ADDRESSING ANTHELMINTIC RESISTANCE IN PARASITIC NEMATODES BY INVESTIGATING THE POTENTIAL OF A NOVEL DRUG TARGET AND THE DYNAMICS OF BENZIMIDAZOLE SELECTION IN A MODEL PARASITIC NEMATODE

by

LEONOR SICALO GIANECHINI

(Under the Direction of Andrew Moorhead)

ABSTRACT

Anthelmintic drugs constitute the primary means for treating and controlling infections with parasitic nematodes both in human and animals. However, ever-increasing levels of anthelmintic resistance are reported in all drug classes, involving virtually all of the most economically important parasites of domestic animals. Anthelmintic resistance is also becoming a significant concern in human medicine, especially with benzimidazole drugs, which are essential components of current therapeutic strategies. To establish sustainable control programs, it is essential to deepen our understanding of the mechanisms driving anthelmintic resistance in parasitic nematodes and, develop drugs with novel modes of action to overcome existing resistance. In chapters 2 and 3, we provide proof of concept and further validation of phosphoethanolamine methyltransferases (PMTs) as novel drug targets in parasitic nematodes. We first conducted the *in vitro* screening of a panel of 15 compounds with inhibitory activity against PMTs in *Haemonchus contortus*. Three PMT inhibitor compounds that showed *in vitro* activity in the lower micromolar range were

then tested *in vivo* using the jird (*Meriones unguiculatus*) – *H. contortus* model. When tested *in vivo*, these compounds achieved relatively high reductions in worm counts, with efficacy ranging from 53.5% to 72.6%. The third study investigated development of benzimidazole resistance in a field population of *H. contortus*, focusing on genotypic changes and their correlation with drug susceptibility. We applied a drug selection protocol involving 20 goats, and monitored drug susceptibility through egg hatch and larval development assays. The results from these tests were correlated with the deep amplicon sequencing and haplotype analysis of beta-tubulin. GLOWORM-FL simulations were performed to predict levels of egg excretion and larval contamination of pastures. Additionally, the archived samples collected throughout our drug-selection field study, will serve as the basis for further studies investigating non-beta tubulin genomic loci under selection from benzimidazole drugs. Overall, this dissertation provides valuable insights into the dynamics of benzimidazole selection under field conditions, and strong evidence supporting the potential of a new drug target in parasitic nematodes. Compounds exhibiting activity against this novel target could serve as a new generation of broad-spectrum anthelmintic drugs for the treatment of nematode infections in animals and humans.

INDEX WORDS: Anthelmintic resistance, benzimidazoles, *Haemonchus contortus*, gastrointestinal nematodes, beta-tubulin mutations, Phosphoethanolamine, novel drugs

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DEDICATION

This dissertation is dedicated to my father, mother, and sisters, who always believed in my ability to achieve anything I set my mind to. To Ignacio, my greatest support in life, and throughout this journey—I couldn't have done it without you. To Ignacio's family, thank you for supporting our dreams.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Nematodes are part of an ancient and diverse group of animals, which have intricate relationships with other species and inhabit a wide array of ecological niches (Maggenti 1981). From blood and tissues of both vertebrate and invertebrate hosts, to all parts of plants and virtually every terrestrial and aquatic landscape, nematodes demonstrate an unparalleled adaptability (Chitwood and Chitwood 1974, Maggenti 1981). The analysis of the nuclear small subunit ribosomal RNA gene (nSSU) has provided significant insights into the phylogeny of nematodes, delineating three principal classes, which can be further divided into five clades (Blaxter, De Ley et al. 1998, De Ley and Blaxter 2002). These lineages include Dorylaimia (Clade I), Enoplia (Clade II), and Chromadoria which comprises, Spirurina (Clade III), Tylenchina (Clade IV), and Rhabditina (Clade V) (Blaxter, De Ley et al. 1998, De Ley and Blaxter 2002, De Ley and Blaxter 2004, Smythe, Sanderson et al. 2006, Donn, Neilson et al. 2011). Among their varied lifestyles, parasitism emerges as a common way of life within this phylum (Blaxter, De Ley et al. 1998). Clade I includes a variety of nematodes found in soil, both as free-living organisms and as parasites of plants. It also includes important parasites of vertebrates such as *Trichinella*, *Trichuris* (whipworms), and *Dioctophyme*, the giant kidney worm (Blaxter, De Ley et al. 1998, De Ley and Blaxter 2004). Enoplia or clade II,

is predominantly composed of free-living nematodes inhabiting aquatic environments, although it also includes several species found in soil as well as plant pests capable of transmitting viruses (Blaxter, De Ley et al. 1998, De Ley and Blaxter 2004). Clade III, Spirurina, includes highly pathogenic parasites of animals and humans, such as filarial worms (*Brugia, Wuchereria, Onchocerca*, *Dirofilaria*), and ascarids (*Ascaris, Ascaridia, Toxocara*) (Blaxter, De Ley et al. 1998, De Ley and Blaxter 2004). In Tylenchina (clade VI), nematode species range from free-living bacterivores, transitory grazing root-hair feeders to the most virulent and economically important root-knot nematode, *Meloidogyne* spp. (Blaxter, De Ley et al. 1998, De Ley and Blaxter 2004). Some of the most economically important parasitic nematodes of domestic animals, however, are classified within clade V (Blaxter, De Ley et al. 1998). In this clade, we can find the Strongylida order, also called bursate nematodes. The principal superfamilies within the bursate nematodes group are the Strongyloidea, Trichostrongyloidea, Metastrongyloidea, Ancylostomatoidea and Diaphanocephaloidea (Chitwood and Chitwood 1974). These include nematodes parasitizing the gastrointestinal tract of ruminant animals (e.g. *Haemonchus, Ostertagia*, *Trichostrongylus, Cooperia*), respiratory system (*Dictyocaulus*) as well as the animal and human hookworms (e.g. *Ancylostoma*, *Necator*).

