highest drug concentrations. The % migration remained at a constant level (above 0%) over the highest 3 to 4 drug concentrations. Hence, for the analysis of the IVM dose-response data we used a non-normalised model in GraphPad (‘top to bottom’), with a variable slope. The output of this analysis provided us with two parameters with which to compare populations: firstly, the % migration at the dose-response plateau (that is, the % of the population unaffected by the highest concentrations of IVM in the assay), and secondly, the IC₅₀ in the remaining proportion of the population that had shown a dose-response to the drug (for instance, if the plateau existed at a level of 40% migration, then the IC₅₀ value defined the response to the drug in the remaining 60% of the worm population alone).
3.3.6 Rhodamine-123 efflux assay

Efflux of rhodamine-123 from larvae following exposure to IVM and LEV was measured using an assay modified from Kerboeuf and Guegnard (2011). Briefly, for each isolate, approximately 20,000 L3 were exposed to IVM (at 0.2 µg/mL and 0.8 µg/mL), LEV at 0.4 µg/mL or 1% DMSO for 3 h, as described above in section 3.3.4. Following exposure to drugs, the worms were centrifuged (3000g, 1 min), the supernatant was discarded, and the worm pellet washed using 1mL distilled water. R-123 solution (2 mL of 1.5 µM) was added to the worm pellet which was then placed on a roller for 15 min in the dark at room temperature (= R-123 accumulation period). At the end of the accumulation period, the worms were centrifuged (3000g, 1 min), followed by washing in 1mL of water. The worm pellet was resuspended in water (1 mL) and placed on a roller-mixer (BTR-5, Ratek®, Australia) in the dark for 60 min (= R-123 efflux period). The worms were sedimented by centrifugation (3000g, 1 min) and the supernatant was collected and stored at room temperature for 60 min in the dark before analysis. R-123 was detected using a fluorescence spectrophotometer (Spectra Max M3, Molecular Devices®, USA) (λ for excitation = 495 nm and λ for emission = 525 nm). Three separate experiments were performed, each with duplicate assays. The concentration of R-123 in each experimental sample was calculated from a standard curve determined using a range of concentrations of R-123. The pooled data (n = 6 for each treatment) were Log10-transformed and analysed using repeated measures ANOVA followed by Fischer’s LSD (GraphPad Prism, USA version 6.01).

3.4 Results

3.4.1 Basal transcription levels of ABC transporters in resistant and susceptible isolates of H. contortus

In the susceptible isolate, transcription of all the P-gp, MRP and ABCF genes was significantly higher (P < 0.05) than for the pgp-1 gene (Fig 3.1A and C). The pattern observed in the resistant isolate was similar, except that pgp-11 and pgp-16 were at equivalent levels to pgp-1 (Fig. 3.1B and C). Transcription of haf-6 was significantly lower (P > 0.05) than pgp-1 in both isolates. Compared to pgp-1, transcription of pgp-9.3 was increased by 136-fold and 83-fold in the susceptible and resistant isolates, respectively. The other markedly higher transcription levels, relative to pgp-1, were for abc-1 (85-fold higher in susceptible, and 52-fold higher in resistant), mrp-5 (68- and 42-fold), abc-2 (61- and 31-fold), pgp-3 (50-fold and 26-fold), and pgp-10 (37- and 29- fold).

A comparison of the transcription levels of the ABC transporter genes in resistant (WAL) and susceptible (Kirby) isolates showed that pgp-1, pgp-9.1 and pgp-9.2 were transcribed at
significantly higher levels in the former isolate ($P = 0.04, 0.03, \text{ and } 0.02$, respectively) (Fig. 3.2). Transcription levels were increased in WAL by 2.5-fold for $pgp$-$I$ and $pgp$-$9.2$, and 3-fold for $pgp$-$9.1$. There were no significant differences in transcription levels for all the other transporters, including MRPs, ABCFs and the $haf$-$6$ gene, between the two isolates.

**Fig. 3.1.** Basal transcription levels of ABC transporter genes in susceptible (A) and resistant (B) isolates of *Haemonchus contortus* compared to the transcription levels of $pgp$-$I$. Data shown as mean ± SEM, $n = 3$ separate experiments each with four technical replicates. Significant differences in the relative transcription of the genes within each isolate are indicated in part (C). Gene transcription levels relative to $pgp$-$I$ are ranked from the highest to the lowest; genes grouped together with vertical lines did not show significant differences in transcription levels (at $P < 0.05$).
Fig. 3.2. Relative transcription levels of ABC transporter genes in resistant (WAL) compared to susceptible (Kirby) isolates of *Haemonchus contortus*. Significant differences in the transcription between the two isolates are indicated by * (P < 0.05). Data shown as mean ± SEM, n = 3 separate experiments, each with four technical replicates.
3.4.2 Transcription levels of ABC transporters in resistant and susceptible isolates of *H. contortus* following exposure to IVM

There were no significant changes in transcription profiles of all 16 ABC transporter genes in the susceptible isolate following 3 or 6 h exposure to IVM at 0.2 µg/mL compared to control worms (exposed to DMSO alone) (Figs. 3.3A and 3.3B). The data at the 6 h time point revealed a degree of variability among different replicates as reflected in the large standard error (SE) bars in some cases in Figure 3B, however, none of the genes showed a statistically significant change relative to controls. In contrast, five P-gp genes and the *haf-6* gene were significantly up-regulated in the resistant isolate after exposure to IVM at 0.2 µg/mL for 3 h (Fig. 3.3C). The magnitudes of these increases were as follows: 12.6-fold for *haf-6* (*P* < 0.0001), 9.1-fold for *pgp-2* (*P* < 0.0001), 6.4-fold for *pgp-9.1* (*P* = 0.0006), 6.4-fold for *pgp-11* (*P* = 0.0004), 3.6-fold for *pgp-1* (*P* = 0.02), and 3.1-fold for *pgp-10* (*P* = 0.02). These increased transcription levels were however quite temporary, as by 6 h of exposure to this concentration of IVM, the resistant isolate showed no changes in transcription of any of the transporter genes relative to DMSO-treated controls (Fig. 3.3D).

The results of IVM-exposure experiments at a concentration of 0.8 µg/mL are shown in Figure 3.4. Again, as seen at 0.2 µg/mL, the only significant instances of altered gene transcription were increases in IVM-exposed resistant isolate larvae at the 3 h time point (Fig. 3.4C). There were no significant changes in any of the genes for the susceptible isolate at 3 or 6 h, however, as noted above for the 0.2 µg/mL data, there was a degree of variability between replicate experiments which resulted in large SEs in some cases, particularly for *pgp-9.1* at the 6 h time point (Fig. 3.4B). The magnitudes of the increases observed for the resistant isolate at 3 h were as follows: *pgp-2* 4.5-fold (*P* = 0.0001), *pgp-9.1* 2.6-fold (*P* = 0.005), *pgp-11* 2.3-fold (*P* = 0.01) and *haf-6* 2.4-fold (*P* = 0.03). By the 6 h time point, transcription levels in the resistant isolate had returned to control levels (Fig. 3.4D), as was observed for the 0.2 µg/mL treatment. Comparison of the 0.2 and 0.8 µg/mL data sets showed that the increases in gene transcription were 2-3 fold lower for the P-gp genes (*pgp-2, 9.1 and 11*) and 6-fold lower for the *haf-6* gene at the higher IVM concentration.
Fig. 3.3. Effects of IVM (0.2 µg/mL) on transcription patterns of ABC transporter genes in the third-stage larvae of susceptible (A at 3 h and B at 6 h) and resistant (C at 3 h and D at 6 h) isolates of *Haemonchus contortus* compared to DMSO controls. Y-axis shows fold-change in gene expression levels in drug-treated vs DMSO-treated larvae. Data shown as mean ± SEM, n = 3 separate experiments, each with four technical replicates. Significant differences in gene transcription levels are indicated by * (P < 0.05).
**Fig. 3.4.** Effects of IVM (0.8 µg/mL) on transcription patterns of ABC transporter genes in the third-stage larvae of susceptible (A at 3 h and B at 6 h) and resistant (C at 3 h and D at 6 h) isolates of *Haemonchus contortus* compared to DMSO controls. Y-axis shows fold-changes in gene expression levels in drug-treated vs DMSO-treated larvae. Data shown as mean ± SEM, n = 3 separate experiments, each with four technical replicates. Significant differences in gene transcription levels are indicated by * (P < 0.05).
3.4.3 Transcription levels of ABC transporters in resistant and susceptible isolates of *H. contortus* following exposure to LEV

Exposure to LEV at 0.4 µg/mL did not affect transcription levels of any of the transporter genes in the susceptible isolate for both the 3 and 6 h exposure treatments (Figs. 3.5A and 3.5B). In contrast, 3 h exposure of the resistant isolate resulted in significant up-regulation of a number of genes: *pgp-1* 3.5-fold (*P* = 0.0003), *pgp-2* 9.6-fold (*P* < 0.0001), *pgp-9.1* 6.5-fold (*P* < 0.0001), *pgp-10* 3-fold (*P* = 0.0007), *pgp-11* 6-fold (*P* < 0.0001), *abcf-1* 2-fold (*P* = 0.03), and *haf-6* 10.3-fold (*P* < 0.0001) (Fig. 3.5C). There was a single instance of down-regulation, with *pgp-16* showing a significant decrease (2-fold) (*P* = 0.04) at 3 h exposure relative to the DMSO control. Interestingly, *pgp-11* remained up-regulated (2.6-fold) after 6 h pre-exposure to LEV at 0.4 µg/mL (Fig. 3.5D), representing the only instance of significantly altered transcription (*P* = 0.04) at this time point across the entire set of IVM and LEV exposure experiments.
**Fig. 3.5.** Effects of LEV (0.4 µg/mL) on transcription patterns of ABC transporter genes in the third-stage larvae of susceptible (A at 3 h and B at 6 h) and resistant (C at 3 h and D at 6 h) isolates of *Haemonchus contortus* compared to DMSO controls. Y-axis shows fold-change in gene expression levels in drug-treated vs DMSO-treated larvae. Data shown as mean ± SEM, n = 3 separate experiments each with four technical replicates. Significant differences in gene transcription levels are indicated by * (P < 0.05).
3.4.4 Functional consequences of increased transcription of transporter genes

3.4.4.1 Larval migration assay

The functional consequences of increased transcription of transporter genes following exposure to IVM and LEV were examined using larval migration assays to measure the ability of IVM and LEV to inhibit migration in larvae of the susceptible and resistant isolates that had previously been exposed for 3 or 6 h to low levels of the two drugs (the same concentrations as used for the gene expression experiments) compared to DMSO-treated controls (Figs. 3.6 and 3.7; Tables 3.1 and 3.2). A feature of the IVM dose-responses in these experiments was the presence of a plateau in the response at the highest IVM drug concentrations (Figs. 3.6A, 3.6B and 3.7A). A proportion of the worm population remained able to migrate through the filter-mesh system at the highest drug concentrations. Our analysis of these dose response curves provided two parameters for comparing the effects of the drug pre-treatments, namely, the % of the population that was unaffected by the high IVM concentrations (the dose-response plateau), and the IC$_{50}$ value derived from the dose–response shown by the remainder of the population. Pre-exposure for 3 h to IVM at 0.8 µg/mL resulted in a significant increase in the % of larvae able to migrate at the highest drug concentrations (plateau increased from 17.6% to 48.1%), alongside no change in the IC$_{50}$ of the remaining population (Fig. 3.6A, Table 3.1). Pre-exposure to IVM at the lower concentration (0.2 µg/mL) resulted in no change to the migration plateau, alongside a decrease in the IC$_{50}$ (to 0.63 of the control). Pre-exposure to LEV (0.4 µg/mL) resulted in a significant increase in the proportion of the population unaffected by the highest drug concentrations (from 17.6 to 30.7%), alongside a decrease in the IC$_{50}$ in the remainder of the population (to 0.57 of the control). The effects of 6 h pre-exposure to IVM at 0.8 µg/mL and LEV at 0.4 µg/mL, were similar to those seen after 3 h, with significant increases in the IVM dose response plateau (from 15.5% in controls to 25.4 and 31.3% in L$_3$ pre-treated with IVM and LEV, respectively), although in this case there was no decrease in the LEV IC$_{50}$ as had been observed after 3 h pre-exposure (Fig. 3.6B, Table 3.1). The response to IVM after 6 h pre-exposure to the same drug at 0.2 µg/mL was similar to that observed after 3 h, with no change in the dose response plateau and a decrease in the IC$_{50}$ for the remaining population (to 0.60 of the control). Overall, two effects were apparent: an increase in the proportion of the population able to migrate at the highest IVM concentrations following exposure to IVM at 0.8 µg/mL and LEV at 0.4 µg/mL, alongside a number of instances of decreased IC$_{50}$ in the remainder of the population, indicating the presence of instances of increased drug tolerance (higher plateau) as well as increased drug sensitivity (lower IC$_{50}$) in different components of the worm populations. After 3h pre-exposure to LEV, these aspects of increased and decreased sensitivity to IVM in the two separate components of the population occurred simultaneously.
LEV IC₅₀ values were significantly decreased following 3 h pre-exposure to IVM (0.2 or 0.8 µg/mL) (to 0.47 or 0.31 of control) and LEV (0.4 µg/mL) (to 0.27 of control) (Fig. 3.6C; Table 3.2). Similarly, following 6 h pre-exposure to IVM or LEV, the L₃ showed increased sensitivity to LEV (IC₅₀ decreased to 0.36 of control for IVM pre-exposure, and 0.27 of control for LEV pre-exposure) (Fig. 3.6D; Table 3.2).

In the susceptible isolate, pre-exposure to IVM (0.2 µg/mL) and LEV (0.4 µg/mL) did not result in any significant changes to the IVM dose response plateau, although the IC₅₀ was reduced following pre-treatment with IVM at 0.2 µg/mL (to 0.72 of control) (Fig. 3.7A; IC₅₀ and plateau data not shown). Pre-exposure to IVM (0.2 µg/mL) resulted in increased sensitivity to LEV (IC₅₀ reduced to 0.29 of control), alongside no change in LEV response following pre-exposure to this same drug (Fig. 3.7B).
Fig. 3.6. Effects of IVM (0.2 and 0.8 µg/mL) and LEV (0.4 µg/mL) pre-exposure on tolerance to IVM (A after 3 h pre-exposure and B after 6 h pre-exposure) and LEV (C at 3 h pre-exposure and D at 6 h pre-exposure) in the third-stage larvae of the resistant isolate of *Haemonchus contortus* in larval migration assays. Each data point represents mean ± SEM, n = 9 (pooled data from three experiments, each with assays in triplicate).
Fig. 3.7. Effects of IVM (0.2 µg/mL) and LEV (0.4 µg/mL) pre-exposure for 3 h on tolerance to IVM (A) and LEV (B) in the third-stage larvae of the susceptible isolate of *Haemonchus contortus* in larval migration assays. Each data point represents mean ± SEM, *n* = 9 (pooled data from three experiments, each with assays in triplicate).
Table 3.1. Response of third-stage larvae of the resistant isolate of *Haemonchus contortus* (Wallangra) to ivermectin following pre-exposure for 3 or 6 h to IVM (0.2 µg/mL and 0.8 µg/mL) or LEV (0.4 µg/mL) in larval migration assays

<table>
<thead>
<tr>
<th>Pre-exposure</th>
<th>IC₅₀</th>
<th>Dose-response plateau</th>
<th>Pre-exposure</th>
<th>IC₅₀</th>
<th>Dose-response plateau</th>
</tr>
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<tr>
<td></td>
<td>Drug conc. a/ µg/mL</td>
<td>95% CI</td>
<td>Drug/ DMSO a,b</td>
<td>Migration c</td>
<td>95% CI</td>
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<td>17.6</td>
<td>12.7-22.5</td>
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<td>0.63*</td>
<td>17.6</td>
<td>13.0-22.0</td>
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<td>IVM 0.8 µg/mL</td>
<td>3.04</td>
<td>2.21-4.19</td>
<td>0.75</td>
<td>48.1*</td>
<td>42.6-53.7</td>
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<td>LEV 0.4 µg/mL</td>
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<td>1.79-2.99</td>
<td>0.57*</td>
<td>30.7*</td>
<td>24.7-36.5</td>
</tr>
</tbody>
</table>

* Within either the 3 or 6 h pre-exposure data sets: * denotes that the IC₅₀ following pre-exposure to anthelmintic was significantly lower than the IC₅₀ following pre-exposure to DMSO, as determined by non-overlap of 95% Confidence Intervals.

b Drug / DMSO = IC₅₀ for IVM following pre-exposure to anthelmintics/ IC₅₀ for IVM following pre-exposure to DMSO

c Within either the 3 or 6 h pre-exposure data sets: * denotes that L3 migration (%) at the dose-response plateau following pre-exposure to anthelmintic was significantly higher than L3 migration (%) at the plateau following pre-exposure to DMSO, as determined by non-overlap of 95% Confidence Intervals.

d Drug/ DMSO = L3 migration (%) at the dose-response plateau for IVM following pre-exposure to anthelmintics/ L3 migration (%) at the plateau for IVM following pre-exposure to DMSO
Table 3.2. Response of third-stage larvae of the resistant isolate of *Haemonchus contortus* (Wallangra) to levamisole following pre-exposure for 3 or 6 h to IVM (0.2 µg/mL and 0.8 µg/mL) or LEV (0.4 µg/mL) in larval migration assays.

<table>
<thead>
<tr>
<th>Pre-exposure</th>
<th>3 hours Pre-exposure</th>
<th>6 hours Pre-exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀[^a] 95% C.I</td>
<td>Drug/ DMSO[^a,b]</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.95 0.81 - 1.11</td>
<td>-</td>
</tr>
<tr>
<td>IVM 0.2 µg/mL</td>
<td>0.47* 0.39 - 0.55</td>
<td>0.5*</td>
</tr>
<tr>
<td>IVM 0.8 µg/mL</td>
<td>0.31* 0.25 - 0.37</td>
<td>0.3*</td>
</tr>
<tr>
<td>LEV 0.4 µg/mL</td>
<td>0.27* 0.22 - 0.32</td>
<td>0.3*</td>
</tr>
</tbody>
</table>

[^a]: Within either the 3 or 6 h pre-exposure data sets: * denotes that the IC₅₀ following pre-exposure to anthelmintic was significantly lower than the IC₅₀ following pre-exposure to DMSO, as determined by non-overlap of 95% Confidence Intervals.

[^b]: Drug/ DMSO = IC₅₀ for LEV following pre-exposure to anthelmintics/ IC₅₀ for LEV following pre-exposure to DMSO
3.4.4.2 Rhodamine-123 efflux assay

The functional consequences of drug pre-exposure were further evaluated by observing the effects of the drug treatments on the ability of L₃ to efflux the fluorescent dye R-123 (Fig. 3.8). In both resistant and susceptible isolates, exposure to IVM (0.8 µg/mL) and LEV (0.4 µg/mL) for 3 h significantly increased \( P < 0.05 \) the efflux of R-123 compared to the DMSO-treated control worms. In contrast, pre-exposure to the lower concentration of IVM (0.2 µg/mL) did not result in any change in R-123 efflux in both the isolates (Fig. 3.8).

