Discovery of new chemicals with anthelmintic activity against the barber's pole worm and other parasitic nematodes

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SUMMARY

Parasitic nematodes cause a substantial disease burden on humans and animals worldwide. A review of the literature (Chapter 1) showed that, on one hand, neglected tropical diseases caused by parasitic nematodes have a devastating, long-term impact on human health; on the other hand, gastrointestinal nematodes are a major constraint to the livestock industries, causing subclinical infections and diseases in animals and leading to a substantial reduction in meat, milk and fibre production. Currently, anthelmintic treatment remains the mainstay of controlling parasitic nematode infections. However, the massive and widespread resistance to the limited number of commercial anthelmintics, particularly in the veterinary and agricultural contexts, demonstrates an urgency to discover new and effective anthelmintics to sustain the economic and health benefits from the application of anthelmintics. Thus, the key focus of this thesis was to discover new chemical entities and/or known drugs with anthelmintic activities against *Haemonchus contortus* and/or other socioeconomically important parasitic nematodes for subsequent development. Whole worm-based phenotypic screening assays were employed, compound collections were obtained *via* product-development-partnerships and/or collaborators, and active compounds were assessed for their potential as anthelmintic candidates.

In this thesis, one new chemical entity (designated SN00797439), two human kinase inhibitors (SNS-032 and AG-1295), 14 AG-1295 (tetrahydroquinoxaline) analogues, one insecticide (tolfenpyrad) and two tolfenpyrad (pyrazole-5-carboxamide) derivatives (a-15 and a-17) with anthelmintic activity in vitro were discovered following the screening of a total of 15,333 chemicals from five distinct compound collections against *H. contortus*. In Chapter 2, a new chemical entity, SN00797439, was identified with activity against a range of parasitic nematodes, including *H. contortus, Ancylostoma ceylanicum, Brugia malayi, Dirofilaria immitis* and/or *Trichuris muris* in vitro, offering a novel, lead-like scaffold for the development of a relatively broad-spectrum anthelmintic.

In Chapter 3, two human kinase inhibitors under pharmaceutical development, SNS-032 (piperidinecarboxamide) and AG-1295 (quinoxaline), were identified to have inhibitory activity on the motility and development of parasitic larvae of *H. contortus* in vitro. AG-1295 had limited cytotoxicity against a normal mammalian epithelial cell line (designated MCF10A).

In Chapters 4 and 5, three pyrazole-5-carboxamides (tolfenpyrad, a-15 and a-17) were shown to possess significant inhibitory effects on *H. contortus* without detectable toxicity on

a human neonatal foreskin fibroblast (NFF) cell line in vitro. All three of these chemicals were shown to inhibit the oxygen consumption in *H. contortus* larvae, a finding that was consistent with the known, specific inhibition of complex I of the respiratory electron transport chain by selected pyrazole-5-carboxamides in arthropods. The evaluation of these hit compounds using various technologies employed in parasitology, drug discovery, chemistry, histology, toxicology, molecular biology and bioinformatics should offer data to support their potential as leads for future drug development and to facilitate the exploration of their mode(s) of action in this and related nematodes.

Encouraged by the findings in Chapter 3 and the detection of a non-wildtype phenotype in treated worms in vitro, Chapter 6 investigated the activities of 14 additional tetrahydroquinoxaline (AG-1295) analogues on *H. contortus*. Qualitative and quantitative assessments of larval motility, development and morphological alterations showed that these 14 chemicals all affected the viability of parasitic larvae and, interestingly, induced an eviscerated larval phenotype and led to cuticular damage and/or stunted growth in in vitro *H. contortus*.

Taken together, Chapters 2 to 6 identified a series of 20 hit compounds, some of which have selectivity against *H. contortus* compared with selected human cell lines tested. In Chapter 7, the research achievements are summarised, and the next steps to be pursued in future research are outlined, including (i) the chemical optimisation of representative chemicals via structure-activity relationship (SAR) evaluations; (ii) assessment of the breadth of spectrum of anthelmintic activity on other parasitic nematodes, such as other strongyloids, ascaridoids, enoplids and filarioids; (iii) detailed investigations of the absorption, distribution, metabolism, excretion and toxicity (ADMET) of optimised chemicals with broad nematocidal or nematostatic activity; (iv) establishment of the modes of action of lead candidates. The findings from the thesis are then put into a broad context and discussed. In conclusion, the present thesis contributes to the fields of parasitology and anthelmintic discovery by identifying compounds with in vitro anthelmintic activity that represent sound starting points for ‘lead’ discovery.

DECLARATION

The work described in the thesis was performed in the Faculty of Veterinary and Agricultural Sciences of the University of Melbourne between September 2015 and November 2018. The scientific work was performed by the author, with the exception of the assistance which has been specifically acknowledged. The thesis is less than 100,000 words in length, exclusive of tables, figures, references and appendices. No part of this thesis has been submitted for any other degree or diploma.

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PREFACE AND DISSEMINATION OF RESEARCH FINDINGS

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Peer-reviewed articles published in international scientific journals:

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Preston, S.*, **Jiao, Y.***, Jabbar, A., McGee, S., Laleu, B., Willis, P., Wells, T.N.C., Gasser, R.B., 2016. Screening of the 'Pathogen Box' identifies an approved pesticide with major anthelmintic activity against the barber's pole worm. *International Journal for Parasitology: Drugs and Drug Resistance* 6, 329-334 (Chapter 4). *Joint first authorship.

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Hofmann, A., Jabbar, A., Gasser, R.B., 2018. Tetrahydroquinoxalines induce an lethal evisceration phenotype in *Haemonchus contortus* larvae in vitro. *International Journal for Parasitology: Drugs and Drug Resistance* 9, 59-71 (Chapter 6).

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TABLE OF CONTENTS

Chapter 1 - Literature review

1.1 Introduction

Parasitic nematodes cause diseases that adversely impact on human health and livestock production (Fenwick, 2012; Fitzpatrick, 2013; Holden-Dye and Walker, 2014). On one hand, more than one billion people in the world, particularly in underprivileged developing countries, suffer from neglected tropical diseases, such as hookworm disease, lymphatic filariasis and onchocerciasis (Hotez et al., 2007; Fenwick, 2012; Hotez et al., 2016a). On the other hand, parasitic nematode infections severely constrain livestock production and represent a major burden to livestock industries worldwide (McLeod, 1995, cf. Roeber et al., 2013; Charlier et al., 2014; Sargison, 2016). Therefore, enormous health and economic benefits can be gained from effectively controlling parasitic nematode infections and associated diseases.

Focussing on economically important livestock production system, the control of parasitic nematodes can be divided into strategies that use anthelmintics and those that do not (Waller, 2006; Sargison, 2012). In particular, anthelmintic treatment remains a dominant feature of the control of parasitic nematode infections (Waller, 1993; Sargison, 2012; Preston et al., 2014). However, the emergence of anthelmintic resistance to the current small numbers of commercial anthelmintics is challenging the efficiency of this dominant control, emphasising the need to discover new anthelmintics (Wolstenholme et al., 2004; Kaplan and Vidyashankar, 2012; Miller et al., 2012).

With regard to anthelmintic discovery, two commonly used strategies are mechanism-based screening and whole worm-based screening, with their individual advantages and disadvantages that need to be considered (Geary et al., 1999; Geary et al., 2015). Besides the screening strategies, the rational selection of compound collections and the application of advanced -omics technologies for screening also play a key role in the early anthelmintic discovery stage (Dandapani et al., 2012; Preston et al., 2016b).

The purpose of this literature review was to critically appraise the current literature on parasitic nematodes, control of parasitic nematode infections, anthelmintic resistance and new anthelmintic discovery, in order to identify knowledge gaps and then to define the research aims of this thesis.

1.2 Parasitic nematodes of socioeconomic importance

Parasitic nematodes exert a heavy disease burden on human health and place a major economic burden on livestock production (Fenwick, 2012; Fitzpatrick, 2013; Holden-Dye and Walker, 2014). Based on various studies, neglected tropical diseases represent a burden of 26 million disability-adjusted life years (DALYs) (Hotez et al., 2014; Hotez et al., 2016a). The DALYs represent losses due to ill-health, disability and early death, which is a tool to measure and compare disease burdens (Mathers et al., 2007). Neglected tropical diseases including hookworm disease, ascariasis, trichuriasis, lymphatic filariasis and onchocerciasis, caused by parasitic nematodes, represent a disease burden of 8.46 million DALYs (Hotez et al., 2007; Hotez et al., 2016a).

1.2.1 Parasitic nematodes of human health importance

Hookworm disease, ascariasis and trichuriasis are three main soil-transmitted helminth infections caused by intestinal nematodes (de Silva et al., 2003; Bethony et al., 2006). Hookworm disease represents 3.23 million DALYs (Hotez et al., 2016a), which is mainly caused by nematodes including *Ancylostoma duodenale* and *Necator americanus* (Crompton, 2000). The public health significance of hookworm disease relates to blood loss caused by the worms' feeding activity in the human gut, leading to the iron-deficiency anaemia (Brooker et al., 2008; Smith and Brooker, 2010). The other two intestinal nematode diseases, ascariasis and trichuriasis, represent 1.32 million and 0.64 million DALYs, respectively (Hotez et al., 2016a). Ascariasis caused by *Ascaris lumbricoides* and trichuriasis caused by *Trichuris trichiura* commonly co-infect people, together with hookworm infection (Bethony et al., 2006), and mainly affect children, resulting in malnutrition, impaired growth, and reduced physical fitness and intellectual development (WHO, 2005; Bethony et al., 2006).

The other two neglected tropical diseases, lymphatic filariasis and onchocerciasis, are caused by filarial nematodes. For lymphatic filariasis (2.78 million DALYs), *Brugia malayi, Brugia timori* and *Wuchereria bancrofti* are the causative agents in humans, with worms developing in the lymphatic systems and microfilariae circulating in the blood system (Molyneux, 2010; Taylor et al., 2010). The invasion and damage of the lymphatic system by filarial worms accounts for a chronic suppression of host immunity, causing permanent disability in patients (Taylor et al., 2010). It is also common for lymphatic filariasis to co-occur with other diseases, in which the suppressive immunomodulatory mechanisms caused by lymphatic filariasis have been identified to modulate protective immune responses for

malaria and tuberculosis (Babu et al., 2009a; Babu et al., 2009b; Metenou et al., 2009). Onchocerciasis (0.49 million DALYs), which is linked to blindness, is caused by another filarial nematode, *Onchocerca volvulus*, with adult worms occupying the subcutaneous layers of the skin and deep tissues, and microfilariae migrating to the skin and the eyes (Burnham, 2010; Taylor et al., 2010).

Neglected tropical diseases commonly occur in underprivileged areas or countries, where affected people live on less than 2 dollars per day (Bethony et al., 2006; Hotez et al., 2007; Houweling et al., 2016; Stolk et al., 2016). The socioeconomic inequality has resulted in a lack of commercial incentive for solutions, such that there is a 13-fold greater probability of a drug being brought to market for the treatment of nervous system disorders or cancer than for a neglected tropical disease (Trouiller et al., 2002), even though the latter diseases rank amongst the world's greatest global health problems (Hotez et al., 2016b; Houweling et al., 2016; Stolk et al., 2016).

1.2.2 Parasitic nematodes of veterinary importance

Parasitic nematodes are amongst the major constraints in livestock production systems (Fitzpatrick, 2013; McRae et al., 2015; Sargison, 2016). Particularly gastrointestinal nematode infections cause reduced meat, milk and fibre production and even the death of animals (Charlier et al., 2014; Preston et al., 2014), leading to annual economic losses estimated at billions of dollars globally (cf. Roeber et al., 2013). Key gastrointestinal nematodes of small ruminants responsible for the substantial economic losses include *Haemonchus contortus, Teladorsagia circumcincta* and *Trichostrongylus* species (see Zajac, 2006; Cantacessi et al., 2012; Roeber et al., 2013). Other gastrointestinal nematodes include *Cooperia curticei*, *Nematodirus spathiger, N. fillicollis, N. abnormalis*, *Oesophagostomum venulosum, Bunostomum trigonocephalum* and *Chabertia ovina* (see Anderson, 2000; Zajac, 2006). Although some species can have relatively low pathogenicity alone, they can contribute substantially to the overall problem of parasitic gastroenteritis in grazing small ruminants in situations with mixed infections (Craig, 1986).

Teladorsagia circumcincta and *Trichostrongylus* spp. are prevalent in cool temperature regions, including parts of Europe, Scandinavia, Asia, New Zealand, America and Australia, with temperate climates being favourable for larval development (O'Connor et al., 2006). The pre-patent period for *T. circumcincta* is 21 days; adult worms are relatively short lived, surviving in their hosts for only a few months. *Teladorsagia circumcincta* inhabits the gastric

glands of the abomasum but does not feed on blood (Anderson et al., 1985). The main pathogenic effects are caused by its larval stages (Zajac, 2006; Cantacessi et al., 2012; Roeber et al., 2013). Larvae form nodules in the abomasal mucosa so as to damage parietal cells which secrete hydrochloric acid to stimulate the conversion of pepsinogen to pepsin (Anderson et al., 1985; McKellar, 1993). Pepsin is essential for protein digestion; thus, heavily infected ruminants can develop anaemia, diarrhoea and death (Holmes, 1985). *Trichostrongylus colubriformis, T. rugatus* and *T. vitrinus* are the three common *Trichostrongylus* species (Beveridge et al., 1989). *Trichostronglus vitrinus* is considered to be the most pathogenic one; the main pathogenic effects are caused by the exsheathed third-stage larvae (xL3s) which induce villus atrophy by disrupting the integrity of the intestinal epithelium and causing plasma loss from the gut (Beveridge et al., 1989). *Trichostronglus colubriformis* is considered to be the intermediate pathogenic worm; heavy infections can cause the enteritis, hypoalbuminaemia and hypoproteinaemia (Barker and Titchen, 1982). *Trichostronglus rugatus* is considered to be the least pathogenic worm (Beveridge et al., 1989).

Haemonchus contortus is recognized as the most highly pathogenic nematode, mainly in tropical and subtropical areas (Miller and Horohov, 2006; O'Connor et al., 2006). Tropical areas include parts of south-east Asia, southern India, central Africa and America, and northern South America, and subtropical areas include eastern Australia, southern Africa, southern North America, South America, and south-east China (cf. O'Connor et al., 2006; Getachew et al., 2007). Various studies have showed that *H. contortus* is not suited to live in cold climates, because of the negative effects of low temperature on the survival and development of the eggs and larvae, and the migratory activities of free-living larvae (Rose, 1963; O'Connor et al., 2006). Individual females of *H. contortus* produce thousands of eggs per day, which can contaminate pastures (Coyne et al., 1991). In sheep, the pre-patent period is 17–21 days, and the life span of adult worms is a few months (Courtney et al., 1983). *Haemonchus contortus* inhabits the mucosa of the abomasum (Parkins and Holmes, 1989). The main pathogenic effect is caused by the fourth-stage larvae (L4s) and adults, which both feed on blood, causing severe anaemia (Baker et al., 1959). Acute infection with large numbers of *H. contortus* usually causes weight loss, with the clinical signs of tanned faeces, anaemia, oedema, fatigue and/or sudden death (Zajac, 2006; Cantacessi et al., 2012; Roeber et al., 2013). Chronic infection decreases small ruminants' food intake, which causes significant weight loss (Kassai, 1999; Taylor et al., 2016).

H. contortus is amongst the experimentally most tractable parasites because of its

fecundity and ease of production (Laing et al., 2013; Geary, 2016). *Haemonchus contortus* has a direct and rapid life cycle which can be divided into free-living and parasitic phases (Fig. 1.1). In the free-living phase, the eggs hatch to first-stage larvae (L1s) in faeces, then moult through second-stage larvae (L2s) to third-stage larvae (L3s); L3s are infective, and migrate from faeces on to pasture, and are then ingested by grazing ruminants, after which the parasitic phase starts. In the parasitic phase, L3s exsheath predominantly in the rumen/reticulum and moult to fourth-stage larvae (L4s) in the abomasum; L4s then develop to adults, and female adults lay eggs in the abomasum, representing the end of the cycle (Veglia, 1916). In addition, *H. contortus* has a close phylogenetic position to the model organism *Caenorhabditis elegans*, and is well-placed for comparisons with *C. elegans* and other related nematodes that infect humans and animals (e.g., Blaxter et al., 1998; Laing et al., 2013; Schwarz et al., 2013).

1.3 Treatment and control of parasitic nematodes

Currently, anthelmintic treatment remains the mainstay of controlling nematode parasites (Waller, 1993; Waller, 2006; Sargison, 2012; Preston et al., 2014). In addition, non-chemotherapeutic means of control and management also play an essential role in livestock production systems (Waller, 1999; Sargison, 2012; Besier et al., 2016; Selemon, 2018; Vercruysse et al., 2018).

1.3.1 Anthelmintic treatment of parasitic nematode infections

Anthelmintic treatment remains a key approach to controlling parasitic nematode infections (Sargison, 2012; Epe and Kaminsky, 2013; Preston et al., 2014; Besier et al., 2016), and the extraordinary success and enormous benefits from using anthelmintic drugs are undeniable (Sargison, 2012; Geary et al., 2015). Currently, five major anthelmintic classes are applied in the field of veterinary medicine: benzimidazoles, imidazothiazoles, macrocyclic lactones, monepantel and derquantel (McKellar and Jackson, 2004; Sargison, 2012; Besier et al., 2016; Harder, 2016). In addition, closantel is a very useful narrow spectrum anthelmintic for only being used to control *H. contortus* (see Hall et al., 1981; Dash, 1986; Besier et al., 2016). Here, individual anthelmintics are reviewed in a chronological order based on their initial introduction to the market (Fig. 1.2).

In the early 1960s, the first benzimidazole drug, thiabendazole, was reported to have broad-spectrum anthelmintic activity against gastrointestinal parasites of domestic animals

(Brown, 1961). Before that, many plant metabolites and chemical compounds with toxic effects on parasitic nematodes, were used to control parasitic nematode infections, whereas most of them were also toxic to mammalian hosts (Sargison, 2012). Benzimidazoles are tubulin-binding anthelmintics, which bind to nematode tubulin in the cell cytoplasm, resulting in the inhibition of the formation of microtubules (Borgers and De Nollin, 1975; Lacey, 1988, 1990; Sargison, 2012). Microtubules are essential to many cellular activities by transporting secretory granules or enzymes within the cell cytoplasm (Borgers et al., 1975; Lacey, 1988; McKellar and Jackson, 2004; Sargison, 2012). Thus, nematodes die of "starvation" after exposure to benzimidazoles (McKellar and Jackson, 2004; Sargison, 2012).

Followed by the introduction of benzimidazoles, the first imidazothiazole drug, levamisole, was reported (Thienpont et al., 1966) and initially approved for use in the early 1970s (Vakil et al., 1970). Imidazothiazoles act as cholinergic agonists at nematode nicotinic neuromuscular junctions, causing sustained muscle contraction and spastic paralysis of nematodes (Van Neuten, 1972; Coles et al., 1975; McKellar and Jackson, 2004; Blanchard et al., 2018). In addition, imidazothiazoles have a narrower therapeutic index than other broad-spectrum anthelmintics, given that imidazothiazoles also act as nicotinic agonists in mammals (Robertson and Martin, 1993; McKellar and Jackson, 2004).

The first macrocyclic lactone, ivermectin, as a natural fermentation product of *Streptomyces avermilitis*, was launched on to the market in the early 1980s, and is a representative of the avermectins (Sutherland and Campbell, 1990; Sargison, 2012). Later, another natural fermentation product of *Streptomyces cyanogriseus*, moxidectin, became the most commonly used drug of the milbemycins (Shoop et al., 1995). The macrocyclic lactones act on ligand-gated ion channels, with the neurotransmitter gamma-aminobutyric acid (GABA)-gated chloride ion channels and glutamate-gated chloride ion channels as the main targets (Arena et al., 1992; Brownlee et al., 1997; Feng et al., 2002; Sargison, 2012). By targeting the ion channels, macrocyclic lactones increase membrane permeability to chloride ions, leading to reduced pharyngeal pumping, paralysis of body muscles and having adverse effects on the uterus (Sutherland and Campbell, 1990; Gill et al., 1991; Geary et al., 1993; Yates et al., 2003; Sargison, 2012). In addition, P-glycoproteins are considered to be essential to the selective toxicity of macrocyclic lactones in nematodes. P-glycoproteins, representing a cell-membrane efflux pump (Broeks et al., 1995), can exclude macrocyclic lactone drugs from distributing into the central nervous system (Lankas et al., 1997; Kerboeuf et al., 2003; McKellar and Jackson, 2004). There are studies showing that P-glycoprotein inhibitors can improve the efficacy of macrocyclic lactones through oral absorption, because P-

glycoproteins are also present in the gut (Lifschitz et al., 2002; McKellar and Jackson, 2004).

A decade ago, the amino-acetonitrile derivative (AAD) drug, monepantel, was licensed in Australia, Europe and Latin America (Kaminsky et al., 2008a; Kaminsky et al., 2008b; Mason et al., 2009). Monepantel was shown to be an allosteric regulator, binding to nicotinic acetylcholine receptor subunits (DEG-3 subfamily), including ACR-20 and ACR-23 in *C. elegans,* and a ACR-23 homologous protein, MPTL-1, in *H. contortus* (see Kaminsky et al., 2008a; Sargison, 2012; Baur et al., 2015), which are gated by betaine and choline (Rufener et al., 2010; Peden et al., 2013; Baur et al., 2015). ACR-23/MPTL-1 is expressed in body wall muscle cells; therefore, monepantel can cause paralysis in treated nematodes (Sargison, 2012; Rufener et al., 2013). The reason for the selective toxicity of monepantel to nematodes but not mammalian hosts is that ACR-23/MPTL-1 is nematode-specific (Rufener et al., 2010; Lecová et al., 2014).

In 2011, as a semi-synthetic member of spiroindoles, derquantel, was introduced to the animal health market, in combination with abamectin (Lee et al., 2002; Little et al., 2010; Little et al., 2011). Derquantel also belongs to the nicotinic acetylcholine receptor antagonists, causing rapid muscle paralysis and death in treated nematodes (Ruiz-Lancheros et al., 2011; Sargison, 2012).

These commercial anthelmintics can assist in controlling infections of different parasitic nematodes and do not compromise the performance of hosts when given at the recommended doses (Sargison, 2012). Benefits to the livestock production system have been excellent, which means that anthelmintic treatment has been and will likely remain a dominant feature of nematode control.

1.3.2 Non-chemotherapeutic control of parasitic nematode infections

In addition to anthelmintic treatment, non-chemotherapeutic approaches also play an important role in controlling parasitic nematode infections. Here, the advantages and disadvantages of five non-chemotherapeutic methods commonly used in the livestock production systems are appraised; these approaches include breeding of livestock for parasite resistance, grazing management, vaccination, supplementary feeding and biological control (Waller, 1997, 1999, 2006; Shalaby, 2013; Besier et al., 2016; Selemon, 2018).

Breeding approaches

Selecting genetically superior animals for breeding has been explored widely in the

livestock industry (e.g., Woolaston and Baker, 1996; Waller, 1997). Through continuous exposure to infected larvae, based on Darwinian principle of 'survival of the fittest', it is reasonable to assume that animals that are naturally resistant to parasitic nematodes exist (Waller, 1997). For instance, the Red Maasai sheep has been reported to be resistant to gastrointestinal nematode infections (Mugambi et al., 1996; Benavides et al., 2015). Other examples of genetic resistant animals are *Bos indicus* cattle, which are resistant to the cattle tick *Rhipicephalus (Boophilid) microplus or australis* (see Nabours, 1912; Riek, 1962; Seifert, 1971; Wagland, 1978; Estrada-Pena et al., 2012; Jonsson et al., 2014), and Javanese thin-tailed sheep, which are resistant to a trematode *Fasciola gigantica* (see Wiedosari and Copeman, 1990). Moreover, given that the performance of an animal's offspring can be predicted according to their individual performance and genetic history, it is reasonable to assume that the introduction of high performance genotypes and/or cross-breeding with indigenous breeds would help improve productivity (Waller, 1997). It is logical to assume that ruminants that are naturally resistant to parasitic nematodes can be selected by breeding, which may be very useful to sustainable parasite control in livestock production system (Waller, 1997).

However, introducing exotic breeds or cross-breeding with indigenous breeds may fail to achieve worm resistance. The major problem encountered for exotic breeds is the adaptation to new environments, which means that it could be difficult for exotic breeds to survive, thrive and reproduce, leading to reduced tolerance to diseases in new environments (Waller, 1997, 1999). In addition to phenotypic traits, such as faecal egg counts and other quantitative traits in sheep and cattle associated with resistance to gastrointestinal nematodes (e.g., Kim et al., 2014; Benavides et al., 2015), many fundamental studies of animals need to be undertaken to define genes or genomic elements responsible for such resistance.

Grazing management

The life cycle of parasitic nematodes of ruminant livestock can be divided into free-living and parasitic phases. The control of parasitic nematode infections through grazing management is based on controlling the free-living stages of parasitic nematodes on pasture (Waller, 1997; Barger, 1999; Waller, 2006). Rotational grazing through subdivision of pasture into small plots and alternation of grazing host species are key approaches of grazing management (Barger, 1999).

Grazing management through interchanging pastures allows farmers to divide one pasture into small plots and rotate animals grazing in these subdivided plots (Waller, 1997; Barger,

1999; Waller, 2006; Besier et al., 2016). The combination of grazing management with anthelmintic treatment has been highly recommended in temperate regions (Levine et al., 1975; Waller, 1997). This strategy not only helps reduce the usage of anthelmintics by decreasing the intensity of infection of parasitic nematodes (Barger et al., 1994) but also helps improve the efficiency of herbage conversion into animal products (Barger, 1997). However, this strategy is affected by a number of factors including the environmental conditions, seasonal conditions, parasite species, infection state and animal grazing behaviour (Waller, 2006; Besier et al., 2016). Moreover, the grazing time and the number of subdivided plots need to be well controlled and planned in order to keep efficacy as well as utilize pastures economically. If the grazing time is too long and/or there are too many subdivided plots, the grazed plot could be severely damaged while the ungrazed plots left senescent; if the grazing time is too short and/or there are too few subdivided plots, rotation length may not be long enough for grazed plots to regrow (Barger, 1999). In this instance, farmers often consider themselves to take more effort in grazing management than simply drenching animals with anthelmintics (Waller, 1997).

Interchanging different species of animals to graze on the same pasture has been stated as a very effective grazing form in controlling parasite infections and increase the efficacy of pasture utilisation (Waller, 1997; Besier et al., 2016). The principle of this method is based on that the same species of nematodes are either usually not able to cross transmit to different species of animals or less pathogenic to different species of animals (Waller, 1997; Barger, 1999; Waller 2006). For example, if sheep graze on one pasture first, the following year would be considered safe for cattle to graze on the same pasture; alternative grazing between cattle and sheep is effective for controlling infection of many parasites of both the cattle and sheep (Waller, 1997).

Other reported grazing management strategies include concurrent grazing two different animal species, increasing stocking rate and the use of bioactive forages, e. g., tanniferous plants, in grazing (Waller, 1997, 2006; Besier et al., 2016). However, grazing mixed species of animals did not necessarily reduce the requirement for anthelmintics (Nari et al., 1996; Waller, 1997). Increasing the stocking rate was shown to fail to improve animal production; there is no clear relationship between stocking rate and parasitism (cf. Waller, 2006). Moreover, the efficacy and mechanism of bioactive forages needs further investigation (cf. Waller, 2006; Hoste et al., 2016).

Vaccination

It would be of substantial benefit to use effective vaccines to control parasitic nematodes of livestock (Hewitson and Maizels, 2014). The use of mathematical modelling to predict the consequences of vaccination has revealed that substantial benefits are likely to be obtained even with 80% efficacy achieved or more than 80% of the flock protected (Barnes et al., 1995; Besier et al., 2016). Much work and enormous funds have been invested to attempt to develop effective vaccines to control parasitic nematode infections.

Currently, no vaccines are available for human use (Hewitson and Maizels, 2014) and only radiation-attenuated vaccines for lungworm *Dictyocaulus viviparus* of cattle (Jarrett et al., 1959) and for hookworm *A. caninum* of dogs (see Miller, 1965) were/have been sold commercially. After some 20 years of work, a promising *H. contortus* vaccine called "Barbervax" containing hidden antigens, H-gal-GP and H11, has been developed and commercialised by the Moredun Research Institute in Scotland (Smith, 2014; Besier et al., 2016). The two vaccine antigens function in the digestion of blood taken up by *H. contortus*, and thus the antigen specific antibodies produced after vaccination can bind to the functional antigens to disturb digestive processes, resulting in worm starvation (Knox et al., 2003; Smith and Zarlenga, 2006; VanHoy et al., 2018). Previous study of assessing H-gal-GP and H11 as candidate vaccine antigens and vaccination trials have proven the efficacy of these two vaccine antigens in sheep (Kabagambe et al., 2000; Smith et al., 2001; Knox et al., 2003; LeJambre et al., 2008; Bassetto et al., 2014; Besier et al., 2016; Nisbet et al., 2016).

However, due to the vaccine antigens reside on the mucosal membrane of the worm's gastrointestinal tract that are hidden to the host immune system, a challenge infection could not boost the immune response effectively and repeated vaccination is required (Smith et al., 2001; LeJambre et al., 2008; Emery et al., 2016). To date, it remains unsuccessful to achieve recombinant protective versions of the two vaccine antigens (Miller and Horohov, 2006; Hewitson and Maizels, 2014; Besier et al., 2016; Gasser et al., 2016). The lack of a detailed understanding of the complexity of the immune system also delays the development of effective vaccines (Grencis et al., 2014; McRae et al., 2015). Despite the development of Barbervax (Besier et al., 2016), much research needs to be done before effective vaccines are developed against parasitic nematodes of livestock (Hewitson and Maizels, 2014; McRae et al., 2015).

Supplementary feeding

Parasitic nematode infections have been stated to decrease voluntary feed intake, induce substantial losses of endogenous proteins, redirect the nutrition to be used for immunological

responses and increase the synthesis of specific proteins to repair the damage caused by infections in hosts (Knox et al., 2006). During parasitic nematode infections, the pain, discomfort and the disrupted hormonal feedback mechanism(s) induced by infections are considered to be reasons for decreased voluntary feed intake in infected hosts (Symons, 1985; Knox et al., 2006). In addition, gastroenteritis caused by most infections could result in the protein losses, leading to reduced productivity, or result in reabsorption and recycling of the nutrients using additional energy (Knox et al., 2006). In addition, nematode infections cause immunological responses which also consume the nutritional resources (McRae et al., 2015); otherwise, nutritional resources could be used in other processes, such as meat growth, wool production and milk production (Knox et al., 2006). The provision of additional protein to infected hosts has also been observed to enhance the immunity to the parasitic nematode infections (Brunsdon, 1964; Coop et al., 1995; Coop and Kyriazakis, 2001), and maintain productivity in livestock (Knox et al., 2006).

The reality is that it is quite complex to apply a supplementary feeding program, with many factors that need to be considered for implementation, including the species of hosts, stages of growth and reproduction of hosts, the seasonal availability of forage, the species of infected parasitic nematodes, and the intensity of infections (Knox et al., 2006). In addition, the cost of supplementary feeding remains an essential consideration for farmers to decide whether they are willing to employ this means to assist in controlling parasites or not (Knox et al., 2006).

Biological control

The aim of biological control is to break the life cycles of parasites by targeting or removing free-living worm stages (Waller, 1997, 2006). It is especially successful in controlling the helminth infections of humans through the extensive use of “pit toilets” (Waller, 1997). An example in the livestock production system is the use of coprophagous invertebrates, e.g., dung beetles, scarabs or earthworms, by which faecal masses are removed from pastures, so that the infective larvae are not able to be ingested by grazing ruminants (Gronvold, 1987; Waller, 2006). In addition, particular birds seek out the coprophagous invertebrates as food. Thus, faecal masses are broken down and dry rapidly, inhibiting the development and survival of free-living larvae and/or faecal masses are dispersed, contributing to the disruption of the life cycle(s) of parasitic nematodes (Waller, 1997, 2006).

Importantly, the use of nematode-destroying fungi to achieve the control of parasitic nematode infections has shown considerable promise (Larsen et al., 1998; Larsen, 1999;

Chandrawathani et al., 2004; Waller, 2006; Waller et al., 2006). With the ability to survive in the digestive system of livestock, a propensity to grow rapidly in fresh dung and a voracious nematophagous capacity, *Duddingtonia flagrans,* attracted considerable attention as a nematode control agent (Larsen et al., 1998; Larsen, 1999; Chandrawathani et al., 2004; Waller et al., 2006), but has not yet been commercialised widely. In terms of the effects of biological control, it has shown to be labile and daily supplementation of fungal material is recommended, in order to achieve optimal results (Waller, 2006; Waller et al., 2006). Thus, compared with anthelmintic treatment, biological control is more time-consuming and can be inconsistent in controlling parasitic nematodes infections (Waller, 2003).

In spite of the development of non-chemotherapeutic means, effective anthelmintics are still needed to assist the control of parasitic nematode infections (Knox et al., 2006). Critically, strategic and integrated control programs incorporating non-chemotherapeutic control and anthelmintic treatment are required (Waller, 1997; Sargison, 2012). Nonetheless, livestock farmers usually consider that they need to invest more efforts and cost into the non-chemotherapeutic methods compared with anthelmintic treatment, such that they prefer to rely on the easiest, inexpensive and most effective anthelmintic treatment. Therefore, anthelmintic treatment remains a predominant component of controlling parasitic nematodes in livestock production systems (Sargison, 2012; Charlier et al., 2014; Preston et al., 2014; Besier et al., 2016).

1.4 Anthelmintic resistance in parasitic nematodes

The irresponsible use of the limited number of anthelmintics has led to anthelmintic resistance, which has become a major problem in veterinary medicine (Prichard et al., 1980; Wolstenholme et al., 2004; Fleming et al., 2006; Miller et al., 2012; Kotze and Prichard, 2016). Currently, resistance to all the above anthelmintic classes (see section 1.3.1) has now been reported, with the exception of resistance to derquantel (McRae et al., 2015). It is noteworthy that the resistance to individual anthelmintics has been reported within a few years after the introduction of these anthelmintics (Kaplan, 2004). The resistance to benzimidazoles was first reported in 1964 (Conway, 1964), followed by imidazothiazoles in 1979 (Sangster et al., 1979) and the macrocyclic lactones in 1988 (van Wyk and Malan, 1988). As the first new anthelmintic on the commercial market in more than 25 years, monepantel was also been reported to induce resistance in 2013 in the field (Scott et al., 2013). Moreover, in the early 1980s, the problem of multiple resistances was identified

(Prichard et al., 1980), and by the 1990s, multiple-drug resistances were relatively widely reported (Kaplan, 2004; Howell et al., 2008; Cezar et al., 2010; da Cruz et al., 2010; Kaplan and Vidyashankar, 2012; Kotze and Prichard, 2016).

With regard to the mechanism of anthelmintic resistance, various mechanisms could be involved, such as changes in the binding sites of drugs, changes in the metabolism of the drugs, and changes in the distribution of drugs in worms (Kerboeuf et al., 2003; Wolstenholme et al., 2004; Fleming et al., 2006). Selection pressure by repeated anthelmintic treatment leads to genetic resistance, which is inherited (Prichard et al., 1980; Gilleard and Beech, 2007). Resistance alleles can relate to pre-existing alleles, novel mutations and recurrent mutations, with pre-existing alleles are suggested to be the main case (Gilleard and Beech, 2007). The features of parasitic nematodes of veterinary importance, including a high level of genetic diversity and rapid rates of evolution, favour the development of anthelmintic resistance (Fleming et al., 2006; Gilleard and Beech, 2007). In terms of population genetics, there are two likely effects on the genetic footprint of resistance (allele selection): gene hitchhiking and meiotic recombination, which still need to be studied in detail (Gilleard and Beech, 2007). In addition to gene mutations, P-glycoprotein as an ATP-binding cassette transporter, which could extrude structurally and functionally unrelated agents, is also reported to be sometimes involved in anthelmintic resistance (Broeks et al., 1995; Kerboeuf et al., 2003; Kotze and Prichard, 2016).

In the following passage, respective mechanisms of anthelmintic resistance are reviewed. Benzimidazole-resistance relates to mutations in the β-tubulin genes, preventing benzimidazoles from binding to β-tubulin (Lubega and Prichard, 1990, 1991; Wolstenholme et al., 2004). In *H. contortus*, utilising restriction map analysis, it was discovered that tyrosine replaced phenylalanine (Phe-Tyr mutation) at codon 200 of isotype 1 ß-tubulin gene (Beech et al., 1994; Kwa et al., 1994; Prichard, 2001; Silvestre and Humbert, 2002). Utilizing transgenic *C. elegans*, the Phe-Tyr mutation at codon 200 of *H. contortus* isotype 1 ß-tubulin gene was shown to confer benzimidazole-resistance, which provided more evidence that this mutation was directly involved in the benzimidazole-resistance mechanism (Kwa et al., 1995; Prichard, 2001). Moreover, the mutation was identified to be present in a range of parasitic nematode species (Prichard, 2001; Silvestre and Humbert, 2002). In addition to the mutations in isotype 1 ß-tubulin gene, mutations in isotype 2 ß-tubulin gene have also been identified to confer benzimidazole-resistance for *H. contortus* in binding assays (Beech et al., 1994; Prichard, 2001). Two other mutations, the Phe-Tyr mutation at codon 167 of the ß-tubulin gene (isotypes 1 and 2) and the Glu-Ala mutation at codon 198 of this gene (isotype 1), have

also been reported to occur in benzimidazole-resistant *H. contortus*, with the Glu-Ala mutation at codon 198 being less frequent (Prichard, 2001; Silvestre and Humbert, 2002; Rufener et al., 2009a; Kotze et al., 2012; Chaudhry et al., 2015; Redman et al., 2015).

In the imidazothiazole class, levamisole is the most widely used compound, and levamisole-resistance was reported in field strains of *T. circumcincta* and *T. colubriformis* as early as 1979 (Sangster et al., 1979). Levamisole is a potent cholinergic agonist on nicotinic acetylcholine receptors in nematode nicotinic neuromuscular junctions (Coles et al., 1975; Harrow and Gration, 1985; Robertson and Martin, 1993; McKellar and Jackson, 2004). The possible mechanism of levamisole-resistance is associated with changes in its binding to nicotinic acetylcholine receptors (Lewis et al., 1980b; Wolstenholme et al., 2004). In early studies of *C. elegans*, levamisole resistance was suggested to involve eleven genes (Lewis et al., 1980a; Lewis et al., 1980b; Prichard, 2001), with five genes, *lev-1*, *lev-8*, *unc-29*, *unc-38* and *unc-63*, encoding nicotinic acetylcholine receptor subunits (Fleming et al., 1997; Blanchard et al., 2018). In *H. contortus*, using equilibrium binding studies, one high-affinity binding component and one low-affinity binding component of nicotinic acetylcholine receptors were revealed; using kinetic studies, the low-affinity binding component was shown to be present more in levamisole-resistant isolates than in levamisole-susceptible isolates (Sangster et al., 1998). In a recent study, through the measurement of gene expression of *H. contortus* which survived exposure to different concentrations of levamisole, a biphasic pattern of levamisole-resistance was shown, in which a non-specific P-glycoproteins-mediated mechanism could confer low levels of resistance and altered nicotinic acetylcholine receptor subunit composition could confer a higher level of resistance (Sarai et al., 2014). The alteration in the nicotinic acetylcholine receptor subunit composition was shown to be regulated by the changes in the gene expression of receptor subunits (*Hco-unc-63a*, *-63b*, *-unc-29* and *-acr-8*) as well as changes in the expression of proteins involved in receptor assembly (*Hco-unc-74, -unc-50, -ric-3.1* and *-ric-3.2*) (Sarai et al., 2014; Blanchard et al., 2018).

Regarding resistance to the macrocyclic lactones, ivermectin-resistance has become widespread, and moxidectin-resistance is increasing (Prichard et al., 2012). Ivermectin-resistance and moxidectin-resistance are not the same (Prichard et al., 2012; Bygarski et al., 2014). The mechanism of ivermectin-resistance is considered to be regulated mainly by an overexpression of P-glycoproteins (Kerboeuf et al., 2003; Wolstenholme et al., 2004; Prichard et al., 2012; Ardelli and Prichard, 2013). In a study using ivermectin-resistant *C. elegans*, P-glycoprotein inhibitors significantly reduced motility and pharyngeal pumping,

indicating a key role of P-glycoproteins in ivermectin-resistance (Ardelli and Prichard, 2013). Recent studies of *C. elegans* have shown that the moxidectin-resistance is mediated in part by P-glycoproteins, with other complex mechanisms still being explored (Bygarski et al., 2014).