Notably, within this clade, alongside nematodes of veterinary and human significance, we also find *Caenorhabditis elegans* (Blaxter, De Ley et al. 1998, De Ley and Blaxter 2002). This soil, free living organism has offered the possibility to study general metazoan biology due to its many useful biological features (Brenner 1974). It can be easily grown in a laboratory setting, taking only 3 1/2 days to develop from egg

to egg-laying adult at 20°C, producing hundreds of offspring (Brenner 1974). Its experimental tractability is paired with an invariant number of somatic cells, making it possible to track the fate of every cell (Sulston, Schierenberg et al. 1983). These precise cell lineages were in fact, the basis for groundbreaking investigations behind the genetic regulation of organ development and programmed cell death. Given the relevance of this work in human diseases, the researchers Sydney Brenner, John Sulston, and H. Robert Horvitz were awarded the Nobel Prize for Physiology and Medicine in 2002 (NobelPrize.org. 2002).

Caenorhabditis elegans was the first multicellular organism to have its genome fully sequenced (Consortium 1998). The genome assembly is of excellent quality, extensively annotated, and highly adaptable to genome editing techniques (Wit, Dilks et al. 2021). Genotypic variation can be correlated with phenotypic traits across a wide diversity of strains and genetic backgrounds, allowing the identification of genetic variants influencing a trait (Cook, Zdraljevic et al. 2017). Consequently, this nonparasitic nematode has also become a model for systematically studying the genetic responses associated with exposure to anthelmintics, especially when pairing it with experimental results from model parasitic nematodes such as *Haemonchus contortus* (Rudolphi, 1803) (Wit, Dilks et al. 2021).

Of all the parasitic nematodes infecting the gastrointestinal tract of livestock, *Haemonchus* (Cobb, 1898) is considered one of the most successful. With 12 recognized species among this genus (Hoberg, Lichtenfels et al. 2004), it demonstrates a broad host range among livestock. Believed to originate in Africa among grazing and browsing antelopes, *Haemonchus* evolved to infect a diverse range of artiodactyl hosts

including 46 genera of Camelidae and Pecora (Hoberg, Lichtenfels et al. 2004). The domestication of cattle, sheep and goats, and the movement of animals due to major human migration events have allowed some species of *Haemonchus* to acquire a cosmopolitan distribution (Hoberg, Lichtenfels et al. 2004, Hoberg and Zarlenga 2016, Sallé, Doyle et al. 2019). Among them, *H. contortus*, *H. placei* (Place, 1893), and *H. similis* (Travassos, 1914) are often found circulating in domestic animals worldwide, but also tend to be the dominant species in North America (Zarlenga, Hoberg et al. 2016).

Haemonchus contortus, also known as the “barber pole” worm, is a highly pathogenic parasite commonly found in small ruminants worldwide (Scott and Sutherland 2009, Zajac and Conboy 2012). Its common name comes from its resemblance to the red and white barbers’ pole when its intestine becomes full following a blood meal. *Haemonchus* fourth-stage larvae and adults feed on host blood with a capacity to extract up to 30 µL of blood daily (Zajac 2006, Emery, Hunt et al. 2016). In severe cases, it can cause acute anemia and subsequent death even before infections become patent (Craig 2009). These cases occur most often in young, immunologically naive animals, or in adults whose immunity has been compromised, and exposed to high levels of infection (Zajac 2006, Craig 2009). However, chronic infections are more common and often go unnoticed by farmers since diarrhea is not a clinical sign associated to the disease (Soulsby 1965). A short prepatent period of 17 to 21 days (Levine 1968), along with a fecundity capacity of up to 10000 eggs per day/female, translates into a rapid accumulation of larvae in the pasture (Zajac 2006). For instance, a flock of 100 animals that are infected on average with 300 female worms each, laying up to 10000 eggs / day, would result in 3 billion eggs being put to the pasture every day.

In the Southeastern United States (US), *Haemonchus* spp. is often the predominant gastrointestinal nematode (GIN) in sheep and goats year-round (Terrill, Miller et al. 2012), giving it an enormous biotic potential during the grazing season. The large effective population sizes and high levels of genetic diversity in *Haemonchus spp.* provide them with a significant ability to develop resistance to all classes of drugs used for treatment (Gilleard and Beech 2007). In fact, *H. contortus* was the first parasite in which anthelmintic resistance (AR) was ever reported (Conway 1964, Drudge, Szanto et al. 1964). Consequently, it has emerged as a model parasitic nematode system for functional and comparative genomics, and for *in vitro* and *in vivo* drug screening (Geary 2016).

Unfortunately, the evolutionary success of parasitic nematodes comes at a cost to livestock, as well as humans. Not only through the huge impact on animal health and productivity, but also the indirect costs associated with parasite control and anthelmintic resistance (Charlier, Rinaldi et al. 2020). Therefore, it is important to deepen our understanding of the mechanisms driving anthelmintic resistance in parasitic nematodes, as well as to develop new drugs with novel modes of action (Nixon, Welz et al. 2020).