The levels of R-123 efflux were equivalent in resistant and susceptible L₃ in the absence of any drug exposure (DMSO-treated) \( P = 0.07 \).
**Fig. 3.8.** The effects of IVM (0.2 and 0.8 µg/mL) and LEV (0.4 µg/mL) pre-exposure for 3 h on R-123 efflux in third-stage larvae of susceptible (A) and resistant (B) isolates of *Haemonchus contortus*. R-123 efflux is expressed relative to controls (DMSO-treated). Significant differences in R-123 efflux compared to controls, within each isolate, are indicated by * (P < 0.05). Data shown as mean ± SEM, n = 3 separate experiments, each with assays in duplicate.
3.5 Discussion

The present study described the relative transcription levels of the suite of drug transporter genes in a parasitic nematode, *H. contortus*. We measured the transcription levels for each ABC transporter gene within a susceptible and resistant isolate and then described these levels relative to *pgp*-1 within each isolate. The ranking of transcription levels from the highest to the lowest was similar, but not identical, in the two isolates. The same set of genes (*pgp*-9.3, *abcf*-1, *mrp*-5, *abcf*-2, *pgp*-10 and *pgp*-3) were transcribed at the highest levels in both isolates. The *haf*-6 gene was transcribed at the lowest levels in both isolates. The protective functions of several of these ABC transporters have been reported previously. Pgp-3 acts in protection against natural toxins in *C. elegans* (Broeks et al., 1995). Issouf et al. (2014) described the specific induction of *pgp*-3 in *H. contortus* exposed to sheep eosinophil granules as suggesting a role in detoxification of host immune cell products. In addition, the expression of some P-gp genes (including *pgp*-3 and *pgp*-9) in intestinal excretory cells of the closely related model organism *C. elegans*, further suggests a role for them in the protection of worms against toxic substances (Ardelli and Prichard, 2013). We also observed relatively high expression levels of ABCF transporters (*abcf*-1 and *abcf*-2) compared to many of the other genes. ABCF transporters lack the transmembrane domains (TMDs) present in other transporter proteins, and their function as transporters is currently unclear. It has been suggested that ABCF transporters are involved in cell physiology (ribosome assembly, translational control and mRNA transport) in arthropods (Dermauw and Van Leeuwen, 2014). Furthermore, it has been reported that absence of ABCF transporters in arthropods results in physical abnormalities as shown by Broehan et al. (2013) who observed the death of L3 and arrested growth, as well as the death as pharate adults, in RNA interference studies with L3 and pupae of *Tribolium castaneum*, respectively. However, the role of these transporters in gastrointestinal nematodes remains to be determined.

The transcription levels for *pgp*-1, *pgp*-9.1 and *pgp*-9.2 were significantly greater in the drug-resistant WAL isolate compared to the drug-susceptible Kirby isolate. This finding is in agreement with the earlier report by Sarai et al. (2013) who described an up-regulation of *pgp*-1 and *pgp*-9 in L3 of the WAL isolate compared to Kirby. This earlier study did not consider the three different homologues of *pgp*-9 (as their existence was unknown at that time) and hence the present study has extended the earlier findings by showing that the increase in transcription of *pgp*-9 reported earlier was most-likely associated with increases for *pgp*-9.1 and 9.2, alongside no change in *pgp*-9.3. The expression patterns of P-gps in nematodes seem to be quite variable, with some reports linking them to anthelmintic resistance and other studies finding no association. Williamson et al. (2011) reported a significantly increased expression of *pgp*-2 and *pgp*-9 in a multi-drug resistant isolate.
compared to a susceptible isolate, alongside a significant decrease in \textit{p}gp-1 transcription. In contrast, Williamson and Wolstenholme (2012) found no changes in P-gp transcription in a laboratory selected IVM-resistant isolate compared to its drug-sensitive parent. In addition, there have been reports describing increased transcription of \textit{p}gp-9 in ML-resistant \textit{T. circumcincta} adult worms, and of \textit{haf}-9 and \textit{mrp}-1 in eggs of IVM resistant \textit{C. oncophora} (Dicker et al., 2011; De Graef et al., 2013). The increased transcription of several P-gps in resistant L3 in the present study suggests that they may play a role in the anthelmintic resistance shown by the multi-drug resistant WAL isolate. However, confirmation of any such association requires studies measuring actual drug efflux from larval and adult life-stages of susceptible and resistant isolates. Our R-123 efflux measurements showed that the rate of efflux was equivalent in the susceptible and resistant L3. This may be expected, despite the up-regulation of several transporter genes in the resistant isolate, as these specific transporters (\textit{p}gp-1, \textit{p}gp-9.1 and \textit{p}gp-9.2) were expressed at low levels compared to most of the other transporter genes (from Fig. 3.1C). Hence, increased transcription of just these three genes would likely have little or no impact on the total efflux activity measured using a general substrate such as R-123.

Substrates of ABC transporters are known to regulate the expression levels of P-gps and other ABC transporters through transcriptional or post-transcriptional mechanisms (Schrenk et al., 2001). There is a great deal of literature available on the inducing effects of various agents on P-gp expression levels in mammals (Lespine et al., 2012; Ménez et al., 2012; Yu et al., 2013). The experimental evidence of up-regulation in nematodes in response to exposure to anthelmintics is limited and inconsistent. Up-regulation of several P-gp genes has been observed in ML resistant \textit{C. elegans} (James and Davey, 2009; Yan et al., 2012; Ardelli and Prichard, 2013). Induction of P-gps was observed in \textit{H. contortus} (\textit{p}gp-2) and \textit{C. oncophora} (\textit{p}gp-16 and \textit{mrp}-1) adult worms after \textit{in vivo} exposure to IVM (Lloberas et al., 2013; Tydén et al., 2014). Furthermore, De Graef et al. (2013) also reported a significant increase (3-5 fold) in transcription levels of \textit{p}gp-11 in \textit{C. oncophora} adult worms 14 days after treatment with IVM or moxidectin compared to non-exposed adults. However, there were no significant differences observed between the expression patterns of P-gp genes before and after IVM treatment in \textit{C. oncophora} and \textit{H. contortus} (Williamson and Wolstenholme, 2012; Areskog et al., 2013).

We examined the effects of exposure to low levels of IVM and LEV on the transcription profiles of ABC transporters in \textit{H. contortus} L3. Exposure to IVM and LEV for 3 h resulted in increased transcription of multiple ABC transporter genes in the resistant isolate only. The patterns of up-regulation were very similar for IVM and LEV, with both drugs resulting in increased transcription of \textit{p}gp-1, -2, -9.1, -10, -11, and \textit{haf}-6, while LEV exposure also resulted in up-
regulation of *abcf-1*. Given the resistance shown by this isolate towards both IVM and LEV, it is noteworthy that two of the genes that responded to drug exposure (*pgp-1* and *pgp-9.1*) were also shown to be constitutively over-expressed in this isolate compared to the susceptible Kirby isolate (Fig 3.2). Surprisingly, the increases in transcript levels following IVM treatment were greater at the lower IVM concentration (0.2 µg/mL) than at the higher concentration (0.8 µg/mL) (compare Figures 3.3C and 3.4C). There are two possible explanations for this. Firstly, as noted in section 2.4, the lower IVM concentration did not affect migration of the resistant larvae, as reported by Raza et al. (2015), while the higher IVM concentration resulted in 12% inhibition of migration. Thus, the larvae exposed to this higher concentration may have been compromised to some degree in their fitness, resulting in a lower gene induction response compared to the larvae exposed to the lower concentration. Secondly, it may be possible that greater increases in transcription occurred with 0.8 µg/mL IVM at an earlier time than the 3 h time point examined in our experiments. Ardelli et al. (2009) showed that *glc-1* transcript increases in *C. elegans* were much more pronounced after exposure to 10 nM IVM compared to 2.5 nM IVM at the 0.5 h time point, while the pattern was reversed after 2.5 h of drug exposure, with the fold increases in transcription being much greater at the lower IVM concentration by that time.

The up-regulation response of the resistant larvae to drug exposure was quite short-lived. By the 6 h time point, gene transcription had returned to the same levels as shown by DMSO-treated controls. The only exception to this was the continued up-regulation of *pgp-11* after 6 h exposure to LEV in resistant L3. Previously, it was observed that more P-gp genes were upregulated after a short period of IVM treatment (0.5 h) compared to the number up-regulated after longer periods of treatment (1.5 and 2.5 h) in *C. elegans* (Ardelli and Prichard, 2013). These transient responses suggest that the worm acts to quickly increase transcription of transporter genes in response to drug exposure, and then returns transcription to basal levels, having initiated the process of synthesising transporter proteins.

The effects of exposure to IVM and LEV were very different for susceptible compared to resistant L3. The former showed no changes in transcription for any of the transporter genes at either the 3 or 6 h time points. Of note however was the marked variability between replicate experiments for the susceptible L3 that resulted in large SE bars, particularly at the 6 h time point. Hence, although there were no significant changes in transcription patterns across the three replicate experiments, the non-significant increases in mean gene transcription values for many of the transporters at the 6 h time point in the susceptible isolate, alongside the greater variability at 6 h compared to the resistant isolate, suggests that some up-regulation may have been occurring in one or more of our separate experiments. It remains possible that the transcriptional response observed
at 3 h with resistant L₃ is delayed in susceptible L₃ such that the 6 h time point represents the early stages of transcriptional response in this isolate. However, this is speculative, and would require an examination of transcription patterns at later time points to confirm. Another possible explanation for the observed variation between replicate experiments for the susceptible isolate might be the slightly toxic effects of the drugs under the experimental conditions. While the lower concentration of IVM and LEV showed about 12% inhibition of larval migration (from Raza et al., 2015), the higher concentration of IVM (0.8 µg/mL) inhibited larval migration by approximately 25%. Hence, the larvae exposed to IVM at these concentrations may have shown a degree of variability in their fitness at the time of sampling for transcription measurements.

We examined the effects of exposure to IVM and LEV on the rate of R-123 efflux by the L₃. Efflux was increased following exposure to the higher concentration of IVM (0.8 µg/mL) and LEV in both resistant and susceptible L₃ despite the fact that the gene up-regulation effects had only been observed with resistant L₃. There were no effects on R-123 efflux in both the isolates following exposure to the lower concentration of IVM (0.2 µg/mL) (Fig. 3.8), in contrast to the effect of this drug treatment in increasing the transcription levels of a number of transporter genes in resistant L₃ (Fig. 3.3C). These differences in the two measurements may be explained by consideration of two aspects of the effects of exposure to xenobiotics on ABC transporters, at the gene and protein levels: firstly, at the gene level, the effects of drug exposure in increasing transcription of transporter genes resulting in the generation of an increased number of efflux proteins (Seelig, 1998), and, secondly, the direct effect of xenobiotics in stimulating the activity of pre-existing transporter proteins (Kerboeuf and Guegnard, 2011). This latter study reported an increase in R-123 transport within a few minutes after addition of anthelmintics in worm eggs. The authors suggested that this was not compatible with up-regulation of P-gp genes in such a short period, but rather was most likely due to increased activity of transporter proteins present in the organism at the start of the drug exposure period. As described above, our gene transcription and R-123 experiments show a number of differences in the responses of the two worm isolates to the two drugs at the different time points. This indicates that the effects of drug exposure on gene transcription and on R-123 efflux are not directly correlated, thus suggesting that the increases in R-123 efflux measured under our experimental conditions were at least partly due to increases in the activity of existing transporter proteins, as observed by Kerboeuf and Guegnard (2011), rather than as a consequence of increased transcription.

We also studied the consequences of exposure to low levels of IVM and LEV in terms of whether such exposure equipped the L₃ with an ability to survive subsequent exposure to higher levels of these same anthelmintics in migration assays. The assays were performed using short
incubation/migration periods (30 minutes for both) compared to the 48 and 24 h periods usually used for this assay (Kotze et al., 2006) in order to avoid any effects of the drugs in possibly inducing transcription of the transporter genes during such long incubation periods. The dose response curves showed the presence of a plateau at the highest IVM concentrations. This was most likely due to the short nature of both the drug incubation and migration phases of the assay, and the subsequent inability to inhibit migration completely in all larvae in this short time frame. Such plateaus are not observed using standard longer time periods (24-48 hrs) (Kotze et al., 2009; Raza et al., 2015). The presence of this plateau allowed us to describe the response of the larvae to IVM in terms of both the % migration at the plateau (the % of the population able to migrate at the high IVM concentrations), as well as the IC₅₀ for the remainder of the population. We found that resistant L₃ pre-exposed to IVM (at 0.8 µg/mL) or LEV showed an increased tolerance to subsequent treatment with IVM in a proportion of the population, as indicated by an increase in the percentage of the population able to migrate at the highest drug concentrations. This apparent tolerance to the drug only occurred in the proportion of the population represented by the dose response plateau, as the IC₅₀ in the remainder of the population was either unchanged or decreased compared to L₃ pre-treated with DMSO alone (Table 3.1). On the other hand, there was no increase in this dose response plateau at the lower IVM concentration (0.2 µg/mL) even though this pre-treatment had resulted in greater fold increases in transporter gene transcription. This contradiction may be at least partly explained by examining the drug exposure data alongside both the gene transcription and R-123 efflux data. Exposure to the higher IVM concentration (0.8 µg/mL) and LEV resulted in increases in both gene transcription and R-123 efflux, alongside an increased ability to tolerate subsequent IVM exposure in a proportion of the worm population (higher dose response plateau). On the other hand, exposure to the lower IVM concentration resulted in increased transcription levels, but no change in R-123 efflux, alongside no increased drug tolerance. This suggests that, under our experimental conditions, both the transcription and protein activity responses are required in order to equip a proportion of the L₃ population with an ability to tolerate subsequent drug exposure. Increases in transcription alone, in the absence of increased activity of existing transporter proteins, as was observed with IVM at the lower concentration, may not provide protection against drugs to a degree measurable in our migration assays. We did not assess the effects of exposure to IVM at 0.8 µg/mL on the subsequent ability of susceptible L₃ to the drug as exposure to this drug concentration was previously shown to reduce migration by 25% (Raza et al., 2015), hence making it difficult to accurately measure the specific effects of subsequent drug exposure. The lack of drug tolerance observed in susceptible L₃ pre-exposed to IVM at 0.2 µg/mL (Fig. 3.7A) would be expected given the lack of any increases in both gene transcription and R-123 efflux (Figs. 3.3, 3.4, 3.5 and 3.8).
In contrast to the protective effects towards IVM of drug pre-exposure in a proportion of the larval population for the resistant isolate, as described above in terms of increases in the dose response plateau, there were a number of instances of either no change or a decrease in the IVM IC$_{50}$ of the remainder of the population. An increase in sensitivity to LEV following pre-treatment with IVM or LEV was also observed in most cases. In the absence of any ABC transporter-mediated protection for at least a portion of the population, as suggested above for IVM, a decreased tolerance to a second drug exposure may be expected due to the cumulative effects of sequential drug treatments. This may be particularly so for sequential treatments with IVM and LEV given that macrocyclic lactones have been reported to interact with nAChRs in vertebrates, arthropods and nematodes (Krause et al., 1998; Raymond et al., 2000; Carmichael et al., 2013; Abongwa et al., 2016). Hence, the increased sensitivity of larvae to IVM or LEV following an initial period of drug exposure to one of these drugs, compared to controls exposed to the second drug alone, may be expected. It is the reversal of this expected outcome, as observed in the cases of increased tolerance to IVM as the second drug (Figs. 3.6A and 3.6B, Table 3.1), that suggests the influence of a protective pathway allowing the worms to show a degree of tolerance towards the second drug. As mentioned above, tolerance to IVM in a component of the larval population of the resistant isolate was accompanied by either no change or an increase in sensitivity in the remainder of the population. This suggests a degree of heterogeneity in the response of the larvae to the drug pre-treatment. If, as we suggested above, increased transporter activity is at least partly responsible for the increased tolerance seen at the dose response plateau, then the unchanged or decreased IVM IC$_{50}$ values observed in the other component of the worm population suggests that the gene expression and R-123 efflux increases may also be quite heterogeneous across the worm population.

The difference in the responses of the resistant isolate to IVM and LEV in the migration assay phase of these experiments (some increased tolerance for IVM, none for LEV) suggests that, despite the fact that pre-exposure to both drugs clearly leads to approximately equivalent up-regulation of a number of transporter genes, and equivalent increases in R-123 efflux, the induced transporters are able to subsequently provide protection only against IVM and not LEV. This may suggest that IVM is a better substrate for the *H. contortus* P-gs than LEV as reported earlier for mammalian P-gps (Efferth and Volm, 1993) and, hence, the induced P-gps are more effective in preventing the IVM interacting with its target site compared to LEV.

In conclusion, the present study provides further evidence that P-gps play a role in the interaction of anthelmintics with *H. contortus*. We have shown that there is a great deal of variation in the relative transcription levels of the different transporter genes in this worm species, thereby suggesting an existence of variation in their specific roles in protecting the worms from a range of
xenobiotics. The constitutive over-expression of *pgp-1, pgp-9.1* and *pgp-9.2* in Wallangra L₃ suggests that these specific P-gps may play a role in the anthelmintic resistance shown by this isolate. Furthermore, the increased transcription of some ABC transporters following exposure to anthelmintics (IVM and LEV) indicates that these anthelmintics interact with a number of specific *H. contortus* ABC transporters and hence are likely to be the substrates for these transporters. The presence of significant levels of increased transcription following 3 h exposure to both IVM and LEV in the resistant isolate only, as well as stimulation of the existing transport proteins, alongside the subsequent ability of a greater proportion of the worm population to tolerate high IVM concentrations in larval migration assays, suggest that the ability to rapidly up-regulate protective pathways in response to drugs may be a component of the drug resistance displayed by this isolate, and possibly by other drug-resistant nematodes. The variability seen in the effects of pre-treatment with the two drugs on the sensitivity of larvae in subsequent migration assays, with instances of increased tolerance as well as increased sensitivity within the various treatment groups, and even within single experimental populations, suggests the presence of some heterogeneity in the observed responses to drug exposure in the resistant isolate. This indicates that simple measurements of ABC transporter gene transcription levels and efflux activities in preparations from whole larvae are not necessarily indicative of the ability of transporter pathways to protect worms from these anthelmintics.
**Supplementary Table**

Table 3.3. Primer sequences of housekeeping and ABC transporter genes used for the quantitative PCR used in this study

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Sequences</th>
<th>Product size</th>
<th>Amplification Efficiency %</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Housekeeping genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Hc GAPDH</td>
<td>F: TGGGTGTGAACCACGAGAC R: GCAGCACCACGTCCCATCA</td>
<td>213</td>
<td>91.17</td>
<td>Sarai et al. (2013)</td>
</tr>
<tr>
<td>2</td>
<td>Hc Actin</td>
<td>F: GAGTCATGGTGATGATGGGAC R: GGAGCTTGGTCTCAAAATAGG</td>
<td>140</td>
<td>88.80</td>
<td>Sarai et al. (2013)</td>
</tr>
<tr>
<td>3</td>
<td>Hc β-Tubulin</td>
<td>F: GCATCCGACTTTGAAACTC R: TGAAGCAGGGAATGGAAC</td>
<td>160</td>
<td>88.41</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td><strong>ABC transporter genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>P-gp 1</td>
<td>F: CCACATGCAGCCACACCTTTTAG R: AGACGACTCCGACGTAGTTCAG</td>
<td>145</td>
<td>84.67</td>
<td>Sarai et al. (2013)</td>
</tr>
<tr>
<td>5</td>
<td>P-gp 2</td>
<td>F: GGACAAGAAGCAAGGAATTGCC R: ACAGAAGAAGCCTACGATACGG</td>
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<td>95.73</td>
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</tr>
<tr>
<td>6</td>
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<td>88.02</td>
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</tr>
<tr>
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<td>F: TCGAAGGGAATCAAGAAATC R: GCCCATATTACGGAAGAAGA</td>
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<td>79.72</td>
<td>This study</td>
</tr>
<tr>
<td>8</td>
<td>P-gp 9.2</td>
<td>F: CCAGTCCACCTCAATTCAC</td>
<td>65</td>
<td>93.82</td>
<td>Issouf et al. (2014)</td>
</tr>
<tr>
<td>9</td>
<td>P-gp 9.3</td>
<td>F: AGAAACAACGAATCGGCAAATC R: TGTGCCACAAACGATACGATG</td>
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<td>82.54</td>
<td>This study</td>
</tr>
<tr>
<td>10</td>
<td>P-gp 10</td>
<td>F: TCAGAAAGATTATGCGCCACGG R: CAGCGTCAAGAGTCCGTAATGC</td>
<td>98</td>
<td>89.94</td>
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<td>11</td>
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<td>This study</td>
</tr>
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<td>93.88</td>
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</tr>
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<td>MRP 5</td>
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<td>125</td>
<td>85.28</td>
<td>This study</td>
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<td>80.62</td>
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<tr>
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<td>92.00</td>
<td>This study</td>
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4. Increased expression of ATP binding cassette transporter genes following exposure of
Haemonchus contortus larvae to a high concentration of monepantel in vitro

4.1 Abstract

There is some evidence that ATP binding cassette (ABC) transporters play a role in resistance to anthelmintics, particularly against macrocyclic lactones. Some anthelmintics, including ivermectin (IVM), have been shown to induce transcription of multiple ABC transporters in nematodes; however, the effects of monepantel (MPL) on transcription of these transporter genes has not been studied. Larvae of two MPL-susceptible isolates of Haemonchus contortus were exposed to MPL at two concentrations (2.5 and 250 µg/ml) for periods of 3, 6 and 24 h. Transcription levels of sixteen ABC transporter genes were measured at the end of the incubation periods. The consequences of MPL exposure were examined by measuring rhodamine-123 efflux from the larvae, and their sensitivity to subsequent treatment with IVM or levamisole. Multiple ABC transporter genes showed significantly higher transcription in both worm isolates following exposure to MPL at 250µg/ml for 3, 6 or 24 h, particularly the P-glycoprotein (P-gp) genes pgp-11, pgp-12 and pgp-14. Of these, only pgp-11 maintained the elevated levels 24 h after the end of the drug exposure period. In contrast, there was only a single instance of low-level upregulation as a result of exposure to MPL at 2.5 µg/ml. Larvae exposed to MPL at 250 µg/ml showed an increased efflux of rhodamine-123 and a proportion of the larval population showed an ability to subsequently tolerate higher concentrations of IVM in migration assays. There was no increased tolerance to IVM following pre-exposure to MPL at 2.5 µg/ml. Exposure of H. contortus larvae to 250 µg/ml MPL results in increased transcription of multiple transporter genes and increased R-123 efflux. The subsequent ability of a proportion of the larvae to tolerate IVM suggests a protective role of ABC transporters across different chemical entities. However, these observations were only made at a concentration of MPL well above that experienced by parasitic life stages in vivo, and hence their significance remains unclear.