Resistance to monepantel seems to be relatively rare, but has been reported recently (Scott et al. 2013; Mederos et al. 2014; Van den Brom et al. 2015; Sales and Love, 2016; Lamb et al., 2017; Martins et al., 2017). Through artificial selection under anthelmintic pressure, the resistance to monepantel has been reported to develop rapidly in *T. circumcincta* (Bartley et al., 2015) and in *H. contortus* (de Albuquerque et al., 2017). The mechanism of monepantel-resistance has not yet been confirmed, but mutations in the *acr-23* gene in monepantel-resistant *C. elegans*, and in the *Hco-MPTL-1* and *Hco-DES-2H* genes in monepantel-resistant *H. contortus* have been identified (cf. Rufener et al., 2009b; Bagnall et al., 2017; de Albuquerque et al., 2017). Clearly, studies need to be conducted to provide an insight into the mechanism(s) involved in monepantel-resistance.

1.5 Anthelmintic drug discovery

The problem of anthelmintic resistance, particularly in gastrointestinal nematodes of small ruminants, is prevalent worldwide, threatening the economic development of livestock industry and animal welfare (Wolstenholme et al., 2004; Kaplan and Vidyashankar, 2012; Miller et al., 2012; Kotze and Prichard, 2016). As reviewed above, there are many reports of anthelmintic resistance and studies of resistance (except to derquantel). It seems that the rapid emergence of resistance is likely to outpace the development of new anthelmintics. In the face of widespread anthelmintic resistance, there is an urgency to discover new anthelmintic candidates, and a necessity to make continual efforts to discover new anthelmintic drugs. The discovery and development of new anthelmintics will preserve economic and health advantages (Geary et al., 1999; Geary et al., 2015).

However, as the starting point, drug discovery has many challenges, which mainly relate to cost, limited available technologies and resources for screening, and cooperation among different areas (including parasitology, drug discovery, medicinal chemistry and safety evaluation) (Geary et al., 2015; Preston et al., 2016b). Generally, from drug discovery through to approval by the United States Food and Drug Administration (FDA), it usually takes 10 -17 years to develop a new medicine at considerable financial cost (an average of $2.9 billion) (DiMasi et al., 2016). Up till now, the pharmaceutical industry has delivered approximately 1,400 new drugs (Munos, 2009; Kinch et al., 2014).

Anthelmintic discovery has been restrained to some extent in large companies due to limited economic returns and limited progress, despite some success through the discovery of, for example, emodepside (Harder et al., 2003; Martin et al., 2012), tribendimidine (Xiao et al., 2005; Steinmann et al., 2008), monepantel (Kaminsky et al., 2008b) and derquantel (Little et al., 2011). Currently, with investments from governmental and non-governmental organisations (e.g., Bill and Melinda Gates Foundation), drug discovery in the parasite realm has evolved through devoted resources (Geary et al., 2015). It is also practicable to adapt veterinary drugs to human health realm, which allows the discovery and development of new drugs for both human and veterinary applications (Geary et al., 2015).

On the other hand, drug-screening technology also plays a key role in discovery. In the past, anthelmintic discovery has been conducted using animal models (Geary et al., 1999; Geary et al., 2015), with the successful discovery of anthelmintics such as levamisole (Thienpont et al., 1966) and ivermectin (Campbell et al., 1983). The strategy was to screen compounds in infected animals to identify which ones were effective, in other words, reduced or eliminated parasite burdens (Geary and Thompson, 2003). However, this approach is too costly and time- and labour-intensive (Gosai et al., 2010; Geary, 2016). Currently, screening in animal models has been abandoned due to these mentioned constraints, with only the most promising compounds being tested in animals (Geary et al., 1999; Geary et al., 2015; Geary, 2016). With the economic pressures to reduce labour and time, and to minimise the amounts of compounds used for primary screening and changes in animal ethics, various in vitro screening strategies have been established (Gosai et al., 2010; Geary et al., 2015). In the following sessions, two current commonly used screening strategies are reviewed.

1.5.1 Mechanism-based screening

The principle of mechanism-based anthelmintic screening is to measure the interaction of the drug with a specific target protein (Kotze, 2012). The advantage of this strategy is that the targets of identified active compounds have already been identified or are already known (Kotze, 2012; Geary et al., 2015). *Caenorhabditis elegans* has been extensively studied and provides a major resource for understanding the biology of parasitic nematodes (Holden-Dye and Walker, 2014; Britton et al., 2016). Thus, this worm can help define potential targets for drug discovery based on the understanding of molecular pathways in nematodes, and chemists feel more confident about designing compounds that selectively bind to a defined target (Geary et al., 1999; Holden-Dye and Walker, 2014). In addition, minimum amounts of

compounds are routinely synthesised in medicinal chemistry, making mechanism-based screening amenable to high through-put screening, so as to improve the efficiency of drug discovery (Geary et al., 1999; Kotze, 2012; Geary, 2016).

However, mechanism-based screening can miss specific targets, and 'unassayable' ones may represent the great majority of potential anthelmintic targets in a worm (Kotze, 2012). Compounds may work on predicted targets, but may not work on whole worms if the compounds are not bioavailable (Kotze, 2012). In addition, the complex biological system also challenges and questions the 'one drug - one target' paradigm as it is rare for drugs to bind to just a single molecular target, with most compounds being involved in off-target interactions in vivo (Hopkins et al., 2006). Moreover, the limited understanding of the biology of parasitic nematodes represents a barrier to the mechanism-based anthelmintic screen (Geary et al., 1999; Kotze, 2012; Geary et al., 2015). Indeed, no compound discovered in mechanism-based screen has yet been commercialised as an anthelmintic (Geary et al., 2015).

1.5.2 Whole-worm screening

Whole-worm screening offers a means of discovering novel drugs for which the target is unknown but ensures that compounds ('hits') identified are active against the whole organism in vitro (Kotze, 2012; Schenone et al., 2013; Geary et al., 2015). Unknown targets might undertake greater potential for being used as new anthelmintic targets than already known, predicted targets (see Kotze, 2012).

Whole-worm anthelmintic screening relies on measuring the viability or behaviour (phenotype) of live parasites in vitro (Geary et al., 2015). Conventional whole-worm screening assays have been utilised for screening anthelmintics and detecting anthelmintic resistance. Most assays use free-living larval stages, including the egg hatch assay (Le Jambre, 1976; Dobson et al., 1986), larval development assay (Kotze et al., 2006), larval feeding assay (Alvarez-Sanchez et al., 2005), larval migration inhibition assay and larval motility assays (Lorimer et al., 1996). Indeed, the first benzimidazole anthelmintic, thiabendazole, was discovered in a trichostrongyloid larval assay (Brown, 1961). However, most of these assays are labour- and time-consuming, and are not suitable to efficiently screen large amounts of compounds; moreover, the subjective and manual recording of nematocidal activity (e.g., motility or larval development) can also influence the measurement of the activity of compounds (Paveley and Bickle, 2013; Buckingham et al.,

2014; Preston et al., 2015).

Nonetheless, there have been some recent improvements in the development of screening assays for parasitic nematodes, mainly focusing on the automated recording of nematode phenotypes (Gosai et al., 2010; Smout et al., 2010; Marcellino et al., 2012; Paveley and Bickle, 2013; Storey et al., 2014; Preston et al., 2015). Particularly, the application of video-imaging system and computer software technology have advanced the development of whole-worm anthelmintic screening, which has become easier, less labour- and time-consuming (Geary et al., 2015).

In a recent study, Preston et al. developed a low-cost whole-organism motility screen using parasitic stages of *H. contortus* (Preston et al., 2015). In this assay, the first step is to dilute experimental compounds and prepare parasitic stages of *H. contortus*; diluted compounds are then added to worms in 96-well flat-bottom plates; the plates will be incubated in a CO_2 incubator at 38 °C and 10% (v/v) CO_2 for 72 h; before plates are imaged, each plate is agitated for 20 min on an orbital shaker at 37 °C; a 5 sec video recording is taken of each well on each plate and the video-recordings are then analyzed by quantifying the changes in light intensity over time to get motility index; the motility index of each well will be normalised by comparison to positive control and negative control; a compound will be recorded as active if it reduces xL3 motility by ≥ 70% (Preston et al., 2016a). This assay can also be adapted to using other parasitic nematodes which can be maintained in vitro (Preston et al., 2015). Given that most anthelmintic drugs are considered to be more potent against parasitic stages than free-living stages (see Kotze, 2012), the whole-organism motility screening assay using parasitic larval stages of *H. contortus* might help identify active compounds that are missed in other assays using free-living larval stages. It is worthy to note that several compounds with in vitro anthelmintic activity have been identified using this assay (Preston et al., 2015; Preston et al., 2016b; Preston et al., 2016c).

1.6 Selection of compound collections for anthelmintic drug discovery

The rational selection of a representative subsets of compounds for screening is a critical aspect of drug discovery and remains a challenge for drug discovery (Dandapani et al., 2012; Preston et al., 2016b). Crafting and curating a successful collection of compounds for screening assists in minimizing false-positive and maximizing true-positive ‘hit’-rates (Huggins et al., 2011; Preston et al., 2016b). Generally, there are three parts in assembling a

collection of compounds for screening: (i) compound sourcing, (ii) compound filtering and (iii) compound selection (Huggins et al., 2011). For compound sourcing, chemical libraries, including natural products and synthetic compounds, are acquired based on their drug-like and lead-like properties (Baurin et al., 2004; Monge et al., 2006; Chuprina et al., 2010; Simpson and Poulsen, 2014). The majority of available compounds are synthetic compounds, with a much lower percentage of natural products currently approved for commercial use (Huggins et al., 2011). For compound filtering, compounds with undesirable physicochemical properties, e.g., low levels of drug absorption, and specific structures that are unsuitable for screening or cause problems later in drug development (e.g., aldehydes, epoxides and/or α-halo ketones) are removed (Walters and Murcko, 2000; Huggins et al., 2011). Following compound filtering, the diversity of chemicals is assessed and compared with those that have known biological activities (Martin et al., 2002; Huggins et al., 2011). Clearly, in primary drug screens, it is important to test panels of compounds with diverse chemical structures (Matter, 1997). Following these steps, a compound collection is established, and chemicals can be selected for screening depending on the drug discovery aims (i.e. discovery of new chemical entities or repurposing of known drugs).

1.6.1 Discovery of new chemical entities

A new chemical entity is a drug that contains no active moiety that has been approved previously by the U.S. FDA for any application (www.fda.com). The discovery of new chemical entities as new anthelmintics, is hoped to overcome (in the first instance) current anthelmintic resistance problems and widen the current chemical spaces (Geary et al., 2015). The available access to large compound collections, collaborations with chemists and the availability of high-throughput screening technology all favour the discovery of new chemical entities (Hergenrother, 2006). For the access to large compound collections, commercial industries, such as ChemBridge, ChemDiv, Enamine Screening Compounds, Maybridge and eMolecules, all offer access to compound collections (Hergenrother, 2006), and some academic facilities and universities, such as *Compounds Australia*, also offer comprehensive compound collections for screening (Simpson and Poulsen, 2014). In addition, the application of high-throughput screening technology (both phenotypic and mechanism-based screening) has decreased instrumentation and running costs of new chemical entity screening (Archer, 2004; Hergenrother, 2006).

Despite the current advantages of discovering new chemical entities, the fact is, only

approximately 1400 new chemical entities have been approved by FDA for use as therapeutics (Trouiller et al., 2002; Munos, 2009; Kinch et al., 2014). It is a one to two-decade process to bring a new chemical entity to the market, which will go through discovery and lead optimisation (2-5 years), preclinical development (1-2 years), clinical development (6-7 years), and registration and marketing (1-2 years) (e.g., Ashburn and Thor, 2004) (Fig. 1.3), at major financial cost (on average: $2.9 billion) (DiMasi et al., 2016). Therefore, repurposing known drugs has become attractive, particularly when safety and bioavailability are known, and the cost of production of a chemical is low (O'Connor and Roth, 2005).

1.6.2 Drug repurposing

Drug repurposing is the process of identifying new indications for existing, failed or abandoned drugs, or advanced clinical candidates apart from their original indicated uses (Ashburn and Thor, 2004; Allarakhia, 2013; Sekhon, 2013). Particularly approved drugs with well-defined in vivo pharmacokinetic parameters, toxicity data and dosing information, could be rapidly tested in validation studies using suitable animal models and/or pilot clinical trials, and then proceed through to clinical trials faster than new chemical entities (Ashburn and Thor, 2004; O'Connor and Roth, 2005). Moreover, the original indications would be helpful in predicting and studying the targets of the repurposed drugs to new indications (O'Connor and Roth, 2005). Obviously, compared with the discovery of new chemical entities, repurposing known drugs has the advantage of reducing safety risk and pharmacokinetic uncertainty, and saving time and cost in development (Ashburn and Thor, 2004; Hergenrother, 2006; Corsello et al., 2017). Therefore, drug repurposing via screening is becoming increasingly popular for drug discovery and development (Ashburn and Thor, 2004; Sekhon, 2013). There are numerous successful examples of repurposed drugs (Ashburn and Thor, 2004; Hergenrother, 2006; Sekhon, 2013), such as the repurposing of the antifungal drug amphotericin for the treatment of leishmaniasis (Mondal et al., 2010), the anti-Parkinson’s disease drug ropinirole for treating restless leg syndrome (Dusek et al., 2010), and the anti-hypertension drug propranolol for migraine prophylaxis (Bidabadi and Mashouf, 2010).

Drug repurposing is fostering collaborations between academic scientists and pharmaceutical companies (Hergenrother, 2006; Allarakhia, 2013). In the field of discovering new therapeutics for neglected tropical diseases, product development partnerships (PDPs) represent a major advance as a non-profit organisational structure that play a key role in

offering compound resources for repurposing investigation. PDPs that was emerged in the late 1990s, target neglected tropical diseases, including a number of organisations, such as Medicines for Malaria Venture (MMV), Drugs for Neglected Diseases Initiative (DNDi) and Global Alliance for TB Drug Development (TB Alliance) (Grace, 2010; Moran et al., 2010; Hussaarts et al., 2017; Preston and Gasser, 2018). The significant role of the PDP model has been demonstrated through the delivery of commercial products, such as paromomycin against leishmaniasis - developed by the Institute of One World Health (Davidson et al., 2009), artemether-lumefantrine (Coartem dispersible) - a child-friendly treatment against malaria - developed by PDP between Novartis and MMV (Premji, 2009), and a new vaccine called MenAfriVac against the bacterial meningitis by the Meningitis Vaccine Project (Butler, 2010; Bishai et al., 2011). Through PDPs, compound collections containing specific compounds that have been explored for use against some neglected disease pathogens, could be accessible and tested on other pathogens. For example, in 2013, a compound collection termed the Malaria Box containing 400 key malaria phenotypic 'hits' was launched by MMV for being tested on other related parasites by researchers around the world (Spangenberg et al., 2013). However, the amounts of known and/or approved drugs are relatively small, such as that there are only ~1200 'small molecule drugs' and ~160 'biological' drugs approved by FDA (Trouiller et al., 2002; Overington et al., 2006; Kinch et al., 2014), which seems to confine a relatively small chemical space for drug repurposing (Hergenrother, 2006).

1.7 The relevance of using advanced molecular and informatic technologies to assist anthelmintic discovery

In the era of "systems biology" (cf. Pujol et al., 2010; Cantacessi et al., 2012; Zuck et al., 2017), the application of genomic, transcriptomic, proteomic and/or metabolomic technologies is furthering our understanding of fundamental biology and the pathogenesis of diseases, and is underpinning the design or development of new interventions against infectious and non-infectious diseases (e.g., Hood and Rowen, 2013; Wasmuth, 2014; Matthews et al., 2016; Sotillo et al., 2017). In particular, the integrative use of such technologies can assist in drug discovery by addressing the challenges relating to target validation, modes of action and the definition of endpoints in clinical studies (Matthews et al., 2016).

Genome-guided drug discovery (McCarthy et al., 2013; Preston et al., 2016) has particular promise and can complement traditional approaches to drug target validation (Debouck and

Metcalf, 2000; Shanmugam et al., 2012; Eder et al., 2014). For instance, the availability of genomic and transcriptomic data sets and information for model organisms, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, allows inferences or predictions to be made about gene function and, importantly, the essentiality of particular genes and their orthologues in other metazoans including parasites (e.g., Stroehlein et al., 2015a; Stroehlein et al., 2015b; Korhonen et al., 2016; Stroehlein et al., 2017). In recent years, numerous draft genomes of parasitic helminths have been characterised (cf. Sotillo et al., 2017; Stroehlein et al., 2018), including those of *Ascaris suum* (see Jex et al., 2011), *Trichuris suis* (see Jex et al., 2014) and *Opithorchis viverrini* (see Young et al., 2014). In 2013, *H. contortus* was the first strongylid nematode to have its genome and transcriptomes sequenced; this now provides an important resource for genetic, biological, ecological and epidemiological investigations and also a solid foundation for research in drug discovery and drug resistance (Laing et al., 2013; Schwarz et al., 2013; Gasser et al., 2016).

Haemonchus contortus is relatively closely related to *C. elegans*, facilitating comparative and functional genomic studies of this and related strongylid nematodes (Blaxter et al., 1998; Gilleard, 2004; Laing et al., 2011; Laing et al., 2013; Cantacessi et al., 2015; Britton et al., 2016). The mining of genomic and transcriptomic data of *H. contortus*, predictions of gene essentiality from functional genomic information for *C. elegans* and the identification of enzymatic chokepoints all assist in identifying target candidates for new anthelmintics (Schwarz et al., 2013). In addition, available genomic and transcriptomic information, together with post-genomic explorations using proteomic, lipidomic and glycomic tools (Ju et al., 2010; Schwarz et al., 2013; Matthews et al., 2016; Sotillo et al., 2017) should open up new avenues for drug target and drug discovery.

The genome and transcriptome of a parasite represent only the first step in understanding its molecular biology, and an examination of the genome may guide biological investigations of parasitism and parasitic diseases, or assist in finding new ways of disrupting such processes (Banks et al., 2000; Dhingra et al., 2005; Viney, 2014; Popara et al., 2015). The proteome is subject to a relatively high degree of post-translational modification, such as phosphorylation with functional implications, and is able to provide information for focused investigations of specific biological pathways that enable cross-corroboration and validation with genomic data sets (Banks et al., 2000; Matthews et al., 2016). For the discovery of new anthelmintics, unknown (orphan) proteins of parasitic helminths are now receiving increased attention (Sotillo et al., 2017). Proteomic investigations are thus particularly pertinent to drug discovery (Page et al., 1999; Dhingra et al., 2005; Sotillo et al., 2017). Recently, the

deployment of advanced technologies (e.g., high performance liquid chromatographic and mass spectrometric methods as well as novel techniques for sample preparation and the development of tailored and cutting-edge bioinformatic tools) and the availability of large data sets in high quality and public databases, such as at WormBase ParaSite (parasite.wormbase.org), are improving the way we approach drug discovery (e.g., Harris et al., 2009; Yook et al., 2011; Hood et al., 2012; Howe et al., 2012; Harris et al., 2013; Wang et al., 2014; Howe et al., 2016; Matthews et al., 2016; Howe et al., 2017; Lee et al., 2017). Taken together, the integrative use of these advanced technologies will likely aid significantly in identifying biological pathways affected by anthelmintic treatment and the modes of action of new (and old) chemical entities (Duke et al., 2013; Wasmuth, 2014; Dos Santos et al., 2016; Matthews et al., 2016; Preston et al., 2016; Sotillo et al., 2017).

1.8 Conclusions and research aims

This literature has shown that parasitic nematodes cause diseases (nematodiases) of major animal and human health importance worldwide. As no highly effective vaccines are readily available to prevent these diseases, anthelmintic treatment remains a cornerstone of controlling these parasites. However, emerging or established resistance to most drugs represents a serious risk to future control, such that there is an urgency to discover and develop new and effective anthelmintics. Although target-based screening has not been successful for discovering novel anthelmintics recently, whole-worm screening shows major promise in the following context. First, an important advance has been the development of a screening method that uses parasitic stages of a nematode (e.g., *H. contortus*) - rather than free-living stages that have been traditionally utilised. Second, numerous well-curated collections of synthetic and natural compounds in different countries provide an untapped resource for the discovery of new chemicals with selective nematocidal or nematostatic properties. Third, knowledge of the genome and developmental transcriptome of *H. contortus* underpins drug discovery, as it allows the mechanisms of action of chemicals in the nematode to be studied at the molecular level. Therefore, the availability of a solid whole-organism screening method, access to banks of curated chemicals and the availability of new molecular tools provide a solid basis for the present thesis.

The research aims of this thesis were: (i) to screen compounds from well-defined, curated collections of chemicals, from Australia and overseas, for inhibitory activity against *H. contortus* and/or other parasitic nematodes; (ii) to identify “hit” compounds and then (iii) to assess the potential of such “hits” as possible “lead” compounds for optimisation and subsequent development (Chapters 2-6) and, finally, to discuss the findings in relation to current knowledge and propose areas of future research (Chapter 7).

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Table 1.1. Anthelmintic resistance to major anthelmintic groups used to control parasitic nematode infections in livestock.

Anthelmintic	Year of initial marketing[a]	Year of initial reported resistance[b]	Proposed mechanism of resistance[c]
Benzimidazoles	1961	1964	Phe-Thy mutation at codon 200 of isotype-1 β tubulin gene is the most common, with mutations at codon 167 (Phe-Tyr) and 198 (Glu-Ala) of isotype-1 β tubulin gene as well as mutations at same codons, except 198, of isotype-2 β tubulin gene also reported to be involved in benzimidazole-resistance
Imidazothiazoles	1970	1979	A non-specific P-glycoproteins-mediated mechanism could confer low levels of resistance, and altered nicotinic acetylcholine receptor subunit composition could confer higher level of resistance
Macrocyclic lactones	1981	1988	The essential mechanism involved in ivermectin-resistance is over-expression of P-glycoproteins; moxidectin-resistance is mediated in part by P-glycoproteins, with other complex mechanisms still being explored
Monepantel	2009	2013	Unknown

[a] The first year of drug approval, despite variation among countries.

[b] The first documented resistance despite earlier published reports of suspected resistance and/or unpublished reports of resistance.

[c] References are consistent with the text.

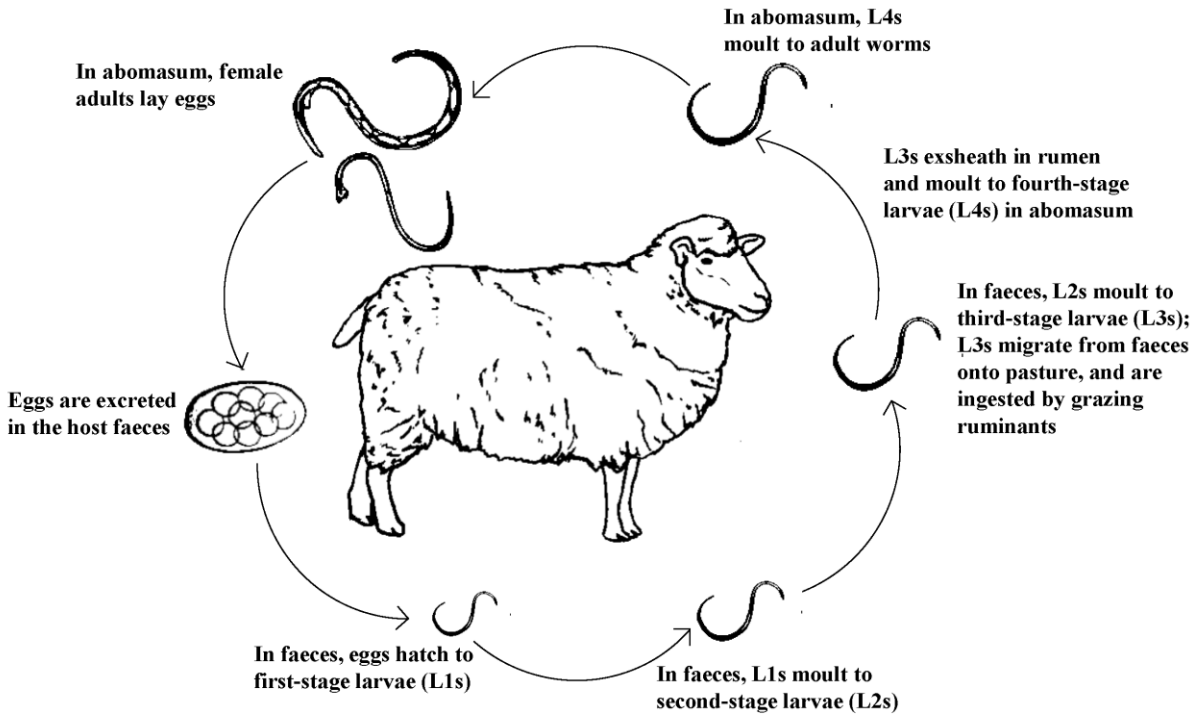

Fig. 1.1. The life cycle of *Haemonchus contortus*. Eggs hatch to first-stage larvae (L1s) in faeces, then moult through second-stage larvae (L2s) to third-stage larvae (L3s); L3s migrate from faeces onto pasture and are then ingested by grazing ruminants; L3s exsheath in the rumen/reticulum and moult to fourth-stage larvae (L4s) in the abomasum; L4s then develop to adult worms and female adults lay eggs in the abomasum.

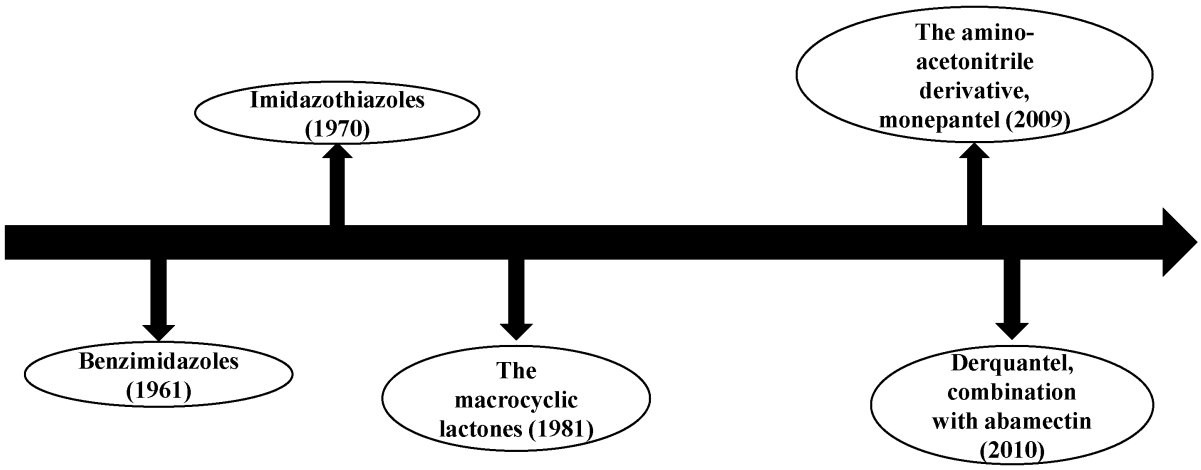

Fig. 1.2. Five currently widely used commercial anthelmintic classes, benzimidazoles, imidazothiazoles, the macrocyclic lactones, monepantel and derquantel. The first benzimididazole drug was introduced in 1961, followed by the introduction of the first imidazothiazole drug in 1970 and the first macrocyclic lactone in 1981; recently, the amino-acetonitrile derivate drug, monepantel, and a semi-synthetic member of spiroindoles, derquentel, were introduced to the market in 2009 and 2010, respectively.

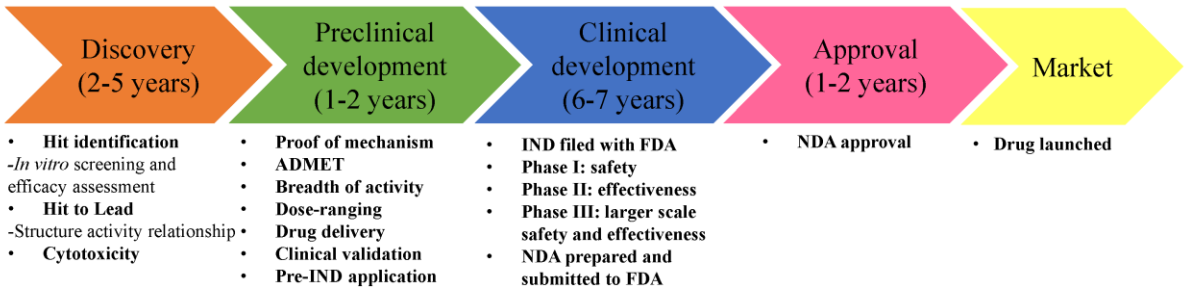

Fig. 1.3. A 10–17 year process to bring a new chemical entity to the market, which will go through discovery (2-5 years), preclinical development (1-2 years), clinical development (6-7 years), and registration and marketing (1-2 years). ADMET = absorption, distribution, metabolism, excretion and toxicity; IND = investigational new drug; FDA= Food and Drug Administration; NDA= new drug application.

Chapter 2 - Identification of a new chemical entity with selective anthelmintic activities against different developmental stages of *Haemonchus contortus* and other parasitic nematodes

Abstract

The discovery and development of novel anthelmintic classes is essential to sustain the control of socioeconomically important parasitic worms of humans and animals. With the aim of offering novel, lead-like scaffolds for drug discovery, *Compounds Australia* released the 'Open Scaffolds' collection containing 33,999 compounds, with extensive information available on the physicochemical properties of these chemicals. In the present study, we screened 14,464 prioritised compounds from the 'Open Scaffolds' collection against the exsheathed third-stage larvae (xL3s) of *Haemonchus contortus* using recently developed whole-organism screening assays. We identified a hit compound, called SN00797439, which was shown to reproducibly reduce xL3 motility by ≥70%; this compound induced a characteristic, "coiled" xL3 phenotype (IC_{50} = 3.46-5.93 µM), inhibited motility of fourth-stage larvae (L4s; IC_{50} = 0.31-12.5 µM) and caused considerable cuticular damage to L4s in vitro. When tested on other parasitic nematodes in vitro, SN00797439 was shown to inhibit (IC_{50} = 3-50 µM) adults of *Ancylostoma ceylanicum* (hookworm) and first-stage larvae of *Trichuris muris* (whipworm) and eventually kill (>90%) these stages. Furthermore, this compound completely inhibited the motility of female and male adults of *Brugia malayi* (50-100 µM) as well as microfilariae of both *B. malayi* and *Dirofilaria immitis* (heartworm). Overall, these results show that SN00797439 acts against genetically (evolutionarily) distant parasitic nematodes (i.e. *H. contortus* and *A. ceylanicum* [strongyloids] *vs. B. malayi* and *D. immitis* [filarioids] *vs. T. muris* [enoplid]), and, thus, might offer a novel, lead-like scaffold for the development of a relatively broad-spectrum anthelmintic. Our future work will focus on assessing the activity of SN00797439 against other pathogens that cause neglected tropical diseases, optimising analogues with improved biological activities and characterising their targets.

2.1 Introduction

Parasitic worms (helminths) of animals and humans cause chronic and often deadly diseases that have a major socioeconomic impact worldwide (Fenwick, 2012; Fitzpatrick, 2013). On one hand, in humans, the disease burden due to parasitic worms represents ~14 million disability-adjusted life years (DALYs) globally, representing half of the DALYs caused by major neglected tropical diseases (Hotez et al., 2014, 2016). On the other hand, in agricultural animals, the annual economic losses due to death, poor health and reduced productivity caused by parasitic worms are estimated at billions of dollars per annum worldwide (cf. Knox et al., 2012; Roeber et al., 2013). In Australia alone, worms of cattle and sheep cause economic losses estimated at 500 million dollars per annum (Lane et al., 2015). As no vaccines are available for the vast majority of these parasites, their control relies predominantly on the use of (usually) one of a small number of medicines (anthelmintics). These anthelmintics are only, at best, partially effective, and their excessive and widespread use, particularly in livestock animals, has led to serious drug resistance problems around the world (Kaplan and Vidyashankar, 2012; Wolstenholme and Kaplan, 2012; Kotze and Prichard, 2016). Therefore, the ongoing development of novel treatments is crucial for the control of parasitic worms of animals.

Despite some success through the discovery of, for example, monepantel (Kaminsky et al., 2008; Prichard and Geary, 2008) and derquantel (Little et al., 2011), limited progress has been made in discovering new drugs against parasitic roundworms (nematodes) (Geary et al., 2015). Major hurdles include achieving anthelmintic efficacy with an acceptable therapeutic index, the ability to develop formulations that deliver the pharmacokinetic profile necessary for efficacy, while maintaining the therapeutic index to support safety, as well as achieving human food safety requirements linked to the use of food animal products and a low cost of manufacturing the drugs (cf. Geary et al., 2015; Campbell, 2016).

In 2008, the Queensland Compound Library (QCL), now called *Compounds Australia*, established a dedicated compound management facility to augment capability in chemical screening and biomedical research, including the discovery of new anti-parasite drugs (Simpson and Poulsen, 2014). A key role of *Compounds Australia* has been to source small molecules, to consolidate them into a central repository that facilitates subsequent screening and to provide these molecules to laboratories around the world to boost drug discovery efforts.

Currently, *Compounds Australia* maintains three main collections: ‘Open Scaffolds’ (with ~33,400 compounds), ‘Open Academic’ (~19,500) and ‘Open Drugs’ (~2,500; Food and Drug Administration [FDA]-approved) (Simpson and Poulsen, 2014). Each collection is well curated and characterized, with extensive information available on the physicochemical properties of compounds, including "rule-of-five" descriptors (chirality, hydrogen bonding acceptor and donors, logP and molecular weight), and chemical fingerprints. The ready availability of these resources provided us with a unique opportunity to evaluate selected compound groups for activity against parasitic nematodes using recently developed whole-organism screening assays. Therefore, we elected to screen prioritised compounds from the largest library, ‘Open Scaffolds’, against parasitic stages of the barber’s pole worm, *Haemonchus contortus* (of ruminants), to identify hit compounds, and then characterise and assess them as nematocidal candidates.

2.2 Materials and methods

2.2.1 The compound library, and the selection and preparation of chemicals for screening

We purchased a prioritised set of compounds from the ‘Open Scaffolds’ collection from *Compounds Australia*, which contains a total of 33,999 chemicals representing 1,226 scaffolds (Simpson and Poulsen, 2014). This collection contains novel, lead-like scaffolds (or chemotypes), with an average of 28 compounds per scaffold to allow meaningful structure-activity relationships (SAR) to be explored. For each compound, the simplified molecular-input line-entry system (SMILES) string was converted to SYBYL line notation (SLN; Homer et al., 2008) using SYBL software (www.certara.com/software/molecular-modeling-and-simulation/sybyl-x-suite/); using this approach, 33,949 compounds were annotated. Then, we selected a subset of representative compounds from this library using the following steps. First, we removed 213 pan assay interference compounds (PAINs) (Baell et al., 2010, 2016). Second, we subjected the remaining chemicals to stringent physicochemical and structural filtering, in order to select compounds with the highest probability of permeating cells, being soluble and being chemically optimised as drugs, using the following criteria: mixtures, metals, isotopes: 0, minimum number of rings: 1, maximum number of rings: 4; minimum molecular weight: 150 Da; maximum molecular weight: 400 Da; hydrogen bond donors: ≤ 3; hydrogen bond acceptors: ≤ 6; minimum number of hydrogen bond acceptors: 1; maximum number of chiral centers: 3; maximum number of rotatable bonds: 10; this process

removed 14,328 compounds, leaving 19,408 compounds. Third, compounds that were > 90% similar to each other in structure were removed, leaving a final set of 14,464 compounds. These 14,464 compounds (Supplementary file 2.1) were individually solubilised in dimethyl sulfoxide (DMSO; cat no. 2225; Ajax Finechem, Australia) to achieve a stock concentration of 5 mM, and then diluted and tested for activity against *H. contortus* (see Subsection 2.2.2).

2.2.2 Screening and evaluation of the effects of compounds on *H. contortus*

Production of parasitic larvae

Haemonchus contortus (Haecon-5 strain) was maintained in experimental sheep in accordance with institutional animal ethics guidelines (permit no. 1413429; The University of Melbourne) as described previously (Preston et al., 2015). To produce exsheathed third-stage larvae (xL3s), L3s were exposed to 0.15% (v/v) of sodium hypochlorite (NaClO) for 20 min at 37 °C (Preston et al., 2015), washed five times in sterile saline (0.9%) and cultured in Luria Bertani medium (LB) and supplemented with final concentrations of 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin (antibiotic-antimycotic, cat. no. 15240-062; Gibco, Life Technologies, USA) (LB*). To produce fourth-stage larvae (L4s), xL3s were incubated in a water-jacketed CO_2 incubator (model no. 2406 Shel Lab, USA) for 7 days at 38 °C and 10% v/v CO_2 or until ≥ 80% of L3s had developed to the L4 stage.

Preparation of compounds for screening

Compounds were supplied from *Compounds Australia* at a concentration of 5 mM. Individual compounds were diluted to 40 µM in LB* containing 1% DMSO and dispensed in 50 µl into the wells of sterile 96-well flat bottom microplates (Corning 3596, Life Sciences, USA) using an automated, multi-channel pipetting platform (Viaflow Assist, Switzerland). LB* containing 1% DMSO and LB* were both included as negative controls in screening assays. The anthelmintics moxidectin (Cydectin®, Virbac, France) and monepantel (Zolvix®, Novartis Animal Health, Switzerland) were used as positive-control compounds in screening assays.

Screening of compounds for their effect on xL3 motility

The whole-organism screening assay developed by Preston et al. (2016a) was used to evaluate the effect of compounds on the motility of xL3s of *H. contortus*. On each 96-well plate, the positive-control compounds (moxidectin and/or monepantel) were arrayed in triplicate. Six wells were used for each negative control (LB* + 1% DMSO and LB* alone).

Test compounds were arrayed in individual wells. Following dispensing into the plates, 300 xL3s in 50 µl of LB* were transferred to each well of each plate (with the exception of perimeter wells) using a multi-channel pipette (Finnpipette, Thermo Scientific). During dispensing, xL3s were kept in a homogenous suspension by bubbling air through the solution using an air pump (Airpump-S100; Aquatrade, Australia). Thus, following the addition of xL3s to individual wells, the final concentrations were 20 µM (compound) and 0.5% (DMSO).

Plates were incubated in a water-jacketed CO_2 incubator at 38 °C and 10% v/v CO_2. After 72 h, the plates were agitated (126 rotations per min) using an orbital shaker (model EOM5, Ratek, Australia) for 20 min at 38 °C. In order to capture the motility of xL3s at 72 h, a video recording (5 sec) was taken of each well on each plate as described previously (Preston et al., 2016a). Each 5 second-video capture of each well was processed using a custom macro in the program Image J (1.47v, imagej.nih.gov/ij), which indirectly measured larvae motility by quantifying the changes in light intensity over time (cf. Preston et al., 2016a).

Screening of compounds for their effect on the motility and development/growth of fourth-stage larvae (L4s)

The motility of L4s was evaluated using the same protocol as for xL3 (Preston et al., 2016a). Following the measurement of larval motility, plates were re-incubated for four more days in a humidified environment (water-jacketed CO_2 incubator) at 38 °C and 10% v/v CO_2. Then, worms were fixed with 50 µl of 1% iodine, and 30 worms from each well were examined at 20-times magnification (DP26 camera, Olympus, Japan) to assess their development based on the presence of a well-developed mouth/pharynx in *H. contortus* L4s (see Sommerville, 1966). The number of L4s was expressed as a percentage of the total worm number (n = 30). Compounds were tested in triplicate on three different days.

Analyses of results from bioassays using *H. contortus*

Raw data were normalised against values of the positive and negative controls to remove plate-to-plate variation by calculating the percentage of motility using the program GraphPad Prism (v.6 GraphPad Software, USA). A compound was recorded as having anti-xL3 activity if it reduced motility by ≥ 70% at 72 h, and was re-screened at 20 µM to confirm its inhibitory properties on motility.