The work presented in this thesis has attempted to investigate the response of a model parasitic nematode to field selection with a benzimidazole drug, and to validate a novel drug target using the same parasitic nematode.

The following specific aims are addressed in this dissertation:

Specific aim 1: Validate Phosphoethanolamine methyltransferases as a viable drug target in parasitic nematodes. To accomplish this, we assessed the *in vitro* activity of 15 candidate PMT inhibitor compounds using two phenotypic assays; the larval development assay (LDA) and the larval motility assay (LMA). Compounds that exerted reasonably potent anthelmintic efficacy *in vitro* were then tested *in vivo*, using the gerbil (*Meriones unguiculatus*) – *H. contortus* model.

Specific aim 2: Study phenotypic, genotypic and genomic changes in *H. contortus* in response to field selection with a benzimidazole drug. To achieve this aim, twenty goats were infected with a drug-susceptible isolate of *H. contortus*, assigned to treatment or control group, and grazed on two paddocks. Subtherapeutic dosages of fenbendazole were administered at intervals of 4-8 weeks to the treatment group. Pre- and post-treatment fecal samples were collected for fecal egg count reduction testing, egg hatch assays (EHA), and larval development assays (LDA). Simultaneously, eggs were obtained from control animals for EHA and LDA. Samples from both groups were archived for genetic analysis. GLOWORM-FL simulations were performed to predict levels of egg excretion and larval contamination of pastures. Deep amplicon sequencing of beta-tubulin isotype-1 gene was paired with haplotype analysis to gain insights into the effects of drug selection on the overall population genetics. Whole genome sequencing will be performed at a later time and is not a component of this dissertation.

Epidemiology and biology of important parasitic nematodes of livestock

Gastrointestinal nematode parasites constitute one of the main constraints for optimal health and production in grazing ruminants (Gibbs 1992, Sykes 1994, Corwin 1997, Sykes and Greer 2003, Zajac and Conboy 2012). Infection with GIN occurs

directly from the pasture forage (Fig. 1) in a fecal-oral cycle, and thus is a natural and inevitable process for grazing livestock. Mixed infections with stomach and intestinal nematodes are called parasitic gastroenteritis (PGE), and most commonly lead to sub-optimal production (Charlier, Höglund et al. 2020). However, severe infections can result in clinical disease and even the death of animals (Charlier, Höglund et al. 2020). Weather, climatic conditions, host immunity, age, stocking density, and nutrition are some of the major factors influencing the magnitude and clinical severity of infections (Scott and Sutherland 2009). Because so many host and environmental factors are involved, the epidemiology of this disease is rather complex.

Strongylid gastrointestinal nematodes (Family: Strongylidae) are one of the most important parasites infecting ruminants (Zajac 2006, Charlier, Höglund et al. 2020). Their eggs are deposited with the feces, and develop to the infective L3 stage within the fecal pat (Fig. 1) (Charlier, Höglund et al. 2020). Once they reach this stage, L3s can withstand harsh environments due to the retention of the L2 sheath, which forms a protective double layer of cuticle. Though this feature improves their survival, this extra cuticle does not allow them to feed. Therefore, they rely on a restricted reserve of stored energy for survival, which they accumulate by feeding during their L1 and L2 larval stages (Rogers 1940). For transmission to occur, larvae need enough energy to endure metabolically challenging conditions such as high environmental temperatures (Rogers 1940), and effectively migrate from the fecal pats to the forage, and between the soil and forage (Armour 1980).