Citation: Raza, A., NH. Bagnall, A. Jabbar, SR. Kopp, AC. Kotze, 2016. Increased expression of ATP binding cassette transporter genes following exposure of Haemonchus contortus larvae to a high concentration of monepantel in vitro. Parasites & Vectors, 9:522.
4.2 Introduction

Monepantel (MPL) (trade name Zolvix®) was the first new anthelmintic for livestock use for over 25 years when it appeared in 2009. It was first introduced in New Zealand for the control of gastrointestinal nematodes (GINs) and associated diseases in small ruminants, and later in Australia and the United Kingdom in 2010. It shows broad-spectrum anthelmintic activity, targeting larval and adult stages of the most important species of sheep GINs (Kaminsky et al., 2008; Lecova et al., 2014). The compound is an amino-acetonitrile derivative (AAD) that acts as a positive allosteric modulator of a nematode-specific clade of nicotinic acetylcholine receptor (nAChR) subunits. Genetic screening of Caenorhabditis elegans revealed nAChR subunit ACR-23 as a target for MPL action (Kaminsky et al., 2008), while Rufener et al. (2009b) suggested that Hco-MPTL-1 and other nAChR subunits of the DEG-3 subfamily are the target for MPL in H. contortus.

Resistance to MPL was first reported by Scott et al. (2013) in New Zealand. The authors reported that MPL showed no efficacy in terms of egg count reduction in goats or reduction of worm burden in sheep, and was ineffective against at least two GINs (Teladorsagia circumcincta and Trichostrongylus colubriformis). There is also evidence of MPL resistance in Teladorsagia spp. and T. colubriformis on a goat farm in New South Wales, Australia (Love, 2014). Resistance to MPL has also been reported in H. contortus in Uruguay, where the authors reported poor efficacy against this nematode with 42% and 82% faecal egg count reduction at two different sheep farms (Mederos et al., 2014). A more recent study has also reported H. contortus population resistant to MPL at a sheep farm in Netherlands (Van den Brom et al., 2015). Early work on MPL resistance in laboratory-selected isolates of H. contortus showed that the resistance was associated with a number of changes to the target site. These mutations resulted in the generation of various truncated versions of the target receptor that would be expected to be non-functional (Rufener et al., 2009b). However, there is no information at present on whether such target site changes are involved in any of the cases of resistance reported from the field. We were interested in whether ABC transporters may interact with MPL in nematodes, as a number of anthelmintics are known to be substrates of these transporters, and increases in efflux activity are associated with anthelmintic resistance in some cases, particularly for macrocyclic lactones (Lespine et al., 2012; Ardelli and Prichard, 2013; Kotze et al., 2014a). As AAD compounds are hydrophobic, they may also be substrates for the drug transport proteins (James and Davey, 2009).

There is a considerable evidence that exposure to anthelmintics results in increased transcription of some ABC transporter genes, both in vitro (James and Davey, 2009; De Graef et al., 2013; Bygarski et al., 2014; Raza et al., 2016a) and in vivo (Lloberas et al., 2013; Heckler et al.,
2014; Tydén et al., 2014). These studies have generally concluded that such a transcriptional response suggests a role for the transporters in the efflux of the transcription-inducing anthelmintic, that is, the anthelmintic is likely a substrate for the transporters. Therefore, as a first step in understanding the interaction of efflux pathways with MPL in nematodes, the present study aimed to explore the effects of MPL exposure on the transcription patterns of ABC transporters in third-stage larvae (L3) of two MPL-susceptible isolates of H. contortus. We further investigated the phenotypic effects of this drug exposure by (i) measuring the efflux of the fluorescent dye rhodamine-123 (R-123) from the L3 pre-exposed to MPL compared to controls, and (ii) examining whether MPL pre-exposure enabled the L3 to tolerate subsequent exposure to ivermectin (IVM) and levamisole (LEV) in larval migration assays.

4.3 Materials and methods

4.3.1 Parasite material

Two isolates of H. contortus were used in the present study:

I. Kirby, a drug susceptible isolate recovered from the field at the University of New England, Kirby Research Farm in 1986 (Albers and Burgess, 1988); and

II. Wallangra (WAL), a multi-drug resistant isolate collected from the New England region of New South Wales (NSW) in 2003; at the time of isolation, it was resistant to benzimidazole, closantel, LEV, IVM and moxidectin (Love et al., 2003). The isolate has been further selected over at least five generations with moxidectin (Cydectin®) and is now unaffected by the recommended dose of moxidectin.

Both isolates were recovered from the field before the introduction of monepantel, and show equivalent sensitivity to this drug in larval development assays (Raza et al., 2016b). Infected animals were housed at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Agriculture FD McMaster laboratory at Armidale, NSW. Faeces were collected from infected animals and sent to the CSIRO Agriculture laboratories at the Queensland Bioscience Precinct, Brisbane, QLD, in zip-lock bags. Third-stage larvae (L3) were harvested from faecal cultures held at 27°C for 7 days. The L3 were stored at 15°C, and used for experiments within four weeks.
4.3.2 *In vitro* monepantel exposure

The commercially available drench product Zolvix® (Novartis Animal Health, Australia) (25 mg MPL/mL) was used as a source of MPL in this study. Multiple separate anthelmintic solutions were prepared by two-fold serial dilutions in dimethyl-sulfoxide (DMSO).

Groups of approximately 30,000 L3 of each isolate were exposed to two concentrations of MPL (2.5 µg/mL and 250 µg/mL) and DMSO (vehicle control) for a range of time periods (3, 6 and 24 h). The DMSO concentration was 1% (v/v) across all samples. The MPL concentrations were chosen based on:

(i) 250 µg/mL: the highest concentration that L3 could be exposed to while maintaining an ability to migrate at equivalent levels to controls in subsequent migration assays, that is, the highest concentration that could be used without compromising the fitness of the larvae as measured by migration assays (migration at 250 µg/mL approximately 90% of controls) (unpublished data); and

(ii) 2.5 µg/mL: as an approximation of the range of MPL concentrations of 2-4 µg/g measured by Lifschitz et al. (2014) in the abomasal contents of sheep within the first 48 h following administration of Zolvix®.

The larvae were kept on a roller-mixer (BTR-5, Ratek, Boronia, Australia) for the entire duration of the drug-exposure period. Three separate experiments were performed. At the end of the incubation period larvae were processed in two ways:

(i) For use in RNA extraction, larvae were centrifuged (3000g, 1 min), and the pellets were washed with 1 mL water, centrifuged again (3000g, 1 min), and the pelleted L3 were stored at -80 °C.

(ii) For use in R-123 and migration assays, larvae were centrifuged (3000g, 1 min), and the pellets were washed with 1 mL water, centrifuged again (3000g, 1 min), and the L3 were resuspended in water and used immediately.

4.3.3 qPCR analysis

RNA extraction, cDNA synthesis and qPCR were performed as described by Raza et al. (2016a). Briefly, total RNA was extracted from 30,000 L3 using the RNeasy mini kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. Turbo-DNase (Ambion, Carlsbad, USA) was used to remove genomic DNA. cDNA was synthesized using DNase-treated RNA with superscript III reverse transcriptase (Invitrogen, Carlsbad, USA) according to the manufacturer’s
instructions. The cDNA samples were diluted to a concentration of 4 ng/ml for downstream applications.

The primers used in this study for quantitative PCR of 11 pgp genes, two multidrug-resistance protein genes (mrp-1 and mrp-5), two genes from the ABCF family (abcf-1 and abcf-2) and one half transporter gene (haf-6) were as reported by Raza et al. (2016a). Three housekeeping genes (GAPDH, actin and β-tubulin) were used as reference genes for the qPCR analysis (see Supplementary Table: Table-4.3). The SYBR Green dye system (Applied Biosystems, Warrington, UK) was used in a Vii A7 thermocycler (Applied Biosystems, USA) under the following PCR cycling conditions: 50°C for 2 min, 95°C for 10 min (stage I), followed by 40 cycles of 95°C for 15 s, 60°C for 1 min (stage II) and a melt curve stage at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s (stage III). Three separate extractions for each treatment were examined, with each PCR run in quadruplicate. Reaction efficiencies, determined by standard curves, were in the range between 80 and 99%. The homogeneity of the PCR products was ensured by (i) analysing the melt-curves in each run, (ii) visualizing a single band for each PCR product on 2% electrophoresis gels, and (iii) cloning of the selected primer products into the vector PCR2.1 (Invitrogen, USA) followed by sequencing (Big-Dye terminator, V3.1; Applied Biosystems, USA) using M13 forward/reverse primers. Expression values for all genes in each sample were normalised to the housekeeping genes using REST 2009 (version v2.0.13) to determine the effects of MPL pre-exposure on the transcription of ABC transporters (drug exposure regimen described above) using DMSO treated samples as control for each isolate. The triplicate expression values were log_{10} transformed and analysed using repeated measures ANOVA with Fischer’s Least Significant Difference (LSD) post-hoc test (P < 0.05) to compare the transcription profiles of control and MPL-exposed samples in GraphPad Prism (version 6.01).

4.3.4 Rhodamine (R-123) efflux assay

R-123 efflux was measured in order to determine the effects of MPL pre-exposure (2.5 µg/mL and 250 µg/mL) on the efflux activity of ABC transporters in Kirby and WAL L3 stage larvae, using the protocols described earlier (Raza et al., 2016a). Briefly, approximately 20,000 L3 were exposed to MPL or DMSO for 3 and 6 h (as described in section 4.3.2) and then placed into a solution of R-123 (2 mL of 1.5 µM) for 15 min in the dark at room temperature on a roller-mixer. The worms were centrifuged (3000g, 1 min) and washed with 1 mL of distilled water. The pellet was resuspended in distilled water (1mL) and placed on a roller for 60 min in the dark. Finally, the worms were centrifuged (3000g, 1 min) and the supernatant was collected and stored in dark for 60 min before use. R-123 concentration in the supernatant was detected by measuring the specific
fluorescence (λ for excitation = 495 nm and λ for emission = 525 nm) using a spectrophotometer (Spectra Max M3, Molecular Devices®, Sunnyvale, CA, USA). The concentration of R-123 in each experimental sample was calculated from a standard curve, determined using a range of R-123 concentrations. Each experiment consisted of duplicate incubations for each treatment. Three separate experiments were performed and data (n = 3 for each treatment) were log10-transformed and analysed using repeated measures ANOVA followed by Fischer’s LSD (GraphPad Prism, version 6.01).

4.3.5 Larval migration assay (LMA)

LMAs were used to measure the changes in tolerance of Kirby and WAL L3 to IVM and LEV following pre-exposure to MPL for 3 or 6 h (MPL exposure as described in section 4.3.2). We were unable to assess whether MPL pre-exposure resulted in tolerance to MPL itself as this drug does not inhibit larval migration, even at high concentrations, and is therefore not suitable for use in LMAs (Raza et al., 2016b). The assay was performed following the procedure as reported by Raza et al. (2016a). This method was based on that described earlier by Kotze et al. (2006), except with the drug exposure and migration periods reduced from 24-48 h to 30 minutes each in order to allow for assessment of migration inhibition without providing any time for drug-induced gene expression changes to occur during the time course of the migration assay itself.

Briefly, MPL pre-treated (2.5 µg/mL and 250 µg/mL) or control (pre-exposure to 1% DMSO only) larvae (3800 L3/ mL of water) were exposed to a range of IVM and LEV concentrations in 96-well microtiter plates for 30 min at 27°C. Final concentrations for IVM and LEV ranged from 100-0.195 µg/mL and 12.5-0.024 µg/mL, respectively. After 30 min, the larvae were collected using a multi-tip pipette and placed into migration plates with 20 µm filters above receiver plate wells (Millipore, Bayswater, Australia). The larvae were allowed to migrate through the filters for a period of 30 minutes, before the filter plates were removed and the larvae in the receiver wells killed using Lugol’s iodine, and counted.

Migration assays consisted of triplicate assay wells at each IVM or LEV concentration. Three separate experiments were performed for each isolate. Data were analysed using non-linear regression in GraphPad Prism (version 6.01). IC50 values and 95% confidence intervals were calculated based on the pooled data from each set of nine assays, and significant differences were determined by the overlap of 95% confidence intervals.

The dose response to IVM in the short-term migration assays used in the present study showed the presence of a plateau in the response at the highest drug concentrations, as described
previously by Raza, et al. (2016a). The percent migration remained at a constant level (above 0%) over the highest 2 to 3 drug concentrations. Hence, for the analysis of the IVM dose-response data, we used a non-normalised model in GraphPad (‘top to bottom’), with a variable slope. The output of this analysis provided us with two parameters with which to compare populations: firstly, the percent migration at the dose-response plateau (that is, the percentage of the population unaffected by the highest concentrations of IVM in the assay), and secondly, the IC$_{50}$ in the remaining proportion of the population that had shown a dose-response to the drug (for instance, if the plateau existed at a level of 30% migration, then the IC$_{50}$ value defined the response to the drug in the remaining 70% of the worm population alone). In addition, to further determine the significant differences between the percentage of larval migration following exposure to DMSO and MPL, the percent migration in MPL-pre-exposed compared to DMSO-pre-exposed worms at each IVM concentration were compared using a $t$-test with Welch’s correction (GraphPad Prism).

4.4 Results

4.4.1 Transcriptional response of ABC transporters to MPL exposure in Kirby larvae

Figure 4.1 shows the transcription patterns of ABC transporters following exposure of Kirby L3 to MPL at 2.5 µg/mL (panels A, B and C) or 250 µg/mL (panels D, E and F) for 3, 6 and 24 h, relative to control larvae (exposed to DMSO only). The fold changes in expression levels in response to 250 µg/mL MPL (relative to DMSO-treated controls) are shown in Table 4.1. There were instances of increased and decreased expression of transporter genes across the various time points in MPL-treated L3 compared to controls, with more instances of increased rather than decreased expression levels, and with increases being generally of a greater magnitude (fold change) than the decreases. (ANOVA: 2.5 µg/mL, 3 h $F_{(16, 32)} = 3.227$, $P = 0.0023$, 6 h $F_{(16, 32)} = 1.070$, $P = 0.4191$, 24 h $F_{(16, 32)} = 2.509$, $P = 0.0131$; 250 µg/mL, 3 h $F_{(16, 32)} = 5.224$, $P < 0.0001$, 6 h $F_{(16, 32)} = 3.037$, $P = 0.0036$, 24 h $F_{(16, 32)} = 69.82$, $P < 0.0001$). The transcriptional responses to MPL 250 µg/ml were greater than for MPL 2.5 µg/ml, in terms of both the number of significant changes, and their magnitude. The only instance of significant gene upregulation following treatment with MPL 2.5 µg/ml was a 1.7-fold increase in transcription level of pgp-11 ($P = 0.002$) at 3 h (Fig. 4.1A). There were no significant changes in transcription patterns of any of the transporter genes after 6 h exposure to MPL 2.5 µg/ml. (Fig. 4.1B); whereas, after 24 h exposure, there was significant down-regulation of pgp-9.1 (1.5-fold; $P = 0.01$) and pgp-11 (1.6-fold; $P = 0.007$) (Fig. 4.1C).

Following exposure to MPL 250 µg/ml for 3 and 6 h, pgp-12 transcription was increased by 5.4-fold ($P = 0.007$) and 9.1-fold ($P = 0.002$), respectively, while pgp-14 showed 8.7-fold ($P = 0.002$) and 4.6-fold ($P = 0.03$) increases, respectively (Figs. 4.1D and 4.1E) (Table 4.1). Exposure to
MPL250 µg/mL for 3 h resulted in significant down-regulation of pgp-2 and haf-6 by 3.4-fold (P = 0.002) and 1.7-fold (P = 0.02), respectively. These decreases in transcription were short-lived, as, by 6 h, transcription levels for these two genes had returned to levels equivalent to DMSO-controls (Figs. 4.1D and 4.1E). Four P-gp genes and mrp-1 were significantly upregulated (P < 0.0001) following 24 h exposure to MPL250 µg/mL; the increases were as follows: 8.8-fold for pgp-11, 2.4-fold for pgp-14, 2.3-fold for pgp-12, 2.2-fold for pgp-2 and 1.8-fold for mrp-1. In addition, there were some instances of down-regulation, with pgp-10 (1.6-fold, P < 0.0001), pgp-16 (2-fold, P < 0.0001) and mrp-5 (1.4-fold, P = 0.009) showing significantly decreased transcriptions relative to DMSO controls (Fig. 4.1F) (Table 4.1). The most consistent changes in larval gene expression following exposure to MPL250 µg/mL were the increased transcription of pgp-12 and pgp-14 at each of the three time points. There was a single instance of inconsistent changes in gene expression values between the two MPL concentrations: after 24 h exposure, pgp-11 showed an increased expression level in response to MPL250 µg/mL (8.8-fold), compared to a decrease in expression in response to MPL2.5 µg/mL (1.6-fold) (compare Fig. 4.1C to 4.1F).

### 4.4.2 Transcriptional response of ABC transporters to MPL exposure in WAL larvae

The effects of MPL on transcription of ABC transporters in WAL larvae are shown in Figure 2 and Table 1 (ANOVA: 2.5 µg/mL, 3 h F_{16,32} = 1.793, P = 0.0782, 6 h F_{16,32} = 1.057, P = 0.4305, 24 h F_{16,32} = 7.058, P < 0.0001; 250 µg/mL, 3 h F_{16,32} = 5.789, P < 0.0001, 6 h F_{16,32} = 4.831, P < 0.0001, 24 h F_{16,32} = 71.99, P < 0.0001). Exposure of WAL L3 to MPL2.5 µg/mL did not result in significant upregulation of any of the transporter genes (Figs. 4.2A to 4.2C). On the other hand, there were a number of instances of significant down-regulation at 3 and 24 h (Figs. 4.2A and 4.2C). The magnitude of these decreases at 3 h were as follows: 1.4-fold for pgp-1 and abcf-1 (P = 0.02; P = 0.04), and 1.3-fold for pgp-10 and haf-6 (P = 0.04; P = 0.03). These decreases in gene transcription were short lived as they had returned to levels equivalent to controls by 6 h exposure (Fig. 4.2B). Following 24 h MPL2.5 µg/mL exposure, pgp-2 (1.4-fold; P = 0.02), pgp-9.1 (1.6-fold; P = 0.002), pgp-11 (2.6-fold; P < 0.0001) and abcf-1 (1.4-fold; P = 0.03) showed significantly decreased transcription relative to DMSO controls (Fig. 4.2C).