For a compound that consistently reduced xL3 motility by ≥ 70%, an 18-point dose-response curve (two-fold serial dilutions; from 100 µM to 0.00076 µM) was produced for

xL3 and L4, to establish its half maximal inhibitory concentration (IC_{50}). For xL3 and L4, motility was measured at 24 h, 48 h and 72 h, and L4 development at 7 days of incubation with each active compound (triplicate) (Subsection 2.2.3). Compound concentrations were transformed using the equation ($x = \log_{10}$ (concentration in nM)) and a log (agonist) *versus* response -- variable slope (four parameter) equation, in GraphPad prism v.6.07 was used to calculate IC_{50} values. For L4 development, IC_{50} values were calculated using the same approach. If a IC_{50} value could not be accurately calculated by the log (agonist) *versus* response -- variable slope (four parameter) equation, a range for the IC_{50} value was given.

2.2.3 Scanning electron microscopy

This microscopy technique was used to assess whether compounds that reduced motility by ≥ 70% caused structural damage to the larval stages of *H. contortus*, as described previously (Preston et al., 2016b). The xL3s and L4s were produced and cultured as described previously (Preston et al., 2015; Subsections 2.2.1-2.2.2). Six replicates of 300 xL3s or L4s were incubated in 100 μM of each compound in LB* for 24 h at 38 °C and 10% v/v CO_2. Larvae were pooled, washed 3 times in 0.9% saline at 9,000 x*g* and resuspended in 1 ml of phosphate-buffered saline (PBS). Subsequently, the larvae were fixed and processed as described previously (Preston et al., 2016b). Larvae were imaged using a field-emission scanning electron microscope (XL30 Philips, Netherlands); six representative images were taken of each sample.

2.2.4 Evaluating compound activity on filarial worms

Procurement of filarial nematodes

The Filariasis Research Reagent Resource Center (Athens, Georgia, USA; http://www.filariasiscenter.org/) has official ethics approval to produce filarial nematodes in experimental animals. From this centre, we obtained fresh, live adults of *Brugia malayi*, produced in *Meriones unguiculatus* (jird) (Michalski et al., 2011; Mutafchiev 2014). Fresh, live microfilariae of *Dirofilaria immitis* (Missouri strain) obtained from the bloods from dogs with patent infection (cf. Michalski et al., 2011; Mutafchiev 2014) were maintained in RPMI-1640 medium (Gibco, Life Technologies, USA).

Assessing compound activity against adults and microfilariae of *B. malayi*

The compound with known activity against *H. contortus* was purchased from ChemDiv (USA) and then dissolved in DMSO to reach a stock concentration of 30 mM, and then tested for its effect on the motility of adult female and male *B. malayi* (see Storey et al., 2014). First, adults were manually separated from each other with forceps, and individuals transferred to single wells of 24-well plates containing 500 µl of RPMI-1640 (Gibco, Life Technologies, USA) supplemented with 10 µg/ml of gentamycin and 2.5 µg/ml amphotericin B (designated RPMI*) at 37 °C. Two-fold dilutions (100 µM to 0.97 µM) of each compound in 500 µl of RPMI* were tested in duplicate (adding the same volume to the wells). RPMI* plus 1% DMSO, but without compound, was added to four negative-control wells. The plates were then incubated at 37°C and 5% v/v CO_2. Subsequently, using the Worminator image analysis system (Storey et al., 2014), the motility of individual adult worms was recorded after 24 h and 48 h of incubation. In brief, at each time point, a video recording (1 min) was taken of each well on each plate until the standard deviation reached zero.

In addition, the effect of compounds on the production and motility of microfilariae originating from individual gravid females of *B. malayi* after 72 h of incubation was assessed. To do this, 200 µl of medium (RPMI* plus 1% DMSO) containing ~100 microfilariae from individual wells were transferred to individual wells of a 96-well plate and recorded for 30 sec (in the same manner as for adult worms) using the Worminator. All assays were repeated thrice, and IC_{50} values were calculated using the log (agonist) *versus* response -- variable slope (four parameter) equation -- in GraphPad Prism (v.6 GraphPad Software).

Evaluating compound activity against microfilariae of *D. immitis*

To assess the effects on the microfilariae of *D. immitis*, compounds were serially diluted two-fold (100 µM to 0.39 µM) in 96-well plates; RPMI* was used as the medium, and each compound dilution was tested in triplicate. Six wells contained RPMI* plus 1% DMSO (negative controls). In test wells, 50 µl of RPMI* containing ~100 microfilariae were added to each well using a multi-channel pipette. Plates were then incubated at 37 °C and 5% v/v CO_2. At 24 h and 48 h, plates were imaged for microfilarial motility using the Worminator (see Subsection 2.5.2). All assays were repeated thrice on different days, and IC_{50} values were determined using GraphPad prism (Subsection 2.2.5).

2.2.5 Assessing compound activity on adult *A. ceylanicum* and *T. muris* L1s

Adults of *A. ceylanicum* were collected from the small intestine of hamsters, which had been infected orally with 150 *A. ceylanicum* L3s for three weeks. For each compound, three worms were placed in each well of a 24-well plate, using 2 wells per compound. Levamisole (50 µM) was used as a positive-control compound. Worms were incubated in the presence of 50 µM of each compound, and culture medium, which was composed of Hanks' Balanced Salt Solution (HBSS) supplemented with 10% foetal calf serum, 25 µg/ml of amphotericin B, 100 U/ml of penicillin and 100 µg/ml of streptomycin. Worms were kept in an incubator at 37°C and 5% v/v CO_2 for 72 h. Thereafter, the condition of the worms was microscopically evaluated.

For *T. muris*, 40 L1s were placed in each well of a 96-well plate and incubated for 24 h at 37°C and 5% v/v CO_2 in the presence of 100 µl RPMI-1640 medium with amphotericin B (12.5 µg/ml), penicillin (500 U/ml), streptomycin (500 µg/ml) and 100 µM of the compound to be tested. Levamisole (100 µM) was used as a positive-control compound. Each compound was tested in duplicate. At 24 h, the total number of L1s per well was counted. The larvae were then stimulated with 100 µl hot water and motile L1s were counted.

2.2.6 Assessing compound cytotoxicity and selectivity

Cell toxicity was assessed in a mammary epithelial cell line (MCF10A), essentially as described previously (Kumarasingha et al., 2016). In brief, MCF10A cells were seeded in black walled, flat bottom 384 well black walled plates (Corning, USA) at 700 cells/well using a BioTek 406 automated liquid handling dispenser (BioTek, Vermont, USA) in a total volume of 40 µl/well. Cells were cultured in DMEM-F12 containing 5% horse serum (Life Technologies, Australia), 20 ng/ml human epidermal growth factor (EGF, Life Technologies, Australia), 100 ng/ml Cholera toxin (Sigma, Australia), 0.5 µg/ml hydrocortisone (Sigma, Australia) and 10 µg/ml insulin (human; Novo Nordisk Pharmaceuticals Pty Ltd, Australia). After an incubation for 24 h at 37 °C, 5% v/v CO_2, the growth medium was aspirated and the cells were treated with test compounds starting at 100 µM, and positive and negative controls (media ± 1% DMSO, monepantel, moxidectin). The chemotherapeutic compound doxorubicin, starting at 10 µM, was also used as a positive control. Compounds were titrated to generate a five-point dose-response curve, in quadruplicate, using an automated liquid handling robot (SciClone ALH3000 Lab Automation Liquid Handler, Caliper Lifesciences, USA) and incubated for a further 48 h. Matched DMSO concentrations for each compound

concentration were also tested separately to account for DMSO induced cell toxicity. To measure cell proliferation, cells were fixed and stained with 4',6-diamidino-2-phenylindole (DAPI; 1:1000), and individual wells imaged at 10-times magnification, covering 16 fields (~90% of well) using a high content imager (Cellomics CellInsight Personal Cell Imager, ThermoFisher Scientific, USA) at a fixed exposure time of 0.12 seconds. Viable cells were counted using the Target Activation BioApplication within the Cellomics Scan software (v.6.5.0, Thermo Scientific, USA) and normalised to the cell density in wells without compound. Toxicity due to DMSO was removed from the normalised cell density counts and IC_{50} values calculated from the variable slope four-parameter equation (v.6 GraphPad Software). Experiments were repeated twice on two different days using four technical replicates for each compound.

2.3 Results and discussion

2.3.1 The identification of compound SN00797439 with activity against parasitic stages of *H. contortus*

In the primary screen, we tested all 14,464 prioritised compounds from the 'Open Scaffolds' collection against *H. contortus* xL3s (Fig. 2.1A). Any compounds that consistently reduced xL3 motility (in independent screens) by > 70% at 72 h was recorded as a "hit" (Fig. 2.1B). Of all compounds tested, a chemical, designated SN00797439 (IUPAC name: N-(4-chlorophenyl)-2-(3-phenyl-1,2,4-oxadiazol-5-yl)-1-pyrrolidinecarboxamide) reduced xL3 motility by ≥ 70% in both the primary and subsequent confirmatory screens (Fig. 2.1B); this compound caused a "coiled" xL3 phenotype (Supplementary File 2.2). The chemical structure and predicted physicochemical properties of SN00797439 are given in Fig. 2.1.

Dose-response curves (two-fold serial dilutions from 100 µM to 0.00076 µM) showed that SN00797439 inhibited xL3 motility, with IC_{50} values of 3.46 ± 0.82 µM (24 h), 10.08 ± 2.06 µM (48 h), and 5.93 ± 1.38 µM (72 h) (Fig. 2.2; Table 2.1); these IC_{50} values were comparable with those of monepantel and moxidectin (positive-control compounds).

Dose-response curves (two-fold serial dilutions from 100 µM to 0.00076 µM) revealed that SN00797439 inhibited the motility of L4s, with IC_{50} values of 6.25 - 12.5 µM (24 h), 0.78 - 1.5 µM (48 h) and 0.31 - 0.78 µM (72 h) (Fig. 2.2; Table 2.1). Subsequently, SN00797439 was tested for its ability to inhibit growth/development from xL3 to L4. The dose-response curves revealed that this compound inhibited L4 development, with an IC_{50} value of 11.04 ± 2.16 µM (Fig. 2.2; Table 2.1). Upon SEM analysis, SN00797439 caused

only minor morphological damage to xL3, but resulted in considerable cuticle alterations to the L4 stage, including multiple, cuticle-'embossed' rings around the worm along its length and a 'scaly' appearance of the worm surface (Fig. 2.3).

2.3.2 Compound SN00797439 also has inhibitory activity on the motility of different developmental stages of other parasitic nematodes

In order to assess whether compound SN00797439 would act against nematodes that are genetically (evolutionarily) very distant from *H. contortus* (order Strongylida), we tested whether these compounds would inhibit the motility of adults and microfilariae of *B. malayi* as well as microfilariae of *D. immitis* in vitro. SN00797439 inhibited the motility of female and male adults of *B. malayi* (Fig. 2.4; Table 2.2), with movement ceasing at 100 µM (24 h) and 50 µM (48 h). This result compares with 1.3 µM (24 h) and 2.9 µM (48 h) for moxidectin (cf. Storey et al., 2014). SN00797439 was then tested for its effect on microfilariae of *B. malayi* (released from females after 72 h in vitro), and inhibited the motility of the microfilariae, with an IC_{50} of ~ 3 µM (24 h) compared with ~ 6 µM for moxidectin (cf. Storey et al., 2014).

Subsequently, SN00797439 was tested against microfilariae of *D. immitis*, a filarial nematode that is related to *B. malayi*, and inhibited the motility of microfilariae, with complete inhibition at 100 µM (24 h and 48 h). For both of these time points, an IC_{50} value of ~ 50 µM was achieved (Table 2.2), which compares with IC_{50} values of 43 µM and 9.3 µM published for ivermectin and moxidectin, respectively (Storey et al., 2014). In addition, when tested on *A. ceylanicum* and *T. muris*, SN00797439 killed adult *A. ceylanicum* at 50 µM and displayed a high nematocidal activity against *T. muris* L1s (90.1% of L1s dead at 100 µM). The positive-control compound (levamisole) killed both nematode species.

2.3.3 Cytotoxicity and selectivity of SN00797439

Using an established proliferation assay, SN00797439 was assessed for its toxicity on mammary epithelial cells (Table 2.3) and was shown to be selective for parasitic larvae of *H. contortus*, with a selectivity index (SI) of ~9 to 128, compared with ~ 67 to 332 (monepantel) and ~ 2 to 300 (moxidectin) for the positive-control compounds (Table 2.3). For filarial worms, SN00797439 was selective only for microfilariae of *B. malayi* (but not *D. immitis*) with an SI of 33.34.

2.4 Conclusion

In the past three decades, only four ‘new’ anthelmintic drugs, emodepside (Martin et al., 2012; Harder, 2016), tribendimidine (Xiao et al., 2005; Steinmann et al., 2008), monepantel (Kaminsky et al., 2008; Prichard and Geary, 2008) and derquantel (Little et al., 2011), have been commercialised. Due the rapid emergence of anthelmintic resistance, even to some recently commercialised compounds (Scott et al., 2013; Van den Brom et al., 2105; Sales and Love, 2016; Cintra et al., 2016), there is an urgent need to identify, validate, optimise and develop novel chemical entities for the treatment of parasitic worms of humans and agricultural animals (Geary et al., 2015).

With this focus in mind, we screened 14,464 prioritised compounds (representing a collection of 33,999 chemicals) to identify potential starting points for the design of new anthelmintics. Under the conditions used in this screening platform, compound SN00797439 was identified as a hit, and exhibited considerable anthelmintic activity against *H. contortus* and induced a “coiled” phenotype (Supplementary File 2.2). SN00797439 was also shown to have anthelmintic activity against a species of hookworm (*A. ceylanicum*; order Strongylida) and two filarioid nematodes (*D. immitis* and *B. malayi*; order Spirurida). Additionally, this compound was also shown to have activity against the mouse whipworm, *T. muris*, which is used as a model to study the genetically related human whipworm (*T. trichiura*; order Enoplida) (cf. Foth et al., 2014) These findings, for multiple representatives of taxonomically, biologically and genetically distinct orders (i.e. Strongylida *vs.* Spirurida *vs.* Enoplida), indicate a relatively broad spectrum of activity of this chemotype against nematodes.

Compound SN00797439 is made up of an oxadiazole and pyrrolidine core. An appraisal of the intellectual property surrounding the sub-structures of this compound (Li and Zhong, 2011, 2010) reveals that similar compounds have been explored as therapeutics for the control of hepatitis C, but not yet as anthelmintics. Following the identification of SN00797439 in the primary screen against *H. contortus*, an evaluation revealed differences in its potency to inhibit the motility, growth and/or development of the parasitic larval stages of this nematode. The results showed that SN00797439 was more potent on L4s than xL3s. This difference in potency might be due to variation in expression of its target(s) in the nematode, a distinction in physiology between these two developmental stages of the worm and/or the nature and extent of the uptake of the compound by the worm. An integrated use of transcriptomic, proteomic and metabolomic techniques (Mikami et al., 2012; Schwarz et al.,

2013; Preston et al, 2016c) might be able to elucidate the physiological and biological pathways in *H. contortus* and/or other worms affected by SN00797439.

In our opinion, identifying a compound with activity against taxonomically distinct nematodes provides a starting point for designing and optimising a lead compound. Thus, we are now eager to critically assess the activity of SN00797439 on various developmental stages of a range of parasitic nematodes of humans, including *Ascaris* sp. (large roundworm) and other common species of hookworm (*Necator americanus* and *Ancylostoma duodenale*) as well as additional filarial worms (*Onchocerca volvulus* and *Loa Loa*) which cause some of the most neglected tropical diseases and collectively affect ~ 1.8 billion people, resulting in a loss of 8.5 million DALYs worldwide (e.g., Hotez et al., 2016). Importantly, future work should also focus on (i) studying the structure-activity relationship (SAR) of derivatives of compounds SN00797439 via medicinal chemistry optimization, to attain a new entity with broad-spectrum and enhanced activity; (ii) on assessing their pharmacological properties (absorption, distribution, metabolism, excretion and toxicity; *ADMET*) of the compound series; and ultimately (iii) on assessing the anthelmintic efficacy of active compounds in vivo in animals.

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Table 2.1. Testing of the effects of the active compound SN00797439 from the *Compounds Australia* 'Open Scaffolds' collection on the motility of exsheathed third-stage larvae (xL3s) as well as the motility and development of fourth-stage larvae (L4s) of *Haemonchus contortus*. A comparison of the 'half of the maximum inhibitory concentration' (IC_{50}) values with those of reference anthelmintic compounds (monepantel and moxidectin); expressed as mean IC_{50} (in µM) ± standard error of the mean or a range.

Time point	SN00797439	Monepantel	Moxidectin
xL3 motility			
24 h	3.46 ± 0.82	0.48 ± 0.23	0.19 ± 0.03
48 h	10.08 ± 2.06	0.26 ± 0.15	0.97 ± 0.84
72 h	5.93 ± 1.38	0.16 ± 0.08	0.08 ± 0.04
L4 motility			
24 h	6.25 to 12.5	0.76 ± 0.29	0.07 ± 0.04
48 h	0.78 to 1.5	0.34 ± 0.18	0.05 ± 0.02
72 h	0.31 to 0.78	0.37 ± 0.32	0.02 ± 0.01
L4 development			
7 days	11.04 ± 2.16	0.075 ± 0.04	3.45 ± 0.75

Table 2.2. Testing the effect of compound SN00797439 from the Open Scaffolds collection on the motility of filarial nematodes *Brugia malayi* and *Dirofilaria immitis*. 'Half maximal inhibitory concentration' (IC_{50}) values of individual compounds are indicated.

Time point	Parasite/stage/sex	SN00797439 IC50 (μM)
Brugia malayi		
24 h	Adult female	25-50
24 h	Adult male	25-50
72 h	Microfilariae	3
Dirofilaria immits		
24 h	Microfilariae	50
48 h	Microfilariae	50

Table 2.3. Cytotoxicity of compound SN00797439 on a normal mammary epithelial cell line (MCF10A) and selectivity of these compounds on *Haemonchus contortus* exsheathed third-stage larvae (xL3) and fourth-stage larvae (L4), *Dirofilaria immitis* microfilariae (mf) and *Brugia malayi* mff and adult filarial worms. Selectivity indices (SIs) were calculated using a recognised formula; (SI = 'half of the maximum inhibitory concentration' (IC_{50}) for MCF10A cells / IC_{50} for nematode).

Compound	MCF10A cells		Selectivity index (SI)[a]						
			H. contortus			Filarioids			
	IC_{50} (µM)	% cell density at 100 µM [c]	xL3 (72 h)	L4 development (7 days)	L4 (72 h)	*B. malayi* females	*B. malayi* males	*B. malayi* mf	*D. immitis* mf (48 h)
SN00797439	50-100	6.20 ± 0.66	16.86	9.07	128.21 [b]	2 [b]	2 [b]	33.34 [b]	2 [b]
Monepantel	24.93 ±11.83	0.10 ± 0.05	155.81	332.40	67.38	n.d.	n.d.	n.d.	n.d.
Moxidectin	< 6	0.05 ± 0.01	75	1.74	300	n.d.	n.d.	n.d.	n.d.

n.d.= not determined.

[a] Selectivity indices were calculated based on the highest value in the IC_{50} range.

[b] Selectivity indices were calculated based on the estimated IC_{50} range from Tables 1 and 2.

[c] Expressed as mean ± standard deviation.

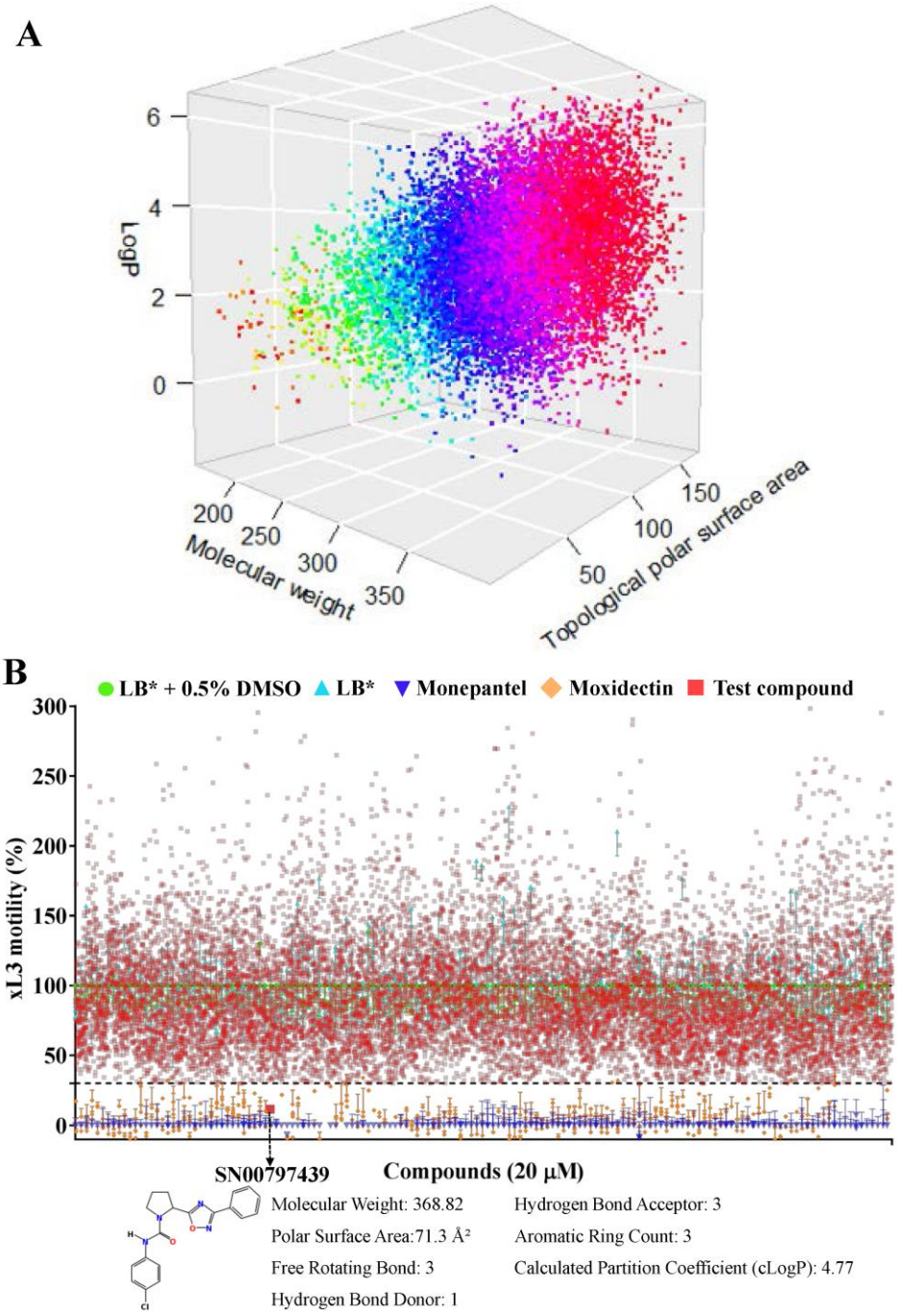

Fig. 2.1. Panel A: Three-dimensional scatterplot displaying the physicochemical properties of molecular weights (in $gmol^{-1}$), calculated partition coefficients (LogP) and topological polar surface areas (in $Å^2$) of all 14,464 compounds prioritised from 33,999 compounds contained within the 'Open Scaffolds' collection. Panel B: Primary screen of the 14,464 compounds at the concentration of 20 µM identified compound SN00797439 to inhibit the motility of exsheathed third-stage larvae (xL3) by ≥ 70% compared with negative (LB* + 0.5% dimethyl sulfoxide, DMSO) and positive controls (monepantel and moxidectin). The chemical structure and predicted physicochemical properties of SN00797439 are listed at the bottom of the image.

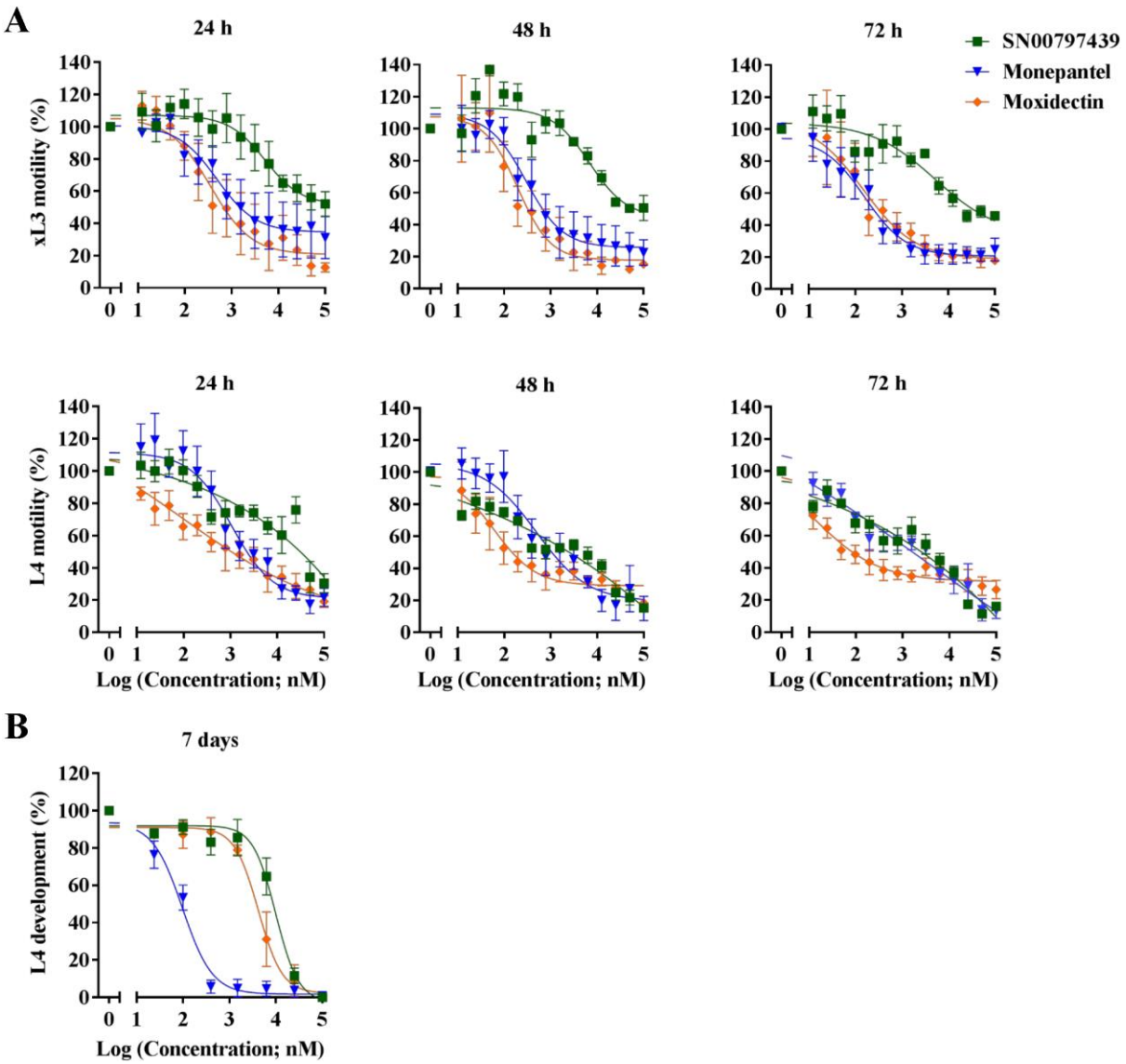

Fig. 2.2. Dose-response curves for compounds SN00797439 on larval stages of *Haemonchus contortus* in vitro with reference to the positive-control compounds monepantel and moxidectin. Inhibition of the motility of third-stage larvae (xL3s) at 24 h, 48 h and 72 h as well as fourth-stage larvae (L4s) at 24 h, 48 h and 72 h for individual compounds (A); and the inhibition of the development of L4s after seven days in vitro culture (B). Each data point represents the mean of three experiments repeated in triplicate on separate days (± standard error of the mean, SEM).

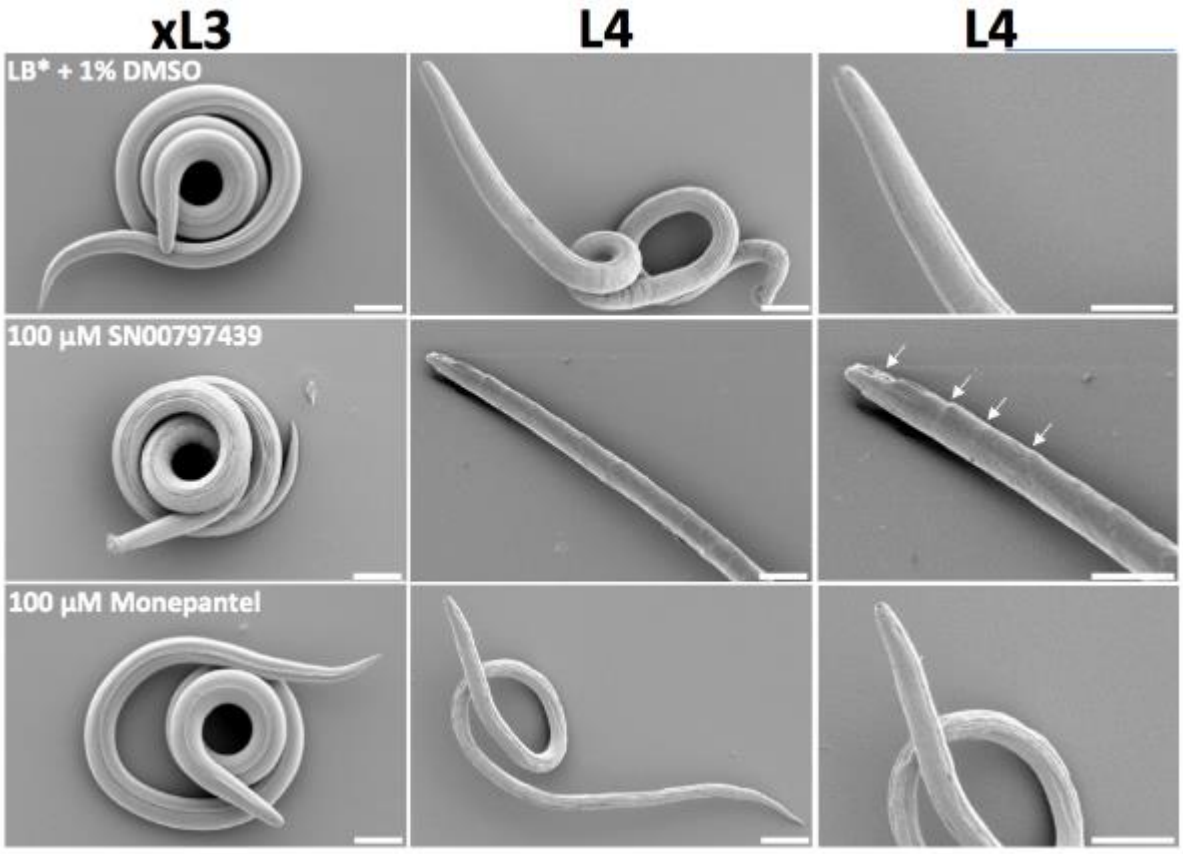

Fig. 2.3. Scanning electron microscopic images of exsheathed third-stage larvae (xL3) and fourth-stage larvae (L4) of *Haemonchus contortus* following exposure to 100 µM of compound SN00797439, monepantel (positive control) or LB* + 1% DMSO (negative control). Arrows indicate the cuticular alterations observed following treatment with SN00797439. Scale = 20 µM.

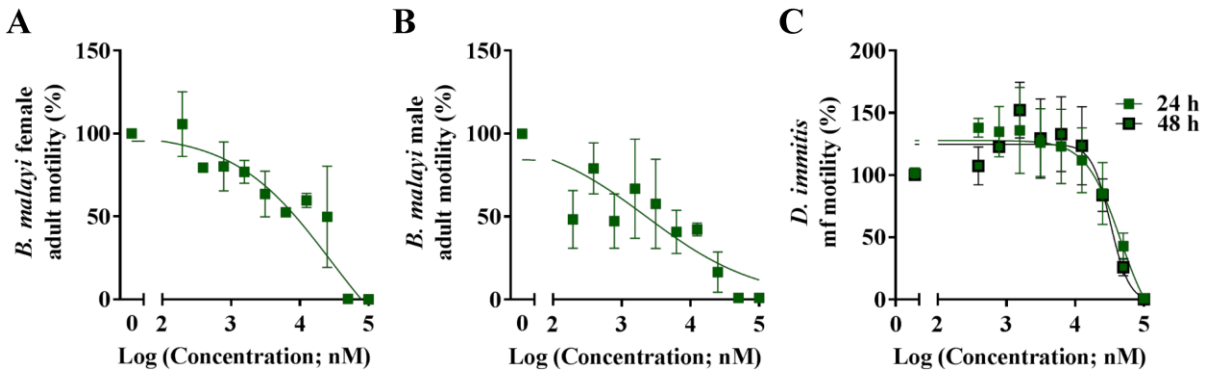

Fig. 2.4. The inhibitory effect of compound SN00797439 from the Open Scaffolds collection on the motility of female and male adult *Brugia malayi* at 24 h (panels A and B). The inhibitory effect of compound SN00797439 (C) on *Dirofilaria immitis* microfilariae (mf) at 24 h and 48 h. Dose-response experiments were performed at 100 µM and serially diluted two-fold. Each data point represents the mean of at least two experiments repeated in duplicate on separate days (± standard error of the mean, SEM).

Chapter 3 - Identification of two kinase inhibitors under pharmaceutical development with activity against *Haemonchus contortus*

Abstract

In partnership with the Medicines for Malaria Venture (MMV), we screened a collection ('Stasis Box') of 400 compounds (which are under pharmaceutical development for illnesses other than infectious diseases) for inhibitory activity against *Haemonchus contortus*, in order to attempt to repurpose some of the compounds to parasitic nematodes. We assessed the inhibition of compounds on the motility and/or development of exsheathed third-stage (xL3s) and fourth-stage (L4) larvae of *H. contortus* using a whole-organism screening assay. In the primary screen, we identified compound MMV690767 (also known as SNS-032) that inhibited xL3 motility by ~ 70% at a concentration of 20 µM after 72 h as well as compound MMV079840 (also known as AG-1295), which induced a coiled xL3 phenotype, with ~ 50% inhibition on xL3 motility. Subsequently, we showed that SNS-032 (IC_{50} = 12.4 µM) and AG-1295 (IC_{50} = 9.92 ± 1.86 µM) had a similar potency to inhibit xL3 motility. Although neither SNS-032 nor AG-1295 had a detectable inhibitory activity on L4 motility, both compounds inhibited L4 development (IC_{50} values = 41.24 µM and 7.75 ± 0.94 µM for SNS-032 and AG-1295, respectively). The assessment of the two compounds for toxic effects on normal human breast epithelial (MCF10A) cells revealed that AG-1295 had limited cytotoxicity (IC_{50} > 100 µM), whereas SNS-032 was quite toxic to the epithelial cells (IC_{50} = 1.27 µM). Although the two kinase inhibitors, SNS-032 and AG-1295, had moderate inhibitory activity on the motility or development of xL3s or L4s of *H. contortus* in vitro, further work needs to be undertaken to chemically alter these entities to achieve the potency and selectivity required for them to become nematocidal or nematostatic candidates.

3.1 Introduction

Parasites of animals cause diseases of major socioeconomic importance globally (Anderson, 2000; Beveridge, 2014). For example, gastrointestinal nematodes of livestock cause subclinical infections and diseases that lead to reductions in meat, milk and fibre production (Charlier et al., 2014; Preston et al., 2014), with ~ AUD $500 million losses per annum in Australia alone (Lane et al., 2015). Currently, anthelmintic treatment remains the mainstay of control for parasitic nematodes (Epe and Kaminsky, 2013; Geary et al., 2015). The occurrence of anthelmintic resistance, together with the limited number of anthelmintics being commercialised, indicates an urgency to discover new and effective anthelmintic compounds (Kaplan and Vidyashankar, 2012; Epe and Kaminsky, 2013; Whittaker et al., 2017).

Product development partnerships (PDPs) are playing a significant role in the development of new medicines for neglected diseases (e.g., Moran et al., 2010; Reeder and Mpanju-Shumbusho, 2016). In the present context, a PDP is usually a collaboration between a non-for-profit organisation, such as the Medicine of Malaria Venture (MMV), Drugs for Neglected Diseases Initiative (DNDi) and Global Alliance for TB Drug Development (TB Alliance), industry and/or academic partners to collectively combat infectious/parasitic diseases (Grace, 2010; Moran et al., 2010). The significant role of the PDP model is demonstrated through the delivery of commercial products, such as paromomycin against leishmaniasis, developed by the Institute of One World Health (Davidson et al., 2009), artemether-lumefantrine (Coartem dispersible) - a child-friendly treatment against malaria - developed by PDP between Novartis and MMV (Premji, 2009), and a new vaccine called MenAfriVac against the bacterial meningitis by the Meningitis Vaccine Project (Butler, 2010; Bishai et al., 2011).

Recently, in a PDP with MMV, we screened compounds in the ‘Pathogen Box’ with known activities against one or more pathogens that cause neglected diseases (including tuberculosis, malaria, sleeping sickness, leishmaniasis, schistosomiasis, hookworm disease, toxoplasmosis and cryptosporidiosis) against *Haemonchus contortus* (see Preston et al., 2016b), an economically important parasitic nematode of ruminants that represents a large order of nematodes called the Strongylida (Schwarz et al., 2013). We identified tolfenpyrad, an approved pesticide with known activity against some kinetoplastid protists (Witschel et al., 2012), which has anthelmintic activity against *H. contortus* (see Preston et al., 2016b). Within this collaborative framework, we were able to source another library, called the ‘Stasis Box’,

from MMV, which contains 400 compounds that have been in clinical development but have not been approved for illnesses; none of them were developed against neglected tropical diseases. The ‘Stasis Box’ contains compounds that have been developed against disorders such as atherosclerosis, restenosis, pulmonary fibrosis, selected cancers, urinary incontinence or depression (Mike Palmer, personal communication). Here, we screened all of these compounds against *H. contortus* using an established whole-organism motility assay (Preston et al., 2016a), with the aim of repurposing some of them as nematocides.

3.2 Methods

Procurement of *H. contortus*

In accordance with institutional animal ethics guidelines (permit no. 1413429; The University of Melbourne), *H. contortus* (Haecon-5 strain) was maintained in experimental sheep as described previously (Preston et al., 2015). To produce exsheathed third-stage larvae (xL3s), infective L3s were exposed to 0.15% (v/v) of sodium hypochlorite (NaClO) for 20 min at 37 °C (Preston et al., 2015), washed five times in sterile saline (0.9%) and cultured in Luria Bertani medium (LB) supplemented with final concentrations of 0.25 µg/ml of amphotericin B, 100 µg/ml of streptomycin and 100 IU/ml of penicillin (antibiotic-antimycotic, cat. no. 15240-062; Life Technologies, USA) (LB*). To produce fourth-stage larvae (L4s), xL3s were incubated in a water-jacketed CO_2 incubator (model no. 2406 Shel Lab, USA) for 7 days at 38 °C and 10% v/v CO_2 or until ≥ 70% of L3s had developed to the L4 stage.

Compound library and screening

From MMV we obtained the 'Stasis Box', which contains 400 compounds that have been under drug development. These compounds were individually solubilised in 10 µl of dimethyl sulfoxide (DMSO) to achieve a stock concentration of 10 mM, and then diluted and tested for activity against *H. contortus*. The compounds were tested using a previously described method (Preston et al., 2016a). In brief, using 96-well flat bottom plates, individual compounds (40 µM) in LB* (50 µl) were added to individual wells in triplicate, with LB*+ 0.5% DMSO as a negative control and a commercial anthelmintic, monepantel (Zolvix, Novartis Animal Health, Switzerland) as a positive control. Subsequently, 300 xL3s in 50 µl LB* were added to individual wells. The plates were then incubated in a 38 °C water-jacketed CO_2 incubator for 72 h. Then, a video recording (5 sec) was made of each well using

a grey-scale camera (Rolera bolt, Q imaging Scientific Coms, Canada) and a motorised X-Y axis stage (BioPoint 2; Ludl Electronics Products, USA). The motility of worms in each well was calculated in a pixel-based algorithm, called motility index (Mi), based on the light intensity changes caused by the worm movement (Preston et al., 2015). For each compound, Mi values were normalized against the positive and negative controls using the program GraphPad Prism (v.6 GraphPad Software, USA). A compound was identified as a "hit" if it reduced worm motility by ≥ 70% or induced a phenotype that differed from wild-type xL3 (i.e. LB* + 0.5% DMSO control). For each compound, each data point represented the mean of a triplicate (± standard error of the mean, SEM).