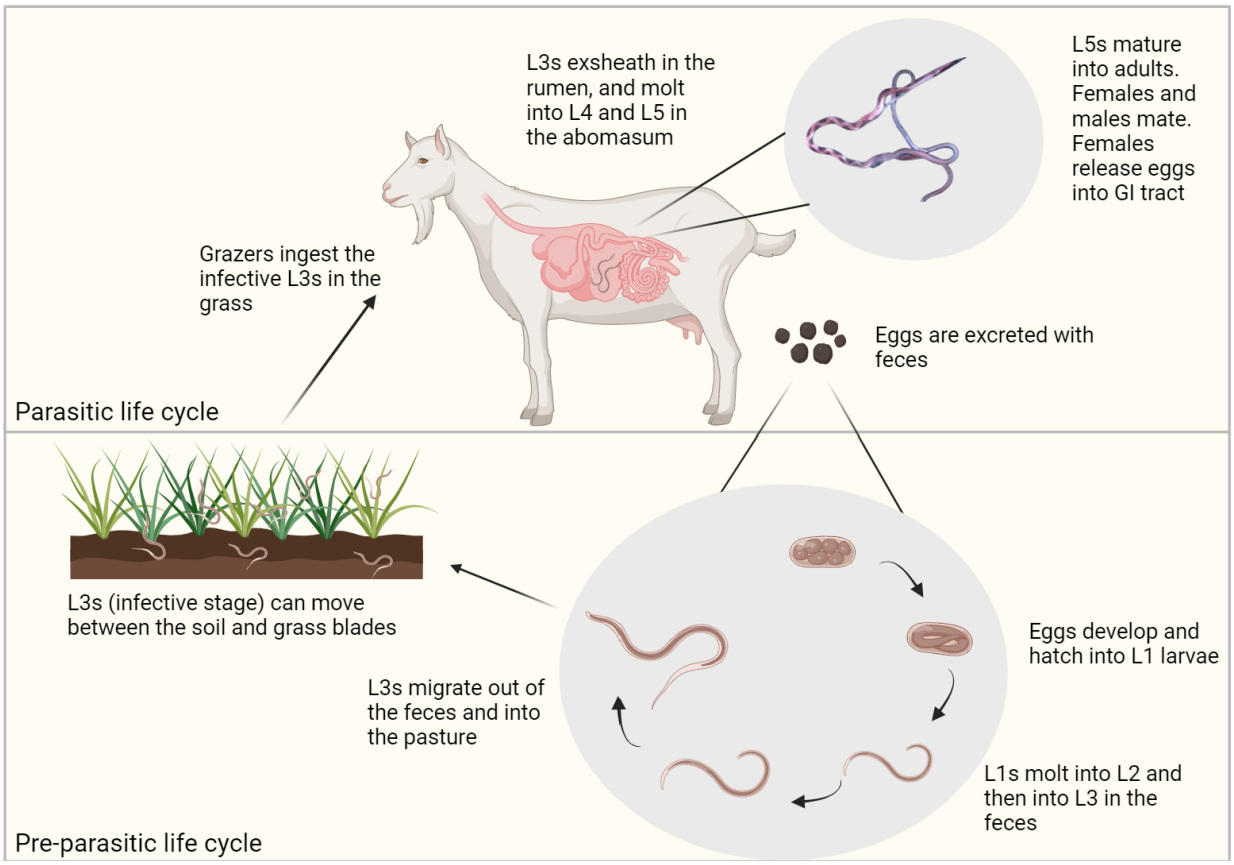

Figure 1. Life cycle of *Haemonchus contortus*. All strongylid nematodes follow a direct life cycle; however, the location of the adult stages within the gastrointestinal (GI) tract will vary according to the species of nematode. Created with biorender.com.

Larval contamination can rapidly increase in the pasture following periods of rainfalls, translating also into high worm burdens in the hosts (Armour 1980). However, the timing and levels of development and survival of free-living stages will vary in different climactic regions. For instance, in the Northern regions of the US, free living stages will be exposed to prolonged freezing temperatures for a substantial part of the year (Hildreth and McKenzie 2020). Worms will need to take advantage of the short summer to infect the animals and build up their numbers in the grass. On the contrary,

in the Southern states, transmission of GIN can occur during the entire year (Terrill, Miller et al. 2012, Navarre 2020). Though, the survival of cold tolerant species will be limited during the warm season and the survival of cold-intolerant species will be limited during the winter (Navarre 2020). Therefore, the prevalence, distribution and seasonal patterns of GIN transmission will be different across region and season. However, the levels of larval contamination on pasture follow predictable seasonal patterns with lowest levels tending to occur early and highest levels tending to occur late in the grazing season (Eysker, van der Aar et al. 1998). As the grazing season progresses, young animals will re-cycle the worms on the pasture, yielding increases in their worm burdens and fecal egg counts (FEC), which then lead to increased levels of pasture contamination (Snyder 1993). As this process continues, these first-year grazers begin to develop immunity, which then leads to reduced worm burdens and FEC (Charlier, Höglund et al. 2020). In this regard, sheep and cattle will usually develop immunity against worms by their second grazing season, whereas goats remain fairly susceptible even as adult animals (Michel 1963, Vercruysse and Claerebout 1997, Nisbet, Meeusen et al. 2016). Immunity can manifest not only as a reduction in worm burdens in the host, but also as stunted growth of adult worms, inhibition of ovulation resulting in a reduced prolificity, and also as resistance to the establishment of newly acquired worms (Michel 1963). Another important epidemiological feature occurs in ewes and does, whereby they experience a temporary immunosuppression that spans a period from 2 weeks prior to lambing/kidding, until around 8 weeks post-partum (Taylor 1935, O'Sullivan and Donald 1970). During this periparturient period, nutrients are prioritized for fetal development, mammary development, and milk production, leading to a decrease in

immunity and subsequent increase in nematode egg shedding, and consequent pasture contamination (Taylor 1935, O'Sullivan and Donald 1970). This periparturient rise in FEC plays an important role in transmission of infective larvae from ewes to lambs during the spring (Gibbs 1982). This phenomenon does not occur in cattle (Snyder 1993). Adult cows will typically shed few GIN eggs (<25 eggs per gram; epg) even when harboring moderate worm burdens (Armour 1980). But the large fecal pats of cattle can act as a reservoir source of infective larvae for a long time. Even in harsh cold climates, larvae of some species of cold-tolerant worms can overwinter in the fecal pats and survive in sufficient numbers to get the cycle started again in the spring (Wang, Avramenko et al. 2020). Yet, other cold-intolerant species like *Haemonchus* will survive the winter arrested in the host, as opposed to inside the fecal pats (Waller, Rudby-Martin et al. 2004). So, depending on the species of worm, different strategies are used that enable them to survive severe environmental conditions in the host, in the pasture, or in both, and start the cycle of infections in the new crop of calves / lambs.