In contrast, exposure of WAL larvae to MPL250 µg/mL for 3 h resulted in significantly increased transcription of five P-gp genes and the haf-6 (Fig. 4.2D) (Table 4.1). Of note here was the greater number of up-regulated genes in WAL compared to Kirby isolate following 3 h exposure to MPL (compare Figs 4.1D and 4.2D). The magnitudes of the increases for WAL were as follows: 7.9-fold for pgp-2 (P = 0.002), 8.4-fold for pgp-9.1 (P = 0.002), 11.7-fold for pgp-11 (P = 0.0004), 9.4-fold for pgp-12 (P = 0.001), 12.2-fold for pgp-14 (P = 0.0002) and 20.5-fold for haf-6 (P = 0.0007).
There were no instances of significant down-regulation at the 3 h and 6 h time points. The increased transcription of pgp-2, pgp-9.1 and haf-6 genes observed at 3 h was short lived as it had returned to DMSO control levels by the 6 h time point for these three genes (Figs. 4.2D and 4.2E) (Table 4.1). In contrast, the up-regulation of pgp-11, pgp-12 and pgp-14 was maintained at the 6 h time point; pgp-11 7.4-fold ($P = 0.0007$), pgp-12 8.2-fold ($P = 0.001$), and pgp-14 5.5-fold ($P = 0.002$). These increases also occurred at the 24 h time point; pgp-11 6.9-fold ($P < 0.0001$), pgp-12 2.9-fold ($P < 0.0001$) and pgp-14 2.6-fold ($P < 0.0001$). For each of these genes, the magnitude of fold change in expression relative to controls decreased over the sequential time points from 3 to 24 h (for example from 12.2-fold to 2.6-fold for pgp-14). Following 24 h exposure to MPL, transcription of pgp-9.3, pgp-10, pgp-16 and mrp-5 were significantly down-regulated at levels of 1.4-fold ($P = 0.003$), 1.7-fold ($P < 0.0001$), 1.3-fold ($P = 0.02$) and 1.3-fold ($P = 0.03$), respectively (Fig. 4.2F).

There were several instances of inconsistent changes in gene expression values between the two MPL concentrations, with expression reduced at the lower MPL concentration and increased at the higher concentration for haf-6 at 3 h, as well as pgp-2 and pgp-11 at 24 h. As described above, this difference in response to the two MPL concentrations was also observed for Kirby with pgp-11 at 24 h.

Overall, for WAL larvae, the most marked effects of MPL exposure were the significant up-regulation of a number of transporter genes at the 3 h time point for MPL$_{250\ \mu g/mL}$, and the sustained nature of these increases for pgp-11, pgp-12 and pgp-14, alongside the absence of any up-regulation by MPL$_{2.5\ \mu g/mL}$. This pattern was very similar to that seen with Kirby larvae, with the exception of the unchanged expression for pgp-11 at 3 h for the Kirby larvae (Table 4.1).

The stability of the observed transcriptional changes was examined by exposing WAL L3 to MPL$_{250\ \mu g/mL}$ for 3 h, rinsing the larvae in water, and then maintaining them in the absence of drug for a further 24 h before recovery for analysis of ABC transporter gene expression patterns. Figure 3 shows that of the gene expression changes measured in WAL L3 after 3 h exposure to MPL (from Fig. 4.2D), only the increased expression of pgp-11 was maintained in these larvae after 24 h in the absence of any drug.

Over this period, the fold increase in this gene in MPL-treated larvae compared to controls decreased from 11.7-fold ($P = 0.0004$) to 3-fold ($P = 0.004$) (compare Figs. 4.2D and 4.3). There was some variability in the expression patterns of pgp-9.1 (as indicated by the large SEM bars), however, statistical analysis across the three replicate experiments showed that the expression level of this gene was not significantly different to controls.
Fig. 4.1. Effect of monepantel on transcription patterns of ABC transporter genes in *Haemonchus contortus* Kirby isolate. Larvae were exposed to MPL at two concentrations for range of time periods: MPL$_{2.5\,\mu g/mL}$ (A at 3 h, B at 6 h and C at 24 h) or MPL$_{250\,\mu g/mL}$ (D at 3 h, E at 6 h and F at 24 h). Y-axis shows fold-change in gene expression levels in drug-treated larvae vs DMSO-treated controls. Data shown as mean ± SEM, $n = 3$ separate experiments each with four technical replicates. Significant differences in gene transcription levels are indicated by * ($P < 0.05$).
Table 4.1. Relative transcription levels of ABC transporter genes in the 250 µg/ml MPL-treated Kirby and WAL larvae compared to DMSO-treated controls. Significant ($P < 0.05$) increases or decreases in transcription in the MPL-treated larvae compared to the DMSO-treated larvae are indicated by * and #, respectively.

<table>
<thead>
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</tr>
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<td>Pgp 11</td>
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</tr>
<tr>
<td>Pgp 12</td>
<td>5.4*</td>
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</tr>
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</tbody>
</table>

# denotes decreased gene transcription following MPL exposure compared to DMSO-treated controls

* denotes increased gene transcription following MPL exposure compared to DMSO-treated controls
Fig. 4.2. Effect of monepantel on transcription patterns of ABC transporter genes in *Haemonchus contortus* WAL isolate. Larvae were exposed to MPL at two concentrations for range of time periods: MPL \(2.5 \mu g/mL\) (A at 3 h, B at 6 h and C at 24 h) or MPL \(250 \mu g/mL\) (D at 3 h, E at 6 h and F at 24 h). Y-axis shows fold-change in gene expression levels in drug-treated larvae vs DMSO-treated controls. Data shown as mean ± SEM, \(n = 3\) separate experiments each with four technical replicates. Significant differences in gene transcription levels are indicated by * \((P < 0.05)\).
Fig. 4.3. Stability of transporter gene transcription following 3h pre-exposure of WAL larvae to monepantel. Larvae were exposed to MPL_{250\mu g/mL} for 3 h, rinsed in water and maintained for 24 h with no drug. Y-axis shows fold-change in gene expression levels in drug-treated vs DMSO-treated larvae. Data shown as mean ± SEM, n = 3 separate experiments each with four technical replicates. Significant differences in transcription levels are indicated by * (P = 0.05).
4.4.3 Phenotypic characterization of the MPL-treated larvae

4.4.3.1 Rhodamine-123 efflux assay

The functional consequences of exposure to MPL were evaluated by measuring the ability of MPL pre-treated Kirby and WAL L3 to efflux the fluorescent dye R-123 (Fig. 4.4). (ANOVA: Kirby 3 h $F_{(2, 4)} = 12.51$, $P = 0.0190$, 6 h $F_{(2, 4)} = 6.004$, $P = 0.0624$; WAL 3 h $F_{(2, 4)} = 4.148$, $P = 0.1058$, 6 h $F_{(2, 4)} = 9.691$, $P = 0.0293$). Exposure of Kirby L3 to MPL2.5 µg/mL and MPL250 µg/mL for 3 h resulted in increased R-123 efflux compared to controls (1.2-fold, $P = 0.009$, and 1.3-fold, $P = 0.017$, respectively) (Fig. 4.4A). By 6 h, increased efflux was still recorded for worms exposed to MPL250 µg/mL (1.2-fold, $P = 0.027$), whereas the efflux had returned to control levels for the MPL2.5 µg/mL treatment (Fig. 4.4B). For WAL larvae, increased efflux was observed for MPL250 µg/mL at both time points (1.2-fold, $P = 0.045$ at 3 h, and 1.2-fold, $P = 0.011$ at 6 h), while efflux was unchanged for the MPL2.5 µg/mL treatment at both time points (Figs. 4.4C and 4.4D).

The levels of R-123 efflux were equivalent in Kirby and WAL L3 in the absence of any drug exposure (DMSO-treated) at both 3 and 6 h ($P = 0.24$ and $P = 0.12$) (data not shown).
Fig. 4.4. Rhodamine efflux from larvae following monepantel pre-exposure. R-123 efflux was measured after exposure of L3 stage larvae to MPL$_{250\mu g/mL}$ or MPL$_{2.5\mu g/mL}$, compared to DMSO-treated controls; Kirby isolate: A at 3 h and B at 6 h; WAL isolate: C at 3 h and D at 6 h. Significant increase in R-123 efflux compared to controls is indicated by *. The columns represent mean ± SEM, $n = 6$ (pooled data from three separate experiments, each with assays in duplicate).
4.4.3.2 Larval migration assay

The functional consequences of changes in transcription of transporter genes following exposure to the two concentrations of MPL were further examined using migration assays. Following 3 h exposure to MPL$_{2.5 \, \mu g/mL}$, there were no significant changes to the tolerance of both Kirby and WAL L3 to IVM and LEV, both in terms of the IVM IC$_{50}$ values and the percent migration at the dose response plateau at the high IVM concentrations (data not shown). Given, this lack of effect of 3 h exposure to MPL$_{2.5 \, \mu g/mL}$, alongside the absence of gene expression changes in 6 h MPL$_{2.5 \, \mu g/mL}$-treated Kirby and WAL larvae (Figs. 4.1B, 4.2B), we did not measure the response of 6 h MPL$_{2.5 \, \mu g/mL}$ pre-exposed larvae to IVM or LEV.

In contrast to the 3 h MPL$_{2.5 \, \mu g/mL}$ data, exposure to MPL$_{250 \, \mu g/mL}$ for 3 or 6 h resulted in significant changes in the IVM dose response curves compared to larvae pre-treated with DMSO alone (Fig. 4.5). The MPL$_{250 \, \mu g/mL}$-treated larvae showed a greater ability to migrate at high IVM concentrations compared to control larvae. This was demonstrated in two ways, first, the significantly higher IVM dose-response plateau for MPL pre-treated larvae compared to controls for WAL larvae at 3 h, and both isolates after 6 h pre-treatment (WAL 3 h 33.79 vs 17.60%; Kirby 6 h 27.08 vs 4.27%; WAL 6 h 37.05 vs 16.75%) (Table 4.2); and secondly, the t-test analysis showing that the percent migration at the highest IVM concentrations was consistently greater ($P < 0.05$) than the percent migration at the same IVM concentration for controls (Fig. 4.5).

In contrast to these changes in response to the highest IVM concentrations, the response of the remaining worm population did not change significantly, as shown by equivalent IC$_{50}$ values, and a lack of statistical differences in percent migration at individual IVM concentrations (t-test analysis) (Table 4.2, Fig. 4.5). That is, the IVM tolerance was only observed in a component of the worm population. The only exception to this, in terms of the t-test analysis, was the decreased migration for MPL treated larvae at an IVM concentration of 0.2 $\mu g/mL$ for WAL larvae (Fig. 4.5C). There were no significant changes in LEV IC$_{50}$ values for these MPL$_{250 \, \mu g/mL}$-treated larvae compared to DMSO controls (Fig. 4.6A).

WAL larvae that had been exposed to MPL$_{250 \, \mu g/mL}$ for 3 h, then washed and held in water for a further 24 h, before being exposed to a range of IVM concentrations in LMAs, showed a similar pattern of IVM tolerance to that described above (Fig. 4.6). The IVM dose-response plateau was increased significantly (from 14.6 to 31.8%), the t-test analysis showed significant differences at the four highest IVM concentrations, and there was no change in the IC$_{50}$ (Fig. 4.6A). There were no significant changes in LEV IC$_{50}$ values for these MPL$_{250 \, \mu g/mL}$-treated larvae compared to DMSO controls (Fig. 4.6B).
Fig. 4.5. IVM sensitivity in Kirby and WAL L3 stage larvae following monepantel pre-exposure. Sensitivity to IVM was measured using migration assays following pre-exposure to MPL_{250µg/mL} in Kirby (A after 3 h pre-exposure and B after 6 h pre-exposure) and WAL (C at 3 h pre-exposure and D at 6 h pre-exposure) L3 stage larvae, compared to L3 pre-exposed to DMSO only. DMSO pre-exposure shown with solid lines and closed symbols, MPL pre-exposure shown with dashed lines and open symbols. The data points marked with * showed significant differences ($P < 0.05$) in percent migration between MPL- and DMSO- pre-exposure groups ($t$-test). Each data point represents mean ± SEM, $n = 9$ (pooled data from three experiments, each with assays in triplicate).
Fig. 4.6. Stability of IVM tolerance in WAL L3 stage larvae pre-treated with monepantel. Sensitivity of WAL L3 stage larvae to IVM (A) or LEV (B) was measured using migration assays following pre-exposure for 3 h to MPL$_{250\mu g/mL}$, followed by 24 h in water only, compared to L3 exposed to DMSO for 3 h, followed by water for 24 h. DMSO pre-exposure shown with solid lines and closed symbols, MPL pre-exposure shown with dashed lines and open symbols. The data points marked with * showed significant differences ($P < 0.05$) in percent migration between MPL- and DMSO- pre-exposure groups ($t$-test). Each data point represents mean ± SEM, $n = 9$ (pooled data from three experiments, each with assays in triplicate).
Table 4.2. Response of *Haemonchus contortus* larvae to ivermectin following pre-exposure to monepantel

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Drug exposure</th>
<th>3 h pre-exposure period</th>
<th>Dose-response plateau</th>
<th>6 h pre-exposure period</th>
<th>Drug/DMSO</th>
<th>IC(_50)</th>
<th>95% CI</th>
<th>Drug/DMSO</th>
<th>IC(_50)</th>
<th>95% CI</th>
<th>Drug/DMSO</th>
<th>IC(_50)</th>
<th>95% CI</th>
<th>Drug/DMSO</th>
<th>IC(_50)</th>
<th>95% CI</th>
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<tr>
<td>Kirby</td>
<td>DMSO</td>
<td>1.6</td>
<td>1.3–1.9</td>
<td>7.3</td>
<td>2.3–12.3</td>
<td>–</td>
<td>–</td>
<td>4.3</td>
<td>-0.06–8.6</td>
<td>–</td>
<td>4.3</td>
<td>-0.06–8.6</td>
<td>–</td>
<td></td>
<td></td>
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<td></td>
<td>MPL</td>
<td>2.5</td>
<td>1.6–3.8</td>
<td>18.3</td>
<td>7.5–29.2</td>
<td>2.5</td>
<td>18.3</td>
<td>7.5–29.2</td>
<td>2.5</td>
<td>18.3</td>
<td>7.5–29.2</td>
<td>2.5</td>
<td>18.3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>DMSO</td>
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<td>17.6</td>
<td>12.7–22.5</td>
<td>–</td>
<td>17.6</td>
<td>12.7–22.5</td>
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<td>17.6</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MPL</td>
<td>6.1</td>
<td>4.2–8.9</td>
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<td>33.8*</td>
<td>25.8–41.8</td>
<td>1.9*</td>
<td>33.8*</td>
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<tr>
<td>WAL</td>
<td>DMSO</td>
<td>1.4</td>
<td>1.2–1.7</td>
<td>4.3</td>
<td>-0.06–8.6</td>
<td>–</td>
<td>4.3</td>
<td>-0.06–8.6</td>
<td>–</td>
<td>4.3</td>
<td>-0.06–8.6</td>
<td>–</td>
<td>4.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MPL</td>
<td>0.9</td>
<td>0.5–1.5</td>
<td>27.1*</td>
<td>18.1–36.1</td>
<td>6.3*</td>
<td>27.1*</td>
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<td>27.1*</td>
<td>18.1–36.1</td>
<td>6.3*</td>
<td>27.1*</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>2.8</td>
<td>2.4–3.3</td>
<td>16.8</td>
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<td>16.8</td>
<td>10.9–22.6</td>
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<td>2.2*</td>
<td>37.1*</td>
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<td></td>
</tr>
</tbody>
</table>

\(a\) Within either the 3 or 6 h pre-exposure data sets, * denotes that the IC\(_50\) following pre-exposure to anthelmintic was significantly higher than the IC\(_50\) following pre-exposure to DMSO, as determined by non-overlap of 95% Confidence Intervals.

\(b\) Drug/DMSO = IC\(_50\) for IVM following pre-exposure to MPL/IC\(_50\) for IVM following pre-exposure to DMSO.

\(c\) Within either the 3 or 6 h pre-exposure data sets, * denotes that L3 migration at the dose-response plateau (%) following pre-exposure to MPL was significantly higher than L3 migration (%) following pre-exposure to DMSO, as determined by non-overlap of 95% Confidence Intervals.

\(d\) Drug/DMSO = L3 migration (%) for IVM following pre-exposure to MPL/ L3 migration (%) for IVM following pre-exposure to DMSO.
4.5 Discussion

In this study, we have demonstrated that exposure of *H. contortus* L3 to a high concentration of MPL (250 µg/mL) results in increased transcription of a number of ABC-transporter genes. Furthermore, exposure to MPL at this concentration results in greater efflux of R-123 from the L3, as well as increased tolerance to IVM in a proportion of the larval population. In contrast, pre-exposure to a 100-fold lower concentration of the compound (2.5 µg/mL) only resulted in a single instance of low-level up-regulation of a transporter gene (*pgp-11*), an increase in R-123 efflux in only one out of four isolate/time point instances, and did not result in any IVM tolerance in subsequent migration assays. Hence, the effects of MPL on gene transcription, R-123 efflux and subsequent IVM tolerance were dependent on the concentration of the compound to which the larvae were exposed. This study is the first, to our knowledge, to indicate an interaction between MPL and ABC transporters in nematodes. However, our data describe the effects of MPL on the regulation of ABC transporter gene expression rather than a direct effect of the drug on the transporter proteins themselves. The demonstration of functional consequences of the gene transcription increases, in terms of R-123 efflux and IVM tolerance, indicates that the transcription increases most likely result in increased transporter protein activity towards R-123 and IVM. Hence, the present study provides evidence of an interaction of MPL with the regulatory mechanism for ABC transporter gene expression, and subsequent protein synthesis, rather than evidence as to whether MPL itself is a substrate for the transporters.

As described above, the transporter gene upregulation/R-123 efflux/IVM tolerance relationships were only observed at the high MPL concentration. This concentration was well above the levels that an adult worm would encounter in the abomasum of a sheep, as measured by Lifschitz et al. (2014) to be 2-4 µg/g of abomasal content 48 hrs after administration of the drug. On the other hand, the lower concentration of MPL used in the present study, which was selected based on it approximating this *in vivo* concentration range, resulted in only one instance of transporter gene up-regulation, and did not result in any IVM tolerance. It is clear that while the present study indicates an interaction between MPL at 250 µg/mL the regulatory mechanism for ABC transporter transcription in *H. contortus*, it does not provide evidence for such a role in *in vivo*. Further experiments would be needed to determine if MPL interacts with transporter in adult worms *in vivo*.

While the patterns of upregulation in Kirby and WAL L3 were quite similar at 6 and 24 h time points, the WAL larvae showed a much greater level of upregulation (in terms of fold increases as well as the number of transporters affected) at the 3 h time point compared to Kirby (compare Figs. 4.2D and 4.1D). Our earlier study reported the upregulation of ABC transporters in
the WAL isolate after exposure to IVM and LEV, alongside no changes in Kirby at the 3 and 6 h time points examined in that study Raza et al. (2016a). This suggested that the rapid upregulation in response to these two drugs in the WAL isolate may be a component of the resistance shown by this isolate to the two anthelmintics. WAL and Kirby are both susceptible to MPL (Raza et al., 2016b), having been isolated from the field before the use of this drug. The present study therefore indicates that this increased responsiveness of WAL larvae also occurs with respect to its response to drugs to which it is not resistant, and to which it has never been exposed. Thus, if the rapid upregulation following exposure to IVM and LEV is a component of the resistance shown towards these drugs as suggested by Raza et al. (2016a), then the present study suggests that this increased responsiveness may be quite general in nature, extending to xenobiotics beyond the anthelmintics that were involved in the original resistance-selection process in the field. Importantly though, while WAL larvae respond more rapidly and to a greater extent than Kirby to MPL under our experimental conditions, the two isolates show equivalent sensitivities to the drug in larval development assays (Raza et al., 2016b). Hence, there is no evidence yet that the increased responsiveness in ABC transporters seen in WAL in the present study provides any subsequent protection against MPL; only protection against IVM has been suggested by our data.