Dose-response assay

Active compounds (99.9% purity; purchased from Selleck Chemicals or Cayman Chemicals, USA), were serially (two-fold) diluted from 100 µM to 0. 76 nM in triplicate in a 96-well flat bottom plate and ~300 xL3s (in 50 µl LB*) added to each well. The plates were then incubated in a 38°C water-jacketed CO_2 incubator and the Mi values of worms measured (Preston et al., 2016a). In addition, following the measurement of xL3 motility, L4 development rates were measured, with 30 worms from each well being examined at 20-times magnification following the addition of 50 µl of 1% iodine to each well after seven days of incubation prior to light microscopic examination at 100-times magnification. Half the maximum inhibitory concentration (IC_{50}) on xL3 motility, L4 motility and L4 development were determined from the dose-response curves using a variable slope four-parameter equation in Graphpad Prism by constraining the top value to 100% and using a least squares (ordinary) fit model. For each curve, each data point represented the mean of two to five experiments repeated in triplicate on different days (± standard error of the mean, SEM).

Assessing cytotoxicity and selectivity

Compounds with activity on *H. contortus* were tested for cell toxicity properties on a non-cancerous ('normal') mammary epithelial cell line (MCF10A; Kumarasingha et al., 2016). In brief, MCF10A cells were dispensed into wells of flat bottom 384-well, black walled plates (Corning, USA) at 700 cells per well (40 µl) using a liquid handling dispenser (BioTek, Vermont, USA). Cells were cultured in DMEM-F12 containing 100 ng/ml cholera toxin (Sigma, Australia), 20 ng/ml human epidermal growth factor (EGF, Life Technologies, Australia), 10 µg/ml insulin (human; Novo Nordisk Pharmaceuticals Pty Ltd, Australia), 5%

horse serum (Life Technologies, Australia) and 0.5 µg/ml Hydrocortisone (Sigma, Australia). Following incubation (24 h at 37 °C and 5% v/v CO_2), the growth medium was aspirated and the cells were treated with test compounds starting at 100 µM as well as positive- (monepantel or moxidectin) and negative- (medium ± 1% DMSO) controls. Compounds were titrated to generate a 5-point dose-response curve (in quadruplicate) employing an automated liquid handling robot (SciClone ALH3000 Lab Automation Liquid Handler, Caliper Lifesciences, USA) and incubated for a further 48 h. For each compound concentration, matched DMSO concentrations were also tested separately to account for DMSO-induced cytotoxicity. To measure cell proliferation, cells were fixed and stained with 4',6-diamidino-2-phenylindole (DAPI; 1:1000) and individual wells imaged at 10-times magnification, covering 16 fields (~90% of well) using a high content imager (Cellomics Cell Insight Personal Cell Imager, ThermoFisher Scientific, USA) at a fixed exposure time of 0.12 sec. Viable cells were counted using the Target Activation BioApplication within the Cellomics Scan software and normalized to the cell density in wells without compound. Toxicity due to DMSO was removed from the normalised cell density counts, and IC_{50} calculated from the variable slope four-parameter equation in GraphPad Prism. Experiments were repeated twice on two different days. The selectivity indices of active compounds were calculated as follows: selectivity index = human fibroblast (MCF10A) cells IC_{50} / *H. contortus* IC_{50} (for xL3 motility, L4 motility and L4 development).

3.3 Results and discussion

In the primary screen of the 400 compounds from the 'Stasis Box' (Fig. 3.1), one compound, MMV690767 (also known as SNS-032), inhibited xL3 motility by ~70% and another compound, MMV079840 (also known as tyrphostin AG-1295 or NSC 380341), induced a coiled larval phenotype and inhibited motility by 50% (Additional file 3.1). No other compound inhibited motility by ≥ 70% or induced a non-wildtype phenotype. The chemical structures and predicted physicochemical properties of SNS-032 and AG-1295 are given in Fig. 3.1.

Compound SNS-032, an N-(5-{[(5-tert-butyl-1,3-oxazol-2-yl)methyl]sulfanyl}-1,3-thiazol-2-yl)-4-piperidinecarboxamide was developed as a cyclin dependent kinase (CDK)-2, -7 and -9 inhibitor for the treatment of B-cell lymphoma by the company Sunesis Pharmaceuticals (California, USA) and entered phase I clinical trials. Compound AG-1295, a 6,7-dimethyl-2-phenylquinoxaline, is a protein tyrosine kinase (PTK) inhibitor targeting the

platelet-derived growth factor (PDGF) receptor kinase. Given their activity against *H. contortus*, compounds SNS-032 and AG-1295 were selected for further evaluation. Dose-response curves on xL3 motility showed that SNS-032 (IC_{50} = 12.4 µM at 72 h) and AG-1295 (IC_{50} = 9.92 ±1.86 µM at 72 h) had a similar potency at inhibiting xL3 motility, without a statistically significant difference (see Table 3.1; Fig. 3.2A). Although neither SNS-032 nor AG-1295 had any detectable inhibitory activity on L4 motility (Table 3.1; Fig. 3.2B), both compounds had considerable potency at inhibiting L4 development, with SNS-032 being less potent at inhibiting larval development than AG-1295 (IC_{50} = 41.24 µM *versus* 7.75 ± 0.94 µM for SNS-032 and AG-1295, respectively) (Table 3.1; Fig. 3.2C). Comparative IC_{50} values for monepantel (control compound) against xL3 motility, L4 motility and L4 development were 0.16 ± 0.08 µM (72 h), 0.37 ± 0.32 µM (72 h) and 0.075 ± 0.04 µM (7 days), respectively (Table 3.1). The testing of the two compounds for toxic effects on breast epithelial (MCF10A) cells revealed AG-1295 to have limited cytotoxicity (IC_{50} >100 µM), whereas SNS-032 was quite toxic to these epithelial cells (IC_{50} = 1.27 µM) and not selective for the parasite (Table 3.2; Fig. 3.2D). The limited inhibitory effect of AG-1295 on the proliferation of MCF10A cells seems to associate with limited expression/transcriptional of genes encoding PDGFR-β in this non-tumorigenic cell line (cf. Kadivar et al., 2017).

SNS-032 is an anti-cancer protein kinase inhibitor that acts as an apoptosis stimulator, cell cycle inhibitor and radio-sensitizer (Gojo et al., 2002; Ali et al., 2007; Kodym et al., 2009). Based on the current literature (Chen et al., 2009; Tong et al., 2010; Mariaule and Belmont, 2014), SNS-032 selectively targets human cyclin-dependent kinases (CDKs), including CDK2, CDK7 and CDK9, suggesting that one or more CDKs of *H. contortus* are target(s) for this compound. This statement is also supported by a recent prediction and prioritization that CDK-7 and CDK-9 homologs (designated *Hc*-PK-002.1 and *Hc*-PK-236.1, respectively) of *H. contortus* are amongst the top-ten kinase drug targets for this nematode (Stroehlein et al., 2015b). The relatively close sequence (79.4%) and structural homologies (root-mean-square deviation, RMSD: 1.79 Å) in the catalytic domain of the latter CDK homologs between *H. contortus* and human (Fig. 3.3) appear to reflect the toxicity of SNS-032 to cells of both organisms and its limited selectivity. In addition, the subtle conformational difference predicted within the ATP-binding site of Hc-PK-236.1 and human CDK9 (Fig. 3.3) might explain a reduced potency of SNS-032 in *H. contortus* with respect to human cells (Tables 3.1 and 3.2). This information indicates that SNS-032 would need to undergo medicinal chemistry optimization to achieve high potency and selectivity for *H. contortus* and/or related nematodes before it could be considered as an anthelmintic candidate.

On the other hand, AG-1295 had more selective and better anthelmintic activity against *H. contortus* than SNS-032 (Tables 3.1 and 3.2), achieving IC_{50} values of 9.92 ± 1.86 µM (xL3 motility) and of 7.75 ± 0.94 µM (L4 development). This selectivity likely relates to limited expression of the target in (normal) MCF10A cells compared with a distinct and moderate activity in developing larvae of *H. contortus* proposed to be associated with relatively high levels of PTK expression (cf. Stroehlein et al., 2015b). AG-1295 is a quinoxaline compound that acts as a platelet-derived growth factor (PDGF) receptor kinase inhibitor (Kovalenko et al., 1994; Gazit et al., 1996; Kovalenko et al., 1997) which has been shown to attenuate porcine and human smooth muscle cell growth in vitro and to possess considerable anti-restenosis effects in pigs (Banai et al., 1998; Levitzki, 2013); this chemical has also been reported to significantly inhibit aortic allograft vasculopathy in rats (Karck et al., 2002) and attenuates the proliferation of rat hepatic stellate cells (Iwamoto et al., 2000). Current evidence shows that AG-1295 selectively inhibits PDGF receptor tyrosine kinase activity apparently without interacting with other protein kinases (Kovalenko et al., 1994; Banai et al., 1998; Levitzki, 2013), and inhibits PDGF-stimulated DNA synthesis with an IC_{50} value of 2.5 µM, without affecting the activity of the epidermal growth factor (EGF) receptor (Kovalenko et al., 1994). Interestingly, although AG-1295 inhibited xL3 motility and L4 development in *H. contortus*, there is presently no evidence of a PDGF receptor kinase in *H. contortus* (cf. Stroehlein et al., 2015b), suggesting an alternative kinase target. Possible targets of AG-1295 in this nematode might be one or more of five related kinases, namely *Hc*-PK-144.1 within the fibroblast growth factor receptor (FGFR) tyrosine kinase family, *Hc*-PK-185.1 and *Hc*-PK-200.1 within the growth factor receptor tyrosine kinase-like family KIN16 (similar to human vascular endothelial growth factor, VEGFR) and *Hc*-PK-319.1 and *Hc*-PK-319.2 within the EGF receptor tyrosine kinase family (cf. Stroehlein et al., 2015b). Further work needs to be done to test these proposals using an integrated experimental-structural biology approach. In conclusion, although two kinase inhibitors (SNS-032 and AG-1295) were shown to have moderate inhibitory activity on the motility or development of xL3s or L4s of *H. contortus* in vitro, substantial further work would need to be undertaken to chemically modify these entities to achieve the potency and selectivity needed for them to become viable nematocidal or nematostatic candidates.

3.4 References

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Table 3.1. Half maximal inhibition concentration (IC_{50}) values for compounds SNS-032 (MMV690767) and AG-1295 (MMV079840) on the motility of exsheathed third-stage larvae (xL3) and fourth-stage larvae (L4) of *Haemonchus contortus* (after 72 h of exposure the compound) and on the development of L4 (7 days of exposure).

Compound	IC_{50} (µM)		
	xL3 motility (72 h)	L4 motility (72 h)	L4 development (7 days)
SNS-032	12.36 [a]	na	41.24 [a]
AG-1295	9.92 ± 1.86	na	7.75 ± 0.94
Monepantel	0.16 ± 0.08	0.37 ± 0.32	0.075 ± 0.04

[a] Half maximal inhibitory concentration could not be accurately calculated by the log (agonist) *versus* response—variable slope (four parameter) equation, a IC_{50} value was estimated; na = no activity.

Table 3.2. Compounds SNS-032 (MMV690767) and AG-1295 (MMV079840) were tested for toxic effects on breast epithelial (MCF10A) cells. Selectivity indices of these compounds on the motility of exsheathed third-stage larvae (xL3) and fourth-stage larvae (L4) of *Haemonchus contortus* (after 72 h of exposure to the compound) and the development of L4s (over 7 days of exposure) were calculated using a recognized formula (Fisher et al., 2014).

Compound	IC_{50} (µM) for MCF10A cells	Selectivity indices for *H. contortus*		
		xL3 motility (72 h)	L4 motility (72 h)	L4 development (7 days)
SNS-032	1.3	nd	nd	0.04
AG-1295	>100	10.1 [a]	nd	10.9 [a]
Monepantel	27.8	173.6	75.1	370.3

[a] Selectivity indices were calculated based on the highest value in the IC_{50} range; nd = not determined.

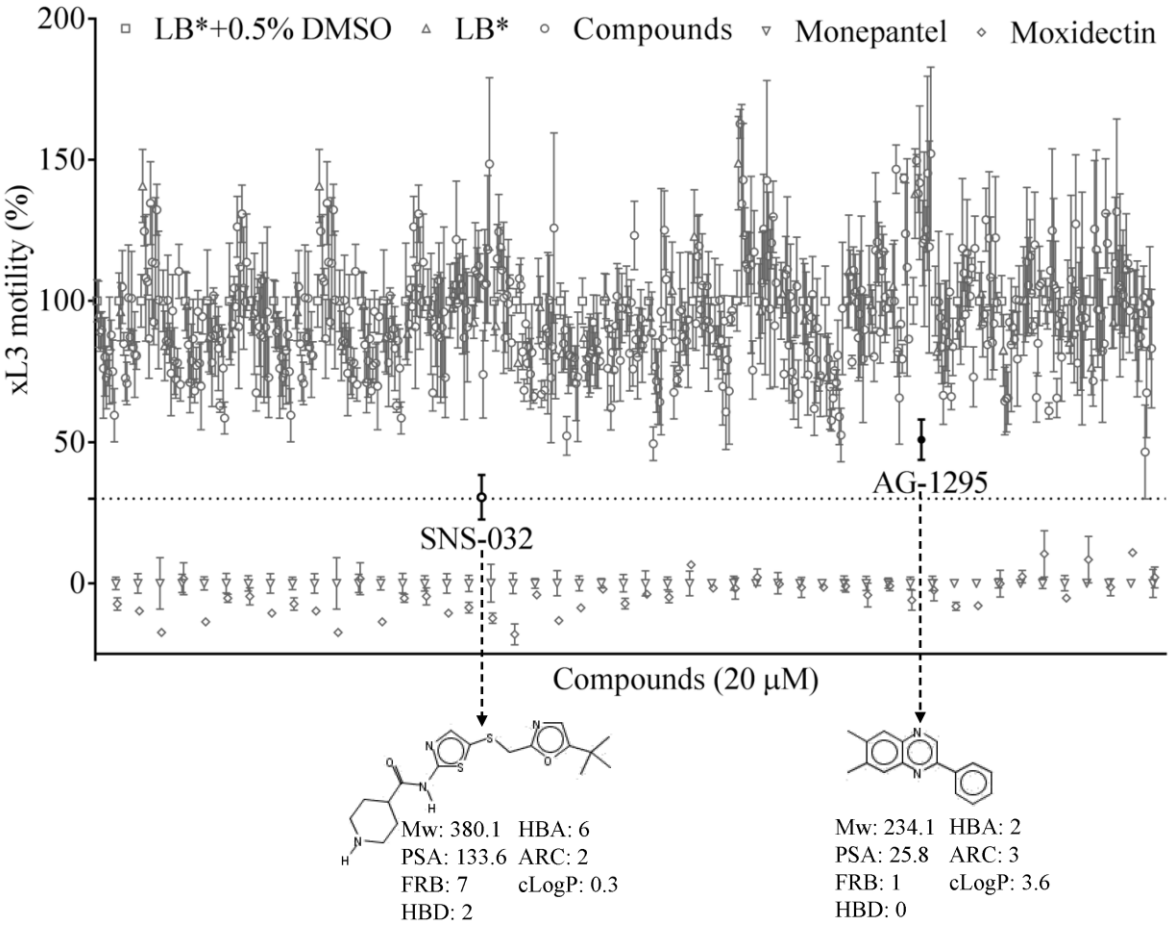

Fig. 3.1. Primary screen of 400 individual compounds from the 'Stasis Box' from the Medicines for Malaria Venture (MMV) at a concentration of 20 µM identified compound SNS-032 (MMV690767) to inhibit the motility of exsheathed third-stage larvae (xL3) of *Haemonchus contortus* (at 72 h) by ≥ 70% compared with negative (LB* + 0.5% dimethyl sulfoxide; DMSO) and positive controls (monepantel). Another compound, AG-1295 (MMV079840), was found to inhibit xL3 motility by ~ 50%, displaying a "coiled" phenotype based on visual inspection of video recordings (see Additional file 3.1). Other compounds with apparent inhibition of ≥ 50% did not exhibit a characteristic phenotype by visual inspection. Each data point represents the mean of a triplicate (± standard error of the mean, SEM). Chemical structures and physiochemical properties of 'hit' compounds SNS-032 and AG-1295 are indicated. Abbreviations: Mw = molecular weight; PSA = polar surface area; FRB = freely rotating bonds; HBD = hydrogen bond donor; HBA = hydrogen bond acceptor; ARC = aromatic ring count; cLogP = calculated partition coefficient.

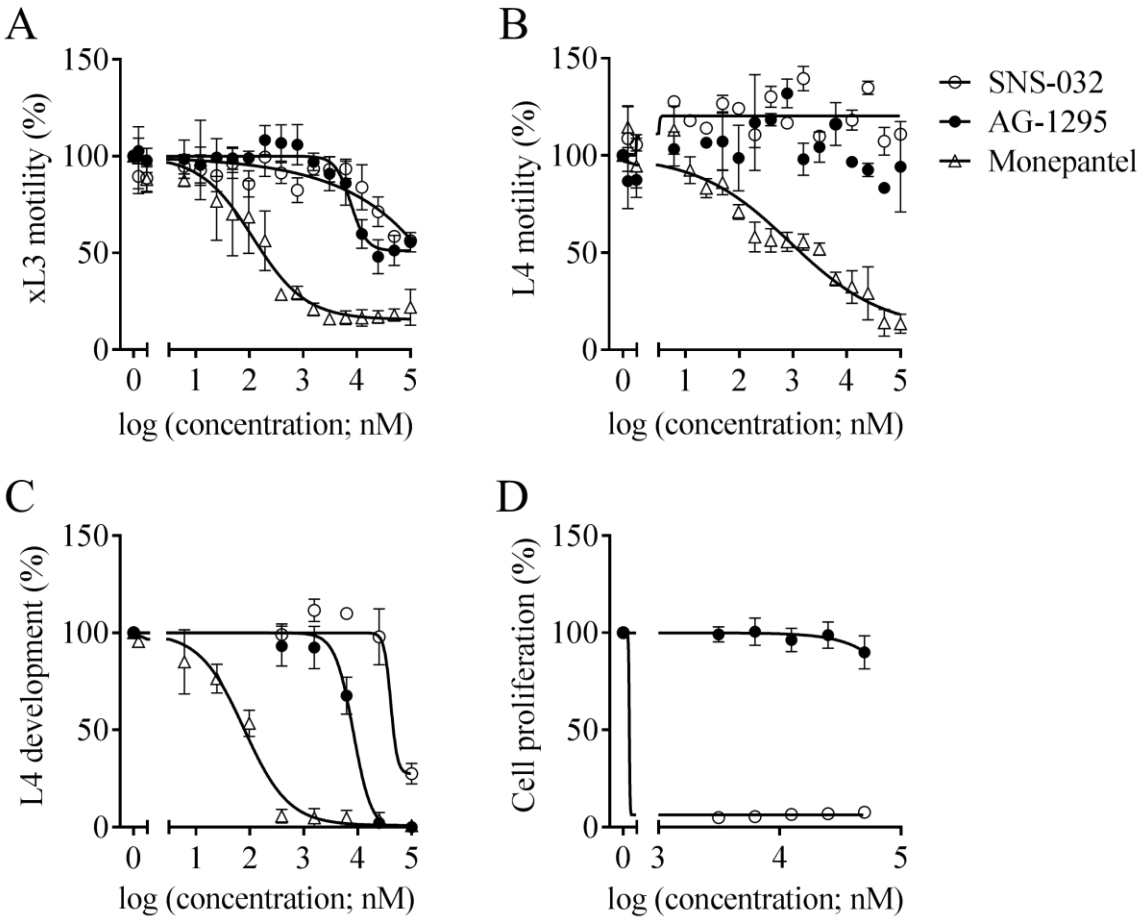

Fig. 3.2. Dose-response curves for compounds SNS-032 (MMV690767) and AG-1295 (MMV079840) on larval stages of *Haemonchus contortus* in vitro with reference to the positive-control compound monepantel. Inhibition of motility of third-stage (xL3) and fourth-stage (L4) larvae at 72 h (panels A and B, respectively) and of development of L4s at 7 days (panel C) of exposure to each of the compound. Assessment of the toxicity of compounds SNS-032 (MMV690767) and AG-1295 (MMV079840) on breast epithelial (MCF10A) cells after 48 h of exposure to each compound in vitro (panel D). Each data point represents the mean of two to five experiments repeated in triplicate on different days (± standard error of the mean, SEM).

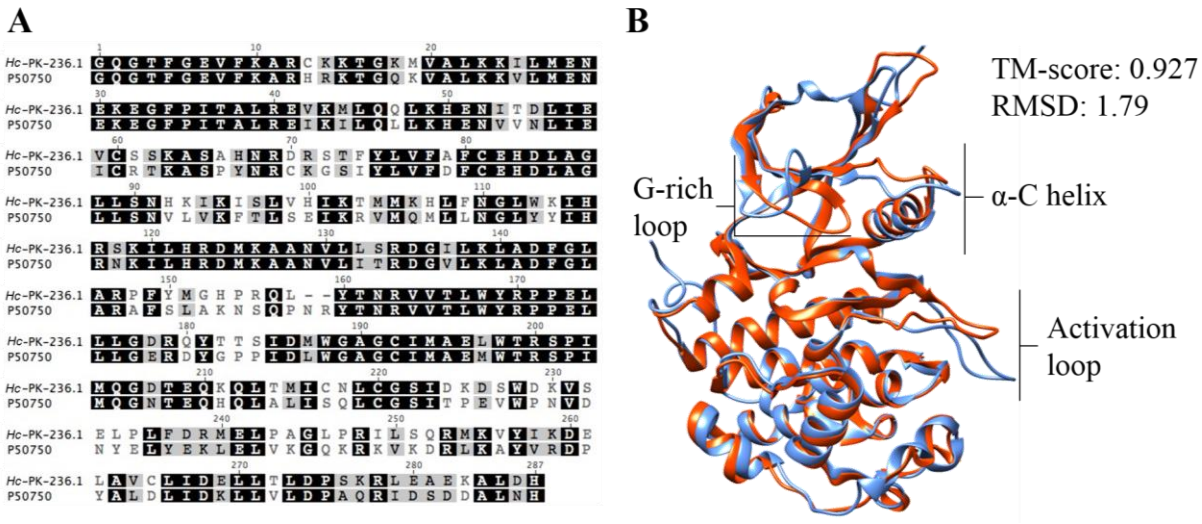

Fig. 3.3. Multiple-sequence alignment showing levels of similarity in the kinase catalytic domains (Pfam identifier: PF00069) of CDK9 homologs between *Haemonchus contortus* (*Hc*-PK-236.1) and human (UniProt accession no.: P50750). The pairwise sequence alignment was constructed using the program MUSCLE (Edgar, 2004) (panel A). Three-dimensional model of CDK9 homolog of *H. contortus* (*Hc*-PK-236.1; orange) superimposed on to the crystal structure of human CDK9 (protein data bank (PDB) identifier: 4or5A; blue). The TM-score and root-mean-square deviation (RMSD) values indicate a high-confidence prediction. Conformational differences predicted in G-rich loop, activation loop and α-C helix between the two structures are indicated. The three-dimensional structure for *Hc*-PK-236.1 was predicted using the program I-TASSER (Yang and Zhang, 2015) using default parameters (panel B).

Chapter 4 - An approved pesticide with major anthelmintic activity against *Haemonchus contortus*

Abstract

There is a substantial need to develop new medicines against parasitic diseases *via* public-private partnerships. Based on high throughput phenotypic screens of largely protozoal pathogens and bacteria, the Medicines for Malaria Venture (MMV) has recently assembled an open-access 'Pathogen Box' containing 400 well-curated chemical compounds. In the present study, we tested these compounds for activity against parasitic stages of the nematode *Haemonchus contortus* (barber's pole worm). In an optimised, whole-organism screening assay, using exsheathed third-stage (xL3) and fourth-stage (L4) larvae, we measured the inhibition of larval motility, growth and development of *H. contortus*. We also studied the effect of the 'hit' compound on mitochondrial function by measuring oxygen consumption. Among the 400 'Pathogen Box' compounds, we identified one chemical, called tolfenpyrad (compound identification code: MMV688934) that reproducibly inhibits xL3 motility as well as L4 motility, growth and development, with IC_{50} values ranging between 0.02 and 3 µM. An assessment of mitochondrial function showed that xL3s treated with tolfenpyrad consumed significantly less oxygen than untreated xL3s, which was consistent with specific inhibition of complex I of the respiratory electron transport chain in arthropods. Given that tolfenpyrad was developed as a pesticide and has already been tested for absorption, distribution, excretion, biotransformation, toxicity and metabolism, it shows considerable promise for hit-to-lead optimisation and/or repurposing for use against *H. contortus* and other parasitic nematodes. Future work should assess its activity against hookworms and other pathogens that cause neglected tropical diseases.

4.1 Introduction

Compounded by massive global food and water shortages and climate change, parasitic illnesses, including neglected tropical diseases (NTDs; WHO, 2015), have a devastating, long-term impact on human and animal health and welfare worldwide, and thus represent a major global challenge. Together, NTDs infect more than one billion people worldwide, resulting in an estimated loss of 26 million disability-adjusted life years (Hotez et al., 2014).

Despite their adverse socioeconomic impact, there are major limitations in the diagnosis, treatment and control of NTDs. Currently, there are no commercial vaccines available against most of these diseases (Pedrique et al., 2013; Hotez et al., 2016), diagnostic methods frequently suffer from insufficient specificity and sensitivity (Utzinger et al., 2012; Assefa et al., 2014), and treatments are often not highly effective and/or are toxic (Castro et al., 2006; Witschel et al., 2012; Molina et al., 2015). In addition, often the small numbers of drugs (or drug classes) frequently used, limited use of combination drug therapies and the implementation of mass drug administration programs bear the risk of drug resistance emerging in some groups of target pathogens (Humphries et al., 2012; Witschel et al., 2012; Webster et al., 2014). Therefore, the development of new drugs is crucial to ensure effective and sustained treatment and control into the future.

In spite of some success through the discovery of, for example, monepantel (Kaminsky et al. 2008; Prichard and Geary 2008) and derquantel (Little et al., 2011), progress in discovering new drugs against parasitic worms of animal health importance has been relatively poor. Likely reasons for limited success beyond the lack of resources include an over-confidence in the validation of molecular targets (enzymes and receptors) and in studying an inappropriate developmental stage of a pathogen. However, key gaps include a lack of readily available curated sets of compounds for targeted screening and subsequent evaluation, limited cooperation among different areas (including parasitology, drug discovery, medicinal chemistry and safety evaluation) which are essential to find starting points for drug discovery, and to bring them to tangible and translational outcomes and outputs.

In the late 1990s, an innovative collaboration model for research and development for neglected diseases emerged in the form of public-private partnerships (PPPs) that came to be known as product development partnerships (PDPs). A key example is the Medicines for Malaria Venture (MMV), created from a desire to catalyse the discovery, development and delivery of new medicines against malaria. Over the last decade, almost seven million

compounds have been tested in phenotypic assays against malaria, and this has resulted in a solid pipeline of new preclinical and clinical candidates. In addition, an open science initiative has made many of these structures available, and a collection of 400 key malaria phenotypic 'hits', called the 'Malaria Box', was launched in 2013. Building on this model, in December 2015, MMV took this a stage further, with an initiative to stimulate the discovery of drugs for neglected parasitic diseases. The 'Pathogen Box' (www.pathogenbox.org), contains 400 diverse drug-like molecules, and is provided at no cost to research groups.

Each of the 400 compounds in the 'Pathogen Box' has confirmed activity against one or more key pathogens that cause some of the most socioeconomically important diseases worldwide, including tuberculosis, malaria, sleeping sickness, leishmaniasis, schistosomiasis, hookworm disease, toxoplasmosis and cryptosporidiosis. In addition, all compounds have been tested for cytotoxicity, with compounds included in the library being at least 5-fold more selective for the pathogen than its mammalian host. The complete set of compounds is dispatched to laboratories around the world to boost drug discovery efforts. This initiative provided us with a unique opportunity to assess these curated compounds for nematocidal activity in a recently developed whole-organism screening assay (Preston et al., 2015, 2016). Our aim was to rapidly screen all 400 compounds against parasitic stages of the barber's pole worm, *Haemonchus contortus*, and to identify hit compounds and characterise/assess them for further evaluation as nematocidal candidates. This worm was used because it is one of the best-studied members of a large order (Strongylida) of socioeconomically important nematodes of animals, including humans, because there is extensive information available on its biology and molecular biology, and because its genome and developmental transcriptome have been characterised in detail (Gasser and von Samson-Himmelstjerna, 2016), providing a foundation for drug discovery efforts.

4.2 Materials and methods

4.2.1 Procurement of *H. contortus*

The Haecon-5 strain of *Haemonchus contortus*, which is partially resistant to benzimidazoles (Dr Jody Zawadzki, personal communication), was maintained in experimental sheep as described previously (Schwarz et al., 2013; Preston et al., 2015) and in accordance with the institutional animal ethics guidelines (permit no. 1413429; The University of Melbourne, Australia). L3s were produced from *H. contortus* eggs by incubating faeces from infected sheep at 27 °C for 1 week (Preston et al., 2015), sieved

through nylon mesh (pore size: 20 µm) to remove debris or dead larvae and then stored at 10 °C for a maximum of 3 months. For screening and basal oxygen consumption measurements (see following sub-sections), L3s were exsheathed and sterilised in 0.15% v/v sodium hypochlorite (NaClO) at 37 °C for 20 min (Preston et al., 2015). Thereafter, xL3s were washed five times in sterile physiological saline by centrifugation at 1700 *g* (5 min) at 22–24 °C. Then, xL3s were immediately suspended in Luria Bertani medium [LB: 10 g of tryptone (cat no. LP0042; Oxoid, England), 5 g of yeast extract (cat no. LP0042; Oxoid) and 5 g of NaCl (cat. no. K43208004210; Merck, Denmark)] in 1 l of reverse-osmosis deionised water). LB was autoclaved and supplemented with 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 2.5 µg/ml of amphotericin (Fungizone, antibiotic – antimycotic; cat. no. 15240-062; Gibco, USA); this supplemented LB was designated LB*. Fourth-stage larvae (L4s) were produced from xL3s in vitro for 7 days at 38 °C and 10% CO_2, as described by Preston et al. (2015, 2016).

4.2.2 Screening of compounds

The Pathogen Box contains 400 compounds representing compounds that are active against one or more of 12 distinct pathogens (http://www.pathogenbox.org/about-pathogen-box/supporting-information). Individual compounds had only been tested to confirm activity against the pathogen for which the compounds were first reported to be active, and have not been tested against the other pathogens represented in the 'Pathogen Box'. All compounds have been tested for cytotoxicity; typically, they are five-fold less potent against a human fibroblast cell line (MRC-5) than the pathogen (cf. Table 4.1); toxicity values are within levels considered acceptable for an initial drug discovery programme (www.pathogenbox.org/about-pathogen-box/supporting-information). Each of the 400 compounds was prepared as described previously (Preston et al., 2015) and screened (in triplicate) at a concentration of 20 µM on xL3s of *H. contortus* in 96-well microculture plates using two reference-control compounds, moxidectin and monepantel (Preston et al., 2015, 2016). In brief, compounds were dissolved to a stock concentration of 10 mM in dimethyl sulfoxide (DMSO, Ajax Finechem, Australia). Compounds were individually diluted to the final concentration of 20 µM using LB*, and dispensed (in triplicate) into wells of the microculture plates using a multichannel pipette. In addition, the negative-controls (LB* and LB* + 0.5% DMSO; six wells each), and positive-controls (final concentration of 20 µM of monepantel [Zolvix, Novartis Animal Health, Switzerland] and 20 µM of moxidectin

[cydectin, Virbac, France]) were dispensed in triplicate wells. Then, xL3s (~300/well) were dispensed into wells of the plate using an automated multichannel pipette (Viaflo Assist/II, Integra Biosciences, Switzerland). Following an incubation for 72 h at 38 °C and 10% CO_2, a video recording (5 sec) was taken of each well of the 96-well microculture plate (containing xL3s) using a grey-scale camera (Rolera bolt, Q imaging Scientific Coms, Canada), and a motorised X-Y axis stage (BioPoint 2, Ludl Electronics Products, USA). Individual videos were processed to calculate a motility index (MI) using an algorithm described previously (Preston et al., 2015, 2016). MIs were normalised to the positive- and negative-controls (to remove plate-to-plate variation) using the program Prism (v.6 GraphPad Software, USA). A compound was recorded as having activity if it reduced xL3 motility by ≥ 70% after 72 h of incubation.

4.2.3 Dose-response assessments of active compounds on xL3 and L4 motility, and L4 growth and development

Anti-xL3 activity of any 'hit' compound was confirmed, and half maximum inhibitory concentration (IC_{50}) values estimated from dose-response curves (24 h, 48 h and 72 h). Compounds that reduced the motility of xL3s were also tested for their ability to inhibit the development of xL3s to L4s, the motility of L4s and/or their ability to retard L4 growth as described previously (Preston et al., 2015, 2016). In brief, growth retardation and morphological alterations in L4s exposed for 48 h to LB* containing either 1% DMSO (negative-control), 100 µM of each tolfenpyrad (test compound), moxidectin (positive-control) or monepantel (positive-control) were assessed microscopically (20-100 x magnification). For each treatment, the mean width of 30 L4s ± the standard error of the mean (SEM) was calculated, and a non-parametric (Kruskal-Wallis) and Dunn's multiple comparisons test was used to calculate statistical difference between treatments. All assays (xL3 motility, and L4 development, growth and motility) were performed in triplicate, between 3-5 times on different days. To determine IC_{50} values, the data from each assay (xL3 motility, L4 motility and development) were converted to a percentage with reference to the negative-control (LB* + 0.5% DMSO), and IC_{50} values determined using a variable slope four-parameter equation, constraining the top value to 100% and using a least squares (ordinary) fit model (v.6 GraphPad Software). Selectivity indices (SIs) were calculated using a recognised formula (SI = human fibroblast (MRC-5) cells IC_{50} / *H. contortus* IC_{50}; Fisher et al., 2014) employing cytotoxicity data linked to the Pathogen Box compounds.

4.2.4 Measurement of basal oxygen consumption

Following standardisation using an established protocol, the basal oxygen consumption ('respiratory') rate in xL3s was measured using the Seahorse XF24 flux analyser (Seahorse Biosciences, USA) (McGee et al., 2011). In brief, 450 µl of LB* + 1% DMSO containing 100 µM of tolfenpyrad ('hit' compound) or 100 µM moxidectin (control; not known to inhibit respiration), or LB* + 1% DMSO alone (untreated control) were transferred in quadruplicate to the wells of a 24-well plate (Seahorse XF24). Then, 1000 xL3s in 50 µl LB* were added to the wells, and the plates pre-incubated for 2 h, equilibrated for 30 min and oxygen consumption measured using the flux analyser. Seven measurements were taken over a 1 h-period (protocol: 2 min-mix, 2 min-pause and 4 min-measure; McGee et al., 2011). Experiments were repeated twice. A two-way repeated measures ANOVA with a Dunnett's multiple comparisons test (v.6 GraphPad Prism) was used to assess the statistical difference in oxygen consumption between treated and untreated xL3s.

4.3 Results

In the primary screen of the 400 compounds (Fig. 4.1; Supplementary file 4.1), one compound (tolfenpyrad; Compound ID: MMV688934; batch: MMV688934-01; pubchem.ncbi.nlm.nih.gov/compound/10110536) was recorded to inhibit xL3 motility by ≥ 70%. Although there are benzimidazole-based compounds in the Pathogen Box, nematocidal activity was not detected using this ≥ 70% threshold, because the Haecon-5 strain of *H. contortus* is partially resistant to this class of chemicals. Subsequent assays using xL3s and L4s of *H. contortus* showed that the potency of tolfenpyrad, measured as IC_{50} values, ranged from 0.02 to 3 µM (Fig. 4.1; Table 4.1). In comparison to moxidectin and monepantel, tolfenpyrad was able to reduce motility earlier than monepantel, with inhibition occurring after 2 h of exposure and an IC_{50} value of 2.3 µM at 24 h compared with 52.1 µM for monepantel (Fig. 4.3). Tolfenpyrad and moxidectin were found to have a similar inhibitory effect on xL3 motility at the time points tested (Table 4.1). Furthermore, when examining the inhibitory activity of the compounds on L4 motility, tolfenpyrad and moxidectin had lower IC_{50} values at 24 h, 48 h and 72 h compared with that of monepantel (Table 4.1). In the L4 development assay, tolfenpyrad had a greater inhibitory effect on the development of xL3 to L4 (IC_{50} of 0.06 µM) than did moxidectin and monepantel (IC_{50} of 12.3 µM and 0.4 µM, respectively). Light microscopic examination of parasitic larvae revealed morphological

damage (shrivelled and granulated appearance) in L4s following exposure *in vitro* (48 h) to tolfenpyrad and monepantel (Fig. 4.2), but not in xL3s (data not shown). L4s exposed to tolfenpyrad were significantly thinner than 'untreated' controls, and had a similar width to moxidectin-exposed, but not as pronounced as monepantel-exposed larvae (Fig. 4.2). Finally, it was assessed whether tolfenpyrad would inhibit respiration in *H. contortus* xL3s, as it does in arthropods by targeting complex I of the respiratory electron transport chain (Song et al., 2013), by measuring oxygen consumption over time (Fig. 4.3). The results showed that tolfenpyrad-treated xL3s consumed substantially ($P<0.05$) less oxygen than both moxidectin-treated and untreated xL3s (Fig. 4.3).

4.4 Discussion

The screening of the Pathogen Box compounds identified one chemical, tolfenpyrad, with major activity against parasitic larval stages (xL3 and L4) of *H. contortus* in vitro; IC_{50} values were comparable with those of two commercially available anthelmintics, monepantel and moxidectin. Tolfenpyrad, a pyrazole-5-carboxamide insecticide, is the International Organization for Standardization – approved name for 4-chloro-3-ethyl-1-methyl-N-[4-(p-tolyloxy)benzyl]pyrazole-5-carboxamide (International Union of Pure and Applied Chemistry, IUPAC), which has the Chemical Abstracts Service number 129558-76-5. Tolfenpyrad was included in the MMV 'Pathogen Box' based on results from a compound screen of agro-chemicals, which showed that tolfenpyrad has potent and selective *in vitro* activity against *Trypanosoma* spp. (Witschel et al., 2012). As a pesticide, tolfenpyrad has relatively broad activity against egg, larval, nymphal and adult stages of various arthropods (including Hemiptera, Coleoptera, Diptera, Lepidoptera, Thysanoptera and Acarina), and has been applied to various infested crops (Song et al., 2012; Yamaguchi et al., 2012). This chemical was developed in Japan and was first approved in 2002; it has been registered for commercial use in several countries other than Japan, including the Dominican Republic, Thailand, the United Arab Emirates, Indonesia and the USA (Yamaguchi et al., 2012).

In the present study, tolfenpyrad was shown to be more potent against L4s than xL3s based on IC_{50} values (motility and development), with substantial cuticular damage to the L4 but not to the xL3 stage. Compared with untreated-control L4s, the width of tolfenpyrad-treated L4s were significantly reduced as were moxidectin- and monepantel-treated control L4s, indicating that treatment suppresses parasite growth and development. It is unclear whether the difference in potency of tolfenpyrad between the two larval stages tested is due

to variation in drug uptake, as at the L4 stage has a functioning pharynx (Sommerville, 1966), or the mode of action. The mode of action of this chemical (as a contact insecticide and fungicide) is likely linked to the specific inhibition of complex I of the respiratory electron transport chain in mitochondria (Lummen, 1998; Song et al., 2013), such that it is effective against various pests that are resistant to insecticides, including organophosphates and carbamates, which have modes of action that are entirely distinct from tolfenpyrad.

To explore whether tolfenpyrad might act as an inhibitor of the electron transport chain in *H. contortus*, oxygen consumption was measured in xL3s in the presence or absence of tolfenpyrad following a pre-exposure for 2 h to the compound. This approach is routinely used to evaluate mitochondrial function in *Caenorhabditis elegans* and mammalian tissue by detecting oxygen consumption in real-time using oxygen-sensitive probes (McGee et al., 2011; Andreux et al., 2014). In the present study, we elected to pre-treat the xL3s of *H. contortus* for 2 h prior to the initial measurement of oxygen consumption, as both tolfenpyrad and moxidectin significantly inhibit motility following immediate exposure. The results revealed a significant decrease in oxygen consumption in xL3s exposed to tolfenpyrad compared with untreated controls. Similarly, oxygen consumption was reduced in tolfenpyrad-treated than moxidectin-treated xL3s, which was also associated with reduced motility following the exposure for 2 h. This discrepancy in oxygen consumption between these treatments indicates that tolfenpyrad is also a complex I mitochondrial electron transport inhibitor in *H. contortus*.