GIN commonly associated with PGE in small ruminants

In the US, the most predominant and pathogenic GIN infecting small ruminants is *H. contortus* (Zajac 2006). This worm feeds directly on the host's blood, resulting in a substantial loss of red blood cells and blood proteins. This loss can lead to the classic symptoms of the disease, including anemia and a condition known as “bottle jaw” (Zajac 2006, Craig 2009). Severe infections leading to death are most commonly observed in young animals during their first grazing season. Other highly pathogenic species, such as *Teladorsagia circumcincta* are found throughout the US but are not as clinically

relevant as *H. contortus* (Zajac 2006). However, in sufficient numbers, *T. circumcincta* can cause clinical manifestations that resemble ostertagiosis in cattle (Levine 1968). These can be very detrimental and an economic burden to the sheep industry in places where *T. circumcincta* is the most prevalent GIN, such as the United Kingdom (Burgess, Bartley et al. 2012). *Trichostrongylus* spp. are another GIN commonly involved in PGE of small ruminants in the US (Zajac 2006, Craig 2009). Some species of *Trichostrongylus* are found in the abomasum (*T. axei*), while others are found in the intestine (*T. colubriformis, T. vitrinus*) (Zajac 2006, Craig 2009). The pathophysiology of this disease is associated with the changes in the abomasal mucosa, or villus atrophy in the anterior small intestine, that leads to appetite loss, lethargy, malabsorption and severe diarrhea. The profuse diarrhea with dark fetid feces, dry, dirty wool will be the predominant clinical sign (Craig 2009), and the reason why “Black scours” is the common name for this PGE.

Though most species of GIN are rather host-specific, there are several species of nematodes that infected both cattle and sheep. The most common of these include *Trichostrongylus axei*, several species of *Cooperia* and *Nematodirus*, and *H. contortus* (Toledo, Porcel et al. 2019). This could potentially be of relevance when co-grazing small and large ruminants, but only rarely leading to infections that are clinically significant. In fact, the complementary foraging behaviors and dietary preferences between species can be used to optimize the utilization of different available resources, sustaining the nutritive value of forage sources throughout the grazing season (d'Alexis, Sauvant et al. 2014, Fraser, Moorby et al. 2014). This, in turn, has been associated with increased body weight gain per animal and per hectare (d'Alexis, Sauvant et al. 2014,

Fraser, Moorby et al. 2014), as well as improved production efficiency (Jerrentrup, Komainda et al. 2020).

GIN commonly associated with PGE in cattle

Parasitic gastroenteritis is commonly associated with a mixed infection of GIN (Charlier, Höglund et al. 2020), located in the abomasum and intestines of their hosts. Subclinical disease is more common, affecting production parameters without manifesting the classical PGE clinical signs such as watery diarrhea, poor body condition, dull hair coat and anorexia (Zajac 2006, Scott and Sutherland 2009, Charlier, van der Voort et al. 2014, Charlier, Höglund et al. 2020).

Ostertagia spp. and *Cooperia* spp. are the most prevalent species of GIN infecting cattle in temperate climates (Scott and Sutherland 2009). *Ostertagia* spp. are the most pathogenic species, causing severe damage in the glandular mucosa of the abomasum as a consequence of invading and developing in the fundic glands and then emerging from them as much larger worms than when they entered (Myers and Taylor 1989, Fox 1993). *Ostertagia* spp. can undergo arrested development in the host when climatic conditions are not optimal for its survival in the environment (Chalmers 1980, Snyder 1993). In the Southern US, arrest will occur in the host during the summer, whereas in the Northern US, this period of arrest occurs during the winter (Myers and Taylor 1989, Snyder 1993, Hildreth and McKenzie 2020). This seasonal accumulation of arrested early 4th stage larvae of *Ostertagia* spp. is termed hypobiosis, and allows the parasite to synchronize its lifecycle with conducive environmental conditions for parasite transmission, ensuring survival of free-living stages (Eysker 1997). These hypobiotic

larvae have low metabolism and can survive for long periods before resuming development (Eysker 1997), and maturing to egg-laying stages.

The synchronous reactivation of *Ostertagia* larvae from the abomasal mucosa following this period of arrest can cause extensive tissue damage and impair the gastric functions of this organ (Fox 1993). This may result in clinical disease marked by significant weight loss, hypoproteinemia, diarrhea, anorexia and even death (Fox 1993). *Cooperia* spp., are found in the small intestine and though they are less pathogenic than *Ostertagia* and *Haemonchus*, they may produce significant negative effects in growing cattle (Stromberg, Gasbarre et al. 2012). Consequently, despite not causing clinical disease to the extent of *Ostertagia*, substantial production losses can result from high levels of infection with *Cooperia*. Over the past few decades, because of the heavy use of macrocyclic lactone drugs (e.g., ivermectin) (Kaplan 2020), and the propensity of this worm to become resistant to this drug class (Benz and Ernst 1979, Egerton, Ostlind et al. 1979, Bisset, Brunsdon et al. 1990), the relative intensity of *Cooperia* compared to other species has risen substantially over the past 40 years. Considering these facts, *Cooperia* should be regarded as an important pathogen in young cattle both in the Northern and Southern regions, during the first grazing season.

In the Southern states, in addition to *Ostertagia* and *Cooperia spp.*, *H. placei* can also have an important impact on the health and productivity of cattle (Navarre 2020). *Haemonchus* spp. feed on blood and may cause anemia, hypoproteinemia, and edema in affected animals. However, these effects are usually much less severe in cattle as compared to the levels of disease observed in small ruminants by *H. contortus*.

Haemonchus spp. can also undergo a period of arrested development, typically during cold winter conditions (Blitz and Gibbs 1972).