The pattern of transcription changes over the time course of the present experiments was quite different to that observed in our earlier experiments with IVM and LEV (Raza et al., 2016a). In this earlier study, exposure to either of the two anthelmintics resulted in ABC transporter transcription increases in WAL larvae at the 3 h time point, however, these had returned to control levels either completely (for IVM) or almost completely (with the exception of one gene for LEV) by 6 h. In contrast, in the present study, the up-regulation response was sustained over the 24 h time period of the experiments. This difference between the two studies is most likely due to the relative concentrations of the drugs used. The concentrations were chosen on the basis of being the highest concentrations that could be examined without effecting the ability of the larvae to migrate in LMAs in the second phase of the drug-exposure experiments, thereby allowing for the consequences of drug exposure to be assessed using LMAs. As MPL is far less potent as an inhibitor of larval migration than IVM and LEV (Kotze et al., 2006; Raza et al., 2016b), the higher of the two concentrations used in the present study was much higher than those used previously by Raza et al. (2016a) for IVM and LEV (250 µg/mL compared to 0.2-0.8 µg/mL).

It has been suggested that nematodes may use gene upregulation as a mechanism to counter the toxic effects of anthelmintics (Ardelli and Prichard, 2013). There is considerable evidence that anthelmintics act as substrates of ABC transporters and have inducing effects on the expression levels of transporter genes in nematodes and mammals (James and Davey, 2009; Menez et al.,
However, nematode transporter genes do not show a consistent pattern of upregulation following exposure to anthelmintics. Significantly increased transcription of several P-gp genes was reported in wild-type and IVM-resistant isolates of *Caenorhabditis elegans* post-exposure to moxidectin (Bygarski et al., 2014). On the other hand, exposure of the same isolates of *C. elegans* to IVM resulted in upregulation of a different set of P-gp genes (Ardelli and Prichard, 2008). Some studies on parasitic nematodes have reported increases in transcription of transporter genes following *in vitro* and *in vivo* exposure to IVM (Dicker et al., 2011; De Graef et al., 2013; Tydén et al., 2014), whereas no transcriptional changes were observed in IVM-resistant *H. contortus* and *Cooperia oncophora* worms collected from animals treated with IVM compared to the worms collected from untreated control animals (Areskog et al., 2013; Alvarez et al., 2015).

We also examined the stability of the observed transcriptional changes by exposing WAL L3 to MPL-250 µg/mL for 3 h, rinsing the larvae in water, and then maintaining them in the absence of drug for a further 24 h. We found that only the transcription level of *pgp-11* remained at significantly higher levels compared to control larvae. We also observed increased tolerance to IVM in a proportion of the WAL larval population 24 h after the removal of MPL following a 3 h pre-treatment period (see Fig. 4.6). There is very little information available on the time course of ABC transporter gene expression patterns after the removal of the inducing agent. Fardel et al. (1996) reported that expression of P-gp (*mdr-1*) in rat liver epithelial cells as measured by qPCR and Western blotting, was significantly higher following exposure to 3-methylcholanthrene for 24 h, with a return to almost basal levels 72 h after removal of the inducing agent. The sustained upregulation of *pgp-11* alone after removal of the MPL in the present study, suggests that this specific ABC transporter may play a more important role in interaction with MPL in WAL larvae than the other transporters. On the other hand, the maintenance of IVM tolerance 24 h after removal of the MPL cannot be linked specifically to the sustained upregulation of *pgp-11* as the tolerance may be due to increased efflux activity of any one of the other ABC transporter proteins that may be present at higher levels as a result of the earlier period of gene upregulation (from Fig. 4.2D).

The presence of plateaus in IVM dose-response curves at the highest IVM concentrations was most likely due to the short nature of both the drug incubation and migration phases (30 minutes for each) of the LMA compared to the 48 and 24 h incubation/migration periods usually used for this assay (Kotze et al., 2006). As described earlier, the assay was modified to avoid any effects of the drugs in possibly inducing transcription of the transporter genes during long (24 and 48 h) incubation periods. These plateaus were likely due to the subsequent inability of IVM to inhibit migration completely in all larvae in the short 30 minute time frame, and were also reported in our earlier study (Raza et al., 2016a). Tolerance to IVM only occurred in the proportion of the
population represented by the dose-response plateau, as the IC$_{50}$ in the remainder of the population was unchanged compared to DMSO pre-treated L3 (see Table 4.2). A proportion of the larvae were clearly better equipped to tolerate IVM following MPL$_{250}$ µg/mL exposure (as indicated by percent migration at plateau and t-test analysis), while the remainder of the population showed no change in IVM sensitivity. Although we have no direct evidence for a role of transporters in the observed IVM tolerance, the known role for ABC transporters in IVM tolerance (Lespine et al., 2012) in parallel with our gene up-regulation/R-123 efflux increases/IVM tolerance observations in the present study, suggest that the MPL-induced transporters were able to subsequently provide protection against IVM in a proportion of the larval population in our experiments. Hence, our data demonstrate the potential role of the transporters in responding to exposure to an anthelmintic, and, in so doing, provide protection against structurally-unrelated compounds. The fact that IVM tolerance was only observed in a proportion of the worm population following MPL pre-exposure may indicate a degree of heterogeneity in the effect of MPL exposure on ABC transporter gene expression across the worm population, as was also suggested by the similar IVM dose-response plateau versus IC$_{50}$ effects following pre-exposure to IVM or LEV in our previous study (Raza et al., 2016a).

There were no significant changes in LEV dose-response experiments following exposure to MPL at both concentrations, suggesting that the transporters induced in response to MPL exposure are able to subsequently provide protection against IVM but not LEV. Raza et al. (2016a) previously showed that pre-exposure to IVM or LEV resulted in tolerance to IVM, alongside no change in the response to LEV. Hence, the earlier and present studies provide evidence to suggest that IVM may be a better substrate than LEV for *H. contortus* ABC transporters, as has been reported for mammalian P-gps (Efferth and Volm, 1993).

### 4.6 Conclusions

In conclusion, the present study has shown that exposure to a high concentration of MPL increases the transcription of multiple ABC transporter genes in two MPL-susceptible isolates of *H. contortus*. The most significant interactions were for *pgp-11*, *pgp-12* and *pgp-14*, based on (i) the magnitude of the transcriptional response, (ii) the occurrence of this response across multiple time points, (iii) the consistency of the response between the two isolates, and (iv) the stability of the response upon removal of the MPL (applicable to *pgp-11* only). The subsequent ability of a proportion of MPL-exposed L3 to tolerate higher levels of IVM provides further evidence that ABC transporters play an important role in protection of worms against this anthelmintic, and illustrates the ability of ABC transporters to interact with different chemical entities. However, while the study
describes interactions of ABC transporters with a high concentration of MPL in vitro, the effects of MPL on efflux pathways in vivo remains to be determined.
### Supplementary Tables and Figure

**Supplementary** Table 4.3. Primer sequences of housekeeping and ABC transporter genes used for the quantitative PCR used in this study.

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Sequences</th>
<th>Product size</th>
<th>Amplification Efficiency %</th>
<th>Source</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Housekeeping genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| 1   | *Hc* GAPDH | F: TGGGTGTGAACCACGAGAC  
R: GCAGCACCACGCTCCATCA | 213 | 91.17 | Sarai et al. (2013) |
| 2   | *Hc* Actin | F: GAGTCATGTTGGTATGGGAC  
R: GGAGCTTCGGTCGTAAGTACG | 140 | 88.80 | Sarai et al. (2013) |
| 3   | *Hc* β-Tubulin | F: GCTTCCGCACCTTTGAAACTC  
R: TGAAGACGAGGGAATGGAAC | 160 | 88.41 | This study |
| **ABC transporter genes** |        |           |              |                             |        |
| 4   | P-gp 1 | F: CCACATGCAGCCCAACCTTTTAG  
R: AGACGACTCCAGCTAGTTTCCG | 145 | 84.67 | Sarai et al. (2013) |
| 5   | P-gp 2 | F: GGACAAAAACGACGAGATGGGC  
R: ACAGACGATGCCTCAACTAGC | 169 | 95.73 | Sarai et al. (2013) |
| 6   | P-gp 3 | F: CCGGCAACTTTGACTCTCAAGGC  
R: TCACTTTGCTCTTCCCCGCAAC | 94 | 88.02 | Sarai et al. (2013) |
| 7   | P-gp 9.1 | F: TCGACGGAATCAAGAAATC  
R: GCCCATCATTACGGAGAAGA | 168 | 79.72 | This study |
| 8   | P-gp 9.2 | F: CCAGTCCACTCAATTCCAC  
R: AACCGCTACGCTCTCTCT | 65 | 93.82 | Issouf et al. (2014) |
| 9   | P-gp 9.3 | F: AGAAACACCGAATCGCAGCT  
R: TGTTGGCCAACAGATAAGGT | 157 | 82.54 | This study |
| 10  | P-gp 10 | F: TCAGAAAGATTATGCACCGACG  
R: CAGGCTTGAAGAGTTCGTAATG | 98 | 88.94 | Sarai et al. (2013) |
| 11  | P-gp 11 | F: ACCACGAAGCAGTGAACGAGAA  
R: CACCAGATGTCAGCCGTC | 150 | 93.20 | This study |
| 12  | P-gp 12 | F: TGATGGTACCAACGCAAAGG  
R: ATGCGGATACGCTCTCTCT | 111 | 93.88 | Sarai et al. (2013) |
| 13  | P-gp 14 | F: GCATTGTCGAGGACATCTG  
R: GCTTTGGAACGAAATGGO | 160 | 89.60 | Sarai et al. (2013) |
| 14  | P-gp 16 | F: AAAAGCGGACAGAAGTGCAGA  
R: TGTTGGTACCATTAGCTTG | 165 | 99.03 | Issouf et al. (2014) |
| 15  | MRP 1 | F: GCCCGATTGTTGTTACTTC  
R: TTTCTCAAGGCTGCTGT-C | 125 | 90.12 | This study |
| 16  | MRP 5 | F: TGTCGGTGAAGCAGGGAAGTG  
R: GCACGGTAAGCAGAAATAGA | 125 | 90.28 | This study |
| 17  | HAF-6 | F: CAATCAAAACCCAGCGGTACAA  
R: CACACAGGCTTGCAAAACAG | 250 | 80.62 | This study |
| 18  | ABCF-1 | F: AAGGTGTCCGCGCCCTAAGAT  
R: TCAGTATGGATGCGCTTGC | 146 | 83.92 | This study |
| 19  | ABCF-2 | F: AGTGTAGCCTITTGTGGTGTC  
R: TTTCAAGTGGGAGCTTCTCG | 157 | 92.00 | This study |
**Supplementary: Fig. 4.7.** Monepantel pre-exposure and LEV sensitivity in Kirby and WAL L3 stage larvae. Effects of MPL_{250µg/mL} pre-exposure on tolerance of Kirby (A after 3 h pre-exposure and B after 6 h pre-exposure) and WAL (C after 3 h pre-exposure and D after 6 h pre-exposure) L3 stage larvae to LEV in larval migration assays, compared to L3 pre-exposed to DMSO only. DMSO pre-exposure shown with solid lines and closed symbols, MPL pre-exposure shown with dashed lines and open symbols. Each data point represents mean ± Standard error of mean (SEM), n = 9 (pooled data from three experiments, each with assays in triplicate).
**Supplementary: Table 4.4.** Response of *Haemonchus contortus* larvae to levamisole following pre-exposure to monepantel

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Drug pre-exposure</th>
<th>3 hours Pre-exposure</th>
<th>6 hours Pre-exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC₅₀</td>
<td>IC₅₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Drug conc.ᵃ (µg/mL)</td>
<td>95% CI</td>
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<td>Kirby</td>
<td>DMSO</td>
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<td>MPL</td>
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<td>0.87-1.10</td>
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<td>WAL</td>
<td>DMSO</td>
<td>0.82</td>
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</tr>
<tr>
<td></td>
<td>MPL</td>
<td>0.64</td>
<td>0.56-0.75</td>
</tr>
</tbody>
</table>

ᵃ Within either the 3 or 6 h pre-exposure data sets, * denotes that the IC₅₀ following pre-exposure to anthelmintic was significantly higher than the IC₅₀ following pre-exposure to DMSO, as determined by non-overlap of 95% Confidence Intervals.

ᵇ Drug/ DMSO = IC₅₀ for LEV following pre-exposure to MPL/ IC₅₀ for LEV following pre-exposure to DMSO
CHAPTER 5

5. Monepantel resistance in *Haemonchus contortus* and ABC transporters

5.1 General introduction

During the course of this study, a monepantel-resistant isolate (MPL-R) became available. This offered opportunities for phenotypic characterisation of the resistance using *in vitro* worm bioassays, and for a study of the role of ABC transporters in the resistance shown by this isolate. The isolate was collected from a property in southwest Queensland, Australia, and represents a field-derived isolate in which only the survivors of a drench treatment have been propagated further in the laboratory.

There have been recent reports describing the emergence of resistance to monepantel (MPL) (Scott et al., 2013; Mederos et al., 2014; Bartley et al., 2015; Van den Brom et al., 2015). A recent study showed that a micro-agar larval development assay was able to describe a dose-response towards MPL in two isolates of *H. contortus* (Stuchlíková et al., 2016). The authors did not examine any reference isolate of known resistance status; hence, the ability of the assay to detect resistance remained unknown. The present chapter, therefore, aimed to examine the ability of the larval development and migration assays to differentiate between the MPL-R isolate and two isolates known to be susceptible to this drug. In addition, this chapter focused on studying the effects of MDRIs on the sensitivity of the MPL-R isolate to MPL by using a combination of third-generation MDRIs with MPL in larval development assays (as had been done in Chapter 2 with IVM and LEV). Moreover, given that Chapter 3 reports that several P-gps are expressed at higher levels in a multidrug resistant isolate of *H. contortus* (WAL) compared to a susceptible isolate, it was of interest to measure the expression patterns of ABC transporters in the MPL-R isolate. Hence in the present Chapter, the expression patterns of ABC transporters in the MPL-R isolate were compared with those of two MPL-susceptible isolates (Kirby and WAL).

This chapter consists of the following sections:

5.2 Larval development assays reveal the presence of sub-populations showing high- and low-level resistance in a monepantel (Zolvix®)-resistant isolate of *Haemonchus contortus*. Presented as published.
5.3. Effects of third-generation P-glycoprotein inhibitors on the sensitivity of a monepantel-resistant isolate of *Haemonchus contortus* to monepantel. This section comprises unpublished data.

5.4. Transcription patterns of ABC transporters in monepantel-resistant isolate compared to Kirby and Wallangra isolates of *Haemonchus contortus*. This section comprises unpublished data.

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5.2 Larval development assays reveal the presence of sub-populations showing high- and low-level resistance in a monepantel (Zolvix®)-resistant isolate of Haemonchus contortus

5.2.1 Abstract

Resistance to the amino-acetonitrile derivative monepantel has been reported in several species of gastrointestinal nematodes over recent years. We were interested in the use of in vitro assays with free-living worm life-stages to detect resistance to this drug. We therefore used larval development and larval migration assays to examine dose response relationships for the drug against two susceptible and one resistant isolate of Haemonchus contortus. The resistant isolate was established by laboratory propagation of the survivors of a field treatment with Zolvix® that had originally resulted in a drug efficacy of over 99%. Drug efficacy against this field –derived laboratory-propagated resistant isolate in vivo was approximately 15%. The larval development assay proved able to discriminate between the susceptible and resistant isolates, with larvae of the resistant isolate showing an ability to develop at higher drug concentrations than the two susceptible isolates. The resistant isolate showed the presence of two distinct subpopulations, separated by a plateau in the dose-response curve. Sub-population 1 (approximately 40% of the total population) showed a low level of resistance with an IC$\text{50}$ increased approximately 7-fold compared to the baseline susceptible isolate, while sub-population 2 (the remaining 60% of the total population) showed an IC$\text{50}$ increased over 1000-fold compared to the baseline susceptible isolate. This level of resistance is unusually high for any gastrointestinal nematode species in drug dose-response in vitro assays. In contrast, the migration assay could not discriminate between the three isolates, with migration not reduced to zero at any of the drug concentrations tested. This study demonstrates that a larval development assay is able to detect resistance to monepantel in H. contortus, and that resistance can exist in two distinct forms. This suggests that at least two separate monepantel resistance mechanisms are acting within the worm isolate studied here, with one or more mechanisms conferring a much higher level of resistance than the other(s).
5.2.2 Introduction

In vitro assays with the free-living larval life stages of gastrointestinal nematode parasites have been developed for measuring sensitivity to anthelmintics. These assays have advantages over in vivo resistance detection by the faecal egg count reduction test (FECRT) (Coles et al., 2006) as they are considerably cheaper and less laborious. The use of in vitro assays for resistance detection depends on the relative sensitivity of the larval stages of different worm isolates reflecting the relative sensitivity of the parasitic adult stages of those isolates to a particular anthelmintic. They also depend on an ability to define the in vitro drug concentration that elicits a particular response in the larval assays as indicative of the presence of resistance in adult worms. The ability of an assay to detect resistance when it exists at low levels is also very important, in terms of both percentage of resistant worms within an isolate, and the degree to which drug sensitivity is altered in the resistant worms.

A number of different assay formats have been described. These include assays measuring the effects of drugs on egg hatch (Le Jambre, 1976), the development of larvae from the egg to the infective larval stage (L3) (Gill et al., 1995), the motility of L3 (Gill et al., 1991), and the ability of L3 to migrate through a mesh (Demeler et al., 2010) or an agar / mesh system (Kotze et al., 2006). The different assays have application with different worm species, life stage and drug groups; for example, the egg hatch assay is useful for benzimidazole drugs and not macrocyclic lactones as only the former are able to penetrate the egg shell and hence prevent hatching; migration and motility assays work with macrocyclic lactones but not benzimidazoles as the latter do not affect worm movement significantly within the time frame of the assays. Drug uptake would be expected to occur via the pharynx as well as across the cuticle during development assays, while only the latter pathway would be relevant for migration and motility assays performed with non-feeding L3 stage larvae. Additionally, and importantly in terms of resistance detection, some of the assays are able to define a dose response relationship to a particular drug, but fail to detect the presence of resistance despite it being present in the parasitic adult stages (as demonstrated by FECRTs). This may be due to the fact that a specific resistance mechanism is expressed in parasitic adult stages but not in the free-living life stages, or may arise in instances where the resistance mechanism is indeed expressed in the larval stage under examination in the assay, however it does not affect the specific phenotype being measured (e.g. hatching, development or migration). An example of this lack of resistance detection in isolates known to be resistant as adult worms is provided by the contrast between the ability of both larval development and migration assays to detect resistance to macrocyclic lactones in *H. contortus* (Gill et al., 1995; Kotze et al., 2006) alongside the inability of both assays to detect resistance in *Teladorsagia circumcincta* (Kotze et al., 2006). Additionally,
some assays are far less sensitive than others with respect to resistance detection with a particular species/ drug combination. Hence, in some cases, where more than one assay is able to detect resistance, the resistance ratios derived from the different assays may differ significantly, for example, the Wallangra isolate of *H. contortus* shows resistance ratios (at the IC₅₀) towards ivermectin of approximately 13-20 fold in larval development assays (Kotze et al., 2014b; Raza et al., 2015), compared to approximately 3-fold in a migration assay (Raza et al., 2015), and zero (no resistance detected) in a motility assay (Smout et al., 2010).