Although mainly used against agricultural pests, tolfenpyrad had not been assessed previously for use against endoparasites, such as parasitic worms, of animals. A report was prepared by the Joint FAO/WHO Meeting on Pesticide Residues at the request of the Codex Committee on Pesticide Residues in 2013 (FAO, 2013). This report extensively reviewed and appraised many aspects of tolfenpyrad, including: (i) acceptable daily intake; (ii) absorption, distribution, excretion and biotransformation; (iii) toxicity studies (acute; short and long-term; carcinogenicity; genotoxicity; reproductive, developmental and neural toxicity), and (iv) studies of metabolites. The main conclusions from this report were: (i) the acceptable daily intake of tolfenpyrad is 0-0.006 mg/kg in mammals; (ii) following oral administration, tolfenpyrad is rapidly absorbed, widely distributed and metabolized by the liver, with 88-93% being excreted in the faeces, and (iii) tolfenpyrad was not found to be carcinogenic, genotoxic or neurotoxic, but was found to have some reproductive and developmental toxicity. Although some intoxications (with excessive exposure/ingestion) have been recorded in humans (e.g., Yamaguchi et al., 2012; Hikiji et al., 2013), well-controlled risk assessment

studies in monogastric mammals (mouse, rat, rabbit and dog) have shown that the no-observed-adverse-effect levels (NOAELs) of tolfenpyrad are between 1-1.5 mg/kg body weight per day, with lowest-observed-adverse-effect levels (LOAEL) of 5-21 mg/kg body weight per day (FAO, 2013). Therefore, based on the evidence presented in this report and associated literature (see FAO, 2013) as well as the high selectivity of tolfenpyrad for *H. contortus* (see Table 4.1), we propose to test tolfenpyrad *in vivo* in sheep against *H. contortus* and/or related parasitic nematodes at dosages between 1 and 5 mg/kg body weight. We also propose to assess its activity *in vitro* against the hookworms *Ancylostoma ceylanicum* and *Necator americanus* of humans and other NTD pathogens (London Declaration, 2012). Nonetheless, it will be important to also extend medicinal chemistry work to establish whether a safer and more effective derivative of this chemical might be synthesized. Given that tolfenpyrad was developed as a pesticide and has already been tested for absorption, distribution, excretion, biotransformation, toxicity and metabolism, there is considerable promise for the repurposing of this chemical for use against *H. contortus* and other parasitic nematodes. Further work should now focus on assessing the activity of tolfenpyrad against hookworms and other worms that cause NTDs.

4.5 References

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Table 4.1. *In vitro* activity of tolfenpyrad on the motility of exsheathed third-stage (xL3) and fourth-stage (L4) larvae of *Haemonchus contortus* (after 24 h, 48 h and 72 h of exposure) and on the development of the L4 stage (after 7 days of exposure) in comparison with values for monepantel and moxidectin, as well as cytotoxicity data for tolfenpyrad.

		Half maximum inhibitory concentration (IC_{50}; μM)		
Bioassay	Time	Tolfenpyrad	Monepantel	Moxidectin
xL3 motility	24 h	2.3	52	2.5
	48 h	3.0	6	2.5
	72 h	3.0	0.4	2.3
L4 development	7 days	0.06	0.4	12.3
L4 motility	24 h	0.13	4.3	2.2
	48 h	0.06	2.2	0.6
	72 h	0.02	3	0.005
Cytotoxicity on human fibroblast (MRC-5) cells[a]		56	na	na

[a] Data from the Laboratory of Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp, provided to Medicines for Malaria Venture (MMV) to accompany the Pathogen Box. na = not applicable.

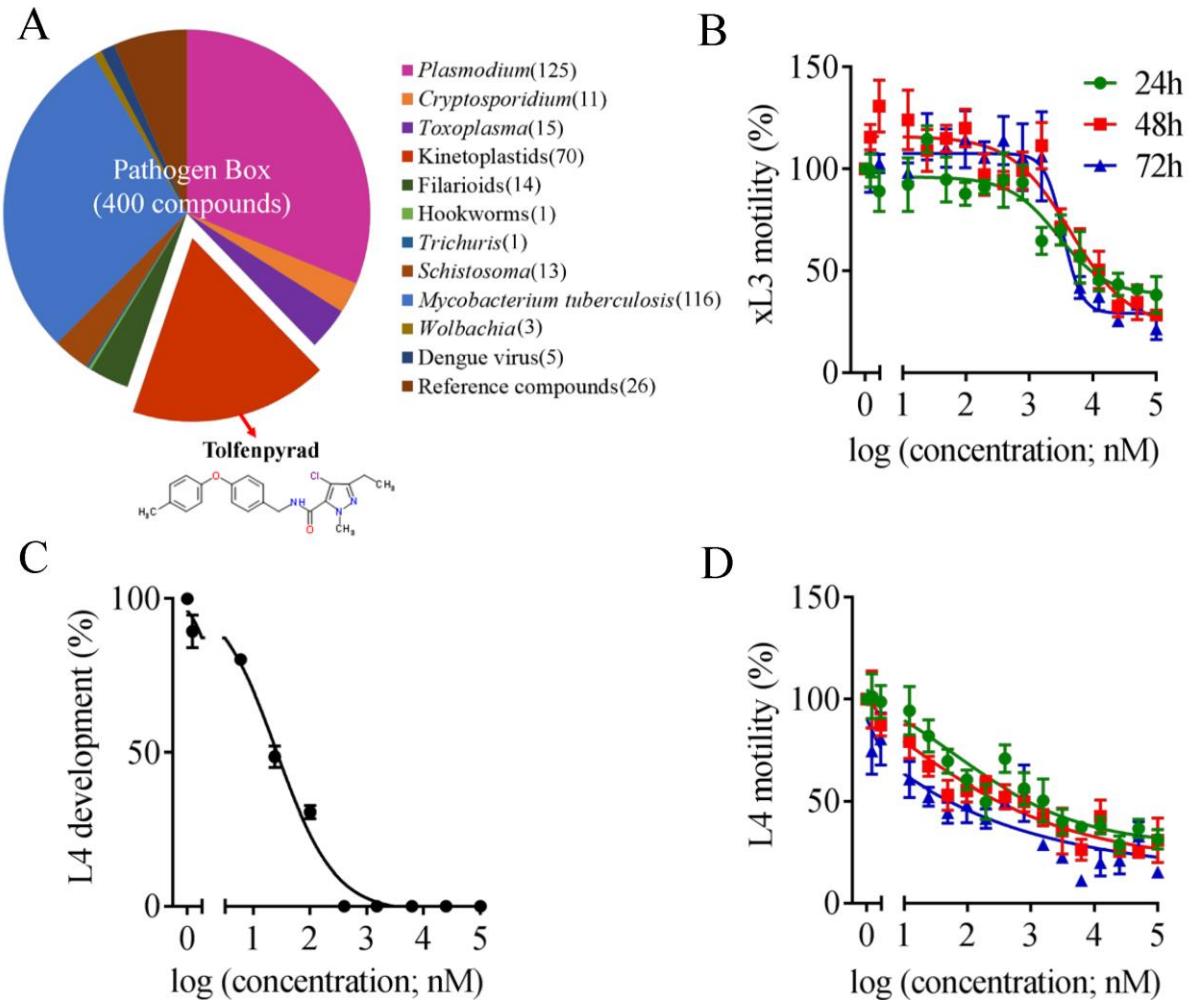

Fig. 4.1. The 'Pathogen Box' from the Medicines for Malaria Venture (MMV) (Panel A) contains 400 diverse drug-like molecules with confirmed activity against one or more key pathogens that cause some of the most socioeconomically important diseases worldwide, including malaria, toxoplasmosis, cryptosporidiosis, trypanosomiasis, leishmaniasis, hookworm disease, trichuriasis, schistosomiasis; numbers of chemicals active against different pathogen/pathogen groups are indicated in parentheses. The graphs (Panels B to D) show the activity of tolfenpyrad on the motility of exsheathed third-stage (xL3) and fourth-stage (L4) larvae of *Haemonchus contortus* (after 24 h, 48 h and 72 h of exposure) and on the development of the L4 stage (after 7 days of exposure), respectively. Activity is given in half maximum inhibitory concentration (IC_{50}). Tolfenpyrad has known activity against *Trypanosoma* spp. (kinetoplastids) (Witschel et al. 2012), and there is no previous report of its activity on nematodes.

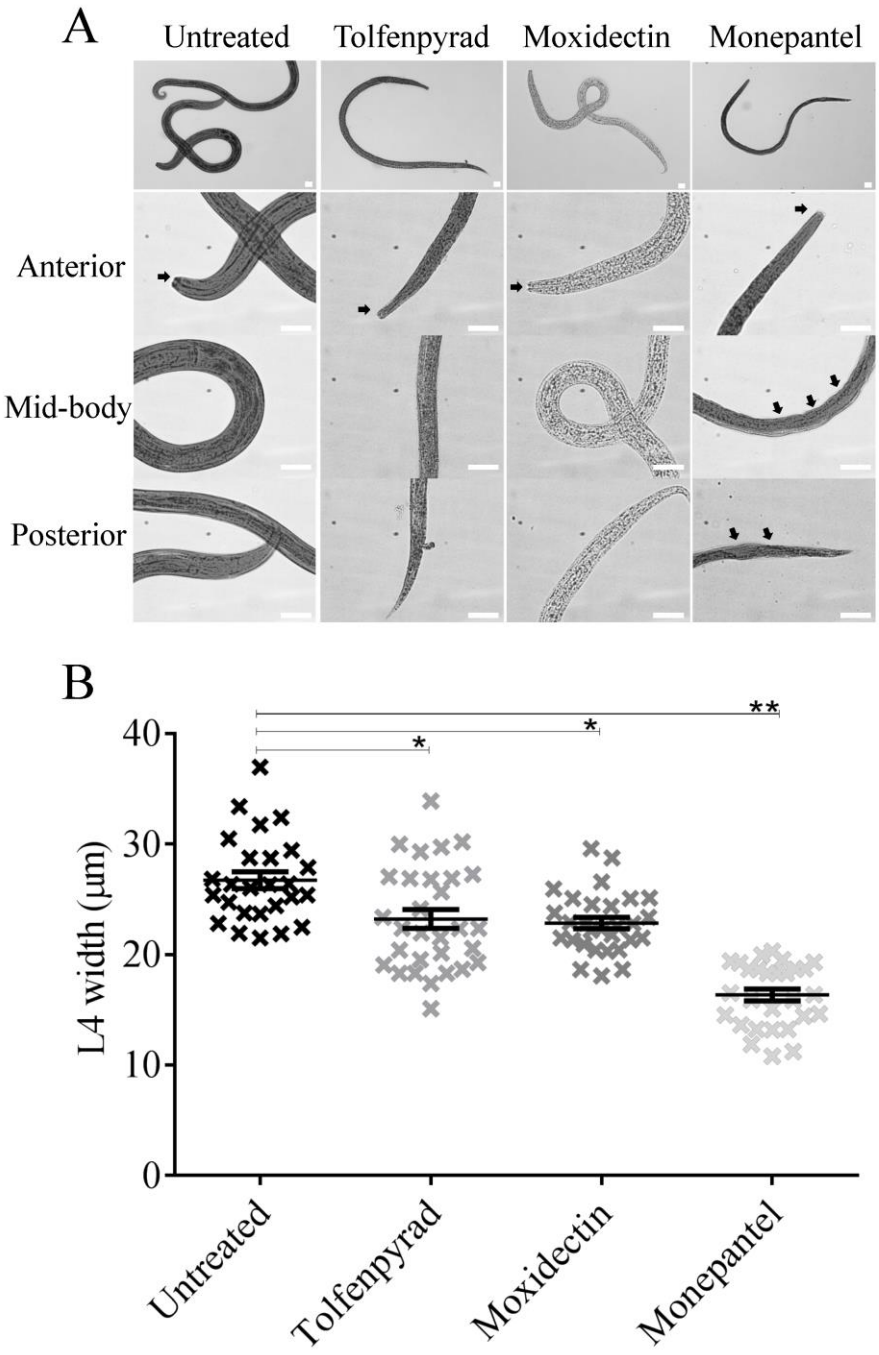

Fig. 4.2. Panel A: Images of fourth stage larvae (L4) exposed for 48 h to in LB* containing either 1% dimethyl sulfoxide (untreated, negative control), 100 µM of each tolfenpyrad, moxidectin (positive-control) or monepantel (positive-control). Displayed are whole worm (L4) or anterior, mid-body and posterior regions (20-100 x magnification; white scale bar: 20 µm). Solid, black arrows show structural damage to the cuticle and mouth of L4s. Panel B: Graph showing the mean widths of L4s under the same experimental conditions. The data points represent the width of 30 individual L4s; the bars represent the mean width ± the standard error of the mean (SEM). Significance between values (mean ± SEM) was determined using a non-parametric (Kruskal-Wallis) and Dunn's multiple comparisons test.

*/** indicate which values are significantly different from one another ($P<0.05$ and $P<0.01$, respectively).

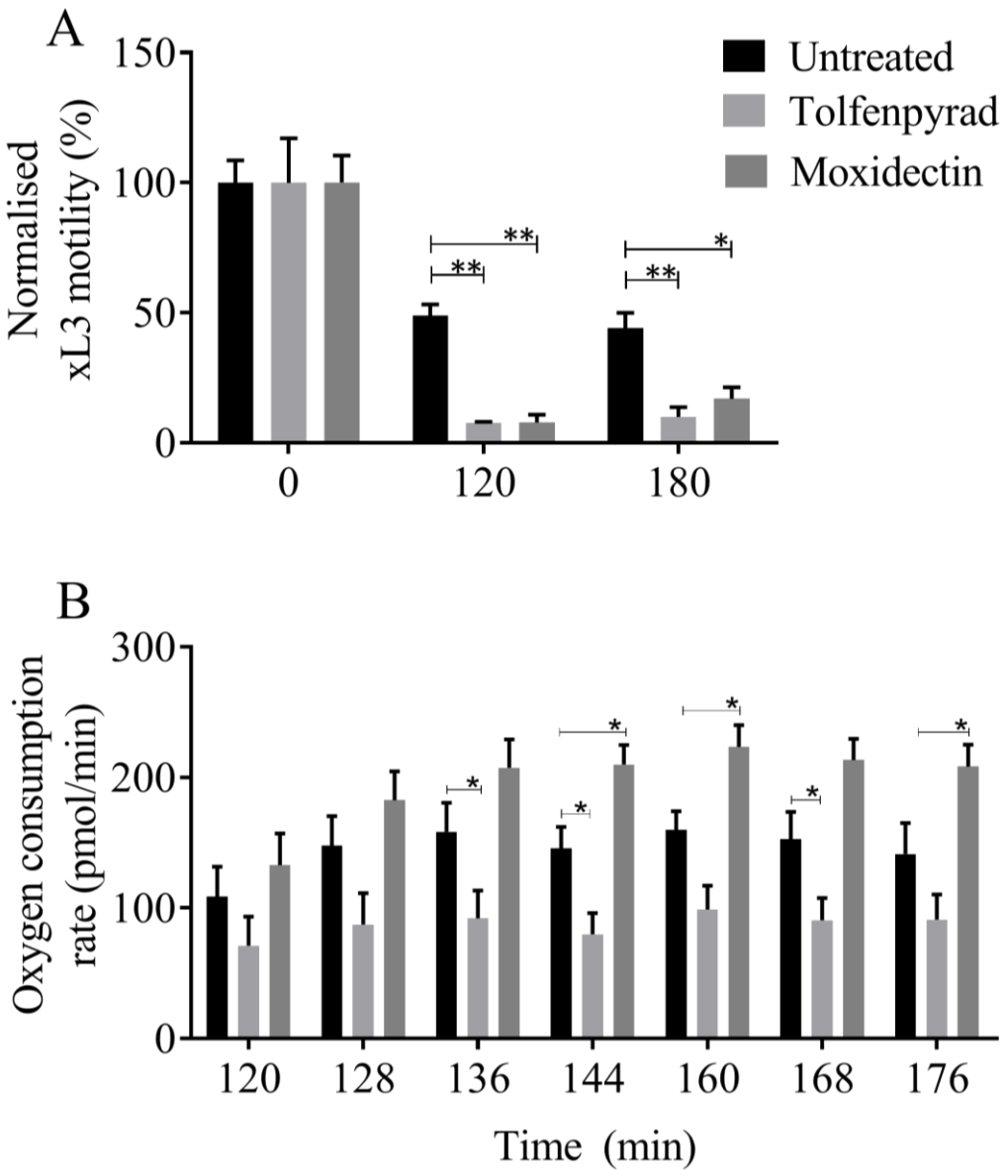

Fig. 4.3. Panel A: Motility of exsheathed L3s (xL3s) of *Haemonchus contortus* incubated in culture medium (LB* + 1% DMSO) (untreated), and in the presence of 100 µM of either tolfenpyrad ('hit' compound) or moxidectin (control) at three distinct time points; at each data point, motility was normalised to time (0 min). Panel B: Based on results in panel A, oxygen consumption was measured seven times (8 min each) over a period of 1 h in untreated xL3s, and xL3s treated with the same concentration (100 µM) of tolfenpyrad ('hit' compound) or moxidectin (control) using a flux analyser. The mean values from 10 replicates (motility) and 8 replicates (oxygen consumption) from two separate experiments are shown, with variation between measurements displayed as a standard error of the mean (SEM). */** indicate values (mean ± SEM) which are significantly different from one another ($P<0.05$ and $P<0.01$, respectively), determined using a two-way ANOVA and Dunnett's multiple comparisons test.

Chapter 5 - Assessing the anthelmintic activity of pyrazole-5-carboxamide derivatives against *Haemonchus contortus*

Abstract

In this study, we tested five series of pyrazole-5-carboxamide compounds (n = 55) for activity against parasitic stages of the nematode *Haemonchus contortus* (barber's pole worm). In an optimised, whole-organism screening assay, using exsheathed third-stage (xL3) and fourth-stage (L4) larvae, we measured the inhibition of larval motility and development of *H. contortus*. Amongst the 55 compounds, we identified two compounds (designated a-15 and a-17) that reproducibly inhibit xL3 motility as well as L4 motility and development, with IC_{50} values ranging between ~ 3.4 and 55.6 µM. We studied the effect of these two 'hit' compounds on mitochondrial function by measuring oxygen consumption. This assessment showed that xL3s exposed to each of these compounds consumed significantly less oxygen and had less mitochondrial activity than untreated xL3s, which was consistent with specific inhibition of complex I of the respiratory electron transport chain in arthropods. The present findings provide a sound basis for future work aiming to identify the targets of compounds a-15 and a-17, and to establish the modes of action of these chemicals in *H. contortus*.

5.1 Introduction

Synthetic pyrazole-5-carboxamide derivatives, such as tebufenpyrad and tolfenpyrad, are important pesticides which are recognized to inhibit complex I of the mitochondrial electron transport (respiratory) chain (Miyoshi, 1998). Tebufenpyrad, which was first discovered by Mitsubishi Kasei Co., Ltd., in 1987, has known activity against selected Homoptera and phytophagous mites (Okada et al., 1991). Tolfenpyrad was discovered by Mitsubishi Chemical Corporation (now Nihon Nohyaku Co., Ltd.) and developed for the control of various agricultural pests, including Acarina, Coleoptera, Diptera, Hemiptera, Lepidoptera and Thysanoptera (Arthropoda); the latter chemical is active mainly upon contact with egg, larval, nymphal and/or adult stages (Okada et al., 1999). Because of the effectiveness of these two pyrazole-5-carboxamides in controlling such agricultural pests (Okada et al., 1996, 1999), there has been a considerable commercial interest in synthesizing various derivatives based on their structures, with changes being made to the pyrazole and/or benzene rings (Okada et al., 1989; Okada et al., 1990; Natsume et al., 1991; Okada et al., 1992; Okada et al., 1996; Okada et al., 1997; Kano et al., 1999; Okada et al., 2002), but little work on altering the chemical bridge between the pyrazole and benzene rings.

Although these pyrazole-5-carboxamide derivatives have been developed to kill arthropod pests, we recently showed, in a compound screen of the 'Pathogen Box' (www.pathogenbox.org) from the Medicines for Malaria Ventures (MMV; www.mmv.org), that tolfenpyrad has an exquisite *in vitro* activity against parasitic stages of the barber's pole worm, *Haemonchus contortus* (Nematoda: Strongylida; Preston et al., 2016). Indeed, tolfenpyrad reproducibly and irreversibly inhibits the motility of exsheathed third-stage (xL3s) and fourth-stage larvae (L4s) of this parasitic nematode, and also the growth and development of L4s, with IC_{50} values ranging between 0.03 and 3.1 µM after 72 h of exposure. We demonstrated that xL3s exposed to tolfenpyrad consumed significantly less oxygen than unexposed xL3s, which was consistent with a specific inhibition of complex I of the respiratory electron transport chain in the mitochondrion (cf. Miyoshi, 1998). In addition, this compound is estimated to be ≥ 18-fold more selective for this nematode than a mammalian cell line (Preston et al., 2016), indicating its potential for repurposing against (at least some) parasitic nematodes and/or hit-to-lead optimisation.

In general terms, this evidence suggested that some pyrazole-5-carboxamide compounds developed as agricultural pesticides (against arthropods of plants) might also be able to be repurposed to other ecdysozoans, such as nematodes, provided that they are sufficiently safe

for application/administration to animals and/or the environment. Therefore, we undertook a search for panels of two published series of novel pyrazole-5-carboxamides (Song et al., 2012, 2013) and three other series of new pyrazole-5-carboxamides analogues. The availability of this small library provided us with an opportunity to extend our recent study (Preston et al., 2016). Here, we evaluated the activity of these pyrazole-5-carboxamide derivatives (n = 55) against parasitic stages of the nematode *H. contortus* and compared their potency with the two original, commercially available chemicals, tolfenpyrad and tebufenpyrad. We used this whole-organism screening assay to measure the inhibition of larval motility and development of *H. contortus* and then investigated the effect of any active compound on mitochondrial function by measuring oxygen consumption in this nematode.

5.2 Materials and methods

5.2.1 Procurement of *H. contortus*

The Haecon-5 strain of *H. contortus* was maintained in experimental sheep as described previously (Preston et al., 2015) and in accordance with the institutional animal ethics guidelines (permit no. 1413429; The University of Melbourne, Australia). L3s were produced from *H. contortus* eggs by incubating humidified faeces from infected sheep at 27 °C for 1 week (Preston et al., 2015), sieved through nylon mesh (pore size: 20 µm) to remove debris or dead larvae and then stored at 10 °C for a maximum of 3 months. For screening and basal oxygen consumption measurements (see following sub-sections), L3s were exsheathed and sterilised in 0.15% v/v sodium hypochlorite (NaClO) at 37 °C for 20 min (Preston et al., 2015). Thereafter, xL3s were washed five times in sterile physiological saline by centrifugation at 1700 *g* (5 min) at 22–24 °C. Then, xL3s were immediately suspended in Luria Bertani medium [LB: 10 g of tryptone (cat no. LP0042; Oxoid, Hampshire, England), 5 g of yeast extract (cat no. LP0021; Oxoid) and 5 g of NaCl (cat. no. 1064045000; Merck, Kenilworth, NJ, USA)] in 1 l of reverse-osmosis deionised water). LB was autoclaved and supplemented with 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin (Fungizone, antibiotic – antimycotic; cat. no. 15240-062; Carlsbad, CA, USA); this supplemented LB was designated LB*.

5.2.2 Pyrazole-5-carboxamide compounds

A total of 55 analogues of tebufenpyrad and tolfenpyrad were derivatised (Supplementary file 5.1). In brief, pyrazole derivatives containing α-hydroxymethyl-N-benzyl carboxamide,

α-chloromethyl-N-benzyl carboxamide and 4,5-dihydrooxazole moieties (a-1 to a-23) (Song et al., 2012), carbohydrazide (b-1 to b-7), imine, oxime ether, oxime ester, and dihydroisoxazoline (c-1 to c-15) (Song et al., 2013), oxazole (d-1 to d-8) and e-1 to e-2 (this study) were designed and synthesized (Supplementary file 5.1). In addition, tolfenpyrad (IUPAC name: 4-chloro-N-[[4-(1,1-dimethylethyl)phenyl]methyl]-3-ethyl-1-methyl-1H-pyrazole-5-carboxamide, cat. no. T535325, Toronto Research Chemicals, Toronto, Ontario, Canada) and tebufenpyrad (IUPAC name: 4-chloro-3-ethyl-1-methyl-N-[4-(p-tolyloxy) benzyl]pyrazole-5-carboxamide, cat. no. T013500, Toronto Research Chemicals, Canada) (99.9% purity) were purchased from commercial suppliers; the former chemical was used as a positive-reference compound (cf. Preston et al., 2016) (Sub-sections 5.2.3 and 5.2.4).

5.2.3 Screening of chemicals, inhibitory concentrations and cytotoxicity assessment

All compounds (Supplementary file 5.1) were prepared as described previously (Preston et al., 2016) and screened (in triplicate) at a concentration of 100 µM on xL3s of *H. contortus* in 96-well microculture plates using two assay-control compounds, moxidectin (Cydectin, Virbac, France) and monepantel (Zolvix, Novartis Animal Health, Basel, Switzerland). In brief, compounds were dissolved to a stock concentration of 10 mM in dimethyl sulfoxide (DMSO, Ajax Finechem, Melbourne, Australia), individually diluted to the final concentration of 100 µM using LB*, and dispensed (in triplicate) into wells of the 96-well microculture plates (cat no. 3596, Corning Life Sciences, Corning, NY, USA) using a multichannel pipette. In addition, the negative-controls (LB* and LB* + 0.5% DMSO; six wells each), and positive-controls (final concentration of 100 µM of monepantel and 100 µM of moxidectin in triplicate wells) were dispensed. Then, xL3s (~300/well) were dispensed into wells of the plate using an automated multichannel pipette (Viaflo Assist/II, Integra Biosciences, Switzerland). Following an incubation for 72 h at 38 °C and 10% CO_2, a video recording (5 sec) was taken of each well of the 96-well microculture plate (containing xL3s) using a grey-scale camera (Rolera bolt, Q imaging Scientific Coms, Canada), and a motorised X-Y axis stage (BioPoint 2, Ludl Electronics Products, Hawthorne, NY, USA). Individual videos were processed to calculate a motility index (MI) using an algorithm described previously (Preston et al., 2015, 2016). MIs were normalised to the positive- and negative-controls (to remove plate-to-plate variation) using the program Prism (v.6 GraphPad

Software, USA). A compound was recorded as having activity if it reduced xL3 motility by ≥ 70% after 72 h of incubation.

Anti-xL3 activity of individual compounds was confirmed, and half maximum inhibitory concentration (IC_{50}) values estimated from dose-response curves (24 h, 48 h and 72 h). Compounds that reduced the motility of xL3s were also tested for their ability to inhibit the development of xL3s to L4s, and the motility of L4s. All assays (for xL3 motility, and L4 development and motility) were performed in triplicate, between 3-5 times on different days. To determine IC_{50} values, the data from each assay (xL3 motility, L4 motility and development) were converted to a percentage with reference to the negative-control (LB* + 0.5% DMSO), and IC_{50} values determined using a variable slope four-parameter equation, constraining the top value to 100% and using a least squares (ordinary) fit model (v.6 GraphPad Software). The toxicity of selected compounds was measured by assessing their inhibition of the proliferation of human neonatal foreskin fibroblast (NFF) cells as described previously (Fisher et al., 2014). Selectivity indices (SIs) were calculated using a recognised formula (SI = human fibroblast (MRC-5) cells IC_{50} / *H. contortus* IC_{50}; Fisher et al., 2014).

5.2.4 Measuring the effects of compounds on respiration

Oxygen consumption (reflecting oxidative phosphorylation) was measured in the medium containing *H. contortus* xL3s (n = 600 per well) in the presence (50 μM or 100 μM) or absence of individual chemical compounds using the Seahorse XFe96 analyzer (Seahorse Biosciences, Agilent Technologies, Santa Clara, CA, USA) as described previously with minor modification (Preston et al., 2016). In brief, xL3s were dispensed into XFe96 cell culture microplates (Seahorse Biosciences, Aligent Technologies) at a density of 600 xL3s per well in 150 μl of XF Base Medium (Seahorse Bioscience, USA), supplemented with 4.5 g/l of glucose, 0.5 mM of sodium pyruvate and 2 mM of glutamine (Sigma-Adlrich, St. Louis, MI, USA) (pH = 7.4). Four wells contained Seahorse medium alone and served as normalisation controls. Subsequently, compounds dissolved in 25 μl of seahorse medium were individually loaded into the injection ports (in quadruplicate), and programmed to dispense into the XFe96 microplate after 5 measurements of respiration at 6 min intervals (2 min-mix; 4 min-measure). Using this approach, xL3s were exposed to compound concentrations of 100 μM, 50 μM and 0 μM. Respiration rates were measured every 6 min for a period of 180 min. Assays were repeated three times on separate days.

5.3 Results

In the primary screen of the 55 test compounds (Fig. 5.1; Supplementary file 5.1), two pyrazole-5-carboxamide derivatives, a-15 and a-17, were recorded to inhibit xL3 motility by ≥70% at concentrations of 50 µM or 100 µM, both revealing a straight phenotype (Fig. 5.1; Supplementary File 5.2). Subsequent assays using xL3s of *H. contortus* showed that the potency of these two test compounds at 72 h of incubation (IC_{50} values = 55.63 ± 0.18 µM and 51.60 ± 1.41 µM, respectively) was considerably less than tolfenpyrad (IC_{50} value = 3.05 ± 0.47 µM) (Table 5.1).

In the L4 motility assay, test compounds a-15, a-17 and tolfenpyrad all had significant inhibitory activities, but IC_{50} values of the test compounds (~ 15-26 µM) at 24 h, 48 h and 72 h were considerably greater than those of the reference control (tolfenpyrad; 0.03 ± 0.02 µM) at the 72 h time point (Table 5.1). In the L4 development assay, a-15 (IC_{50} of 3.97 ± 0.35 µM) and a-17 (IC_{50} of 3.42 ± 0.50 µM) had significantly less inhibitory effect on the L4 development than did tolfenpyrad (IC_{50} of 0.08 ± 0.01 µM) at 7 days using a one-way ANOVA ($F\ (2, 6)$ = 35.52, P = 0.0005) and Dunnett's multiple comparisons test (P = 0.0004 for a-15 and P = 0.001 for a-17). Interestingly, while tolfenpyrad had the expected inhibitory effect on the motility of xL3 and L4 stages (cf. Preston et al., 2016), tebufenpyrad reduced the motility and development of L4s, but not of xL3s. Using available cytotoxicity information (cf. Table 5.2), selective indices (SIs) of a-15 (1.2, 2.5 and 16.8), a-17 (i.e. 1.4, 4.6 and 21.1) and tolfenpyrad (i.e. ~ 33, 3,333 and 1,250) for xL3 motility, L4 motility and L4 development, respectively, were selective and relatively high for the L4s. Subsequently, it was assessed whether a-15 and a-17 would inhibit mitochondrial respiration in xL3s of *H. contortus* by measuring oxygen consumption over time (Fig. 5.3). The results showed that a-15-, a-17- and tolfenpyrad-treated xL3s consumed significantly less oxygen than untreated xL3s at 50 µM and 100 µM using a one-way ANOVA ($F(5, 12)$ = 30.18, P < 0.0001 at 50 µM; $F(5, 12)$ = 34.57, P < 0.0001 at 100 µM) and Dunnett's multiple comparisons test (P = 0.0001, P = 0.0001 and P = 0.0012 for a-15, a-17 and tolfenpyrad at 50 µM compared to untreated, respectively; P = 0.0001, P = 0.0001 and P = 0.0016 for a-15, a-17 and tolfenpyrad at 100 µM compared to untreated, respectively) (Fig. 5.3). As expected, based on its distinct mode of action (cf. Shoop et al., 1995), monepantel resulted in an inhibition of larval motility and development, but it did not significantly reduce oxygen consumption at the time-point measured. Similarly, tebufenpyrad did not inhibit respiration.

5.4 Discussion

The screening of 55 novel pyrazole-5-carboxamide derivatives identified two compounds (designated a-15 and a-17) with major activity against parasitic larval stages (xL3 and L4) of *H. contortus* in vitro; IC_{50} values were compared with tolfenpyrad, a pyrazole-5-carboxamide insecticide, which was recently shown, for the first time, to have substantial anthelmintic activity (Preston et al., 2016).

In the present study, compounds a-15 and a-17, like tolfenpyrad, were shown to be more potent against L4s than xL3s based on IC_{50} values (larval motility and development). Although it is not clear whether the potency difference of each of these two compounds between xL3s and L4s is due to variation in drug uptake, metabolism and/or mode of action, but it is likely that uptake of the chemicals is considerably greater in the L4 stage, as it has a functional pharynx and has a substantial nutrient requirement at this stage of development (Sommerville, 1966). It may also be that the target(s) of these chemicals is/are expressed at a higher level in L4s than xL3s, achieving greater binding and inhibitory effects in the nematode. Although compounds a-15 and a-17 have less anthelmintic effect than tolfenpyrad on *H. contortus* (Table 5.1), these compounds, which contain α-hydroxymethyl-N-benzyl carboxamide, still have considerable anthelmintic activity and appear to have a reasonable level of selectivity. A comparison with results from a previous study (Song et al., 2012) shows that both of the test compounds with anti-*H. contortus* effect(s) also have considerable activity against some plant-parasitic insects. This finding is in accord with that of tolfenpyrad, which is reported to have a relatively broad spectrum of activity against some arthropods of plants (Hollingworth, 2001) and nematodes (Preston et al., 2016).

Published information has implied that the mode of action of some pyrazole derivatives, such as tolfenpyrad (fungicide or insecticide/acaricide), also relates to a specific inhibition of complex I in the respiratory electron transport chain in mitochondria (e.g., Lümmen, 1998; Song et al., 2013). Therefore, to provide support for the hypothesis that compounds a-15 and a-17 act to disrupt or interrupt mitochondrial function, resulting in a loss of parasite motility and viability, oxygen consumption (via oxidative phosphorylation) was measured in real-time in xL3s of *H. contortus* using oxygen-sensitive probes (cf. McGee et al., 2011; Andreux et al., 2014). Both compounds were shown (within 180 min) to reduce, in a dose-dependent manner, oxygen consumption in *H. contortus*. This reduction in oxygen consumption preceded the inhibition of motility and development in this nematode, indicating that each of the two compounds significantly decreases oxidative phosphorylation, resulting (directly

and/or indirectly) in a substantial inhibition of larval motility and development. That these two compounds induce a similar phenotype to that caused by tolfenpyrad (Preston et al., 2016) appears to provide a basis for future studies to confirm that the electron transport chain is indeed the target. Such future studies might be conducted in the free-living nematode *C. elegans* (which is related to *H. contortus*), because it is very amenable to gene knockdown or knockout experiments, in contrast to *H. contortus* (see Britton et al., 2012, 2016; Holden-Dye and Walker, 2014). In addition, other investigations could be conducted in *C. elegans* to establish whether any other pathways or (e.g., stress) responses are involved in enabling or exacerbating the anthelmintic effects of these compounds.

Interestingly, a previous study (Omura et al., 2001) had identified nafuredin as a nematocide against *H. contortus* in vivo in sheep, which selectively targets mitochondrial complex I. Moreover, nafuredin has been shown to selectively inhibit mitochondrial complex I of *Ascaris* by > 1000-fold than the same complex in rat liver cells (Omura et al., 2001; Murai and Miyoshi, 2016). However, the latter drug was not of practical use or commercialised as a nematocide, because it had been shown chemically unstable in air (Shiomi et al., 2005). Adapting to the developmental and environmental alterations, parasitic worms use mitochondria complex I in respiratory chain with rhodoquinone and ubiquinone as electron receptors in parasitic stages (anaerobic) and free-living larvae stages (aerobic), respectively, but not mammalian complex I, indicating that structure features of mitochondria complex I in worms differ considerably from those of the same complex in mammalian mitochondria (Kita et al., 2003; Kita et al., 2007; Murai and Miyoshi, 2016; Harder, 2016). Thus, based on evidence from the present and previous studies, it appears that elements of mitochondrial respiratory chain in parasitic worms seem to have considerable potential as targets for nematocides.

Conclusions

The present findings provide a sound basis for future work, aimed at identifying the targets of compounds a-15, a-17 and tolfenpyrad, and establishing the modes of action of these chemicals in *H. contortus*. In addition, medicinal chemistry-based structure activity relationship (SAR) studies of pyrazole-5-carboxamides will enhance understanding of which features of the pyrazole-5-carboxamides skeleton are vital for anthelmintic activity.

5.5 References

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Table 5.1. Comparison of in vitro activity of test compounds a-15 and a-17 with tolfenpyrad and tebufenpyrad on the motility of exsheathed third-stage (xL3) and fourth-stage (L4) larvae of *Haemonchus contortus* (after 24 h, 48 h and 72 h of exposure) and on the development of L4 (after 7 days of exposure).

Compound	Half maximum inhibitory concentration (IC_{50} µM)						
	xL3 motility			L4 motility			L4 development
	24 h	48 h	72 h	24 h	48 h	72 h	7 days
a-15	—	—	55.63±0.18	—	—	26.31[a]	3.97±0.35
a-17	—	—	51.60±1.41	—	—	15.58 [a]	3.42±0.50
Tolfenpyrad	2.98±0.50	3.85±1.33	3.05±0.47	0.12±0.07	0.06±0.02	0.03±0.02	0.08±0.01
Tebufenpyrad	na	na	na	>100	>100	>100	6.70 [a]

[a] Half maximum inhibitory concentration could not be accurately calculated by the log (agonist) versus response -- variable slope (four parameter) equation, a IC_{50} value was estimated, na = no activity; — = indicates where IC_{50} values were not determined if the log(agonist) vs. response --variable slope (four parameters) model could not be used to fit the curve.

Table 5.2. Compounds a-15 and a-17 were tested for toxic effects on a human neonatal foreskin fibroblast (NFF) cell line. Selectivity indices of these compounds on the motility of exsheathed third- and fourth-stage larvae (at 72 h) (xL3s and L4s) and the development of L4s (at 7 days) were calculated using a recognised formula (Fisher et al., 2014; see Sub-section 2.3).

Compound	IC50 (µM) for NFF cells	Selectivity index (SI) for *H. contortus*		
		xL3 motility	L4 motility	L4 development
a-15	66.72±10.04	1.20	2.54	16.81
a-17	72.15±1.61	1.40	4.63	21.10
Tolfenpyrad	>50	>16.40	>1666.70	>625

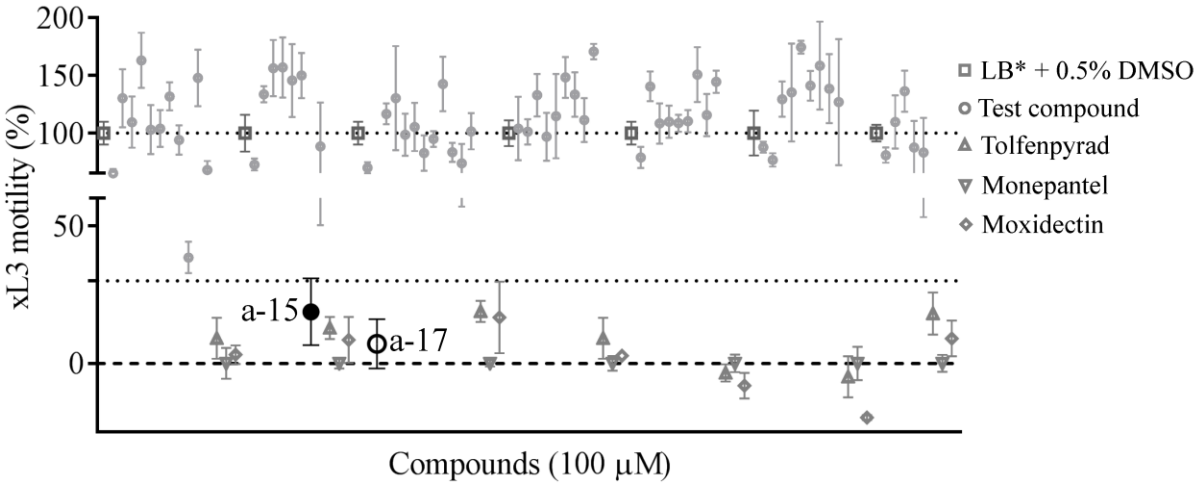

Fig. 5.1. Primary screening of 55 synthetic pyrazole-5-carboxamides for inhibition of motility of exsheathed third-stage larvae (xL3) of *Haemonchus contortus* at a concentration of 100 µM (after 72 h of exposure). Three commercial compounds, tolfenpyrad, monepantel and moxidectin, were also included as control compounds. Tolfenpyrad was used as the positive reference-control compound, as it has known activity against xL3s and L4s of *H. contortus* (see Preston et al., 2016). Two active compounds, a-15 and a-17, inhibited xL3 motility by ≥ 70%. Supplementary file 5.2 shows the "straight" phenotype in larvae exposed to a-15 and a-17, similar to that of the tolfenpyrad control.