Strategies for controlling infection with gastrointestinal nematodes; the role of anthelmintic drugs

The goal of a sound parasite control program for livestock should focus on eliminating clinical presentations of parasitism, minimizing sub-clinical losses, and preserving the effectiveness of dewormers (Stromberg and Gasbarre 2006). It is not desirable nor advisable to pursue complete “elimination” of gastrointestinal nematodes. This is not possible, and will only accelerate the pace at which resistance to anthelmintic drugs develops (Kaplan 2020). Infections with GIN are a natural part of being a grazing ruminant, and in many animals cause little harm. Hence, maintaining an equilibrium between tolerable levels of infection with GIN and the resulting production losses is crucial (Charlier, Velde et al. 2015). Our goal then is to minimize production losses and clinical disease at the herd level. Though commonly performed, whole-herd deworming treatments are not needed to achieve this. The administration of highly efficacious anthelmintics to animals that would benefit from treatment will achieve this, and is more sustainable in the long term rather than frequent whole-herd treatments with a partially effective anthelmintic (Leathwick, Miller et al. 2008, Leathwick, Hosking et al. 2009, Bartram, Leathwick et al. 2012, Leathwick 2012, Leathwick and Besier 2014). However, since the introduction of safe and broad spectrum anthelmintics 60 years ago (Gordon 1961), parasite control of production animals has relied almost exclusively on the widescale use of these drugs (Kaplan 2020). Unfortunately, this approach has heavily selected for multiple drug resistant GIN, resulting in a reduced

effectiveness of pharmaceutical control (Sutherland and Leathwick 2011). Consequently, the aim is to achieve a control program that is both effective and sustainable. However, sustainable parasite control strategies will not be addressed in detail here but have been reviewed elsewhere (Scott and Sutherland 2009, Leathwick and Besier 2014, Burke and Miller 2020).

Pharmaceutical-based parasite control

There are only four major classes of anthelmintic drugs available on the global veterinary market, including the benzimidazoles (BZs), macrocyclic lactones (ML), nicotinic acetylcholine receptor agonists (nAChR), and amino-acetonitrile derivatives (AADs) (Nixon, Welz et al. 2020). The introduction of the BZs and pro-BZs in the 1960s (Gordon 1961) to veterinary medicine fundamentally changed the way parasite control was practiced over the next 60 years. This new broad spectrum, highly efficacious, and safe drug class offered major advantages over previously used chemicals.

Benzimidazoles (BZ)

Benzimidazoles have a broad spectrum of action, being used to treat infections with nematodes, cestodes, trematodes and some protozoa (O'handley, Olson et al. 1997). Their broad spectrum of efficacy, along with wide safety margins has made this drug class very attractive in human and veterinary medicine. Structurally, BZs are a bicyclic ring system with benzene fused to the 4 and 5 positions of an imidazole molecule (Townsend and Wise 1990). Moreover, modifying positions 2 and 5 of the BZ ring system, has led to the development of highly efficacious derivatives, including fenbendazole (Baeder, Bähr et al. 1974), oxfendazole (Averkin, Beard et al. 1975), and

albendazole (Theodorides, Gyurik et al. 1976). Some of which have also high efficacy against inhibited larval stages of GIN (Baeder, Bähr et al. 1974, Theodorides, Gyurik et al. 1976). But their lack of solubility in water affects drug formulation, thus most of the products are sold as oral suspensions or pastes.

Benzimidazole and pro-BZ compounds exert their mechanism of action by selectively binding to parasite tubulin and disrupting the polymerization of tubulin dimers that form the cell microtubules (Cleveland and Sullivan 1985, Lacey 1988) (Fig. 2). Microtubules are a crucial component of the eukaryotic cytoskeleton and play an essential role in cell division, intracellular transport, and structural support (Desai and Mitchison 1997). They are polar structures formed by the association of alpha and beta-tubulin heterodimers (Amos and Klug 1974). Microtubules undergo dynamic instability (Mitchison and Kirschner 1984), a process characterized by phases of rapid polymerization (growth) and depolymerization (shrinkage) at their ends. The rapid growing end is referred to as the plus end, while the slower growing end as the minus end (Allen and Borisy 1974). During polymerization, tubulin subunits assemble onto the growing microtubule end in a process called "treadmilling," extending the microtubule length. However, this growth phase can abruptly switch to depolymerization, during which tubulin subunits disassemble from the microtubule end, causing rapid shrinkage (Fig. 2). This dynamic behavior allows microtubules to explore cellular space, search for cellular targets, and respond rapidly to intracellular signals. Benzimidazoles bind to beta-tubulin, disrupting the microtubule assembly and dynamics (Lacey 1988).