Hence, it is important to examine the use of the various assays with each new target worm species and drug combination to determine which is best at detecting the specific resistance. With the recent reports of resistance to monepantel in gastrointestinal nematodes of livestock (Scott et al., 2013; Mederos et al., 2014; Bartley et al., 2015; Van den Brom et al., 2015) we were interested in the use of *in vitro* assays to detect resistance to this compound. Stuchlíková et al. (2016) recently showed that a micro-agar larva development assay was able to describe a dose response towards monepantel in two isolates of *H. contortus*. This allowed them to define the response in terms of IC₅₀ values, however, they did not examine any resistant isolates and, hence, the ability of the assay to detect resistance remained unknown. The present study therefore aimed to examine a monepantel- resistant isolate of *H. contortus* using a larval development assay and a migration assay in order to determine if either assay could discriminate between this isolate and two isolates known to be susceptible to this chemical.

### 5.2.3 Materials and methods

#### 5.2.3.1 Parasites

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

i) **MPL-R**: isolated from a property in southwest Queensland, Australia, in 2014. Zolvix® had been the preferential treatment on this property since 2010. The isolate was not resistant to monepantel when tested in the field as defined by World Association for the Advancement of Veterinary Parasitology criteria (Coles et al., 2006), with Zolvix® showing an efficacy of 97.9% (faecal egg count reduction) when tested in 2013, and 99.2% when tested in April 2014. *H. contortus* larvae were cultured from faeces collected from animals that had shown clinical signs of scouring after the drench treatment in 2014. 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 two more housed animals. Eggs recovered from the faeces of these two animals were used for the present study. This isolate therefore represents a field-derived isolate in which only the
survivors of a drench treatment have been propagated further. Zolvix® efficacy against the isolate in the initial housed animal was found to be approximately 15%. The field isolate (prior to propagation of drench survivors) showed the following responses to drugs when assessed in 2013: moxidectin efficacy 66%, levamisole 65%, closantel 92%, and albendazole 40%.

ii) **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).

iii) **Wallangra**: isolated from the New England region of Northern New South Wales in 2003; at the time of isolation from the field it was resistant to benzimidazoles, closantel, levamisole and ivermectin (Love et al., 2003). It was isolated from the field prior to the introduction of Zolvix®. The isolate has been further selected using moxidectin (Cydectin®) over at least five generations, and is now unaffected by a full registered dose of moxidectin. This isolate represented a macrocyclic lactone-resistant / monepantel-susceptible control for the present study.

### 5.2.3.2 Anthelmintics

The commercially-available drench product, Zolvix®, was used as a source of monepantel. The drench (concentration of 25 mg monepantel/mL) was serially diluted 2-fold in dimethyl sulfoxide (DMSO) to produce a series of working solutions. Technical grade ivermectin and thiabendazole were purchased from Sigma Chemical Co. Stock solutions were prepared at 10 mg/mL in DMSO, followed by two-fold serial dilutions in DMSO to produce working solutions.

### 5.2.3.3 Larval development assay

The ability of anthelmintics to inhibit the growth of *H. contortus* larvae from the egg to the L3 stage was determined using larval development assays (LDA) as described by (Kotze et al., 2009). Aliquots of the drug solutions (2 µL) were added to the wells of 96-well plates and 200 µL of molten agar (Davis Gelatin Co) was added to each well, and allowed to solidify. Controls received 2 µL of DMSO only (final concentration 1% v/v), followed by agar. Drug concentrations ranged from 12,500-0.4 ng/mL (26,400-0.84 nM) for monepantel, 40-0.08 ng/mL (46-0.09 nM) for ivermectin, and 12,500 -0.4 ng/mL (62,000-2.0 nM) for thiabendazole.

Worm eggs were recovered from faeces following the standard protocols described by Kotze et al. (2009). The faeces were passed through two fine filters (250 µm followed by 75 µm), and the filtrate added to a 2-step sucrose gradient (10% and 25%) prior to centrifugation. The eggs were recovered from the interface of the two sucrose layers and washed over a 25 µm sieve with water to remove the sugar. Finally, the eggs were treated with a solution of sodium hypochlorite (8.4 mg/L)
for 12 min, followed by thorough washing with water. The eggs were diluted in distilled water at a concentration of 38 eggs/10 µL after the addition of amphotericin B (final concentration 25.0 µg/mL) and tylosin 2-3-dihydroxybutanedioate (final concentration 800 µg/mL). This egg suspension was aliquoted into the assay plate wells (30 µL per well), and the plates incubated overnight at 27 °C. The next day, when the eggs had hatched, 10 µL of growth medium (live culture of *Escherichia coli* in a nutrient solution, from Kotze et al. (2009)) was added to each well. The plates were incubated for a further 6 days. At the end of this period, the larvae in each well were killed by addition of 10 µL of Lugol’s iodine, and the number of fully grown infective stage larvae (L3) was counted in each well.

Each assay consisted of triplicate wells at each concentration of drug, as well as a minimum of 12 control wells per plate. Three separate experiments were performed for each drug with each worm isolate.

5.2.3.4 Larval migration assay

A larval migration assay (LMA), as described by Kotze et al. (2006), was used to characterize the effects of monepantel and ivermectin on the three worm isolates. The assay utilised 96-well multiscreen mesh filter plates (Millipore, Australia) with each well fitted at its base with a 20 µm mesh. L3 stage larvae were recovered from faecal cultures and separated from debris by passage through a 20 µm filter. The L3 were stored at 15 °C and used for migration assays within 3 weeks of collection. On the day that assays were established, a sample of larvae were diluted to a concentration of 3-3.5/µL, amphotericin B (250 µg/mL) was added at a rate of 100 µL/mL, and Penicillin/Streptomycin (P 10,000 U and Streptomycin 10,000 µg/mL) added at 10 µL/mL. Aliquots of each drug solution (1 µL) were added to each well of 96-well assays plates (control wells received 1 µL of DMSO), followed by water (70 µL) and the larval suspension (30 µL). The final DMSO concentration was 1% (v/v). Plates were placed into zipper plastic bags (to prevent evaporation) and incubated for 48 h at 27 °C. Each drug concentration was present in triplicate, with 12 control wells, per plate. Final concentration ranges were 30-0.5 µg/mL (63-1.05 µM) for monepantel, and 25-0.1 µg/mL (28.7-0.11 µM) for ivermectin.

After 48 h, the drug exposed worms were transferred to the filter plates fitted over receiver plates. The wells of these receiver plates had previously received 4 µL of each drug concentration, and 300 µL of water. The control wells received only DMSO (4 µL) and water. These plates were placed at room temperature until required. A layer of soft agar was added above the filter meshes by addition of 75 µL of 0.1% agar (w/v) to each well of the multiscreen mesh filter plate. The plates were covered and allowed to sit at room temperature for approx. 2 h. The filter / agar plates were
then lowered into the receiver plates (Millipore, Australia) and left for an hour on the bench to allow the drugs to equilibrate through the agar.

The drug exposed worms were then transferred using multi-tip pipettes to their respective filter/ agar plates, as described by Kotze et al. (2006). The filter/ agar/ receiver plates were then bagged in clear plastic, and placed under a light at 27 °C. After 24 h, the filter plates were removed, and the worms that had migrated down into the receiver plate wells were killed by Lugol’s iodine (10 µL) and counted.

5.2.3.5 Data analyses

For each experiment, the number of L3 larvae in each well of development or migration assay plates was expressed as a percentage of the mean number in multiple control (no drug) wells. The data were then analysed using non-linear regression with GraphPad Prism® software (GraphPad Software Inc., USA, version 6.07). The model used to fit the data was based on a normalised response (dose response curve from 100% to 0%) and a variable slope. This allowed for the calculation of IC₅₀ values and 95 % confidence intervals. An exception to this was for analysis of the response of the MPL-R isolate to monepantel. As two separate phases in the response curve were evident with this data set, we divided the response into two separate regions covering either the low or high range of drug concentrations, and then analysed each region separately using a model based on non-normalised data. In this way, the analysis allowed for the calculation of IC₅₀ values and 95% confidence intervals for the two separate populations, as well as defining the dose response plateau that separated the two populations. Data from each set of nine assays (3 experiments, each with triplicate assay wells) were pooled and used to calculate IC₅₀ values and 95% confidence intervals (CI) for each isolate / drug combination. Significant differences between IC₅₀s among the isolates (within a drug) were determined by overlap or non-overlap of 95% confidence intervals. Resistance ratios were calculated for each isolate / drug combination as: IC₅₀ resistant isolate (Wallangra or MPL-R)/ IC₅₀ Kirby isolate.

5.2.4 Results and discussion

All three isolates showed dose responses ranging from full development to 100 % inhibition of development across a range of increasing concentrations of monepantel, indicating that the assay was able to define the response of *H. contortus* larvae to the drug (Figure 5.1A). The response of the MPL-R isolate was clearly shifted to the right of the two susceptible isolates, indicating that the larvae of this isolate were resistant to the effects of the drug. Hence, the development assay
outcome reflects the presence of resistance at the adult life stage that resulted previously in a drug efficacy of just 15% after administration of Zolvix® to infected sheep (as described in section 2.1).

While the Kirby and Wallangra isolates showed dose response curves shaped as expected, the response of the MPL-R isolate was clearly separated into two phases. As the drug concentration in the assay increased, larval development decreased and then plateaued at a level of approximately 60% development, followed by a decrease to zero at the highest drug concentrations. We suggest that this dose response indicates that two distinct populations are present in this isolate. We have defined these two populations as population 1, showing a resistance ratio relative to Kirby (at the IC50) of 7.3-fold (Table 5.1), and population 2, showing a resistance ratio of 1080-fold. The position of the plateau at the 60% development level indicates that the highly-resistant population 2 represents approximately 60% of the larvae in the total population of larvae in this isolate.

The Wallangra isolate showed no resistance to monepantel (Figure 5.1A, Table 5.1) as would be expected as it was isolated from the field before monepantel was introduced for worm control. The MPL-R isolate showed a low level of resistance towards ivermectin (resistance ratio of 2.8-fold at the IC50) compared to the higher level resistance shown by Wallangra (resistance ratio of 17) (Table 5.1, Figure 5.1B). These relative levels of ivermectin resistance may be expected given the known differences between the two isolates in susceptibility to moxidectin: zero efficacy against Wallangra compared to 85% efficacy against MPL-R (see section 2.1). The MPL-R isolate showed approximately equal resistance towards thiabendazole as Wallangra (resistance ratios 17-22) (Figure 5.1C, Table 5.1). Benzimidazole resistance would be expected in any field isolate of this species in Australia.
Fig. 5.1. Larval development assay dose responses for the MPL-R, Wallangra and Kirby isolates of *H. contortus* towards monepantel (A), ivermectin (B), and thiabendazole (C). The legend shown on part B also applies to part C. Each data point represents mean ± SEM, *n* = 9 separate assays (pooled data from three experiments, each with assays in triplicate).
Table 5.1. Dose responses of MPL-R, Wallangra and Kirby isolates of *H. contortus* towards monepantel, ivermectin and thiabendazole in larval development and migration assays.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Isolate</th>
<th>Development assay</th>
<th>Migration assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC₅₀ (ng/mL)</td>
<td>95% CI</td>
</tr>
<tr>
<td>Monepantel</td>
<td>Kirby</td>
<td>4.31</td>
<td>3.88 – 4.79</td>
</tr>
<tr>
<td></td>
<td>MPL-R population 1</td>
<td>31.5*</td>
<td>14.7 – 67.3</td>
</tr>
<tr>
<td></td>
<td>MPL-R population 2</td>
<td>4640*</td>
<td>3310 – 6500</td>
</tr>
<tr>
<td></td>
<td>Wallangra</td>
<td>5.18</td>
<td>4.50 – 5.97</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>Kirby</td>
<td>0.23</td>
<td>0.21 – 0.26</td>
</tr>
<tr>
<td></td>
<td>MPL-R</td>
<td>0.55*</td>
<td>0.48 – 0.62</td>
</tr>
<tr>
<td></td>
<td>Wallangra</td>
<td>3.92*</td>
<td>3.40 – 4.58</td>
</tr>
<tr>
<td>Thiabendazole</td>
<td>Kirby</td>
<td>13</td>
<td>12 – 14</td>
</tr>
<tr>
<td></td>
<td>MPL-R</td>
<td>220*</td>
<td>190 – 254</td>
</tr>
<tr>
<td></td>
<td>Wallangra</td>
<td>283*</td>
<td>229 - 351</td>
</tr>
</tbody>
</table>

<sup>a</sup> within a drug and within an assay type, IC₅₀ values marked with * were significantly different in the MPL-R and Wallangra isolates compared to the Kirby isolate.

<sup>b</sup> RR = resistance ratio = IC₅₀ of MPL-R or Wallangra/ IC₅₀ Kirby, within each drug

<sup>c</sup> within a drug and within an assay type, RR values marked with # are derived from IC₅₀ values that were significantly different from each other
The behaviour of monepantel in the migration assay was in stark contrast to that observed with the development assay (Figure 5.2A). The drug did not reduce migration to zero at any of the drug concentrations tested for any of the isolates, and hence no IC$\text{}_{50}$ values could be calculated. This highest concentration shown in Figure 5.2A was approximately 30 µg/mL, almost 7,000-fold higher than the IC$\text{}_{50}$ for the Kirby isolate in larval development assays (from Table 5.1). Higher concentrations of monepantel resulted in the appearance of crystals and a coloured residue in the wells of the migration assay plates, and hence we did not test at these drug levels. The migration of Kirby larvae did decrease to approximately 25% at a drug concentration of 8 µg/mL, followed by an increase in migration as the drug concentration increased further. This pattern is similar to the response reported for several species of trichostongyloid nematodes in motility assays with levamisole and pyrantel (for example, Kopp et al., 2008) in which motility has been observed to decrease over a range of drug concentrations before increasing again as the drug concentration increases further. The response to monepantel shown in Figure 5.2A clearly demonstrates that this assay is not suitable for detecting resistance to this drug in the MPL-R isolate. The ivermectin migration assay showed a significant difference between the Wallangra and Kirby isolates, with a resistance ratio of 2.8 (Figure 5.2B and Table 5.1), with the MPL-R isolate showing a very similar response to Wallangra (resistance ratio 3.5). The resistance ratio for Wallangra in this assay was over 6-fold less than for the LDA (17 compared to 2.8).

The presence of two populations in the MPL-R isolate displaying low and high levels of resistance suggests that at least two resistance mechanisms are functional in this isolate, with one (or more) conferring a much higher level of resistance than the other(s). Rufener et al. (2009b) reported that laboratory-selected monepantel-resistant isolates of H. contortus showed the presence of a number of mutations in the gene coding for the putative target site of the drug, mptl-1, resulting in the formation of various truncated forms of the target protein in the resistant worms. These various mutations resulted in the absence of a functioning drug target in the resistant worms. H. contortus worms are able to metabolise monepantel via S-oxidation and hydrolytic pathways (Stuchlíková et al., 2014), however, there is no evidence that these pathways are increased in resistant worms. The interaction of monepantel with drug efflux pathways (e.g. P-glycoproteins) has not been reported. At present, the molecular basis of the resistance seen in MPL-R populations 1 and 2 is unknown.
Fig. 5.2. Migration assay dose responses for the MPL-R, Wallangra and Kirby isolates of *H. contortus* towards monepantel (A) and ivermectin (B). Each data point represents mean ± SEM, *n* = 9 separate assays (pooled data from three experiments, each with assays in triplicate).
The presence of subpopulations showing different levels of resistance within an isolate of *H. contortus*, as observed here, is similar to that reported recently for larvae of the Wallangra isolate with respect to their response to levamisole *in vitro* (Sarai et al., 2014). These authors described enhanced P-gp gene expression in larvae showing a low level of resistance, as well as reduced target site (nicotinic acetylcholine receptor subunit) gene expression in more highly resistant larvae, suggesting that these two mechanisms were responsible for the two observed levels of resistance. These *in vitro* observations paralleled levamisole resistance reports from the field that described ‘moderate level resistance’ and ‘high level resistance’ based on larval development assay data (Drenchrite Users Guide, 1996, Horizon technology, Australia). Similarly, the present study suggests that two resistance mechanisms are acting to provide two distinct levels of resistance to monepantel in the MPL-R isolate in our *in vitro* assays. Whether these two levels of resistance at the larval life-stage also translate into the existence of adult worms showing two distinct levels of resistance is unknown at present. Although speculative, it is possible that the 85% of the adult worm population that survived a Zolvix® treatment (drug efficacy 15%, from section 5.2.3.1) represents the highly resistant larval population 2, while the 15% of the population that was removed by the drench represents population 1.

The resistance ratio shown by population 2 towards monepantel is extraordinarily high (at 1080-fold) compared to the resistance ratios generally reported in the literature for *in vitro* assays with the free-living stages of gastrointestinal parasites. For example, while Wallangra adults are unaffected by a full dose of moxidectin *in vivo*, the present study found that this isolate showed a resistance ratio in the larval development assay with ivermectin of only 17, while an earlier study reported a resistance ratio of only 4-fold toward moxidectin itself (Kotze et al., 2014b). The egg hatch assay resistance ratios reported for *H. contortus* isolates showing resistance to benzimidazoles generally range up to about 20-fold (for example, von Samson-Himmelstjerna et al., 2009). The high resistance ratio for MPL-R population 2 and monepantel is however similar to that reported for levamisole against two resistant isolates of this species, which showed resistance ratios of 690- and 900-fold in larval development assays (Sarai et al., 2013), and also similar to the high resistance ratio of 870-fold reported for the UGA/2004 isolate towards thiabendazole (Williamson et al., 2011). The very high resistance ratio seen in the present study is in accord with the fact that when resistance to monepantel has been reported from the field it is usually at very high levels, with drug efficacy reduced to zero in most cases (Scott et al., 2013; Mederos et al., 2014; Van den Brom et al., 2015).

In conclusion, the present study has shown that the larval development assay is able to define the sensitivity of the free-living life stages of *H. contortus* to monepantel. The larvae of a resistant
isolate showed an ability to develop to the L3 stage at drug concentrations significantly greater than those which prevented development in larvae of susceptible isolates. The assay indicated the presence of a sub-population showing a very high level of resistance alongside a smaller sub-population showing low level resistance. The study suggests that the larval development assay may be a useful tool for detection of monepantel resistance in field isolates of this species, and that the shape of the response may be utilised in order to indicate the relative prevalence of individuals showing the two levels of resistance. It will be important to use such diagnostic tools to monitor for the emergence of monepantel resistance as reports from the field to date indicate that such resistance can emerge quickly and render the compound completely ineffective. Hence, early detection will be important to allow management strategies, such as drench rotation, to be implemented promptly once resistance is first detected on a property.
5.3 Effects of third-generation P-glycoprotein inhibitors on the sensitivity of a monepantel-resistant isolate of *Haemonchus contortus* to monepantel

5.3.1 Introduction

The availability of a field-derived laboratory-propagated MPL-resistant isolate provided an opportunity to study the effects of third-generation P-gp inhibitors on the sensitivity of this isolate to monepantel. It has been reported that third-generation P-gp inhibitors increased the sensitivity of a drug-resistant isolate (WAL) to IVM and LEV (Chapter 2, published as Raza et al., 2015). Such an increase in sensitivity to a drug in the presence of a P-gp inhibitor would be expected if ABC transporters were playing a role in the resistance. On the other hand, co-administration of these inhibitors with MPL resulted in reduced sensitivity of WAL larvae to MPL (section 2.3), which suggested that ABC transporters may be involved in MPL transportation to its target sites within the body of the nematode. It was therefore of interest to evaluate the effects of third-generation P-gp inhibitors on MPL sensitivity in an isolate of *H. contortus* showing resistance to this drug. The larval development assay had shown an ability to define the response of MPL-R *H. contortus* larvae to MPL (section 5.1; published as Raza et al., 2016b), and hence, this assay was used to measure the effects of zosuquidar and tariquidar on sensitivity to MPL in the MPL-R isolate.

5.3.2 Materials and methods

5.3.2.1 Parasite isolate and chemicals

A field-derived laboratory-propagated MPL-R isolate of *H. contortus* was used in this study. The drug resistance profile, origin of the isolate and protocol to harvest eggs from faeces are described in section 5.1.