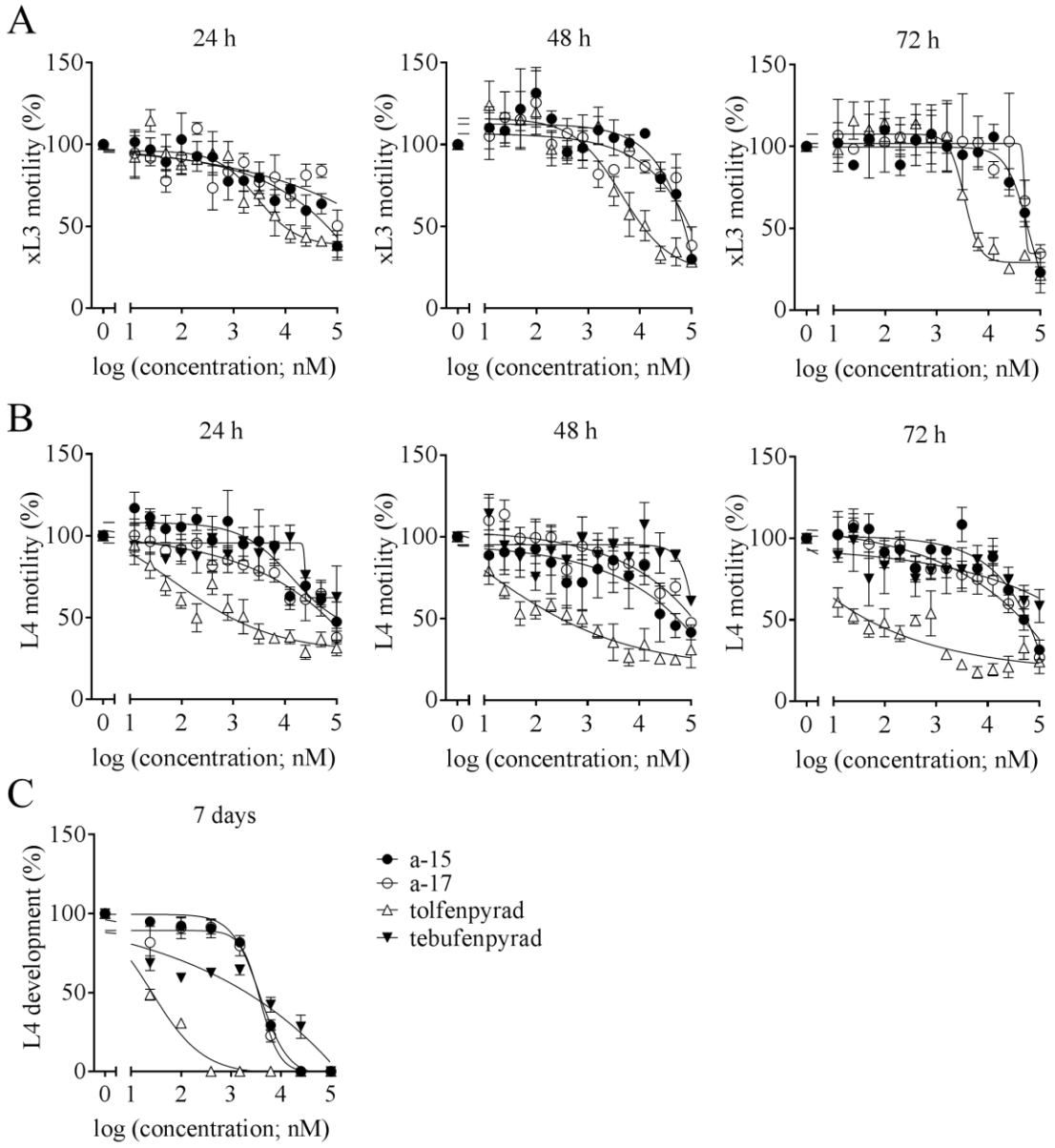

Fig. 5.2. Dose response curves of the effects of the active pyrazole-5-carboxamide test compounds, a-15 and a-17, tolfenpyrad and tebufenpyrad (reference positive-control compound) on parasitic stages of *Haemonchus contortus in vitro*. Inhibition of the motility of third-stage larvae (xL3s) at 24 h, 48 h and 72 h (a) for individual compounds motility (b) and inhibition of development (c) of fourth-stage larvae (L4s) after seven days. Each data point represents the mean of three experiments (± standard error of the mean, SEM).

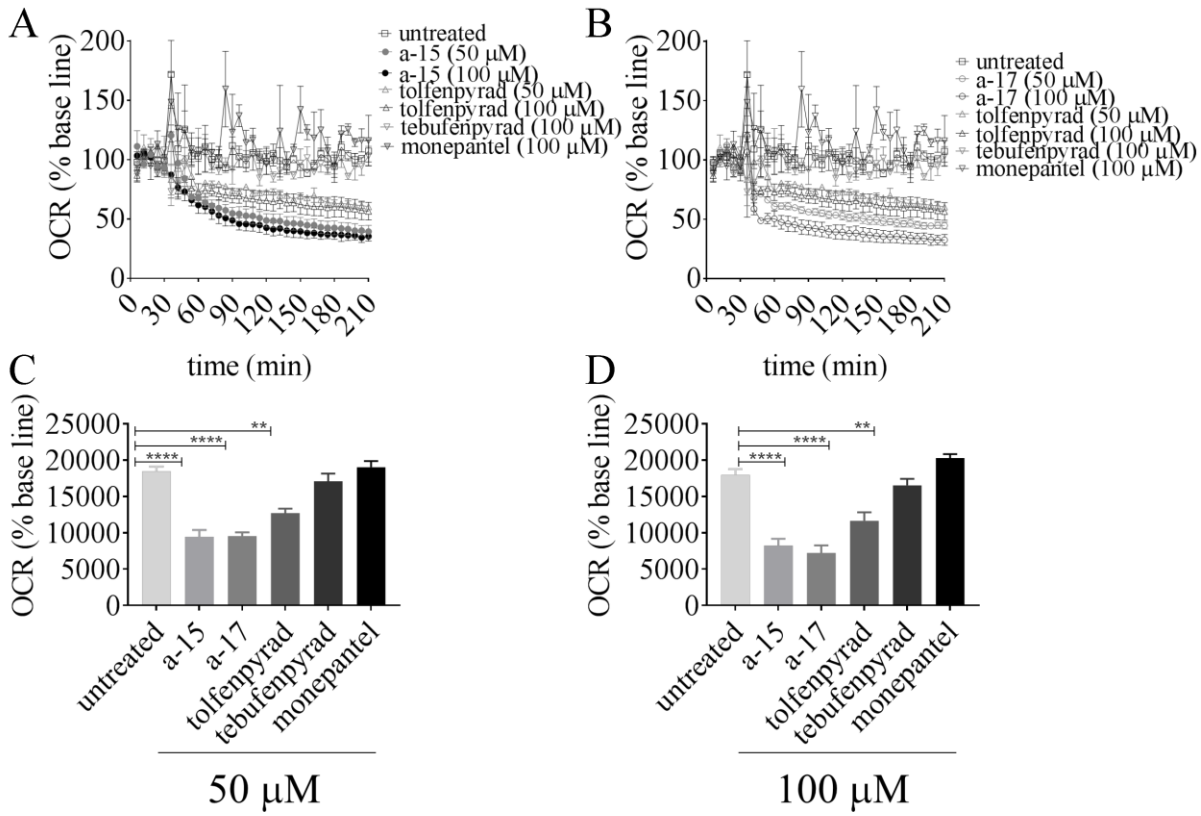

Fig. 5.3. Respiration rates of *Haemonchus contortus* treated with test or control compounds in vitro. Panel a and b show the individual curves of oxygen consumption rate (OCR) of third larvae (xL3s) (n = 600 per well) following exposure to individual test compounds (a-15 and a-17) and the reference control compound (tolfenpyrad, tebufenpyrad and monepantel). The OCR data were measured 35 times (2 min-mix 4 min-measure) for 30 min before and 180 minutes after exposure to each compound using a Seahorse XFe96 flux analyser. Three separate experiments were conducted using 4 replicates in each experiment. Panel c and d show the total oxygen consumption of xL3s (n = 600 per well) following exposure to individual test compounds (a-15 and a-17) and the reference control compounds (tolfenpyrad, tebufenpyrad and monepantel) by calculating the area under the curve (AUC). Variation was expressed as the standard error of the mean (SEM). Significance between values (mean ± SEM) was determined using a one-way ANOVA and Dunnett's multiple comparison test. Asterisks indicate values that are significantly different from one another (**$P < 0.01$; ****$P < 0.0001$).

Chapter 6 - Tetrahydroquinoxalines induce a lethal evisceration phenotype in *Haemonchus contortus* in vitro

Abstract

In the present study, the anthelmintic activity of a human tyrosine kinase inhibitor, AG-1295, and 14 related tetrahydroquinoxaline analogues against *Haemonchus contortus* was explored. These compounds were screened against parasitic larvae - exsheathed third-stage (xL3) and fourth-stage (L4) - using a whole-organism screening assay. All compounds were shown to have inhibitory effects on larval motility, development and growth, and induced evisceration through the excretory pore in xL3s. The estimated IC_{50} values ranged from 3.5 to 52.0 μM for inhibition of larval motility or development. Cytotoxicity IC_{50} against human MCF10A cells was generally higher than 50 μM. Microscopic studies revealed that this eviscerated (Evi) phenotype occurs rapidly (< 20 min) and relates to a protrusion of internal tissues and organs (evisceration) through the excretory pore in xL3s; severe pathological damage in L4s as well as a suppression of larval growth in both stages were also observed. Using a relatively low concentration (12.5 μM) of compound m10, it was established that the inhibitor has to be present for a relatively short time (between 30 h and 42 h) during in vitro development from xL3 to L4, to induce the Evi phenotype. Increasing external osmotic pressure prevented evisceration and moulting, and xL3s remained unaffected by the test compound. These results point to a mode of action involving a dysregulation of morphogenetic processes during a critical time-frame, in agreement with the expected behaviour of a tyrosine kinase inhibitor, and suggest potential for development of this compound class as nematocidal drugs.

6.1. Introduction

Parasitic worms cause substantial morbidity and mortality in humans and animals, and major losses to food production globally (cf. Roeber et al., 2013; Hotez et al., 2014). In particular, some parasitic roundworms (nematodes) of the order Strongylida, also called strongylids, or bursate nematodes, cause some of the most important gastrointestinal diseases of livestock worldwide, affecting hundreds of millions of animals (including sheep, goats, cattle and/or pigs), with economic losses estimated at tens of billions of dollars per annum (Roeber et al., 2013; Thamsborg et al., 2013; Lane et al., 2015). These pathogens can cause gastroenteritis, anaemia and/or associated complications (depending on species), and death in severely affected animals, and many of them cause marked productivity losses through subclinical infections. These nematodes are transmitted orally from contaminated pastures to the host through a direct life cycle (Beveridge and Emery, 2014): eggs are excreted in host faeces; the first-stage larvae (L1s) develop inside the egg, usually hatch within 1 day at environmental temperatures of $\geq 10^{\circ}$ C and then develop to the second- and third-stage larvae (L2s and L3s) in about one week; the infective L3s are then ingested by the host, exsheath (xL3) and develop through fourth-stage larvae (L4) to dioecious adults (often within 3-4 weeks) in the gastrointestinal tract of the animal.

Currently, the control of these nematodes relies largely on the use of a limited number of anti-parasitic drug classes. However, drug resistance is now widespread (Kotze and Prichard, 2016), and no vaccines are available for the vast majority of these worms (Hewitson and Maizels, 2014), such that the development of new drugs is crucial to ensure sustained and effective control into the future. Although the development of monepantel (Kaminsky et al., 2008; Prichard and Geary, 2008) and derquantel (Little et al., 2011) has introduced novel classes of nematocides, success in discovering new anthelmintic drugs has been limited.

We have been focusing on identifying synthetic chemical entities with nematocidal or nematostatic activity (Preston et al., 2016b; Jiao et al., 2017a; Jiao et al., 2017b; Preston et al., 2017), working toward optimising their potency and selectivity. Recently, we obtained a collection of compounds (‘Stasis Box’) from Medicine for Malaria Venture (MMV); this collection contains 400 compounds which were under pharmaceutical development, and about which there is published information in the scientific literature or patents. Utilizing a whole-worm motility screening assay (Preston et al., 2015), we identified in the ‘Stasis Box’ a platelet-derived growth factor (PDGF) receptor kinase inhibitor (Kovalenko et al., 1994; Gazit et al., 1996; Kovalenko et al., 1997) called AG-1295 (6,7-dimethyl-2-

phenylquinoxaline) with relatively potent anthelmintic activity against *H. contortus* in vitro (Jiao et al., 2017a). Dose-response evaluations showed that AG-1295 inhibits the motility of exsheathed third-stage larvae (xL3s) and the development of fourth-stage larvae (L4s), with half maximum inhibition concentration (IC_{50}) values of 9.9 ± 1.9 μM and 7.8 ± 0.9 μM, respectively (Jiao et al., 2017a). Moreover, AG-1295 was not toxic (IC_{50}>100 μM) to normal human breast epithelial (MCF10A) cells when tested in vitro (Jiao et al., 2017a).

Interestingly, AG-1295 had been under development as a medicine for restenosis treatment (Levitzki, 2013). This kinase inhibitor has been reported to (i) attenuate porcine and human smooth muscle cell growth in vitro (Banai et al., 1998), (ii) block the proliferation of rat hepatic stellate cells in vitro (Iwamoto et al., 2000) and (iii) inhibit aortic allograft vasculopathy in rats (Karck et al., 2002). However, currently nothing is known about how AG-1295 affects or acts on *H. contortus* or any other nematode. Considering that AG-1295 has a well-defined drug-like structure (Gazit et al., 1996), has suitable pharmacokinetic properties (Banai et al., 1998; Levitzki, 2001, 2010) and has dramatic effects on both motility and development of *H. contortus* in vitro (Jiao et al., 2017a), this compound or one or more analogues thereof might have potential for being repurposed as anthelmintics. In the present study, we extend previous work (Jiao et al., 2017a) to (i) explore the anthelmintic activities of 14 AG-1295 analogues (tetrahydroquinoxalines) on motility and development of larval stages of *H. contortus* in vitro, (ii) start investigating their mode of action by qualitatively and quantitatively assessing the morphological alterations that occur in larval stages of *H. contortus* following exposure to these analogues in vitro, and (iii) discuss how this worm is affected by representative compounds.

6.2 Materials and methods

6.2.1 Procurement of *H. contortus*

Haemonchus contortus (Haecon-5 strain; partially resistant to benzimidazoles) was maintained in Merino sheep in approved facilities in The University of Melbourne with institutional animal ethics approval (permit no. 1413429). Larval stages of *H. contortus* were produced as described previously (Preston et al., 2015). In brief, faeces were collected from infected sheep four weeks following inoculation and were then incubated at 27 °C for one week. Then, L3s were isolated from faeces and sieved through two layers of nylon mesh (pore size: 20 μm; Rowe Scientific, Australia). Exsheathed L3s (xL3s) were produced by incubating L3s in 0.15% (v/v) sodium hypochlorite (NaClO) at 37 °C for 20 min, and

immediately washed five-times in sterile physiological saline by centrifugation (1700 x*g*, 5 min). The xL3s were resuspended in LB* [10 g of tryptone (cat. no. LP0042; Oxoid, England), 5 g of yeast extract (cat. no. LP0021; Oxoid, England) and 5 g of NaCl (cat. no. 1064045000; Merck, Denmark) in 1 l of reverse-osmosis deionized water; supplemented with 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin (antibiotic-antimycotic; cat. no. 15240062; Gibco, USA)]. L4s were produced by incubating xL3s in LB* at 38 °C and 10% (v/v) CO_2 for 7 days; larvae were not used unless ≥ 80% of xL3s developed to L4s - identified based on the appearance of a mouth and pharynx (Veglia, 1916; Sommerville et al., 1966).

6.2.2 AG-1295 analogues

A total of 14 tetrahydroquinoxaline compounds whose structures are closely related to AG-1295 were sourced from two co-authors (JBB and SV) (Supplementary file 6.1). All compounds were synthesized to form the arylated 5,6,7,8- tetrahydroquinoxaline N-oxide intermediates by palladium catalyzation, and the N-oxide moiety was reduced to yield the final chemicals (Supplementary file 6.1). AG-1295 was purchased from a commercial company (IUPAC name: 6,7-dimethyl-2-phenylquinoxaline, cat. no. A425200, 99.9% purity, Toronto Research Chemicals, Canada).

6.2.3 Compound screening

Compounds were screened against xL3s of *H. contortus* using an established approach (Preston et al., 2016a). In brief, compounds were individually dissolved in dimethyl sulfoxide (DMSO, Ajax Finechem, Australia) to a stock concentration of 20 mM. To test their activity against *H. contortus*, individual compounds were diluted in LB* to the final concentration of 100 µM. All compounds were tested in triplicate; a commercial anthelmintic, monepantel (Zolvix, Elanco Animal Health, Switzerland) was used as a positive control (in triplicate), and LB*+0.5% DMSO was used as a negative control (in sextuplicate). All compounds were tested using in a 96-well plate (Corning 3596; Life Sciences, USA) with 300 xL3s per well for 72 h at 38 °C and 10% (v/v) CO_2. Following incubation, worms were agitated using an orbital shaker at 37 °C for 20 min, and five-second video recordings were taken of individual wells. Each video capture of each well was processed using a custom macro in the program Image J (1.47v, imagej.nih.gov/ij); larval motility in each well was expressed as a motility index (Mi), calculated as described previously (Preston et al., 2015). A compound was

identified as active against *H. contortus* ('hit') if it reduced the Mi value of xL3s by ≥ 70%, or if a phenotype was observed that differed from wildtype xL3 (i.e. LB* + 0.5% DMSO control).

6.2.4 Dose-response assessments of active compounds on xL3 and L4 motility, and L4 development

Active compounds were tested against *H. contortus* larvae at a range of concentrations to determine the half maximum inhibitory concentration (IC_{50}) values of each compound as described earlier (Preston et al., 2015). Stocks of individual compounds (20 mM) were diluted in LB* (50 µl) to 200 µM, and then serially titrated (in two-fold steps) across a 96-well flat bottom plate. Subsequently, 300 worms (either xL3s or L4s) in 50 µl of LB* were added to individual wells and the plates were incubated in a water-jacketed CO_2 incubator at 38 °C and 10% (v/v) CO_2. After 24 h, 48 h and 72 h of incubation, worm motility index (Mi) values were measured and processed to generate an 18-point dose-response curve using v.7 GraphPad Prism software. Following motility measurement, the xL3 plates were re-incubated for four more days to assess the L4 development. IC_{50} values for each dose-response curve (xL3 motility, L4 motility and L4 development) were calculated by fitting a variable slope four-parameter equation, constraining the top value to 100%.

6.2.5 Assessment of cytotoxicity

A 'normal' human breast epithelial cell line (MCF10A) was used to test the cytotoxic activity of compounds (Jiao et al., 2017a). In brief, in a 384-well flat bottom, black walled plate (Corning, USA), 700 MCF10A cells in 40 µl DMEM-F12 [100 ng/ml cholera toxin (Sigma, Australia), 20 ng/ml human epidermal growth factor (EGF, Life Technologies, Australia), 10 µg/ml insulin (human; Novo Nordisk Pharmaceuticals Pty Ltd, Australia), 5% horse serum (Life Technologies, Australia) and 0.5 µg/ml hydrocortisone (Sigma, Australia)], were dispensed into wells using a liquid handling dispenser (BioTek, Vermont, USA). Plates were incubated at 37 °C and 5% (v/v) CO_2 for 24 h. Meanwhile, test compounds were diluted in the cell growth media from 250 µM to 15.6 µM in a two-fold serial way using an automated liquid handling robot (SciClone ALH3000 Lab Automation Liquid Handler, Caliper Lifesciences, USA). The diluted compounds (10 µl) were then added to cultured cells, with doxorubicin as a positive control and DMEM-F12 + 0.5% DMSO as a negative control. Following an incubation for 48 h, cells were fixed and stained with 4'6-diamidino-2-

phenylindole (DAPI; 1:1000). Subsequently, cell nuclei present in each well were quantified using a high content imager (Cellomics Cell Insight Personal Cell Imager, Thermofisher Scientific, USA) at 10-times magnification (with a fixed exposure time of 0.12 sec) and the Target Activation BioApplication within the Cellomics software. IC_{50} values for inhibition of MCF10A cell proliferation were calculated from dose-response curves, using a variable slope four-parameter equation in GraphPad Prism. Each assay was repeated two times in quadruplicate on different days. The selectivity indices (Si) of hit compounds were then calculated using the formula: Si = IC_{50} for MCF10A cells / IC_{50} for *H. contortus*.

6.2.6 Morphological examination of the eviscerated (Evi) phenotype induced by incubation with individual test compounds

Light microscopy was used to examine morphological alteration(s) in larvae caused by test compounds after 7 days (Subsection 6.2.4). At each compound concentration tested, 30 larvae (fixed with 1% iodine) were classified as either 'normal xL3s', 'eviscerated xL3s' or 'L4s', using a light microscope (DP26 camera, Olympus, USA). The concentration at which each test compound induced maximum numbers of eviscerated larvae was recorded. Compounds were also tested for their ability to inhibit larval growth by measuring worm length and width microscopically (20-times magnification), using a corresponding concentration of DMSO as a negative control. For each treatment, the mean length and width of ≥ 30 larvae ± standard error of mean were calculated, and a one-way ANOVA with Tukey's test was used to measure the statistical differences between different treatments.

6.2.7 Scanning electron microscopy (SEM)

Larvae were also examined by SEM. Briefly, in a 96-well flat bottom plate, 300 worms (xL3s or L4s) in 50 µl of LB* were exposed to the concentration of test compound that induced the greatest number of larvae with an Evi phenotype, with a corresponding concentration of DMSO as a negative control (in sextuplicate). Subsequently, the plate was incubated at 38 °C and 10% (v/v) CO_2 for 72 h. For each treatment, worms were then pooled together and washed three times in 0.9% saline at 9,000 *xg*. For SEM analysis, worms were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde for 2 h, rinsed three times in 0.1 M sodium cacodylate trihydrate for 10 min, and then post-fixed in 1% osmium tetroxide at room temperature for 2 h. Fixed worms were rinsed again three times in distilled water for 15 min. Following fixation, 200 µl of concentrated worms were incubated on poly-L-lysine coated

glass coverslips; the slips were pre-prepared by immersing them in poly-L-lysine solution for 10 min, drying in an oven at 60 °C for 10 min, immersing again in poly-L-lysine solution for 10 min and then drying them at room temperature. The coverslips with adhered nematodes were dehydrated in a graded ethanol series (30%, 50%, 70%, 90% and 100%) for 17 min, dried in a critical point dryer (EM CPD300, Leica, Wetzlar, Germany), mounted on to aluminium stubs (25 mm) with double-sided carbon tabs and then coated with gold. Worms were imaged using a JEOL JCM-6000 Plus NeoScope scanning electron microscope (Jeol, Tokyo, Japan); at least six worms were examined for each treatment group.

6.2.8 Transmission electron microscopy (TEM)

Individual xL3s and L4s were sliced twice to give three even (anterior, mid and posterior) body portions in 3.2% paraformaldehyde and 0.2% glutaraldehyde with 0.15 M sodium cacodylate trihydrate (pH 7.2) (cf. Kovacs, 2015), and ruthenium red (RR) solution was added to stain individual pieces. After 2 h of primary fixation, worm portions were rinsed three times in 0.125 M sodium cacodylate trihydrate for 2 h, and then post-fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide in the dark for 2 h. Fixed worm portions were rinsed again three times in 0.125 M sodium cacodylate trihydrate for 2 h and then embedded in 1.5% agar while keeping them straight. The agar blocks containing worm portions were washed two times in distilled water for 2 h and dehydrated in a graded ethanol series (50%, 70%, 90%, 95% and 100%) for 2 h on a platform rocker, rinsed two times in 100% acetone for 45 min, rocking, and then embedded in Spurrs resin. Ultrathin sections were cut with an EM UC7 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) and contrasted with lead citrate and aqueous uranyl acetate. Sections were imaged using a transmission electron microscope (JEOL 1011, Tokyo, Japan).

6.2.9 Evaluation of the dynamics of the compound-induced Evi phenotype in xL3s

To determine the kinetics of the anterior protrusion and evisceration in xL3s, a time course experiment was performed using the compound concentration that caused maximum numbers of worms with the Evi phenotype. At various time-points (every 6 h for the first 4 days and then every 24 h for the next 3 days) after treatment, aliquots (50 µl) of 150 larvae were fixed in 1% iodine, and 30 worms from each aliquot were scored as either ‘normal xL3s’, ‘eviscerated xL3s’ or ‘L4s’ under a compound microscope using 100-times magnification.

For each time point, the percentage of larvae in each category was calculated, and a two-way ANOVA with Tukey's test was used to calculate statistical differences between groups. This assay was repeated at least two times in triplicate on different days.

6.2.10 Assessment of the reversibility of effect of test compounds on xL3s

To determine whether the effect of test compounds on xL3 was reversible, a previously published method was used (Kumarasingha et al., 2016). In brief, xL3s were exposed for 18 h, 24 h, 27 h, 30 h, 36 h and 48 h to 12.5 µM of test compound in triplicate in 96-well plates. Following exposure, at each of these time points, compounds were removed by washing larvae three times in 0.9% saline, 100 µl of LB* were added to individual wells, and the plate was then re-incubated in a water-jacketed CO_2 incubator at 38 °C and 10% (v/v) CO_2. After 72 h, the percentages of the eviscerated xL3s and the L4s were calculated. Controls included xL3s continually incubated with 12.5 µM of test compound (in triplicate) for 72 h and xL3s incubated in LB* alone. This assay was repeated two times on different days. Differences in percentage of eviscerated xL3s between treatments were evaluated using a one-way ANOVA with Tukey's test.

Subsequently, xL3s were cultured first in LB*, and test compound (to 12.5 µM) was added to individual wells at two different time-points (39 h and 42 h). Again, worms were scored microscopically at 72 h. A matched compound concentration, added at 0 h, was used as a positive control, and the corresponding concentration of DMSO was used as a negative control. This assay was repeated two times on different days. A two-way ANOVA with Tukey's test was used to measure statistical differences between treatments in the percentages of eviscerated xL3s and developed L4s.

6.2.11 Influence of external osmotic pressure on compound-induced Evi phenotype in xL3s

To examine whether an osmotic pressure gradient was involved in the induction of the Evi phenotype in xL3s, compound-treated and untreated xL3s were cultured in the presence of increasing concentrations of sorbitol (0 M, 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M and 1.0 M in LB*). After 72 h of incubation at 38 °C and 10% (v/v) CO_2, the percentages of the eviscerated xL3s and the developed L4s were calculated at each concentration. This assay was repeated two times on different days.

Since hypertonic sorbitol itself was observed to inhibit xL3 moulting, the reversibility of this effect was also tested. To do this, xL3s cultured in the presence of 0.6 M sorbitol for 48 h were washed three times in 0.9% saline and then placed in fresh LB*, and worms were examined microscopically after 7 days in culture. The controls included xL3s continually incubated in 0.6 M sorbitol (in triplicate) for 7 days, and xL3s incubated in LB* alone for the same period.

The reversibility of compound effect on xL3s, while xL3 moulting was inhibited by hypertonic sorbitol, was also assessed. To do this, xL3s were exposed to 12.5 µM of test compound in the presence of 0.6 M sorbitol, washed at 48 h and examined microscopically after 7 days in culture. Unwashed xL3s exposed to 12.5 µM of test compound in the presence of 0.6 M sorbitol for 7 days were used as a control for complete osmotic protection, and xL3s exposed to a matching concentration of test compound in LB* represented the control for a lack of osmotic protection. A two-way ANOVA with Tukey's test was used to calculate statistical differences between treatment groups. This assay was repeated two times on different days.

6.3 Results

6.3.1 Inhibitory effects of AG-1295 analogues on xL3 motility, L4 motility and L4 development

Fourteen AG-1295 analogues (Supplementary file 6.1) and AG-1295 itself (reference compound) were tested against *H. contortus* in the established whole-worm screening assay. The reduction of Mi values of xL3s treated for 72 h with 100 µM of each of the analogues ranged from 11% to 65%, and an Evi phenotype was observed (Fig. 6.1C and D). At this concentration and after 7 days, L4 development was fully inhibited (100%) by all compounds, except m13 (48.6%).

Dose-response evaluations of xL3 motility established the potencies of individual compounds at 24 h, 48 h and 72 h. Four compounds (m6, m7, m13 and m14) gave full dose-response curves, with IC_{50} values ranging from 9.8 µM to 50.1 µM at 72 h (Table 6.1; Fig. 6.2A).

Ten analogues (m1, m2, m3, m4, m6, m8, m9, m10, m11 and m12) displayed an inhibitory effect on L4 motility, with IC_{50} values (at 72 h) ranging from 6.9 ± 2.8 µM to ~52.0 µM (Table 6.1; Fig. 6.2B). Compound m10 was most potent, with an IC_{50} value of 6.9 ± 2.8 µM.

At shorter incubation times, individual compounds tended to be less potent at inhibiting L4 motility (Table 6.1; Fig. 6.2B).

In the test for inhibition of L4 development after 7 days of incubation, all compounds (except those that were less potent: m2, m12 and m13) demonstrated IC_{50} values ranging from 3.5 ± 0.9 µM to 31.8 ± 0.7 µM (Table 6.1; Fig. 6.2C). Like in the L4 motility assay, compound m10 was also most effective at inhibiting L4 development, with an IC_{50} value of 3.5 ± 0.9 µM.

Overall, these findings revealed that different members of this chemical series are able to inhibit xL3 motility, L4 motility and L4 development with potencies in the tens of µM; compound m10 was most potent at inhibiting the development from xL3 to L4 and the motility of the L4 stage (Table 6.1; Fig. 6.2).

6.3.2 Cytotoxicity of AG-1295 analogues on MCF10A cells

The next step was to estimate the toxicity and selectivity of the 14 AG-1295 analogues on MCF10A cells with reference to doxorubicin - which reduced the final cell density to 0.3 ± 0.03 % of the untreated control at the highest concentration tested (10 µM), with an IC50 of 1.2 ± 0.1 µM (Table 6.2). Consistent with results from a previous study (Jiao et al., 2017a), AG-1295 displayed very limited inhibition of MCF10A cell proliferation, resulting in a final cell density of 90.2 ± 3.9 % of the control at 50 µM. The cytotoxicity of the analogues in this assay varied markedly, from those which kept cell density to 25% of control to those that allowed it to reach 87% at 50 µM (Table 6.2; Fig. 6.2D). Of the 14 test compounds, the four most potent chemicals (m6, m8, m9 and m10) in this assay showed IC50 values ranging from 25 µM to 50 µM (Table 6.2; Fig. 6.2D). Based on these values, selectivity indices of 0.6 to 14.1 for the inhibition of L4 motility and L4 development were calculated; thus, compounds m10 and m11 appeared to be the most measurably selective compounds for *H. contortus* in vitro (Table 6.2).

6.3.3 Microscopic characterisation of the Evi phenotype in xL3s exposed to test compounds

While assessing the inhibitory effects of individual AG-1295 analogues on xL3 motility, we observed a non-wildtype phenotype in a large percentage of worms exposed to each of the 14 compounds (100 µM, 72 h), in contrast to wildtype xL3s incubated in LB* with 0.5% DMSO (negative control) (Fig. 6.1C, 6.1D). Non-wildtype xL3s failed to moult to L4s,

whereas wildtype xL3s in LB* containing 0.5% DMSO developed unhindered to L4s with discernible mouthparts and pharynx (Fig. 6.1C).

Detailed light microscopic examination (100-times magnification) with phase-contrast optics revealed that the Evi phenotype in xL3s was characterised by a protrusion of the alimentary tract and surrounding tissues through or around the excretory pore (Fig. 6.3A), while the excretory canal was clearly visible in untreated xL3s in the same area (Supplementary file 6.2A). An examination of timed video recordings revealed that evisceration was a gradual but relatively rapid process (< 20 min) (Supplementary file 6.2B). Initially, a few globular structures emerged from the excretory pore (109.5 ± 1.9 µm from the anterior tip of the xL3), followed by an expulsion of the anterior and, sometimes, the posterior part of the intestine, forming an external 'balloon', with ingesta and body fluids flowing to the exterior of the worm (Fig. 6.3A; Supplementary file 6.2B). At this later stage, the breach in the body wall seemed to expand, and the eviscerated mass ended up being located about 80-90 µm from the anterior tip of the worm (Fig. 6.1C; Fig. 6.3A; Supplementary file 6.2B). This Evi phenotype was observed exclusively in xL3s (but not in L4s) exposed to any of the 14 compounds.

A SEM study provided a detailed insight into the protrusion of the alimentary tract and surrounding tissues, and into the wrinkled cuticular surface of treated xL3s (Fig. 6.3B). Although the Evi phenotype was not observed in L4s, detailed SEM examination revealed a shrivelling in the cuticular surface of such larvae, compared with the smooth cuticular surface of untreated L4s (Fig. 6.3B); xL3s or L4s exposed to LB* containing the corresponding concentration of DMSO (negative control) were unaffected. A TEM examination revealed a release of the hypodermis from the undulated cuticle of treated xL3s, with pathological alterations in internal organs and tissues, including excretory canals, whereas in untreated xL3s the hypodermis was consistently connected to the cuticle, and no anatomical changes were evident (Fig. 6.3C). The cuticular shrivelling seen in L4s by SEM is likely related to the pathological alterations observed by TEM in compound-treated larvae (detached hypodermis; myodegeneration; cell lysis; and the appearance of electron-dense vesicles) (Fig. 6.3C). Such alterations were not observed in untreated L4s maintained in culture for the same time.

Members of this compound series were also shown to inhibit larval growth. After measuring 30 xL3s treated with 12.5 µM m10 or LB* + 0.5% DMSO for 72 h, it was shown that compound treatment reduced xL3 length from 641 ± 4.6 µm to 456 ± 11.2 µm. Width was similarly reduced from 18 ± 0.2 µm to 16 ± 0.4 µm (Supplementary file 6.3). Statistical analyses, using a one-way ANOVA with Tukey's test, showed the differences to be

significant (Supplementary file 6.3). Similarly, the lengths and widths of treated L4s (601 ± 9.6 µm and 18 ± 0.3 µm) were reduced with respect to untreated L4s (624 ± 12.2 µm and 21 ± 0.3 µm), with the difference in width being statistically significant ($P < 0.0001$; Supplementary file 6.3). Thus, members of this compound series suppress larval growth, in addition to inhibiting larval motility and development.

6.3.4 Determining the optimum compound concentrations to induce the Evi phenotype in xL3s

The percentages of eviscerated xL3s following 7 days of exposure to each of the 14 compounds were assessed using a ten-step, two-fold serial dilution (100 µM to 0.4 µM) (Table 6.3; Fig. 6.2E). The maximum percentages of such xL3s ranged from 5.0 ± 2.3 % to 82.9 ± 3.1 %. The most potent compound to induce this phenotype was m10, resulting in evisceration in 82.9 ± 3.1 % of xL3s at 12.5 µM. Interestingly, there was an optimum concentration for each inhibitor to induce the Evi phenotype, most clearly seen for m10, but discernible for most compounds; exposure to higher or lower concentrations resulted in lower percentages of eviscerated larvae (Table 6.3; Fig. 6.2E).

Efficacy at inducing the Evi phenotype was clearly related to compound inhibitory potency in the larval motility or development assay. As all data sets were normally distributed, according to the D'Agostino-Pearson normality test, the Pearson correlation coefficient (r) was compared between the potencies of individual compounds to induce the Evi phenotype (xL3s) and the IC50 values measured either in the L4 motility assay ($r = 0.73$) or in the L4 development assay ($r = 0.80$) (Fig. 6.3; data normalised by defining the maximum percentage of eviscerated xL3s as 100% for each compound). Thus, the more potent a compound was at inhibiting larval motility or development, the lower the concentration required to eviscerate a maximum number of xL3s.

6.3.5 Dynamics of evisceration in xL3s

We recorded phenotypic changes in xL3s over time to determine the kinetics of evisceration. Compound m10 was selected for this purpose, as it was the most potent chemical at inhibiting larval motility and development and at inducing evisceration; m7 (a less potent compound) was also selected for comparison with m10. Concentrations of m10 (12.5 µM) and m7 (50 µM) that achieved maximum numbers of eviscerated xL3s at 7 days of exposure were used (Table 6.3). We measured the percentage of xL3s with an anterior

protrusion every 6 h for the first 4 days and then every 24 h for the next three days (Fig. 6.5). Evisceration was microscopically detected first at 42 h of exposure to compounds m7 and m10. This was the time at which the first L4s were detected in untreated control wells (Fig. 6.5). The percentage of eviscerated xL3s increased gradually over time, from 3-16% at 42 h to 65-78% at 72 h, and 83-87% at 7 days. From 66 h onwards, the percentages of eviscerated larvae paralleled the number of developed L4s in unexposed control wells (Fig. 6.5). According to results from a two-way ANOVA with Tukey's test, there were no significant differences between the percentage of eviscerated xL3s after m10 treatment and the percentage of L4s in untreated control wells, except at one time point; there were also no significant differences in the percentage of eviscerated xL3s between m7 and m10 treatments over time, except at three time points (Fig. 6.5). To increase the time resolution of observations made between 36 h and 48 h, an independent examination of video recordings, taken every 20 min following treatment with m10, showed that the percentages of eviscerated xL3s increased gradually from 42 h onwards (Supplementary file 6.4), supporting previous observations.

6.3.6 Reversibility of compound-induced effects and sensitivity time-window

To investigate when the critical process inhibited by the compounds took place during the 7-day development period from xL3 to L4, xL3s were exposed to 12.5 µM of m10 and then washed free of compound at different times and re-incubated in LB* for up to 72 h. Compound was removed after 18 h, 24 h, 27 h, 30 h, 36 h or 48 h of larval treatment, or not at all in the treated control. The percentages of eviscerated xL3s at 72 h were not significantly different from zero when the compound was removed at early time points, up to 36 h. Once larvae had been exposed for at least 36 h, the number of eviscerated larvae began to increase significantly until the end of the experiment (one-way ANOVA with Tukey's test) (Table 6.4; Fig. 6.6A). Therefore, the sensitivity window for this particular phenotype opens sometime between 30 h and 36 h. To estimate when it closes, m10 was added to the larvae in culture medium after different times of incubation. When added at 39 h, substantial numbers of eviscerated xL3s were still produced (42.4 ± 3.4 %), but compound addition at 42 h had a significantly reduced effect (15.4 ± 2.8 % of eviscerated larvae) (Table 6.4, Fig. 6.6B). Of note, while most xL3s treated at 42 h were not eviscerated, they were, nevertheless, blocked from developing to L4s (Table 6.4, Fig. 6.6B). Thus, the action of m10 blocks xL3 to L4 development, induces pathological changes to the worm (Subsection 6.3.3) and inhibits

motility of the L4 stage. It additionally induces a mechanistically interesting Evi phenotype in xL3s, when added between 30 h and not much later than 42 h, during development of *H. contortus* xL3s to L4s in vitro. This time window seems to coincide with the first appearance of L4s in untreated controls (Fig. 6.4), suggesting that the Evi phenotype and moulting are linked.