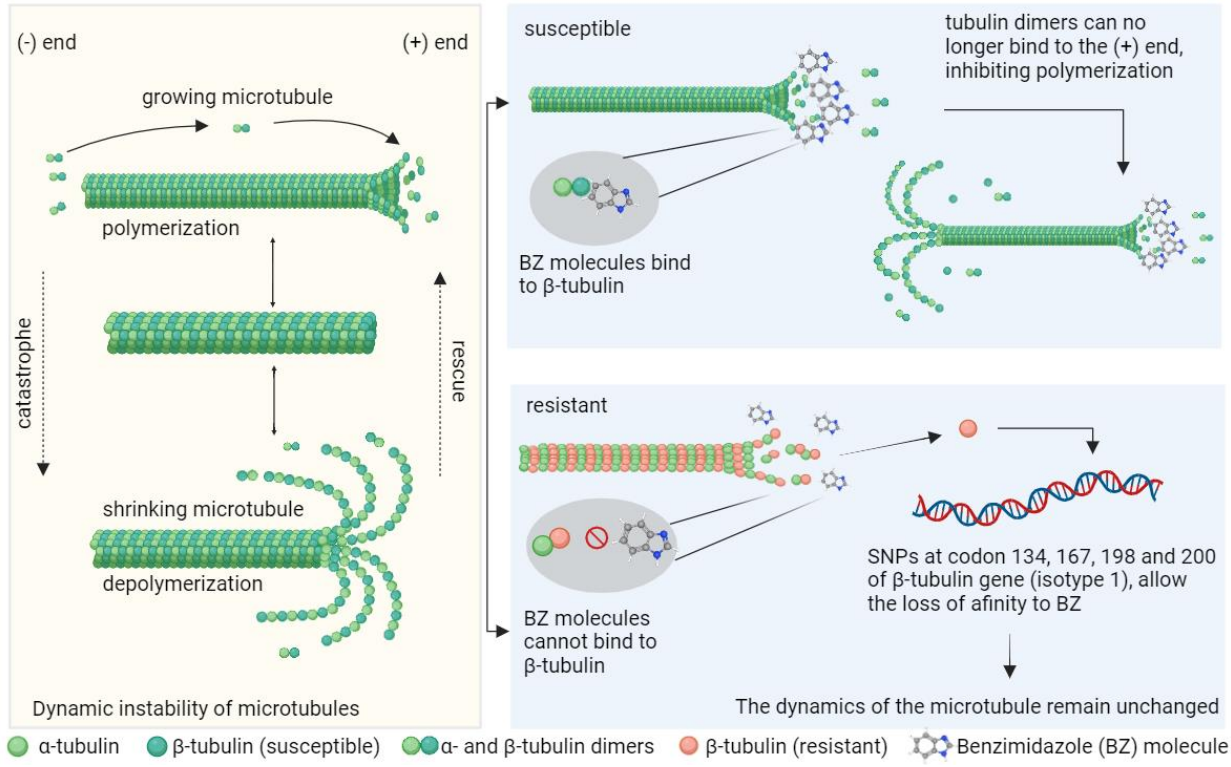

Figure 2. Mechanism of action of benzimidazole drugs. Created with biorender.com

Benzimidazole methylcarbamate drugs (e.g. albendazole and fenbendazole), undergo a two-step S-oxidation process, producing sulphoxide and sulphone metabolites, in which liver enzymes play a fundamental role (Virkel, Lifschitz et al. 2004, Lanusse, Canton et al. 2018). Furthermore, the sulphoxide metabolites have less anthelmintic potency compared to their parent drugs, while sulphones are nearly inactive (Lanusse, Canton et al. 2018). Therefore, liver oxidation significantly decreases the efficacy of the original compounds. That is why *in vivo* interference with the liver oxidative metabolism has led to significant changes in the pharmacokinetics and/or increased systemic availability of active benzimidazole compounds (Virkel, Lifschitz et al. 2004). This in turn has been shown to improve efficacy against resistant isolates of

sheep gastrointestinal nematodes (Benchaoui and McKellar 1996). In the case of pro-BZ (e.g. febantel), the drug administered is converted to the active metabolites responsible for the anthelmintic action by metabolic processes in the host animal (Lanusse and Prichard 1993). Pro-benzimidazoles have better solubility properties than other forms, and some, have been developed to overcome the absorption problems seen with the directly active drugs (Lanusse and Prichard 1993). The difference in solubility, even when small, may greatly influence the absorption of the drug, and the overall efficacy of the product (McKellar, Harrison et al. 1990, Lanusse and Prichard 1993). Less soluble drugs, such as fenbendazole, reach maximal plasma concentrations 24 hours post administration in cattle (Lanusse and Prichard 1993). In sheep, fenbendazole and its metabolites, fenbendazole sulphoxide (FBZSO) and fenbendazole sulfone ($FBZSO_2$) reach maximal plasma concentration at 24, 30, and 36 hours, respectively when administered orally (Marriner and Bogan 1981). In the abomasum, peak concentrations of fenbendazole, FBZSO and $FBZSO_2$ were achieved at 30, 48, and 72 hours, respectively. In cattle treated with fenbendazole, the same pattern of metabolites is found in plasma, however the ratio of fenbendazole to FBZSO is slightly different (Prichard, Hennessy et al. 1985).

Furthermore, the rate of residence time in the gut has a profound effect on the absorption and bioavailability of BZs (McKellar, Harrison et al. 1990). Therefore, animals with lower volume of content in their guts (e.g. monogastric vs ruminant) may require repeated doses of BZs to achieve optimal efficacy (Marriner 1986). In ruminants, it has been shown that fasting the animals for periods of 12 – 14 hours prior to drenching, reduced the digesta flow rate improving the anthelmintic activity of BZs (Ali and

Hennessy 1995). This effect might be greater in the case of fenbendazole, since it counts with reduced solubility in abomasal content when compared to albendazole or oxfendazole (Lanusse and Prichard 1993). Therefore, the slow release of fenbendazole and other BZ drugs to the abomasum, can result in higher bioavailability of the parent drug and its metabolites (Lanusse and Prichard 1993). Since BZs enter parasites through passive diffusion (Mottier, Alvarez et al. 2006), extending the contact time with the drug can lead to increased absorption, and efficacy.

However, the rumen not only acts as a drug reservoir, but also the place for metabolic reduction of the sulfoxide metabolites back to the parent drugs (Lanusse, Nare et al. 1992). As a result, albendazole sulfoxide and FBZSO can be converted back to their respective parent drugs by ruminal and intestinal microorganisms, serving as a source of albendazole and fenbendazole in the GI tract (Lanusse, Nare et al. 1992, Lanusse and Prichard 1993). Consequently, the bacteria-mediated reduction may play a key role in enhancing drug efficacy against GIN.