The commercially available drench product Zolvix® was used as a source of MPL as described in section 5.2.3.2. The details of zosuquidar and tariquidar procurement, preparation of stock solutions and multiple dilutions are described in section 2.2.3.2.

5.3.2.2 Larval development assay

The inhibitory effects of MPL-alone or in combination with MDRIs on the growth of *H. contortus* larvae from the eggs to L3 were examined using the LDA as described in section 2.2.3.3.

The data were analysed using non-linear regression with GraphPad Prism® software (GraphPad Software Inc., USA, version 5.03).
5.3.3 Results and discussion

The concentrations of zosuquidar and tariquidar used in combination with MPL were the same as used for WAL isolate in Chapter 2 and are shown in Table 5.2. The MPL dose-response curves either alone or in combination with MDRIs clearly indicated the presence of two distinct populations in this isolate (Figs. 5.3 and 5.4), as has been described in section 5.1. The two populations were defined as subpopulation 1 and 2 based on their resistance ratios compared to Kirby isolate (section 5.1; published as Raza et al., 2016b).

The results of LDAs with MPL alone or in combination with zosuquidar are described in Table 5.2, with dose-response curves shown in Fig. 5.3. The presence of the higher concentration of zosuquidar (40 µg/mL) had no effects on the larval development of subpopulation-1 (Fig. 5.3A). On the other hand, the MPL IC₅₀ value significantly increased for subpopulation-2 (7.03 µg/mL compared to 4.12 µg/mL), as indicated by non-overlap of 95% CI. There was a small shift of response-curve to the right with a SR of 1.7 (Fig. 5.3A; Table 5.2). The combination of MPL with the lower concentration of zosuquidar (20 µg/mL) is shown in Fig. 5.3B. For subpopulation-1, there was no shift in the MPL dose-response curve with the IC₅₀ value (0.057 µg/mL) within the range of 95% CI for MPL-alone (0.02-0.07). For subpopulation-2, a slight shift in the response curve to the right was accompanied with a small increase in IC₅₀ of 1.5-fold (6.30 µg/mL compared to 4.12 µg/mL); however, it was not possible to determine the significance of this change as the CI value for population-2 was designated as ‘very wide’ by the GraphPad analysis (Table 5.2; Fig. 5.3B). These wide CIs most likely arose due to the steepness of the dose-response curve, with larval development decreasing from no effect to 100% inhibition for subpopulation-2 over a range of 2 or 3 drug concentrations. Hence, there is no evidence that the presence of zosuquidar at 20 µg/mL resulted in any significant changes in IC₅₀ values.

Co-administration of tariquidar did not show any significant effects on MPL toxicity to subpopulation-1 (Fig. 5.4A and B), as indicated by overlap of 95% CI (Table 5.2). For tariquidar at 40 µg/mL, there was a slight shift in the dose-response curve to the right (IC₅₀ value of 6.45 µg/mL compared to 4.12 µg/mL), with a SR of 1.5 in subpopulation-2 (Fig. 5.4A; Table 5.2). However, the 95% CI range was very wide, so it was not possible to judge the significance of this increase in IC₅₀. The lower concentration of tariquidar (20 µg/mL) resulted in a small shift of response curve to the right (Fig. 5.4B), and an increase in IC₅₀ value at 1.6-fold (6.71 µg/mL compared to 4.12 µg/mL). However, this increase in IC₅₀ value was not significant as indicated by non-overlap of 95% CI. (Table 5.2).
**Fig. 5.3.** Larval development assay dose responses for the MPL-R isolate of *H. contortus* towards monepantel in the presence of zosuquidar at 40 µg/mL (A) and 20 µg/mL (B). In both A and B, MPL-alone populations 1 and 2 shown with solid line and closed symbols, and MPL plus zosuquidar shown as dotted lines and open symbols. Each data point represents mean ± SEM, n = 9 separate assays (pooled data from three experiments, each with assays in triplicate). Zq: zosuquidar; pop: population.
Fig. 5.4. Larval development assay dose responses for the MPL-R isolate of *H. contortus* towards monepantel in the presence of tariquidar at 40 µg/mL (A) and 20 µg/mL (B). In both A and B, MPL-alone populations 1 and 2 shown with solid line and closed symbols, and MPL plus tariquidar shown as dotted lines and open symbols. Each data point represents mean ± SEM, n = 9 separate assays (pooled data from three experiments, each with assays in triplicate). Tq: tariquidar; pop: population.
Table 5.2. Larval Development Assay: IC$_{50}$ and Synergism ratios (SRs) for monepantel either alone or in the presence of different concentrations of MDRIs, with MPL-R isolate of *H. contortus*

<table>
<thead>
<tr>
<th>MPL-R population</th>
<th>MPL-alone</th>
<th>Zosuquidar</th>
<th>Tariquidar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (ng/mL)</td>
<td>95% CI</td>
<td>IC$_{50}$ (µg/mL)</td>
</tr>
<tr>
<td>Subpopulation-1</td>
<td>0.044</td>
<td>0.02-0.07</td>
<td>0.044 (0.01-0.12)</td>
</tr>
<tr>
<td>Subpopulation-2</td>
<td>4.12</td>
<td>3.35-5.07</td>
<td>7.03* (5.38-9.20)</td>
</tr>
</tbody>
</table>

$^{a}$ Within a population, * denotes that the IC$_{50}$ in the presence of the MDRI was significantly greater than the IC$_{50}$ for the monepantel alone, as determined by non-overlap of 95 % Confidence Intervals.

$^{b}$ Synergism ratio = IC$_{50}$ for anthelmintic in the absence of MDRI/ IC$_{50}$ for anthelmintic in the presence of MDRI

$^{c}$ SR values denoted by * are derived from IC$_{50}$ values significantly increased by the presence of the MDRI
Overall, with respect to subpopulation-2, the presence of the inhibitors resulted in one instance of increased IC50, with the significance of the other observed 1.5-1.6-fold increases being uncertain due to the wide CIs reported by the GraphPad analysis. However, it was clear from Fig. 5.3 and Fig. 5.4 that the % development at a MPL concentration of 6.25 µg/mL (log10 = 0.796 µg/mL) was consistently higher in the presence of each of the four separate MDRI treatments. These differences were confirmed by t-test analysis of the data at this MPL concentration within each separate MDRI data set (P = 0.02 and 0.002 for zosuquidar and P = 0.004 and 0.002 for tariquidar at lower and higher concentrations, respectively).

The results observed above clearly demonstrate that combinations of third-generation MDRIs with MPL did not increase the MPL-sensitivity in both resistant populations, as may have been expected if increased drug efflux was acting as a MPL-resistance mechanism. In contrast, the presence of these inhibitors (zosuquidar and tariquidar) resulted in increased sensitivity of a multidrug resistant isolate (WAL) of *H. contortus* to IVM, LEV and thiabendazole (Chapter 2; published as Raza et al., 2015). Hence, while the data in Chapter 2 indicates a role for ABC transporters in the resistance shown by the WAL isolate (to IVM, LEV and thiabendazole), the data in Chapter 5 do not support such a role for transporters in the resistance shown by the MPL-R isolate to MPL.

On the other hand, there was evidence of reduced sensitivity in subpopulation-2 larvae co-treated with an MDRI. This evidence consisted of a single instance of increased IC50, alongside a consistent reduction in sensitivity to an MPL concentration of 6.25 µg/mL (log10 = 0.796 µg/mL) in the presence of an MDRI. This is consistent with the earlier observations (Chapter 2, section 2.3) that these MDRIs reduced the sensitivity of the WAL isolate to MPL (up to 5-fold). The reduced sensitivity of subpopulation-2 to MPL in the presence of the MDRIs further supports the hypothesis that ABC transporters may be involved in pharmacokinetics and pharmacodynamics of MPL in *H. contortus*, as was suggested previously in Chapter 2, section 2.3.
5.4 Transcription patterns of ABC transporters in monepantel-resistant isolate compared to Kirby and Wallangra isolates of *Haemonchus contortus*

5.4.1 Introduction

As described in Chapter 3, the multi-drug resistant isolate (WAL) showed overexpression of three P-gp genes compared to a drug-susceptible isolate (Kirby). This variation in gene expression was considered to be a component of the observed resistance in the WAL isolate. As described above in section 5.1, the MPL-R *H. contortus* population was divided into two distinct sub-populations in terms of the LDA response, suggesting that at least two separate MPL resistance mechanisms were acting in this isolate. Thus, an experiment was designed to examine the transcription patterns of ABC transporters in this isolate, and whether these transporters showed different expression patterns compared to the WAL and Kirby (both susceptible to monepantel). This study, therefore, aimed to measure the transcription patterns of ABC transporter genes in the MPL-R isolate, and compare them with the patterns shown by the Kirby and WAL isolates, using real-time PCR.

5.4.2 Materials and methods

5.4.2.1 Parasites

Three isolates of *H. contortus* MPL-R, Kirby and WAL were used in this study. The details on origin and susceptibility status of each isolate are described in section 5.2.3.1.

5.4.2.2 Molecular biology

Molecular techniques including RNA extraction, synthesis of complementary DNA (cDNA) (reverse transcription) and real time PCR were performed as described in Chapter 3, section 3.2.2. Expression values for all genes in each sample were normalised to the three housekeeping genes using REST 2009 (version v2.0.13) and the data were analysed using repeated measures ANOVA with Fischer’s LSD as multiple comparison test in GraphPad Prism® software (GraphPad Software Inc., USA, version 5.03) as described in Chapter 3, section 3.2.2.

5.4.3 Results and discussion

A comparison of the transcription levels of ABC transporter genes between MPL-R and Kirby isolates showed that *pgp-11* was transcribed at significantly lower levels (*P < 0.0001*) in the former isolate (Fig. 5.5A). The decrease in *pgp-11* transcription was 4-fold compared to the Kirby isolate. There were no significant differences in transcription levels of any of the other transporter genes between the two isolates.
On the other hand, transcription levels of three P-gp genes (pgp-2, pgp-9.2 and pgp-11) and mrp-1 were significantly decreased in the MPL-R isolate compared to those in WAL (Fig. 5.5B). The magnitudes of these decreases were as follows: 1.5-fold for pgp-2 ($P = 0.02$), 2-fold for pgp-9.2 ($P < 0.0001$), 3-fold for pgp-11 ($P < 0.0001$) and 1.6-fold for mrp-1 ($P = 0.004$).

**Fig. 5.5.** Relative transcription levels of ABC transporter genes in monepantel-resistant isolates compared to Kirby (A) and WAL (B) isolates of *Haemonchus contortus*. Significant differences in the transcription between the isolates are indicated by * ($P < 0.05$). Data shown as mean ± SEM, $n = 3$ separate experiments, each with four technical replicates.
The results described above show that the only instances of altered gene transcription in the MPL-R isolate were the decreases in this isolate compared to the two monepantel-susceptible isolates. Four transporter genes showed reduced expression in MPL-R isolate compared to WAL, while only one gene was transcribed at lower levels compared to Kirby isolate. This difference might be expected because it has been shown that the WAL isolate exhibits overexpression of several P-gp genes compared to the Kirby isolate (Sarai et al., 2013; Raza et al., 2016a). That is, the MPL-R isolate may be expected to show lower expression levels than WAL as some transporters have been implicated in the IVM resistance displayed by the latter isolate. However, it is clear that there is no increased expression of any specific ABC transporter genes in the MPL-R isolate compared to the other two isolates. Hence, there is no evidence that the resistance displayed by the MPL-R isolate is due to increased drug efflux pathways.

The inhibition of efflux activity of ABC transporters by the co-administration of MDRIs with MPL resulted unexpectedly in increased tolerance of WAL larvae to MPL in LDAs (Chapter 2, section 2.3). Similarly, increased tolerance was observed in one out of four instances of MPL/MDRI combinations with the MPL-R isolate (section 5.2). As described in Chapter 2, there could be two possible explanations for this antagonism of MDRIs to MPL; firstly, it is possible that P-gps may normally act to remove MPL from the cells before its detoxification by metabolising enzymes. Therefore, inhibition of P-gps may result in an increased time of drug retention in cells and hence an increased opportunity for the enzymes within the cell for detoxifying the drug. Secondly, it may be possible that ABC transporters are involved in transportation of MPL to its target sites within the body of worms. Although it is speculative to draw a connection between the two data sets, the gene expression data presented here is compatible with these suggested interactions of transporters and monepantel. If the transporters were involved in the toxic action of the drug (based on the efflux of active drug before it can be detoxified by metabolising enzymes and/ or movement towards receptors), then reduced expression of transporter genes (as observed in Fig. 5.5) may be expected in a resistant isolate; however, this is speculation and warrants further testing.

It is noteworthy that the only gene to be significantly downregulated in the MPL-R isolate relative to WAL and Kirby was *pgp-11*. This gene was also observed to respond most strongly to MPL exposure in the induction experiments (Chapter 4). Further research is needed to reveal the functional role of ABC transporters, particularly *pgp-11* in the MPL-R isolate, and to unravel the genetic basis of the two resistance mechanisms observed in this isolate.
CHAPTER 6

6. General discussion, conclusions, and future research directions

This thesis aimed to investigate the role of ABC transporters in anthelmintic resistance (AR) using *Haemonchus (H.) contortus*. The main objectives of the thesis were:

(i) To assess effects of MDRIs (particularly third-generation inhibitors) on the sensitivity of three isolates of *H. contortus* (drug susceptible (Kirby), multi-drug resistant (WAL) and monepantel resistant (MPL-R) isolates) to ivermectin (IVM), levamisole (LEV), thiabendazole (TBZ) and monepantel (MPL),

(ii) To determine variation in the expression levels of different ABC transporter genes between drug-susceptible and -resistant isolates of *H. contortus*,

(iii) To measure effects of IVM, LEV and MPL on the expression patterns of ABC transporters in Kirby and WAL isolates, and

(iv) To evaluate phenotypic characterization and expression patterns of ABC transporter genes in the MPL-R isolate.

This chapter presents the major findings of this thesis, shows how the results of this project increase our understanding of the mechanisms involved in AR, and suggests future research directions.

6.1 Research achievements and discussion

6.1.1 Multi-drug resistance inhibitors and anthelmintic sensitivity of *Haemonchus contortus*

In Chapter 2, the effects of different MDRIs (selected first, second, and third generation inhibitors) on *in vitro* sensitivity of *H. contortus* to anthelmintics were investigated. Larval development and migration assays were used to measure the sensitivity of larvae to anthelmintics alone, or in the presence of the MDRIs. The results showed that most of the MDRIs were able to increase the sensitivity of *H. contortus* larvae to anthelmintics in both assays. Members of the third-generation of MDRIs (zosuquidar and tariquidar) and a tyrosine-kinase inhibitor ‘crizotinib’ showed more marked effects on the sensitivity of *H. contortus* larvae compared to first and second generation inhibitors in migration assays. These inhibitors reduced the IVM IC$_{50}$ value of a resistant field isolate (WAL) to below that measured for a sensitive reference isolate (Kirby) with IVM-alone, and hence effectively rendered the WAL larvae more sensitive to IVM than Kirby larvae. Additionally, the evidence on the ability of MDRIs to increase the sensitivity of GINs to
Anthelmintics was limited to the members of first and second generation inhibitors (Bartley et al., 2009; Demeler et al., 2013). The synergistic effects of third-generation inhibitors (zosuquidar, elacridar, and tariquidar) in increasing the efficacy of praziquantel had only been reported for adult Schistosoma mansoni (Kasinathan et al., 2014). The present study, therefore, has significantly extended these earlier reported studies by highlighting the synergistic effects of a number of third generation MDRIs in an economically-important species of GIN. In addition, the present study was the first report describing the effects of crizotinib in potentiating the toxicity of IVM towards nematodes.

Several of the inhibitors showed synergistic effects by increasing the sensitivity of both the drug-resistant and -susceptible isolates, while others had significant effects on the resistant isolate only. Therefore, the study highlighted two aspects of the role of P-gps: (i) instances where P-gps are active in both isolates, and presumably represent intrinsic defence pathways, and (ii) instances where P-gps are more active in resistant larvae and may, therefore, be a contributing factor to the observed resistance. The latter scenario is of particular interest with respect to extending the longevity of anthelmintics in the face of developing field resistance.

In migration assays, only third-generation MDRIs revealed synergistic effects with LEV (in WAL larvae; SR up to 3-fold). On the other hand, six out of seven MDRIs increased IVM toxicity to WAL larvae (SR up to 6-fold). These results suggest that ABC transporters may play an important role in resistance to IVM, but are perhaps less important in LEV resistance. The role of ABC transporters in IVM resistance may be specific to WAL isolate, therefore, further examination of a broader range of isolates from wider regions is required to confirm this association of ABC transporters to IVM resistance.

The effect of inhibitors on sensitivity to monepantel was in stark contrast to that observed with IVM and LEV. Verapamil and ascorbic acid slightly increased MPL sensitivity of Kirby and WAL isolates (up to 1.8-fold). On the other hand, the presence of all the other inhibitors resulted in increased tolerance of WAL larvae to MPL (that is, a shift in the dose-response curve to the right). These effects were more obvious with third-generation inhibitors, with 2 to 5-fold increases in MPL IC\(_{50}\). These results with WAL larvae were quite unexpected and were not observed with the Kirby isolate. This antagonism can plausibly be explained in two ways: firstly, it is possible that inhibition of P-gps by MDRIs reduces the rate of efflux of the drug from cells, and hence increases the drug resident time within cells. This in turn may provide an increased opportunity for detoxification enzymes to metabolise the MPL into non-toxic metabolites. Secondly, the efflux from cells in some locations within the nematode’s body may result in movement of the drug towards other locations.
within the body in which the MPL-receptors are located, and hence, inhibition of this efflux may result in less MPL reaching its target site. However, further studies are required to investigate these possibilities.

Overall, these results indicate that third-generation P-gp inhibitors and crizotinib clearly increase the sensitivity of resistant worms to IVM, and to a lesser extent to LEV, and in some instances this is also true for susceptible isolates. The effects were much marked with the resistant isolate compared to susceptible isolate. The unexpected interaction of the MDRI with MPL highlights the need for further study of the interaction of ABC transporters with this anthelmintic.

6.1.2 Expression levels of ABC transporters in drug-susceptible and -resistant isolates of *H. contortus*

Transcription levels for each known ABC transporter within a susceptible and a multi-drug resistant isolate were measured using real-time PCR (Chapter 3). Transcription was compared to the levels of *pgp-1* within each isolate, and levels of transporters were compared between the WAL and Kirby isolates. The same set of six genes (*pgp-3, pgp-9.3, pgp-10, abcfl-1, abcfl-2, and mrp-5*) were transcribed at the highest levels in both isolates. Overall, the expression patterns of ABC transporters were similar but not identical in the two isolates. ABC transporters have been described as having various protective functions within nematodes, for example, *pgp-3* protects *C. elegans* worms against natural toxins (Broeks et al., 1995), and acts in the protection of *H. contortus* against host immune cell products (Issouf et al., 2014). A great deal of variation in expression levels of different ABC transporters observed in this study supports the specific protective roles for each transporter or a combination of transporters against different chemical entities. Also observed in this study were high expression levels of ABCF transporters compared to many other genes. It has been reported that ABCF transporters are involved in cell physiology in arthropods (Dermauw and Van Leeuwen, 2014), and play an important role in insect development (Broehan et al., 2013). The function of ABCF proteins as transporters is currently unclear, and their role in GINs remains to be determined.

The resistant isolate (WAL) revealed higher expression levels of three P-gp genes (*pgp-1, pgp-9.1* and *pgp-9.2*) compared to the susceptible isolate (Kirby). This study has extended the previous findings of Sarai et al. (2013) who described an upregulation of *pgp-1* and *pgp-9* in WAL larvae as compared to Kirby larvae. The increased transcription of *pgp-9* reported earlier was most likely due to increases for *pgp-9.1* and *pgp-9.2*, alongside no changes in *pgp-9.3*, because the existence of three different homologues of *pgp-9* was not known at the time of the Sarai et al. (2013) study. The expression patterns of P-gps in nematodes seem to be quite variable, with some
reports linking them to AR (Dicker et al., 2011; Williamson et al., 2011) and other studies finding no association (Williamson and Wolstenholme, 2012; Areskog et al., 2013). The increased transcription of several P-gp genes in the drug-resistant WAL larvae suggests that these particular P-gps may play a role in the resistance phenotype shown by this isolate.