6.3.7 Influence of external osmotic pressure on the evisceration of xL3s

We reasoned that the most likely driving force behind the dramatic Evi phenotype was an osmotic pressure gradient between the medium and the larval pseudocoelomic fluid, such that it might be possible to protect xL3s to some extent by reducing the pressure differences between the inside and outside of the larvae. To test this hypothesis, xL3s were treated with 12.5 µM m10 in the presence of increasing concentrations of sorbitol (an inert sugar) in LB* (Fig. 6.7A, left). The osmotic pressure in nematode pseudocoelomic fluid has been reported as 323-400 mOsm/L (Harpur and Popkin, 1965; Fuse et al., 1993; Davey, 1995), and it is 240 mOsm/L in LB (Rothe et al., 2012); antimicrobials in LB* add less than 1 mOsm/L to the medium. Here, it was found that increasing external osmotic pressure with sorbitol indeed protected compound-treated larvae from being eviscerated, commencing at 0.2 M sorbitol in LB* (440 mOsm/L) (Fig. 6.7A, left). We also observed that sorbitol inhibited untreated larvae from moulting (Fig. 6.7A, middle), an osmotic effect described previously (Sommerville, 1976). Interestingly, protection of treated xL3s from evisceration by sorbitol mirrored the inhibition of moulting in untreated larvae (Fig. 6.7A, right). This inhibition was reversible; the removal of sorbitol at 48 h led to a recovery of L4 development to ~ 80%, comparable with the no-sorbitol control at 7 days (Fig. 6.7B, left). While moulting was inhibited by sorbitol, compound m10 had no effect on xL3 viability (Fig. 6.7B, left), and the removal of both compound and sorbitol at 48 h led to a resumption of L4 development, with only 7% of larvae eviscerating (Fig. 6.7B, right). However, in the no-sorbitol control, the removal of m10 at 48 h still induced the Evi phenotype in a large fraction of xL3s, with only a small percentage (~ 18%) reaching the L4 stage by 7 days (Fig. 6.7B, right). Collectively, these findings show that compound m10 (at 12.5 µM) did not affect xL3s, unless they entered the moulting process.

6.4 Discussion

Extending a study (Jiao et al., 2017a) that identified a quinoxaline compound, AG-1295, as being inhibitory to *H. contortus* motility, we sought to investigate whether the anthelmintic activity of AG-1295 was a unique occurrence to this particular compound or a property of the quinoxaline chemical series that could be chemically modulated. The results of the present investigation show that AG-1295 is not a singleton and that a series of 14 analogues have varying degrees of activity, paving the way to structure-activity relationships through future chemical optimisation. We recorded marked inhibitory effects of individual test compounds on larval motility or development and a striking Evi phenotype in xL3s in vitro.

In the primary screen, these compounds were identified as 'hits' based on their induction of a unique larval phenotype and their ability to inhibit L4 development, unlike in previous screens, in which 'hit' compounds were identified by a ≥ 70% reduction in xL3 motility (Preston et al., 2015). Subsequent compound evaluation confirmed a relatively limited potency of these compounds at inhibiting xL3 motility, with only four of them achieving IC_{50} values in the range of 10 µM to 50 µM. These findings indicated that other complementary endpoints, such as morphological phenotype, in addition to xL3 motility, could be employed to refine the detection of 'hits' through in vitro screening.

Most (n = 11) of the test compounds were shown to be more potent at inhibiting the motility of L4s than xL3s (Table 6.1). Differences in compound penetration, target expression level, metabolism, detoxification and/or efflux (cf. Gill et al., 1991; George et al., 2017) between xL3s and L4s might explain the different potencies of these compounds between these two larval stages. Importantly, L4s possess a pronounced mouth and functional pharynx (for blood feeding in vivo) which likely facilitate drug uptake. Nonetheless, three select compounds (m7, m13 and m14) as well as AG-1295 itself all reduced xL3 motility, but not L4 motility. To fully understand the reasons for varying potencies, a study of structure-activity relationships by chemical modification and drug target identification should be undertaken in this or model nematodes.

Cytotoxicity was tested against a human, non-malignant mammary epithelial cell line (MCF10A), and different members of this series exhibited varied, generally low cytotoxicities (Table 6.2). Furthermore, there was no correlation between the potency against *H. contortus* and the cytotoxicity against MCF10A cells (Tables 6.2 and 6.3). Although the present selectivity (0.6 to 14.1) is lower than desired, variation among the different compounds indicates that selectivity can be chemically modulated during lead optimisation. In later stages, in vivo toxicity should be investigated to establish the relationship between in

vitro and in vivo systems, as they may not correlate perfectly (cf. McKim, 2010; Sayes, 2014).

The initial screening hit and parent compound, AG-1295, has been described as a receptor tyrosine kinase inhibitor (Kovalenko et al., 1994; Gazit et al., 1996; Kovalenko et al., 1997). Whether this is also the mode of anthelmintic action needs to be investigated, but here we conducted a detailed morphological analysis of the effects of the most potent analogue, m10, against xL3s and L4s, using light and electron microscopy. At the relatively low concentration of 12.5 µM, the m10 analogue was observed to cause widespread morphological damage in both larval stages (Fig. 6.3; Supplementary file 6.2), but treated xL3s, in particular, suffered from a lethal process (i.e. evisceration), by which an initial small protrusion, located at the level of the excretory pore, rapidly progressed to an extrusion of the intestinal tube plus other pseudocoelomic components (Fig. 6.3A). Interestingly, all analogues were able to induce this phenotype at an optimum concentration, related to their potency in the anthelmintic assays (Table 6.3; Fig. 6.2E; Fig. 6.4), suggesting that the inhibition of larval motility or development and the induction of the Evi phenotype all result from an inhibition of the same target(s) in *H. contortus*. Nevertheless, while the degree of inhibition of motility or development increased monotonically with compound concentration, the Evi phenotype became less pronounced above an optimum concentration, indicating that some physiological processes have to be disrupted, but not fully inhibited, for evisceration to take place. This supports a mode of action involving alterations in signalling mechanisms, in agreement with the described activity of AG-1295 and the multiple protein kinases involved in nematode moulting (see Lazetic and Fay, 2017). Another explanation could be compound insolubility above a particular concentration, but this would have also reduced the inhibition of motility or development at the highest concentrations tested, and it did not (Fig. 6.2A and B).

We also found that there was a particular time window in which the Evi phenotype could be induced in xL3s. Larvae exposed to test compounds for the first 30 h of culture only, before ecdysis started, or to compound added after 42 h, once the first L4s appeared, did not show eviscerations. However, while early treatment, followed by compound removal, did not seem to affect larvae, worms treated after 42 h in culture appeared as morphologically damaged xL3s and L4s. One possible interpretation is that test compounds can only gain access to the larvae once ecdysis starts and the cuticle sloughs, and the morphogenetic process that makes larvae vulnerable to evisceration occurs in a time window of 30 h to 42 h of in vitro culture. An alternative explanation might be that the target(s) for this chemical

series is essential only after the beginning of ecdysis. We found that xL3s do survive compound exposure when placed in hypertonic medium, but we also found that, as previously published (Sommerville, 1976), hypertonic media prevent larval moulting, so it is still unclear whether the target(s) first appears in L4s, or whether it is always present but the compounds only access the larval interior after ecdysis commences. In support of this latter hypothesis, AG-1295 and analogues m6, m7, m13 and m14 seem able to affect xL3 motility after prolonged exposure (72 h) (Table 6.1); this might also explain why the kinetics of emergence of the Evi phenotype in xL3s parallel those of moulting (Subsection 6.3.5).

From a fundamental biological perspective, it would be significant to elucidate the signalling mechanism required to produce the intestinal prolapse through the excretory pore. Supplementary file 6.2 shows intestinal content moving but being diverted to the pore, not to the mouth or the anus, as if, at this stage, these two openings would not allow passage of material. Clearly, morphogenetic processes are being disrupted, with catastrophic consequences for the worm, indicating that the compounds will have nematocidal activity against larval stages, although activity against adult worms needs to be assessed.

In conclusion, we have established the in vitro anthelmintic activity of this tetrahydroquinoxaline chemical series, a family of compounds derived from a protein tyrosine kinase inhibitor, AG-1295, a known inhibitor of PDGF receptor kinase in humans (Kovalenko et al., 1994; Gazit et al., 1996; Kovalenko et al., 1997). This tyrosine kinase might have been of interest as a drug target in this nematode, but the PDGF receptor kinase family had not been identified in *H. contortus* (Stroehlein et al., 2015; Jiao et al., 2017a). However, other related tyrosine kinase families may serve as a target for the quinoxalines and, thus, have potential as anthelmintic drug targets. If this turns out to be the case, a useful anthelmintic could be developed with a totally novel mode of action, and its further development would benefit from the wealth of knowledge accumulated for compounds targeting protein kinases.

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Table 6.1. In vitro activities of 14 tetrahydroquinoxaline compounds against *Haemonchus contortus*. Half maximum inhibitory concentration (IC_{50}) values of test compounds on the motility of exsheathed third-stage (xL3) and fourth-stage (L4) larvae after 24 h, 48 h and 72 h of exposure, and on L4 development after 7 days of exposure, in comparison with values for monepantel and AG-1295 (± standard error of mean). Each assay was repeated at least two times in triplicate on different days.

Compound	**Half maximum inhibitory concentration (IC_{50} µM)**						
	xL3 motility			**L4 motility**			**L4 development**
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**	**7 days**
m1	–	–	–	–	52.8±3.8	34.1±9.1	18.0±2.0
m2	–	–	–	–	–	81.2±16.6	–
m3	–	–	–	32.4±1.6	23.3±1.6	~12.6	26.5±1.4
m4	–	–	–	~49.5	33.1±1.5	17.7±5.1	11.4±2.5
m5	–	–	–	–	–	–	21.2±3.3
m6	–	–	~9.8	–	–	27.7±6.7	11.8±1.2
m7	–	–	~11.4	–	–	–	12.0±1.7
m8	–	–	–	–	~48.2	41.5±7.8	16.4±1.1
m9	–	–	–	–	42.4±7.3	36.6±7.4	21.5±1.6
m10	–	–	–	–	6.9±4.8	6.9±2.7	3.5±0.9
m11	–	–	–	24.3±3.8	15.9±3.4	14.4±4.6	8.4±0.2
m12	–	–	–	–	–	~51.9	–
m13	–	–	~50.1	–	–	–	–
m14	–	–	~43.1	–	–	–	31.8±0.7
AG-1295	–	–	9.9±1.9	–	–	–	7.7±0.9
Monepantel	0.5±0.2	0.3±0.1	0.2±0.1	0.8±0.3	0.3±0.2	0.4±0.3	0.07±0.04

'~' indicates where half maximum inhibitory concentration could not be accurately calculated by the log (agonist) versus response - variable slope (four parameter) equation and IC_{50} value was estimated; '–' = no activity.

Table 6.2. In vitro cytotoxicity of tetrahydroquinoxaline compounds against human breast epithelial cells (MCF10A). Comparison of 14 tetrahydroquinoxalines with AG-1295 and doxorubicin (reference and positive-control compounds) for inhibition of MCF10A cell proliferation in vitro (± standard error of mean). Selectivity indices of these compounds on the motility of fourth-stage larvae (L4s; at 72 h) and development of L4s (at 7 days) were calculated using the formula: Si= IC_{50} for MCF10A cell/ IC_{50} for *H. contortus*. Each assay was repeated two times in quadruplicate on different days.

Compound	**Cell density (%) at 50 µM**	**IC_{50} (µM) for MCF10A cells**	**Selectivity index (SI) for *H. contortus***	
			L4 motility	**L4 development**
m1	79.6±2.2	>50	>1.5	>2.8
m2	75.7±2.9	>50	>0.6	nd
m3	51.3±1.6	>50	>4.0	>1.9
m4	48.2±1.1	>50	>2.8	>4.4
m5	62.9±3.9	>50	nd	>2.4
m6	39.6±1.9	35.7±3.1	1.3	3.0
m7	70.3±3.8	>50	nd	>4.2
m8	23.8±1.4	30.9±7.0	0.7	1.9
m9	30.2±1.5	25-50	0.7-1.4	1.2-2.3
m10	38.1±1.1	25-50	2.6-7.3	7.1-14.1
m11	70.8±2.4	>50	>3.5	>5.9
m12	80.7±4.0	>50	>0.96	nd
m13	87.2±1.8	>50	nd	nd
m14	73.9±1.2	>50	nd	>1.6
AG-1295	90.2±3.9	>50	nd	>6.4
Doxorubicin	0.2±0.03 (at 10 µM)	1.2±0.1	nd	nd

nd = not determined due to a lack of half maximum inhibitory concentration (IC_{50}) values needed to calculate a selectivity index (SI).

Table 6.3. Frequency of the eviscerated third-stage larvae (xL3s) of *Haemonchus contortus* exposed to individual test compounds (m1 to m14) and the control compound (AG-1295) at different compound concentrations for 7 days (± standard error of the mean). This assay was repeated two times in triplicate on different days.

Compound	Eviscerated xL3s (%) when exposed to different compound concentrations (µM)										Maximum (%)	Optimum concentration (µM)
	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0		
m1	63.9±2.7	73.8±3.2	44.9±3.9	2.8±0.3	0	0	0	0	0	0	73.8±3.2	50
m2	63.3±1.8	72.3±1.1	14.9±1.5	0	0	0	0	0	0	0	72.3±1.1	50
m3	51.5±2.1	64.6±3.6	24.7±5.8	0	0	0	0	0	0	0	64.6±3.6	50
m4	24.9±5.4	55.8±4.5	59.8±8.7	62.9±7.1	6.1±2.0	0	0	0	0	0	62.9±7.1	12.5
m5	21.2±6.0	22.1±2.0	18.0±2.8	1.1±1.1	0	0	0	0	0	0	22.1±2.0	50
m6	41.5±1.4	50.6±8.4	56.3±3.2	11.1±3.6	0	0	0	0	0	0	56.3±3.2	25
m7	64.2±3.2	69.7±3.9	50.6±4.9	6.5±1.2	0	0	0	0	0	0	69.7±3.9	50
m8	18.2±2.1	67.0±2.5	50.5±4.7	12.8±3.4	0	0	0	0	0	0	67.0±2.5	50
m9	56.5±0.4	63.8±4.3	25.2±5.2	0	0	0	0	0	0	0	63.8±4.3	50
m10	29.1±2.1	35.2±2.6	38.3±3.2	82.9±3.1	42.9±6.5	3.9±1.1	0	0	0	0	82.9±3.1	12.5
m11	0	14.8±2.6	61.2±4.5	57.5±2.1	12.9±3.0	0	0	0	0	0	61.2±4.5	25
m12	26.6±3.1	58.4±1.5	10.7±4.5	0	0	0	0	0	0	0	58.4±1.5	50
m13	5.0±2.3	0.8±0.8	0	0	0	0	0	0	0	0	5.0±2.3	100
m14	66.3±2.2	25.1±5.7	0	0	0	0	0	0	0	0	66.3±2.2	100
AG-1295	35.3±3.9	21.8±4.2	14.1±4.0	0	0	0	0	0	0	0	35.3±3.9	100

Table 6.4. Reversibility of the inhibitory property of selected tetrahydroquinoxaline, 12.5 µM of m10, on the exsheathed third-stage larvae (xL3s) of *Haemonchus contortus*. The compound was washed off or added at the times indicated on the table, with corresponding concentration of DMSO as negative-control (± standard error of mean). This assay was repeated two times in duplicate on different days.

Compound washed at	**Eviscerated xL3s (%)**	
	At time of washing	**After 72 h**
18 h	0	0
24 h	0	3.1±2.2
27 h	0	3.9±3.9
30 h	0	6.8±2.0
36 h	0	38.1±4.8
48 h	10.7±2.9	52.3±5.5
No wash	na	66.8±3.0
Untreated control	na	0

Compound added at	**Eviscerated xL3s (%) at 72 h**	**L4 development (%) at 72 h**
0 h	65.0±1.6	0
39 h	42.4±3.4	11.9±1.3
42 h	15.4±2.8	13.4±1.1
Untreated control	0	58.7±2.6

na = not applicable.

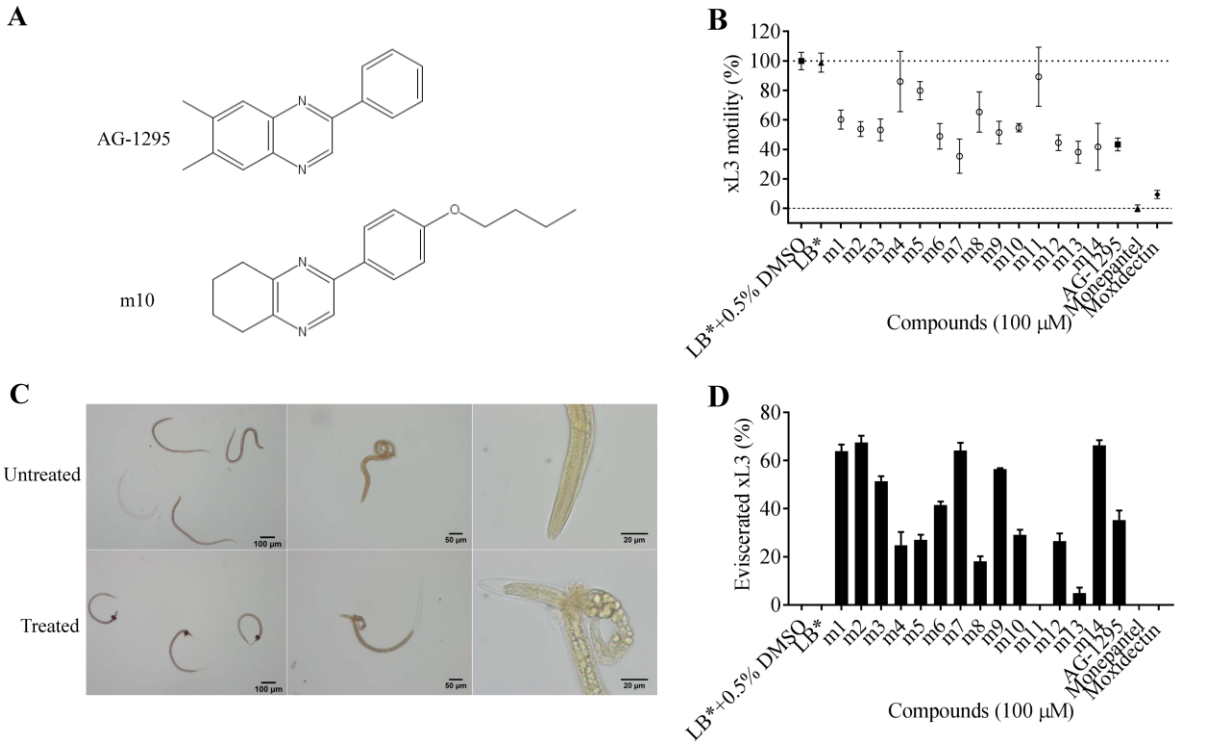


Fig. 6.1. Screening of 14 tetrahydroquinoxalines against exsheathed third-stage larvae (xL3s) of *Haemonchus contortus*. Panel A: Chemical structures of AG-1295 and one representative tetrahydroquinoxaline analogue m10. Panel B: Primary screen for inhibition of xL3 motility after 72 h of compound treatment. Panel C: The compound-induced Evi phenotype in xL3s after 7 days of compound treatment. Panel D: Percentages of eviscerated xL3s induced by individual compounds. Positive controls were monepantel and moxidectin, and negative controls were corresponding concentrations of dimethyl sulfoxide (DMSO).

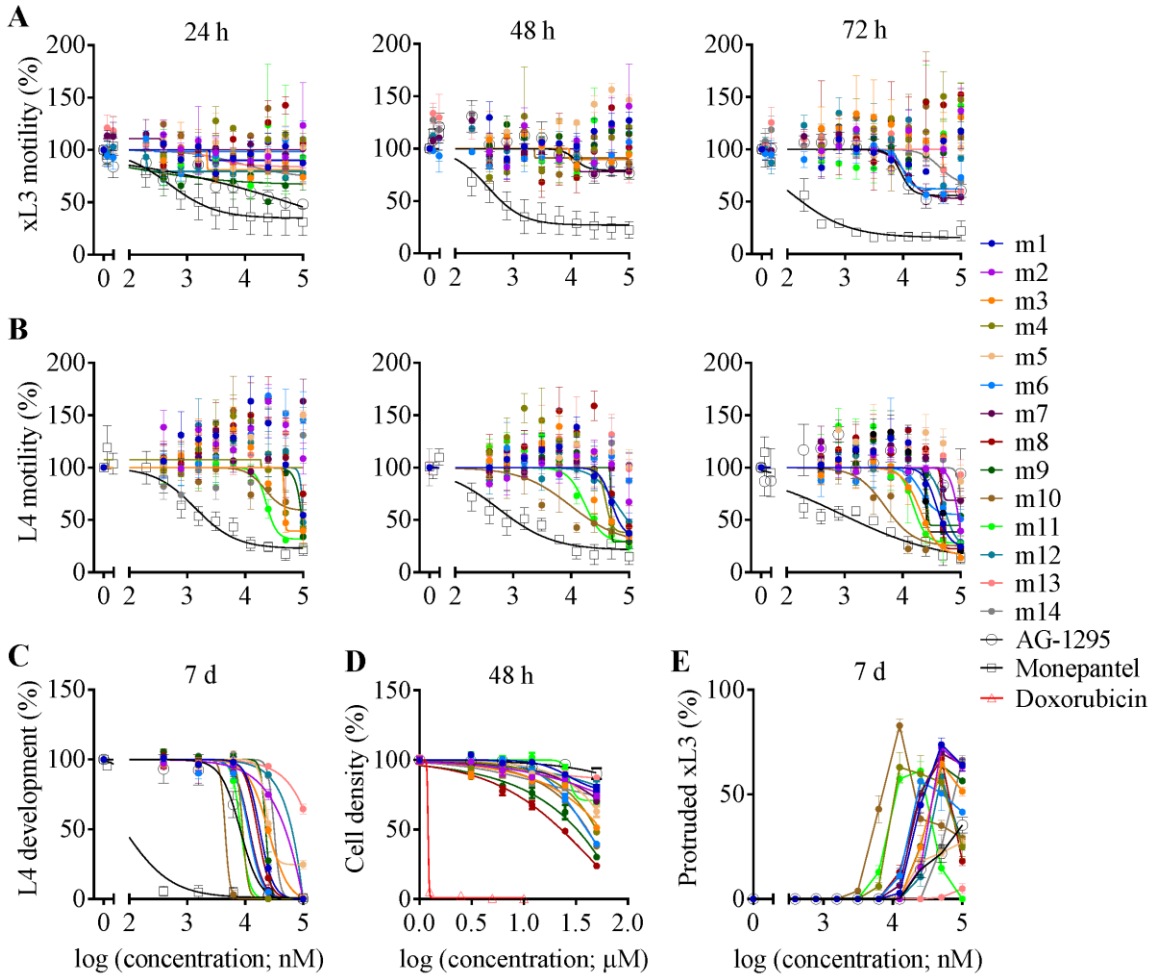
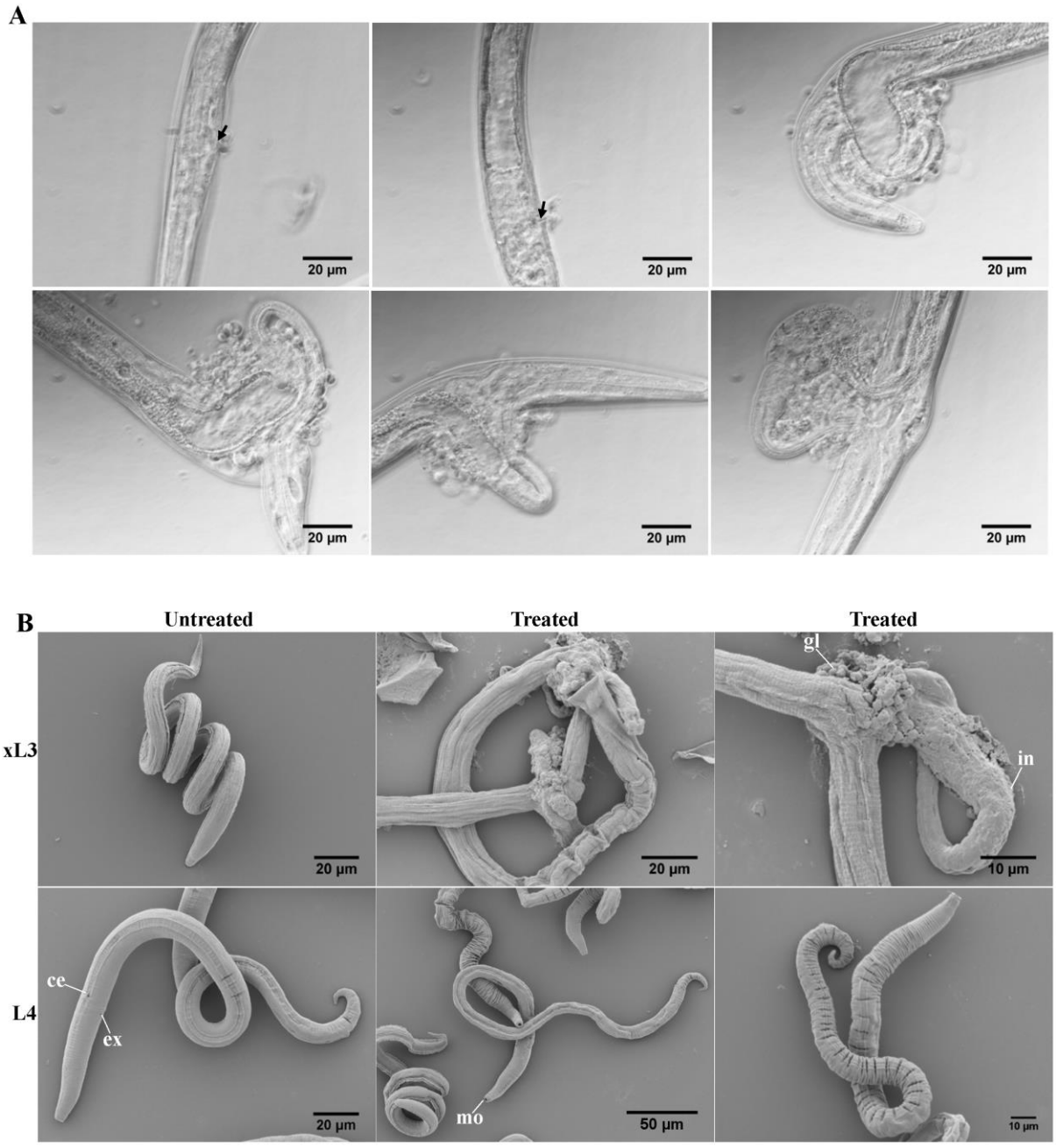


Fig. 6.2. Dose-response curves of the effects of 14 AG-1295 analogues against *Haemonchus contortus* in vitro. Panel A: Inhibition of the motility of exsheathed third-stage larvae (xL3s) at 24 h, 48 h and 72 h. Panel B: Inhibition of the motility of fourth-stage larvae (L4s) at 24 h, 48 h and 72 h. Panel C: Inhibition of the development of L4 development after 7 days. AG-1295 was used as a reference compound. Panel D: Inhibition of the proliferation of normal human breast epithelial (MCF10A) cells; doxorubicin was used as the reference positive-control. Panel E: Commencement of evisceration in xL3s (%) after 7 days. Data points represent at least two independent experiments conducted in triplicate, presented as the mean ± standard error of the mean.

(Fig. 6.3)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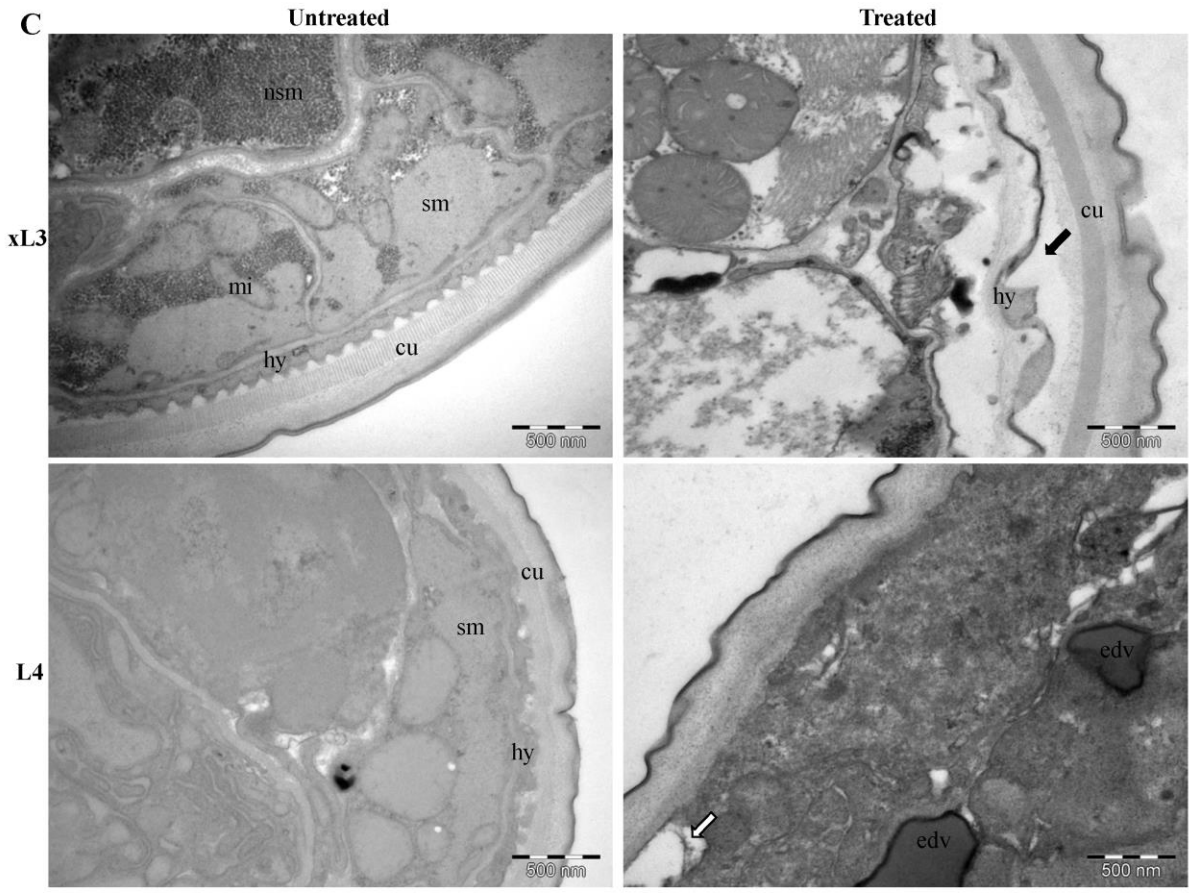

Fig. 6.3. Microscopy of representative exsheathed third-stage (xL3s) and fourth-stage (L4s) larvae of *Haemonchus contortus*. Panel A: Phase-contrast light microscopy of xL3 following exposure to 12.5 µM of m10. Black arrows indicate excretory canals in xL3. Panel B: Scanning electron microscopy of xL3 (top) and L4 (bottom) stages, following exposure to 12.5 µM of m10 or 0.0625% DMSO (untreated control). Abbreviations: ce = cervical papillae; ex = excretory pore; gl = globules; in = intestine; mo = mouthparts. Panel C: Transmission electron microscopy of xL3 (top) and L4 (bottom) stages following exposure to 12.5 µM of m10 or 0.0625% DMSO (untreated control). A black arrow indicates a release of the hypodermis from the cuticle (appearing undulated) in treated xL3s. A white arrow indicates myo-degeneration and cell lysis in treated L4s. Abbreviations: cu = cuticle; hy = hypodermis; sm = striated muscle; nsm = non-striated muscle; mi = mitochondria; edv = electron-dense vesicles.

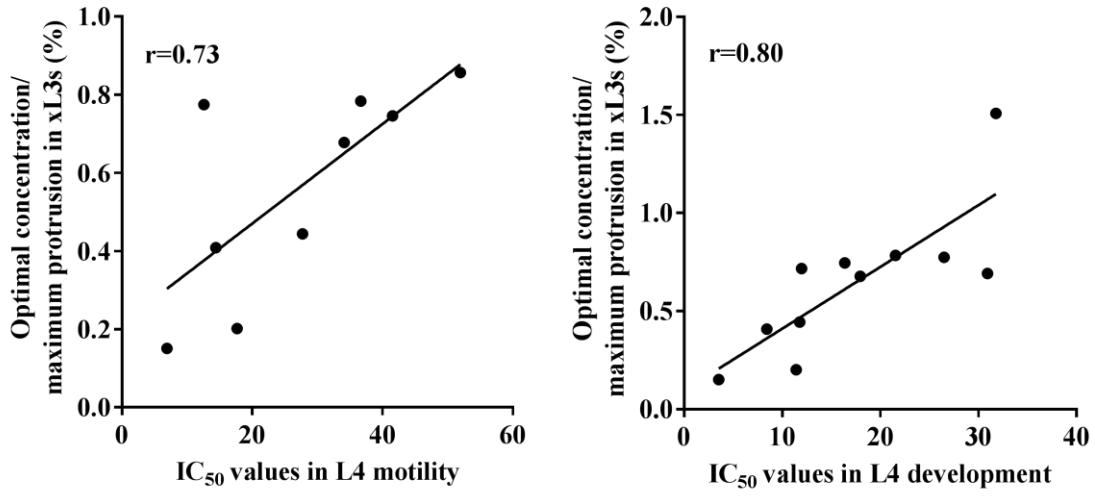

Fig. 6.4. Pearson correlation coefficients (r) between half maximum inhibitory concentration (IC_{50}) of the motility of fourth-stage larvae (L4s) of *Haemonchus contortus* and the normalised, optimum compound concentration inducing maximum evisceration (%) in the exsheathed third-stage larvae (xL3s) (left panel); Pearson correlation coefficients (r) between IC_{50} of L4 development and the normalised optimum compound concentration inducing maximum evisceration in xL3s (%) (right panel). The optimum compound concentration was normalised by defining the maximum evisceration in xL3s as 100% for individual compounds.

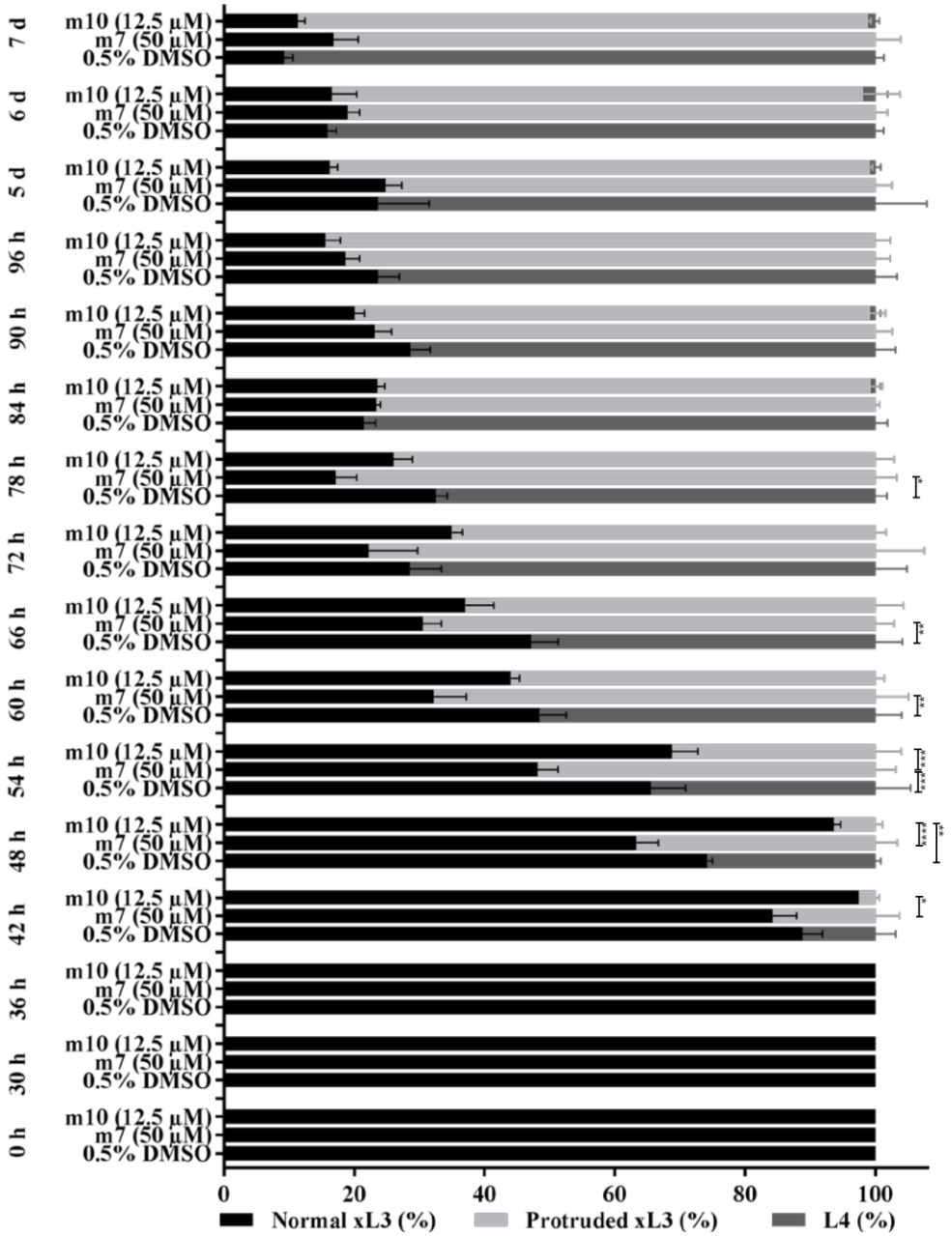

Fig. 6.5. The dynamics of the compound-induced Evi phenotype in exsheathed third-stage larvae (xL3s) of *Haemonchus contortus*. After exposure to 50 µM of compound m7 or 12.5 µM of compound m10 or only LB* containing 0.5% DMSO (negative-control), the normal xL3s, eviscerated xL3s and fourth-stage larvae (L4s) were examined by light microscopy (20-100 × magnification) at the times indicated on the left. For each time point, the mean percentage of eviscerated larvae in 30 xL3s ± the standard error of the mean was calculated, and a two-way ANOVA with Tukey's test was used to calculate statistical differences between different groups. Asterisks indicate values that are significantly different from one another (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001). This assay was repeated at least two times in triplicate on different days.