6.1.3 Effects of anthelmintics on the expression patterns of ABC transporters in *H. contortus*

An important finding of this project was the significantly increased transcription of multiple ABC transporter genes following exposure to IVM (at 0.2 µg/mL and 0.8 µg/mL) and LEV (at 0.4 µg/mL) in the drug-resistant WAL larvae only (Chapter 3). On the other hand, the increased transcription of multiple ABC transporters was observed in both drug-resistant (WAL) and -susceptible (Kirby) isolates following exposure to a high concentration of MPL (250 µg/mL) (Chapter 4).

The pattern of upregulation in WAL larvae was very similar for IVM and LEV, with both drugs upregulating transcription of five P-gp genes and haf-6, while LEV exposure also resulted in upregulation of *abcf-1* following 3 h exposure. These increases in gene transcription were quite short lived, and by the 6 h time point, gene transcription had returned to control levels. The only exception to this was the sustained upregulation of *pgp-11* after 6 h exposure to LEV. These effects on gene transcription were not observed in the Kirby isolate. There have been previous reports showing upregulation of ABC transporter genes after macrocyclic lactone (ML) exposure in free-living (Ardelli and Prichard, 2013) and drug-resistant parasitic nematodes (De Graef et al., 2013; Lloberas et al., 2013; Tydén et al., 2014); however, the present study is the first to demonstrate that this upregulation occurs over a short time frame (3 h) in a drug-resistant isolate only, which suggests that an ability to rapidly upregulate protective pathways in response to drugs may be a component of the resistance displayed by the WAL isolate.

As a consequence of drug exposure, a proportion of the WAL larval population was equipped with an ability to tolerate higher IVM concentrations in subsequent migration assays. This increased tolerance may be associated with the observed increased transcription of multiple ABC transporter genes. On the other hand, IVM pre-exposure resulted in increased sensitivity of larvae to LEV. This might be expected due to cumulative effects of sequential drug treatments given that MLs have been reported to interact with nAChRs in nematodes (Abongwa et al., 2016). Such an increased susceptibility to LEV following IVM/ moxidectin selection has also been observed in *C. elegans* (Menez et al., 2016). These authors proposed that since LEV and IVM bind at different types of gated ion channels, operating on excitatory and inhibitory circuits respectively, it is likely that resistance to one of these drugs will increase the susceptibility to the other. Another phenotypic
consequence of the drug exposure was increased R-123 efflux in both WAL and Kirby isolates, exposed to IVM at 0.8 µg/mL, which was in contrast to the isolate-specific transcription changes (Chapter 4). This suggests that drug exposure also stimulated the activity of existing transport proteins in both isolates. On the other hand, exposure to IVM at the lower concentration (at 0.2 µg/mL) resulted in increased transcription for multiple transporters, but no change in R-123 efflux suggesting that this lower IVM concentration was unable to stimulate the activity of existing transporters. The tolerance to IVM in subsequent migration assays was only observed for the higher IVM pre-exposure treatment (0.8 µg/mL), suggesting that both the increased gene transcription and stimulated activity of existing transporters are required to enable a proportion of the larval population to tolerate higher IVM concentrations. These findings add to the growing body of literature that anthelmintics such as IVM are substrates of ABC transporters and exhibit transcription-inducing effects on these transporters (James and Davey, 2009; Kerboeuf and Guegnard, 2011; De Graef et al., 2013; Bygarski et al., 2014). These studies generally concluded that such a transcriptional response suggested a role for the transporters to eliminate the transcription-inducing anthelmintic, that is, the anthelmintic is likely to be a substrate for the transporters.

Prior to this work, there was no published report available describing the interaction of MPL with ABC transporters in GINs. Therefore, as a first step in understanding whether this drug interacts with ABC transporters, the effects of MPL exposure (at 250 and 2.5 µg/mL) on the transcription patterns of ABC transporters, and phenotypic consequences (R-123 efflux and sensitivity to IVM and LEV) of drug exposure were measured in third-stage larvae of two MPL-susceptible isolates of H. contortus (Chapter 4). In contrast to the isolate-specific gene upregulation following exposure to IVM and LEV (as described in Chapter 3), multiple ABC transporter genes were transcribed at significantly higher levels in both Kirby and WAL isolates following exposure to the higher concentration of MPL (250 µg/mL) for 3, 6 and 24 h. These increases in gene transcription were consistent for pgp-11, pgp-12 and pgp-14 across all the time points in both isolates. Of these, elevated transcription level was maintained for pgp-11 only, 24 h after the end of 3 h drug exposure period. WAL larvae showed much greater levels of upregulation at the 3 h time point compared to Kirby in response to MPL at 250 µg/mL, while the pattern of gene upregulation in Kirby and WAL were quite similar following 6 and 24 h MPL exposure. WAL and Kirby are both susceptible to MPL, having been isolated from the field before the introduction of this drug. The present study, therefore, indicated that the increased responsiveness of WAL larvae compared to Kirby (observed with respect to IVM and LEV exposure in Chapter 3) also occurs with respect to its response to drugs to which it is not resistant. MPL exposure at this high concentration resulted in
greater efflux of R-123 from the larvae, as well as increased tolerance to IVM in a proportion of the larval population in both isolates. The higher concentration of MPL was well above the levels that an adult worm would encounter in the abomasum of a sheep, as measured by Lifschitz et al. (2014) to be 2-4 µg/g of abomasal contents 48 hrs after administration of the drug. On the other hand, the lower concentration of MPL used in the present study, which was selected based on it approximating this in vivo concentration range, resulted in only one instance of transporter gene up-regulation, and did not result in any IVM tolerance. It is clear that while the present study indicates an interaction between MPL at 250 µg/mL and ABC transporters in *H. contortus*, it does not provide evidence for such a role in vivo. Further experiments would be needed to determine if MPL interacts with the transporters in adult worms in vivo.

It has recently been shown that selection with IVM or moxidectin over a number of generations results in cross-resistance to several other ML drugs in *C. elegans* and *H. contortus* (Menez et al., 2016). Although, in the present study, larvae were exposed to drugs only once for a short period (as compared to laboratory selection over a number of generations), a proportion of the larval population showed higher tolerance to IVM following exposure to IVM or LEV (Chapter 3) as well as MPL (Chapter 4). It was not possible to assess whether the MPL-exposure resulted in increased tolerance to MPL itself, as this drug is not suitable for use in migration assays, as it does not inhibit larval migration even at high concentrations (Chapter 5).

**6.1.4 Phenotypic characterization and expression patterns of ABC transporters in monepantel-resistant isolate of *H. contortus***

The availability of the MPL-R isolate offered an opportunity for phenotypic characterization of the resistance using larval development assays, as well as comparison of expression levels of ABC transporters with the two MPL-susceptible isolates (Kirby and WAL) (Chapter 5). *In vivo* drug efficacy against this field-derived laboratory-propagated resistant isolate was approximately 15%, indicating a high level of resistance in the adult life stages. The results of this study showed that the LDA was able to discriminate between the MPL-resistant and -susceptible isolates. The dose-response curve for the MPL-R isolate was clearly shifted to the right of the two susceptible isolates, indicating that this isolate was resistant to the effects of this drug. The resistant isolate showed the presence of two distinct subpopulations that were defined as subpopulation-1 (approximately 40% of the total population), showing a resistance ratio relative to Kirby of 7.3-fold (at the IC₅₀), and subpopulation 2 (approximately 60% of the total population), showing a resistance ratio of 1080-fold. The MPL-R isolate showed a low level of resistance towards IVM (2.8-fold at IC₅₀) compared to higher level resistance shown by WAL isolate (RR 17-fold), and approximately equal resistance towards thiabendazole as WAL (RR 17-22). The presence of two populations in the
MPL-R isolate displaying low and high levels of resistance indicates that at least two resistance mechanisms are functional in this isolate, with one or more conferring a much higher level of resistance than the other(s). The presence of subpopulations showing different levels of resistance was previously reported in larvae of WAL isolate with respect to their response to LEV in vitro (Sarai et al., 2014). These authors described increased P-gp gene expression in larvae showing a low level of resistance, as well as reduced target site (nAChR) gene expression in highly resistant larvae, suggesting that these two mechanisms might be responsible for the two observed levels of resistance.

Monepantel resistance has previously been associated with truncated forms of the target protein due to a number of mutations in the coding gene in laboratory-selected MPL-resistant *H. contortus* (Rufener et al., 2009b). At present, the molecular basis of the resistance seen in MPL-R subpopulations-1 and 2 is unknown. The results of Chapter 4 suggest that MPL may act as a substrate for ABC transporters, and it was therefore of interest to compare the expression patterns of ABC transporters in the MPL-R isolate with MPL-susceptible isolates (Kirby and WAL) to determine if drug efflux may be a component of the resistance mechanism (Chapter 5). This study showed that none of the transporters were increased in the MPL-R isolate compared to the susceptible isolates. There is therefore no evidence that increased efflux plays a role in the resistance to MPL displayed by the MPL-R isolate. However, an interesting finding of Chapter 5 was the significantly decreased transcription of *pgp-11* in MPL-R larvae compared to the Kirby isolate. Moreover, transcription of three P-gp genes and *mrp-1* were significantly decreased in MPL-R isolate compared to those in WAL larvae. In addition, the toxicity of MPL to the WAL isolate was decreased when efflux activity of ABC transporters was reduced by MDRIs. The presence of MDRIs increased MPL-IC$_{50}$ up to 5-fold, which suggests that the interaction of MPL with its target site was reduced in the presence of MDRIs (Chapter 2). This reduced sensitivity of WAL isolate to MPL in the presence of MDRIs can be plausibly explained in two ways; firstly, it is possible that P-gps may normally act to remove MPL from the cells before its detoxification by metabolising enzymes. Therefore, inhibition of P-gps may result in an increased time of drug retention in cells and hence an increased opportunity for the enzymes within the cell for detoxifying the drug. Secondly, these transporters may be involved in transporting MPL to its target site within the worm’s body, hence inhibition of transporters activity resulted in reduced interaction of MPL with its target sites. These effects were not observed in the Kirby isolate, which might be due to the fact that (i) Kirby isolate exhibit lower expression of some P-gps as compared to WAL, and (ii) the combination of different MDRIs with IVM and LEV revealed greater effect in increasing the sensitivity of WAL isolate compared to Kirby (in terms of number of MDRIs showing synergism
and synergism ratio values). Taken together, decreased transcription of pgp-11 in MPL-R isolate compared to Kirby larvae further supports the possibility that decreased transcription (particularly pgp-11) (Chapter 5) or inhibition of ABC transport proteins may be playing a role in tolerance to MPL (Chapter 2). However, this is speculative, and further work is required to better explore these findings.

6.2 Limitations of the study

This research was planned carefully and has achieved all its aims set at the start of the project. However, there are a number of limitations to the study in terms of its scope and the techniques used. Consideration of these limitations will be important in the planning of future studies on the interaction of drugs with nematode transporters, and the role of transporters in anthelmintic resistance. Below is a description of some of these limitations of the present study:

1. The study involved one drug-susceptible (Kirby) and two drug-resistant (WAL and MPL-R) isolates of H. contortus. The observed effects of MDRIs in increasing the toxicity of anthelmintics to these isolates, particularly to WAL, may not translate to other isolates that show other resistance phenotypes. Further experimentation using a wide range of isolates from different regions is required to confirm the findings of the present study. In addition, the study was limited to the use of free-living third-stage larvae and the results may not translate directly to the parasitic adult life stage. Measuring the anthelmintic/MDRI interactions in adult worms would provide greater insight into this type of interaction in vitro and in vivo with the actual parasitic stage that is the target of chemotherapeutic approaches to worm control in the field.

2. The study did not provide an explanation for the unexpected effects of monepantel/MDRI combinations, particularly in the WAL isolate. As suggested in section 2.3, there may be an interaction between drug metabolism and drug efflux systems, or involvement of transport proteins in transporting the monepantel towards its target sites. Apart from ABC transporters, a plethora of proteins have been designated as putative multidrug resistance transporters on the basis of homology (Higgins, 2007). There may be activation of a backup mechanism to compensate for the reduced drug efflux through ABC transporters by the activation of another transporter family, e.g. drug metabolite transporters family (DMTs). Although DMTs have not been associated with resistance in nematodes, such a mechanism could be the focus of future studies.

3. A lack of information about which specific ABC transporters were inhibited was another limitation of the study. The study relied on extrapolation of knowledge around mammalian MDRIs, in the absence of nematode specific MDRIs. Structural comparison of nematode ABC
transporters with mammalian transporters would provide a good platform for using specific mammalian transporter inhibitors and also discovering nematode-specific MDRIs.

4. The use of bulk-worm analysis in gene expression experiments was a limitation of the research. Bulk-worm analysis may have masked some of the drug exposure effects as resistance acts at the level of an individual, hence using a pool of larvae may not have represented the actual impact of drug exposure. Working with individual L3s in drug exposure and gene expression experiments is technically difficult. This would have been possible if the experiment was performed on individual adult worms, however, was not possible with the much smaller freeliving larval stages. In addition, the qPCR experiments were limited to single dye system (SYBR green). A more extensive examination of gene expression patterns could have been achieved using Taqman probe PCRs, however this would have been a considerable task given the number of separate transporter genes examined here.

5. The study used larval migration assays and rhodamine-123 assays to evaluate the activity of upregulated or stimulated ABC transporters. The use of the R-123 assay in this way has previously been reported (Kerboeuf et al., 2011), and the LMA also worked well in quantifying the response of drug exposed larvae in terms of increased tolerance to IVM. However, a more direct means of examining transporter activity would have been beneficial to quantify the amount of drug present in the larvae over time, in the presence and absence of MDRIs. This could have been done using an analytical technique, such as High Performance Liquid Chromatography (HPLC), to measure drug levels. Alternatively, a commercially-available ELISA assay kit could be used to measure drug levels, at least for IVM. Such an approach should be considered for future studies.

6. The assessment of the effects of drug exposure on ABC transporters in this study was limited to gene expression and some indirect phenotypic measures (R-123 and migration assays) only, with no measurements of actual ABC transporter protein levels. As an additional approach, proteomic analysis could be used to characterise the effect of drug exposure on levels of transporter proteins. Transcriptomic analysis may further provide greater insights into the changes in a range of structures, including target receptors for different drugs, members of the ABC transporter family, as well other multidrug resistance transporter families.

6.3 Conclusions, implications, and future directions

This study highlights the need for future research on many aspects of drug efflux systems and AR in nematodes including: (i) in vivo use of anthelmintic/MDRI combinations to increase the efficacy of currently available anthelmintics, to which nematodes have developed resistance (ii) the development of chemical compounds that interact specifically with nematode transporters, (iii) the
study of the role of ABC transporters in cell physiology, and exploring their potential as drug targets, and (iv) the study of interactions of ABC transporters with MPL.

The potential of first and second generation P-gp inhibitors to potentiate drug efficacy \textit{in vitro} has been recognised for many years (reviewed by Lespine et al., 2012). Results from this thesis raise the possibility that some third generation inhibitors and crizotinib may also be considered as potential candidates for combination therapies to overcome drug efflux pathways in resistant parasitic nematodes. These anthelmintic/MDRI combinations are important in two ways; (i) the combinations which render resistant worms equally or more sensitive than susceptible worms can be used as a ‘resistance breaking’ strategy, and (ii) some MDRIs which also increase the sensitivity of susceptible worms to anthelmimtics have potential to reduce the recommended dose of an anthelmintic while maintaining 100% efficacy against susceptible worms.

Further studies with adult worms would be required to assess the practical applicability of the AR-reversing ability of different MDRIs, since this is the target life stage of most chemotherapeutic approaches for parasite control at this time. There are, however, significant barriers to such an approach. There might be potential side effects in the host as a result of inhibiting host animal efflux proteins alongside the nematode transporters (Lespine et al., 2008). Initially, though, \textit{in vitro} studies with adult worms using sensitive assays may be informative as to compound concentrations (ratios) required in order to observe synergistic effects with the adult life-stage. It may be that therapeutically safe doses for the host species are still able to exert an appropriate effect upon resident nematodes. Furthermore, structural comparison of nematode P-gps to host animal P-gps would provide a framework to develop MDRIs that bind selectively to the nematode P-gps. Using such drug delivery systems that minimize or inhibit the absorption of MDRIs in the gastrointestinal tract (GIT) and increase the resident time within GIT (Ponchel and Irache, 1998; Wu et al., 2015), is another strategy that may result in increased MDRI concentration within the GIT without inhibiting host P-gps, thereby protecting the host animal against the possible side effects. A second important barrier to \textit{in vivo} application of MDRIs is the high cost of such compounds. These combination therapies will not be a cost-effective option while cheaper drugs remain effective, however, in the future, such cost constraints may diminish.

The present study has built upon our understanding of the interaction of anthelmimtics with ABC transporters in nematodes. Great diversity in the transcription levels of different ABC transporters in \textit{H. contortus} suggests a variety of specific roles in protecting worms against a range of xenobiotics, as has previously been suggested (Prichard and Roulet, 2007). The overexpression of several P-gp genes in a resistant field isolate compared to a susceptible reference isolate allows
one to imagine that these specific P-gps might be contributing to the observed drug resistance in this particular resistant isolate. In addition, the induction of increased transcription of multiple ABC transporter genes in the resistant isolate following exposure to IVM and LEV suggests that these drugs interact with a number of *H. contortus* ABC transporters, and hence are likely to be the substrates for these transporters. An important outcome of this work was the observation that worms previously exposed to drugs have increased tolerance to higher concentrations of IVM. This suggests that the increases in transcription of multiple ABC transporters and stimulation of existing efflux proteins (as measured by R-123 efflux) lead to an increased ability of worms to tolerate IVM. In addition, this research has also demonstrated that exposure of both resistant and susceptible worms to MPL leads to subsequent upregulated transcription of transporter genes and increased tolerance of these worms to IVM. It is worth noting, however, that these observations were only made following exposure to MPL at a concentration well above that experienced by parasitic life-stage *in vivo* (Lifschitz et al., 2014), and hence, the practical implications remain unclear. Importantly, though, this was the first report to indicate that MPL interacts with worm ABC transporters. This raises the possibility that efflux pathways could be contributing to the recently reported resistance to MPL in field isolates of *H. contortus* and other species.

The characterization of MPL sensitivity in the MPL-R isolate using the LDA suggests that this assay may be a useful tool for detection of MPL resistance in field isolates of this species. It will be important to use such diagnostic tools to monitor for the emergence of MPL resistance as reports from the field to date indicate that such resistance can emerge quickly and render the compound completely ineffective. Hampering surveillance against emerging field resistance is the fact that the molecular basis of MPL resistance remains unknown. This thesis has provided evidence that multiple mechanisms might be at play in MPL resistance, but also evidence that increased efflux by ABC transporters may not play a role in resistance to MPL in the isolate examined in this study. However, reduced sensitivity of WAL isolate larvae to MPL in the presence of MDRIs suggests that these transporters may be involved in some way in determining the sensitivity to MPL. While these observations have advanced the knowledge frontier, there is still much work to be done in better characterising phenotypic and genetic aspects of MPL resistance in helminths of veterinary importance.

In conclusion, this thesis enhances our understanding of the interactions of anthelmintics with nematode ABC transporters, and on the mechanisms of AR. In the future, *in vivo* and *in vitro* studies with adult worms to explore the effectiveness of anthelmintics/MDRI combination therapies, and the consequences of drug exposure in terms of modification of transporters and subsequent drug tolerance will build upon this work to provide greater insights into understanding...
the role of ABC transporters in nematodes. Finally, further characterisation of the physiological functions of ABC transporters may expose their vulnerability as potential drug targets in nematodes, revealing another chemotherapeutic approach to the control of GINs.


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