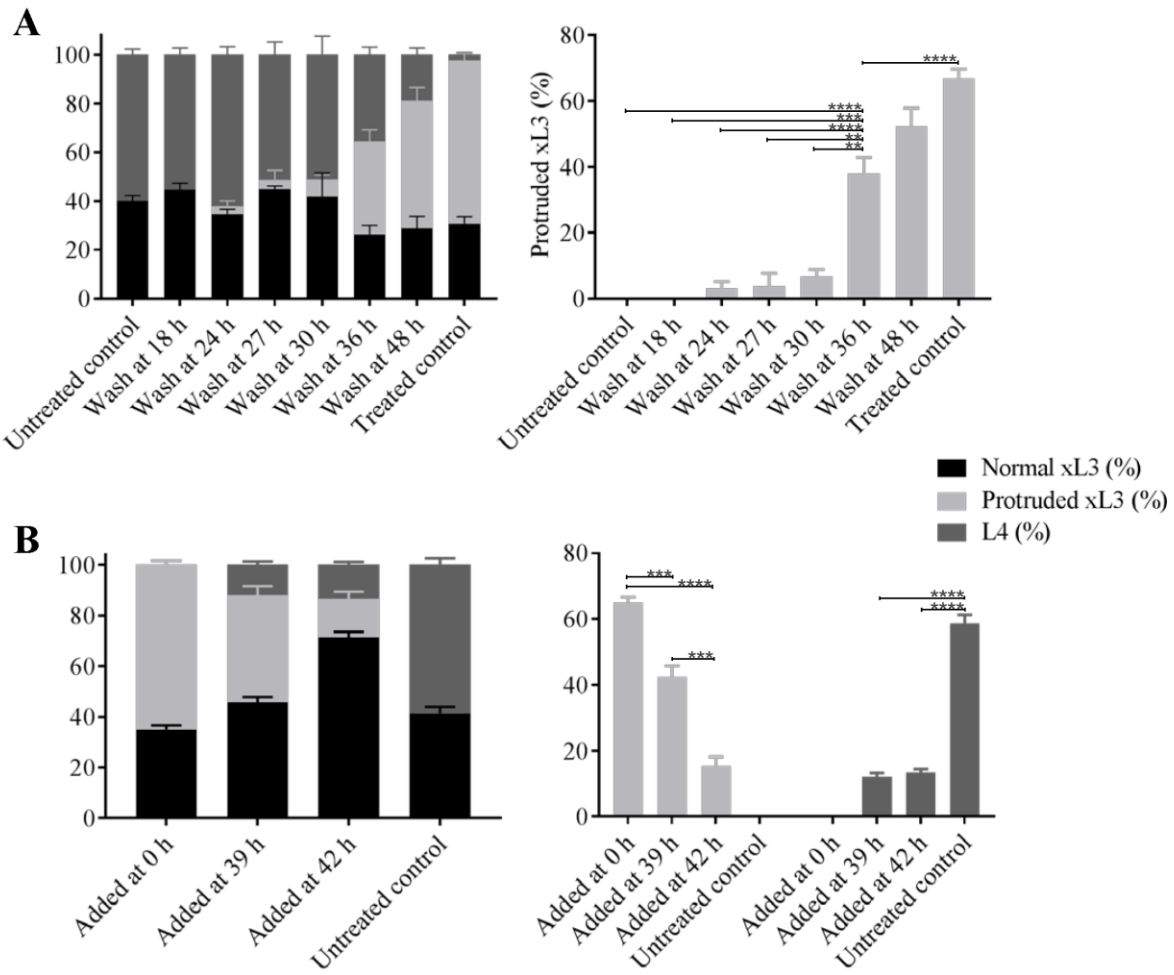

Fig. 6.6. Reversibility of the effect of individual AG-1295 analogues on the exsheathed third-stage larvae (xL3s) of *Haemonchus contortus*. Panel A: At different time points (18 h, 24 h, 27 h, 30 h, 36 h and 48 h following compound treatment), test compounds (12.5 µM of m10) were removed by washing from wells. At 72 h of exposure, the percentage of xL3s with an Evi phenotype and the rate of development of fourth-stage larvae (L4s) was assessed (left). The controls included xL3s continually exposed to 12.5 µM of individual test compounds for 72 h (treated control) and xL3s incubated in LB* alone (untreated control). A one-way ANOVA with Tukey's test was used to calculate statistical differences in the percentage of eviscerated xL3s between treatments (right). Panel B: Compounds were added at 39 h and 42 h after first culturing larvae; the percentage of xL3s with an Evi phenotype and rate of L4 development were assessed at 72 h. A two-way ANOVA with Tukey's test was used to calculate statistical differences between groups. This assay was repeated two times times in duplicate on different days. Asterisks indicate values (mean ± the standard error of the mean) that are significantly different from one another (**$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

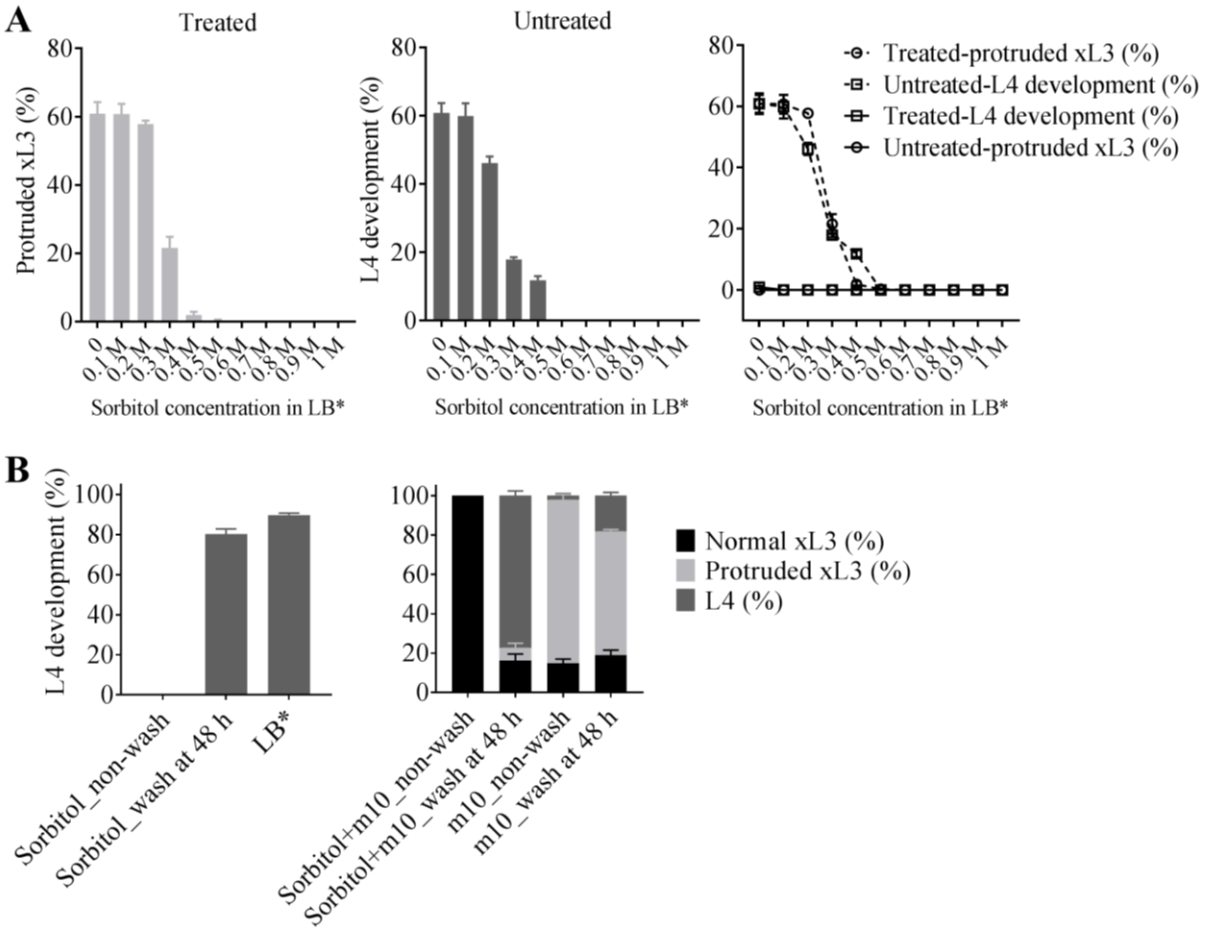

Fig. 6.7. Effect of external osmotic pressure on the compound-induced Evi phenotype in exsheathed third-stage larvae (xL3s) of *Haemonchus contortus*. Compound m10 was used to treat xL3s at the concentration of 12.5 µM. Panel A: Compound-treated xL3s (left) and untreated xL3s (middle) were cultured in the presence of increasing concentrations of sorbitol in LB*, as indicated in the figure. At 72 h, the percentage of xL3s with an Evi phenotype and the rate of development of fourth-stage larvae (L4s) were evaluated. Increased osmotic pressure in the culture medium reduced evisceration in treated xL3s (%), mirroring the inhibition of moulting in untreated xL3s (right). Panel B: xL3s cultured in the presence of 0.6 M of sorbitol were washed at 48 h, and the rate of L4 development was counted at 7 days to test the reversibility of sorbitol in inhibiting xL3 moulting (left); the controls included xL3s continually incubated in 0.6 M of sorbitol for 7 days, and xL3s incubated in LB* alone for the same time period; xL3s exposed to 12.5 µM of m10 in the presence of 0.6 M of sorbitol were washed at 48 h and examined microscopically after 7 days, to test the effect of test compound on xL3s when xL3 moulting was inhibited (right); the controls included unwashed xL3s exposed to 12.5 µM of compound m10 in the presence of 0.6 M of sorbitol, and xL3s exposed to 12.5 µM of m10 in LB*. This assay was repeated two times in duplicate on different days. Each value represents the mean ± standard error of the mean.

Chapter 7 - General discussion

The control of parasitic nematodes is seriously challenged by widespread anthelmintic resistance (Kaplan and Vidyashankar, 2012; Wolstenholme and Kaplan, 2012; Geary et al., 2015; Kotze and Prichard, 2016). Therefore, the discovery and development of new and effective anthelmintics are essential to sustain the economic and health benefits from their application to control parasites (Geary et al., 2015; Campbell, 2016).

The research aims of this thesis were (i) to use whole-organism screening of compounds from well-defined, curated collections of chemicals for inhibitory activity against *H. contortus* and/or other parasitic nematodes; (ii) to identify 'hit' compounds, and (iii) to assess these compounds as possible 'leads' for optimisation and subsequent development. These aims were achieved in that 20 compounds with in vitro-activity against *H. contortus*, including one new chemical entity (designated SN00797439), two human kinase inhibitors (SNS-032 and AG-1295), 14 AG-1295 (tetrahydroquinoxaline) analogues, one insecticide (tolfenpyrad) and two tolfenpyrad (pyrazole-5-carboxamide) derivatives (a-15 and a-17) (Chapters 2-6). Collectively, these achievements offer a sound starting point for the development of new anthelmintics.

The purpose of the present chapter is to discuss the achievements in a broader context, considering aspects of screening technologies (Subsection 7.1), compound libraries (7.2), the evaluation of 'hit' compounds (7.3) and their potential as 'lead' compounds in future anthelmintic development (7.4), and to propose lines of future research (7.5 and 7.6).

7.1. Screening technologies

In this project, established phenotypic screening assays employing parasitic stages of *H. contortus* were used (Preston et al., 2015), with the advantage of being able to assess nematostatic or nematocidal effects of chemicals on whole worms in real time. This approach circumvented some of the hurdles of mechanism-based screens (Geary et al., 1999), where compounds only bind to selected targets and are often not assessed on other molecular targets or whole organisms (cf. Kotze, 2012; Geary et al., 2015). However, compared with mechanism-based screens, where the target/s is/are defined, in whole-worm screens, the molecular targets are usually not known in the first instance. Nonetheless, the modes of action of active compounds identified in whole-worm screening assays can be studied at the molecular level, for instance, using knowledge of the genome, transcriptome and proteome of the target organism (e.g., Laing et al., 2013; Schwarz et al., 2013; Geary, 2016; Harder,

2016). In addition, for known drugs destined for repurposing to parasitic worms, their modes of action might be extrapolated to the 'new' organism to which the drug is being repurposed, although an emphasis needs to be placed on critically evaluating any possible side effects on the host organism (O'Connor and Roth, 2005).

Motility reduction as a measure to detect hits and its limitations. The assays employed in this thesis were designed to measure a reduction in 'motility' of larvae in order to identify active compounds (Preston et al., 2015). However, some compounds, such as AG-1295 and its analogues (Chapter 6), did not reduce motility, but rather caused a phenotypic change in the larvae. Thus, motility reduction is not always a suitable measure and can lead to 'false-negative' results. In the present study, close inspection of treated larvae by microscopy identified a number of 'hit' compounds based on their induction of non-wildtype phenotypes, such as the 'coiled', 'straight' and 'anterior protrusion' phenotypes, following exposure of *H. contortus* to SN00797439, tolfenpyrad and AG-1295 analogues. In addition, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) sometimes provided useful insights into compound-induced morphological changes at the tissue and cellular levels.

Therefore, in the future, the phenotype of treated worms should be recorded as a complementary endpoint to improve the identification of 'hit' compounds. Although the current assays allow for worm phenotypes to be recorded by visual inspection of five-second videos, the workload associated with this step, particularly when screening large numbers of compounds, such as the 'Open Scaffolds' library, restricts screening throughput. Hence, it will be important to develop an assay that can quantitatively assess both worm motility and worm phenotype to avoid 'false-negative' results and to increase screening efficiency. It is worth mentioning that quantitative tracking of phenotypes has already been applied to worms such as *C. elegans* (see Geng et al., 2004) and *Schistosoma mansoni* (see Singh et al., 2018), utilizing mathematic algorithms and automatic imaging technology. As the motility dynamics of parasitic nematodes is distinct from *C. elegans* and *S. mansoni*, new algorithms will need to be developed and assessed to ensure reliability for hit discovery.

The importance of extending screening to adult worms. Traditionally**,** conventional whole-worm screening assays, such as the egg hatch assay (Le Jambre, 1976; Dobson et al., 1986) and larval development assay (Kotze et al., 2006), have been applied to free-living larval stages. Compared with such conventional assays, the present motility assays (Preston et al.,

2015) were an improvement through the use of ‘parasitic’ larvae, xL3s and L4s, both produced in vitro. A substantial advantage was also the ability to store L3s for long periods (up to 6 months). The ability to directly exsheath L3s in vitro and raise xL3s and then L4s circumvented the need for experimental animals to produce L4 and adult stages in vivo in sheep. Nonetheless, considering that the adult stages of gastrointestinal (strongylid) nematodes are usually the target stage for most anthelmintics, the inclusion of adults in future in vitro screening of chemical libraries would be advantageous, and would allow a comparative assessment of potency among developmental stages.

7.2. Considerations regarding compound libraries

Numerous well-curated compound collections in different countries provide an untapped resource with potential for the discovery of new anthelmintics. In the present project, five different compound collections were obtained from different sources. The ‘Open Scaffolds’ library was sourced from *Compounds Australia*, the ‘Stasis Box’ and the ‘Pathogen Box’ were both acquired through a product-development partnership (PDP) with Medicine for Malaria Venture (MMV), and the pyrazole-5-carboxamide collections and tetrahydroquinoxaline collections were both obtained from academic institutions.

Compounds Australia sources small molecules and consolidates them into a central repository that facilitates subsequent screening and provides these molecules to laboratories around the world, in order to boost drug discovery efforts (Simpson and Poulsen, 2014). The ‘Open Scaffolds’ library, screened in the present project, is one of the main collections maintained by *Compounds Australia* (Chapter 2). This collection, containing 33,999 compounds (Simpson and Poulsen, 2014), is much larger than other compound collections that were screened, e.g., the ‘Stasis Box’ and the ‘Pathogen Box’, each with 400 compounds. Generally, the relatively small number of known compounds is considered to limit the chemical space for drug repurposing (Hergenrother, 2006). However, the ‘hit’ rate from the latter two boxes (0.5% for ‘Stasis Box’; 0.25% for ‘Pathogen Box’) for possible repurposing was higher than that for the discovery of new chemical entities (0.0029%) from the ‘Open Scaffolds’ library. This finding can be explained by the smaller compound collections containing advanced compounds (cf. Sekhon, 2013), e.g., with known pharmacokinetic parameters and/or biological activities. Thus, careful consideration needs to be given to the selection of compound libraries for screening on parasites.

Fortunately, 14 tetrahydroquinoxaline analogues of the active compound from the 'Stasis Box' (AG-1295) could be sourced from a collaborator, Professor Jonathan Baell, which enabled early investigations of potency on xL3 and L4 stages of *H. contortus* (Chapter 6) and a foundation for future mode of action studies. Similarly, 55 tolfenpyrad (pyrazole-5-carboxamide) analogues were sourced through collaborators in China from the State Key Laboratory of Elemento-organic Chemistry in Nankai University, China (Chapter 5), allowing potency and cytotoxicity evaluations. Clearly, further medicinal chemistry optimisation is required to enable further assessment and development of these chemical entities.

7.3. Evaluation of 'hit' compounds

Following the identification of hits through screening and dose-response experiments, compound evaluation can guide the identification of lead candidates (cf. Goldstein et al., 2008).

Inhibition of larval motility and development. In this project, the assessment of inhibition of larval motility and development in *H. contortus* was consistently used to evaluate the potency of 'hit' compounds, offering essential information for the selection of compounds for further study. Of the twenty 'hit' compounds, the IC_{50} values for xL3 motility inhibition (72 h) ranged from 3 µM to 56 µM, the IC_{50} values of L4 motility reduction (72 h) ranged from 0.03 µM to 26 µM, and the IC_{50} values of L4 development inhibition (7 d) ranged from 0.08 µM to 41 µM. The in vitro potency of some of these 'hit' compounds, e.g., tolfenpyrad, was comparable with that of the commercial anthelmintic monepantel (IC_{50} values of 0.2 µM, 0.4 µM and 0.07 µM, respectively). Based on IC_{50} values, tolfenpyrad was identified as the most potent compound at inhibiting larval motility and development (Chapter 4) and, thus, may be a candidate for further optimisation. Nevertheless, other hit compounds (i.e. SN00797439, a-15, a-17, SNS-032, AG-1295 and 14 tetrahydroquinoxaline compounds) also show promise for hit-to-lead optimisation, given that they have lead-like scaffolds and/or have already been developed or proposed as drugs.

The kinase inhibitors SNS-032 and AG-1295 did not show any detectable inhibitory activity on L4 motility, even though the L4 stage has a functional pharynx, which allows the uptake of chemicals (Chapter 3). This information suggests that the expression of the kinase target(s) in L4s is markedly lower than that in xL3s, and may explain the difference in

potency between the two stages. In contrast, for the other hit compounds with more potency in L4s than in xL3s, it remains unclear whether the difference in potency between the two stages is due to the level of expression of target(s) and/or variation in drug uptake and/or metabolism.

Cytotoxicity testing. With regard to cytotoxicity and selectivity of 'hit' compounds, mammalian cell proliferation was used as an 'endpoint assay' - which has been used extensively for the screening of drugs for use in humans (Li et al., 2006; McKim, 2010; Fisher et al., 2014). The in vitro cytotoxicity test provides important information for selecting compounds with (apparent) low risk profiles for further development and for the 'de-prioritisation' of compounds with high risk profiles prior to expensive preclinical and clinical trials, thus reducing early risks and improving the probability of success in the development pipeline (McKim, 2010; Sayes, 2014). However, in vitro cytotoxicity tests may be unreliable in predicting in vivo toxicity due to the pronounced differences between in vitro and in vivo systems relating, for example, to differences in metabolic activities, protein binding and/or compound solubility (McKim, 2010). Therefore, results from an in vitro cytotoxicity test cannot be used to make a final decision about the toxicity of a compound in vivo, such that extensive animal toxicity testing must be undertaken to verify the safety of a drug under consideration as a lead candidate, and is a requirement for registration.

7.4. Potential of identified compounds as 'leads' in future anthelmintic development

Pyrazole-5-carboxamides. Of the 'hit' compounds identified in this project, the pyrazole-5-carboxamide insecticide, tolfenpyrad, has the highest in vitro anthelmintic potency against *H. contortus*. As insecticides or acaricides, pyrazole-5-carboxamides are considered to target complex I of the electron transport chain in arthropods (Hollingworth, 2001). Here, through measuring the mitochondrial oxygen consumption rate (OCR), tolfenpyrad and its two pyrazole-5-carboxamide derivatives, a-15 and a-17, were shown to significantly reduce OCR in *H. contortus* (Chapters 4 and 5), indicating that this compound series might also target complex I of the electron transport chain in *H. contortus*. In the electron transport chain, parasitic larvae and adult worms of *H. contortus* are predicted to employ a NADH-fumarate reductase system, in which complex I is the NADH-rhodoquinone reductase that is different from mammalian animals using NADH-ubiquinone reductase as complex I (Kita et al., 1997;

Harder, 2016). The difference in complex I between *H. contortus* and mammals suggests that it may be a suitable and selective drug target for anthelmintic discovery (Kita et al., 2003; Harder, 2016), a proposal supported by cytotoxicity data showing that tolfenpyrad is more selective for *H. contortus* than mammalian cells (Chapters 4 and 5).

The energy production in adult worms via mitochondria appears to be mainly linked to reproduction and a complex regulation of the neuromuscular system (Harder, 2016). Indeed, there are only few anthelmintics targeting the energy production system in nematodes, with most currently used anthelmintics targeting the nematode neuromuscular system (Kotze, 2012; Holden-Dye and Walker, 2014). For instance, imidazothiazoles act as cholinergic agonists at nicotinic neuromuscular junctions (Coles et al., 1975; Prichard, 1990; McKellar and Jackson, 2004), macrocyclic lactones target neurotransmitter gamma-aminobutyric acid-gated chloride ion channels and glutamate-gated chloride ion channels (Arena et al., 1992; Brownlee et al., 1997), monepantel binds to nicotinic acetylcholine receptor subunits (Kaminsky et al., 2008; Sargison, 2012; Baur et al., 2015) and derquantel represents nicotinic acetylcholine receptor antagonists (Ruiz-Lancheros et al., 2011; Sargison, 2012). Here, tolfenpyrad shows some promise as a new anthelmintic chemical, but needs to be explored further, optimised and tested for safety and efficacy in animals.

Kinase inhibitors. By screening compound collections obtained through a PDP with MMV, besides tolfenpyrad, two human kinase inhibitors (SNS-032 and AG-1295) were identified to have in vitro anthelmintic activity but showed less motility inhibition than tolfenpyrad. The discovery of kinase inhibitors as therapeutics is a relatively 'hot' topic in the field of drug discovery, particularly in relation to anti-cancer drugs (Sebolt-Leopold and English, 2006; Zhang et al., 2009). Clearly, kinases play a crucial role in all cellular processes, and the dysregulation of kinase activities is related to many diseases (Goldstein et al., 2008). With the success of imatinib (Manley et al., 2002; Baselga, 2006; Collins and Workman, 2006; Larson et al., 2008), the discovery of kinase inhibitors has attracted considerable attention (Sebolt-Leopold and English, 2006; Verweij and de Jonge, 2007; Zhang et al., 2009). Moreover, for drug repurposing, imatinib has been evaluated for activity against *S. mansoni*, and has pronounced schistosomicidal activity in vitro but not in vivo (Katz et al., 2013). Kinome studies of socioeconomically important parasitic worms, including *H. contortus*, *S. haematobium*, *Trichinella spiralis*, *T. pseudospiralis* and *Trichuris suis* (see Cantacessi et al., 2010; Beckmann et al., 2014; Preston et al., 2015; Stroehlein et al., 2015a; Stroehlein et al., 2015b; Stroehlein et al., 2016), can deliver important resources for

genome- or transcriptome-guided anthelmintic discovery. In this context, the identification of SNS-032 with anthelmintic activity against *H. contortus* indicates the potential of bioinformatic-guided prioritisation of targets and chemicals via kinome investigations, because the targets of SNS-032, CDK-7 and CDK-9 have been predicted *in silico* to be prioritised kinase targets in *H. contortus* (see Stroehlein et al., 2015b).

SNS-032 has been developed as an anti-cancer drug (Ali et al., 2007; Kodym et al., 2009) and AG-1295 has been developed as an anti-restenosis drug (Banai et al., 1998). Therefore, the two human drugs were specifically designed to bind to human kinase targets rather than those of *H. contortus*. Marked differences in sequences and three-dimensional structures between the human kinase targets of SNS-032 and their corresponding *H. contortus* homologs (Jiao et al., 2017) appear to help explain variations in potency and selectivity of this kinase inhibitor at inhibiting worms *versus* mammalian cells, and suggest that there is an opportunity to chemically optimise SNS-032 and AG-1295 (tetrahydroquinoxaline) analogues to bind specifically to targets in *H. contortus*.

Compound SN00797439. The new chemical entity, SN00797439, has a novel, lead-like scaffold containing a 1, 2, 4-oxadiazole and a 1-pyrrolidinecarboxamide. The 1, 2, 4-oxadiazole has been relatively widely used as a drug for different applications (Bora et al., 2014), such as an anti-inflammatory agent (Dalhamn, 1969), an analgesic (Kumar et al., 2012) and a muscarinic receptor agonist (Street et al., 1990). The 1-pyrrolidinecarboxamide has been rarely used, except as cisanilide ((2R, 5S)-rel-2, 5-dimethyl-N-phenyl-1-pyrrolidinecarboxamide), a now obsolete herbicide (Frear and Swanson, 1975; Frear and Swanson, 1976).

SN00797439 achieved IC_{50} values of 0.3 µM in inhibiting larval motility, and is more potent than other 'hit' compounds, except tolfenpyrad. In particular, the efficacy of this compound was also characterised by a 'coiled' xL3 phenotype in *H. contortus* and considerable cuticular damage in L4s in vitro. Evaluations on other parasitic nematodes showed that SN00797439 possesses relatively broad-spectrum anthelmintic activity, and has potential to be developed as a new anthelmintic. In this project, several genetically (evolutionarily) distant and socioeconomically important parasitic nematodes (i.e. *H. contortus* and *A. ceylanicum* [strongyloids] *vs. B. malayi* and *D. immitis* [filarioids] *vs. T. muris* [enoplid]) were tested (Chapter 2). In the future, the activity of SN00797439 might be assessed against other worms that cause neglected tropical diseases.

7.5. Future work toward anthelmintic development

Although in vitro nematocidal/nematostatic activity and cytotoxicity data for the 'hit' compounds identified in this project provide some promise for their development as new anthelmintics, future work needs to focus on detailed investigations of absorption, distribution, metabolism, excretion and toxicity (ADMET), following their optimisation via structure-activity relationship (SAR) assessments. This step is essential for compounds to enter further preclinical and then clinical development stages prior to registration and commercialisation (Ashburn and Thor, 2004; Lombardino and Lowe, 2004; McKim, 2010; Hughes et al., 2011; Geary et al., 2015; Andrade et al., 2016; Campbell, 2016; Matthews et al., 2016). This is an involved process, with many risks along the way, which explains why many anthelmintic candidates have not reached commercialisation (Hughes et al., 2011; Geary, 2016). Indeed, drug development is extremely challenging, with only 1 in 10,000 chemicals in the discovery process ever reaching the market (McKim, 2010; Matthews et al., 2016). 'Chemical death' occurs particularly in the latter stages of the development pipeline (Kola, 2008). Clearly, late-stage failures contribute substantially to the cost of bringing new drugs to market (Kola, 2008; McKim, 2010).

Given the challenges facing the pharmaceutical industry, it is crucial to prevent such attrition (Kola and Landis, 2004; Bowes et al., 2012; Jakovljevic and Ogura, 2016). Factors that can lead to drug development attrition are indicated in Fig. 7.1, with the two principal reasons for this failure being inefficacy and toxicity (cf. Kola and Landis, 2004; Kola, 2008; Hughes et al., 2011; Waring et al., 2015). In compiled attrition data for small molecule drug candidates destined for development in four large pharmaceutical companies (AstraZeneca, Eli Lilly, GlaxoSmithKline and Pfizer) between 2000 and 2010 (Fig. 7.1), toxicology terminations were most prominent in the preclinical phase (59.27%) and clinical phase I (25.48%), and a lack of efficacy was most prominent in clinical phase II (34.83%) (Waring et al., 2015). Thus, given the major pressure that pharmaceutical companies are under to circumvent drug development failures (Kola, 2008; Waring et al., 2015), the focus should be on overcoming the two 'key' factors/challenges (Kola and Landis, 2004; Bowes et al., 2012). In addition, drug formulation, bioavailability, manufacturing costs and commercial viability are also important aspects that need to be considered in the drug development process (Kola and Landis, 2004; Waring et al., 2015). For example, formulation is essential for the compound to be dissolved and absorbed at a particular pH.

Although drug discovery and development are long, complex, expensive and risky processes (Ashburn and Thor, 2004; Lombardino and Lowe, 2004; McKim, 2010; Geary et al., 2015; Campbell, 2016), there is an urgency to develop new and effective anthelmintics to circumvent established and emergent resistance problems in parasitic nematodes of animals (particularly ruminant livestock), to sustain parasite control into the future. In the present project, the identification and validation of 20 'hit' compounds offer a sound starting point for the translation of these chemicals into new anthelmintics (Fig. 7.2).

Chemical optimisation. In the drug development stage, chemical optimisation plays an essential role in assisting in maximising potency and minimising side effects of 'hit' compounds (cf. Lombardino and Lowe, 2004; Hughes et al., 2011). Medicinal chemists will decide on which analogues should be synthesised to explore structure-activity relationships, in an effort to maximise the desired activity (Lombardino and Lowe, 2004). In preliminary work, pyrazole-5-carboxamide derivatives have been synthesised and tested in SAR for enhancing potency and reducing toxicity. In the first instance, with the application of medium- to high-throughput screening, chemical optimisation relies heavily on data obtained from in vitro testing. Further optimisation and validation are required to assess whether the desired biological effect in vivo, e.g. in sheep, is achieved or not, and detect any side effects (cf. Lombardino and Lowe, 2004; Hughes et al., 2011).

Pharmacokinetic properties. In order to assess the compounds in vivo, the pharmacokinetic properties (absorption, distribution, metabolism, excretion and toxicity; ADMET) of test compounds would need to be defined (Hughes et al., 2011). In particular, the assessment of toxicity should be conducted once active analogues are obtained, and the next screen should eliminate any compounds with a high-risk profile (cf. Lombardino and Lowe, 2004; McKim, 2010). In the present project, all 'hit' compounds, except SNS-032, showed limited toxicity to normal breast epithelial cells (MCF10A) in vitro; however, further work needs to assess these compounds and their active analogues for toxicity in vivo as well as non-clinical toxicity, e.g., genotoxicity and mutagenicity.

Spectrum of activity. In order to develop a new anthelmintic with broad-spectrum activity, the compounds with most promise should be critically assessed for their activity on a range of socioeconomic-important parasitic nematodes (e.g., hookworms, ascaridoids, filarioids and whipworms). Although there is a lack of commercial incentives (by companies) to develop,

for example, chemotherapies against neglected tropical diseases affecting 1.8 billion people in developing countries (Hotez et al., 2016; Houweling et al., 2016), this step obviously would have major philanthropic and societal importance. SN00797439 has already shown potential for this purpose, considering that it acts against a range of parasitic nematodes including *A. ceylanicum* (hookworm of dogs, cats and humans), *T. muris* (whipworm of mice) and *B. malayi* (filarial worm of humans and some animals) as well as *D. immitis* (heartworm of canids).

Mode of action and resistance development. Other critical steps will be studies of the mode of action of any new anthelmintic entity and its potential to induce drug resistance (Vanden Bossche, 1990; Harder, 2016; Kotze and Prichard, 2016). Investigating modes of action will be important to support further work on lead compounds (Swinney and Anthony, 2011; Duke et al., 2013; Dos Santos et al., 2016). The use of advanced molecular, informatic and functional genomic (e.g., gene knockdown and knockout methods) technologies will be important to assist in exploring drug targets of new anthelmintics in the future.

7.6. Concluding remarks

The present thesis makes a contribution to the field of anthelmintic drug discovery and offers prospects for anthelmintic development, in light of the massive and widespread anthelmintic resistance problems. This general discussion shows that the future focus should now be on key areas including: (i) chemical optimisation of representative chemicals *via* structure-activity relationship (SAR) evaluations; (ii) assessment of the breadth of spectrum of anthelmintic activity on various parasitic nematodes, such as other strongyloids, ascaridoids, enoplids and filarioids; (iii) detailed investigations of the absorption, distribution, metabolism, excretion and toxicity (ADMET) of optimised chemicals with (relatively) broad nematocidal or nematostatic activity; (iv) establishment of the modes of action of lead candidates; (v) assessment of drug resistance development to lead compounds; and (vi) passage through pre- and clinical development phases, in which the dosage, bioavailability, therapeutic index, safety, formulation and other parameters of lead candidates need to be established. Clearly, collaborations among scientists from different fields (including parasitology, drug discovery, medicinal chemistry, clinical sciences and pharmacy and/or biotechnology) will be central to achieving these goals and toward the translation and subsequent commercialisation of effective new anthelmintic compounds.

7.7 References

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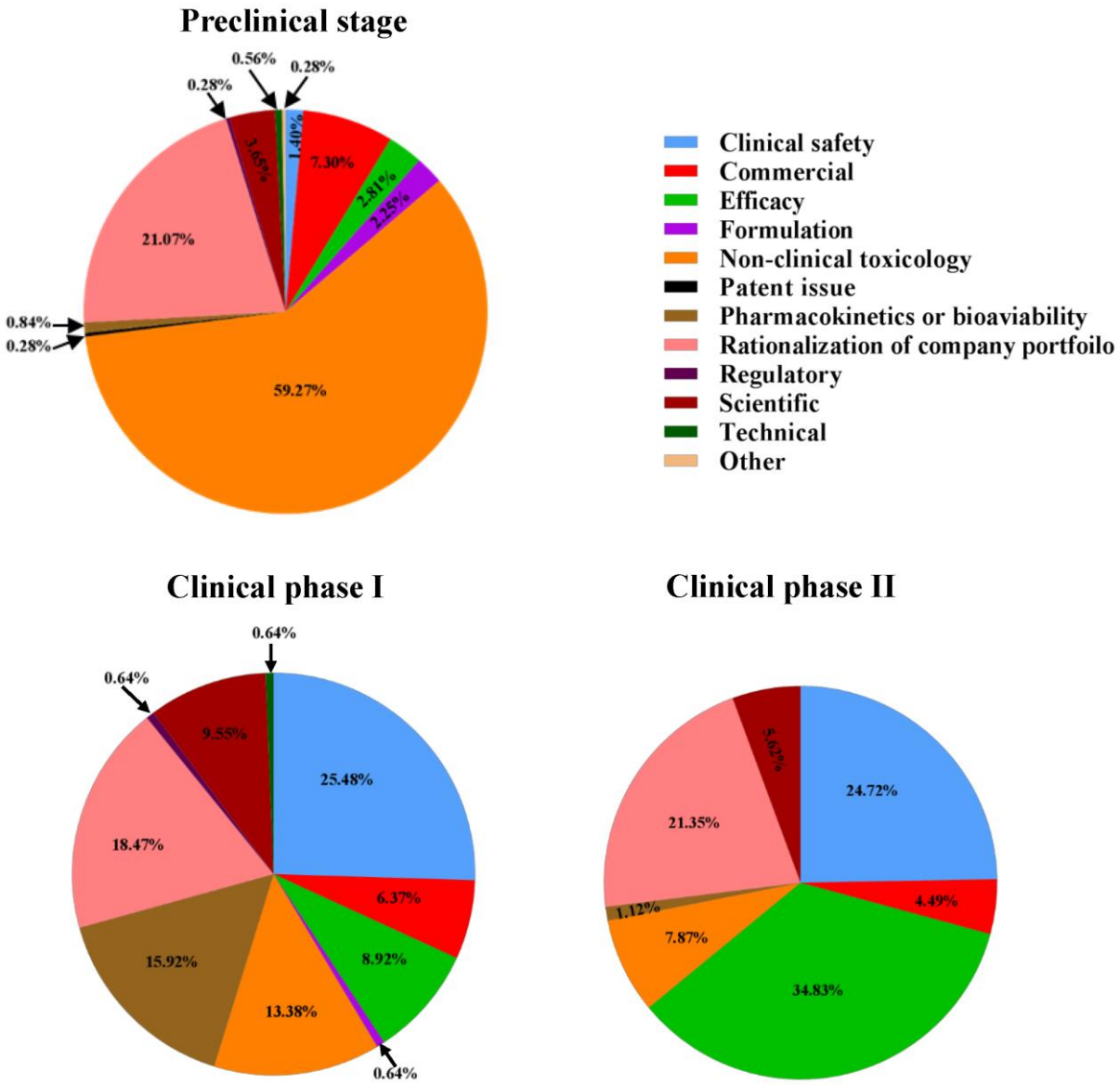

Fig. 7.1. Reasons for drug failure in preclinical, clinical phase I and phase II development. Data coming from an analysis of the attrition of drug candidates from four major pharmaceutical companies-AstraZeneca, Eli Lilly and Company, GlaxoSmithKline and Pfizer (Waring et al., 2015).

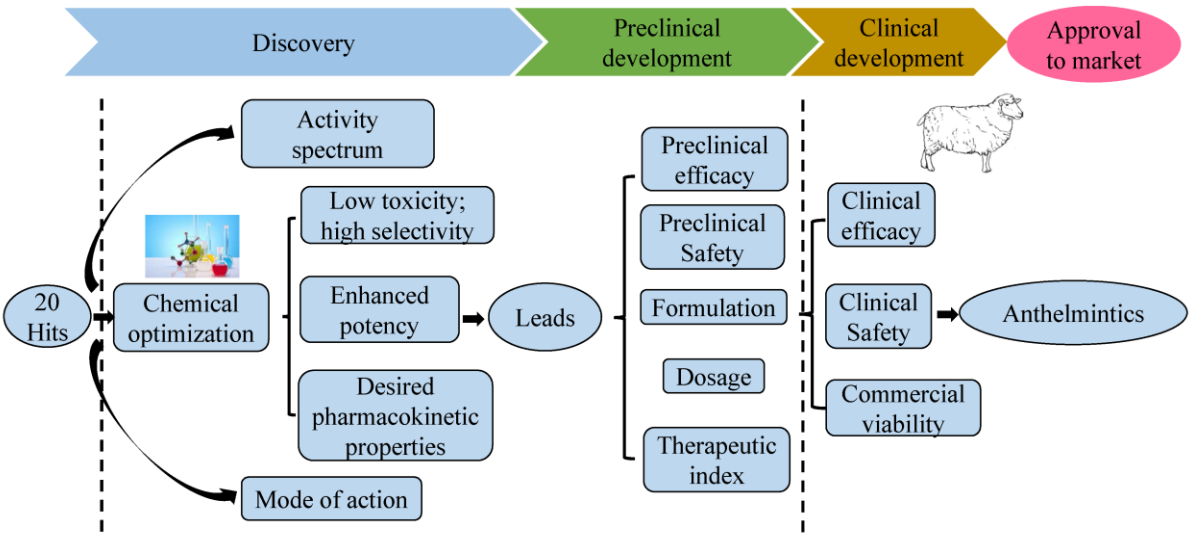

Fig. 7.2. Proposed development pipeline for new anthelmintics using *Haemonchus contortus* as a model. 20 Hits represent active chemicals identified with in vitro anthelmintic activities in this thesis, including one new chemical entity (designated SN00797439), two human kinase inhibitors (SNS-032 and AG-1295), 14 AG-1295 (tetrahydroquinoxaline) analogues, one insecticide (tolfenpyrad) and two tolfenpyrad (pyrazole-5-carboxamide) derivatives (a-15 and a-17).

LIST OF SUPPLEMENTARY FILES

CHAPTER 2

Supplementary file 2.1. Physicochemical properties of chemicals compounds in the 'Open Scaffolds' collection screened against exsheathed third-stage larvae (xL3s) of *Haemonchus contortus*.

Supplementary file 2.2. Five-second digital movies of live exsheathed third-stage larvae exposed to 20 μM of SN00797439 and 20 μM of each of the two positive-control compounds (i.e. monepantel and moxidectin); the negative control (LB* + 0.5% DMSO) does not contain compound.

CHAPTER 3

Supplementary file 3.1. Five-second video recordings of exsheathed third-stage larvae (xL3s) of *Haemonchus contortus* displaying the 'coiled' phenotype induced by exposure to AG-1295 (MMV079840; 100 μM). Videos of xL3s exposed to the same concentration of SNS-032 (MMV690767), monepantel, or no-compound were also included for comparison.

CHAPTER 4

Supplementary file 4.1. The 400 compounds in the 'Pathogen Box'.

CHAPTER 5

Supplementary file 5.1. The 55 pyrazole-5-carboxamide derivatives synthesized *de novo*.

Supplementary file 5.2. Five-second video recordings of *Haemonchus contortus* exsheathed third-stage larvae (xL3s) showing the "straight" phenotype induced by exposure to the pyrazole-5-carboxamides a-15, a-17 (test compounds) or tolfenpyrad (100 μM). Videos of xL3s exposed to the same concentration of tebufenpyrad, monepantel or moxidectin, or not exposed to any compound (LB* + 0.5% DMSO) were also included for comparison.

CHAPTER 6

Supplementary file 6.1. Organic synthesis and characterization of 14 tetrahydroquinoxaline derivatives of compound AG-1295.

Supplementary file 6.2. Video recordings of functional excretory canal (black arrow; pumping ~2.4 times per second) in untreated exsheathed third-stage larvae (xL3s.) of *Haemonchus contortus* (Panel A) and the Evi phenotype in xL3s treated with 12.5 µM of m10 (Panel B), employing phase-contrast light microscopy.

Supplementary file 6.3. Mean lengths (left) and widths (right) of exsheathed third-stage (xL3s) and fourth-stage (L4s) larvae of *Haemonchus contortus* under the same experimental conditions. Untreated xL3s and treated xL3s were measured at 0 h and after 7 days of exposure to 12.5 µM of m10, respectively; untreated L4s and treated L4s were measured at 0 h and after 72 h of exposure to 12.5 µM of m10, respectively. The data points represent the mean lengths or widths of 30 individual larvae ± the standard error of the mean. Significance of differences between values (mean ± standard error of the mean) was determined using a one-way ANOVA with Tukey's test. Asterisks indicate values that are significantly different from one another (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). This assay was repeated two times on different days.

Supplementary file 6.4. Detailed dynamics study of compound-induced Evi phenotype in the exsheathed third-stage larvae (xL3s) of *Haemonchus contortus* between 36 h and 48 h following compound exposure. Larvae were counted microscopically every 20 min. Corresponding concentration of DMSO was used as a negative control.