Macrocyclic lactones (ML)

The introduction of ivermectin in the 1980s (Chabala, Mrozik et al. 1980) marked a significant advancement in parasitic treatment. In addition to high efficacy and a high safety profile, this new drug class (macrocyclic lactones; ML) counted with endectocide activity, transforming antiparasitic therapies for production animals, heartworm treatment for companion animals, and filarial chemotherapy for humans (Shoop and Soll 2002). Ivermectin, the first ML formulated for animal use, is produced by the fermentation of *Streptomyces avermitilis* (Shoop and Soll 2002). Structural modifications, and microbial

fermentation research, allowed the ML family to proliferate in response to the market needs (Campbell 2016).

The ML basic chemical structure is characterized by a 16-member cyclic pharmacophore coupled to a spiroketal group, a benzofuran ring and disaccharide functionalities (Campbell 1989). It is this unique pharmacophore, that gives all MLs the capacity to bind to the same receptors, and exert the same spectrum of action (Shoop and Soll 2002). While the pharmacophore of both classes of MLs (avermectins and milbemycins) remains identical, these count with different substitutions at positions C-13, C-22,23, and C-25. For example, avermectins have a sugar moiety termed bisoleandrosyloxy at C-13, whereas milbemycins lack a substituent at that position (Shoop and Soll 2002).

They are believed to exert their mechanism of action by binding to glutamate-gated chloride channels in the membranes of pharyngeal muscles, motor nerves, female reproductive tracts, and the excretory/secretory pores of nematodes and in nerve and muscle cells of insects and crustaceans (Campbell 1989, Prichard, Ménez et al. 2012). These ion channels are crucial for regulating the flow of chloride ions across cell membranes, leading to hyperpolarization of the membrane, inhibition of neuronal signaling and subsequent paralysis of the nematode's musculature (Prichard, Ménez et al. 2012). The effect in susceptible GIN is concentration dependent, and includes paralysis of pharyngeal pumping and of body-level motility, resulting in starvation, paralysis, and expulsion of the worms from their location (Kass, Wang et al. 1980, Turner and Schaeffer 1989, Geary, Sims et al. 1993). In addition to the paralytic effect, MLs can also impact the reproductive capabilities of certain parasites. This is achieved

by reducing oviposition rates or inducing abnormal oogenesis (Li, Rush et al. 2014, Ballesteros, Tritten et al. 2016). This drug class is remarkably potent, reaching therapeutic activity at doses of 200 - 300 µg/kg in ruminants, pigs, and horses, yielding C_{max} plasma concentrations of 11 - 54 ng/mL (González Canga, Sahagún Prieto et al. 2009, Martin, Robertson et al. 2021). But only 0.5 - 1 ng/mL plasma concentrations, are required for optimal anthelmintic activity against most gastrointestinal and lung nematodes in cattle (Lifschitz, Virkel et al. 1999).

When ivermectin is administered via subcutaneous injection in ruminants, it is able to distribute effectively into lipid compartments, resulting in a three-fold increase in its residence time and a prolonged half-life of 114 to 216 hours (Marriner, McKinnon et al. 1987, Lanusse, Lifschitz et al. 1997, Toutain, Upson et al. 1997). The C_{max} in peripheral plasma of cattle, in this case, occurs ~96 hours (Lanusse, Lifschitz et al. 1997, Toutain, Upson et al. 1997). When administered orally, ivermectin and doramectin exhibit a high association of approximately 97-99% with the rumen particulate matter (Ali and Hennessy 1996, Hennessy, Page et al. 2000), functioning as a gastrointestinal reservoir for exchange of drug into gastrointestinal fluid (Hennessy and Alvinerie 2002). High levels of ivermectin in gastrointestinal content following oral administration, have been associated with higher levels reaching *C. oncophora* in the intestine (Leathwick, Miller et al. 2020). However, when using the subcutaneous route, higher levels of ivermectin were observed in the abomasal mucosa, and correlated to higher levels of drug in *O. ostertagi* (Leathwick, Miller et al. 2020). Therefore, the route of administration will influence the distribution of the drug in the different compartments (Hennessy and

Alvinerie 2002), and the level of exposure of different GIN to the drug (Leathwick, Miller et al. 2020).

The high lipophilic profile and potency of this drug class has also allowed the adoption of topical formulations (Hennessy and Alvinerie 2002). The ease of administration and the broad-spectrum endectocide activity has played a major role in the frequent use of MLs in the livestock industry. Though convenient and easy to administer, the concentration of drug that reaches target organisms, has been shown to be more variable with pour-on formulations (Bisset, Brunsdon et al. 1990, Laffont, Bousquet-Mélou et al. 2003). Additionally, poor application technique is common, further decreasing the amount of drug that is absorbed by the animal. The lower than optimal tissue drug levels that are prone to occur in some animals treated with pour-on products increases the risk of suboptimal doses reaching the worms (Forsyth, Gibbon et al. 1983, Sallovitz, Lifschitz et al. 2005, Bousquet-Mélou, Jacquiet et al. 2011, Leathwick, Miller et al. 2016). This will place high levels of drug selection on worm populations by allowing more resistant genotypes to survive treatment (Sargison, Wilson et al. 2009, Sutherland and Leathwick 2011).

Imidazothiazoles (Levamisole)

In the 1960's, Janssen Pharmaceutica engaged in a research program that ended up discovering the first imidazothiazole derivative, tetramisole hydrochloride (Thienpont, Vanparijs et al. 1966, Janssen 1976). The primary anthelmintic activity of this racemate lies within its levorotatory isomer, levamisole, which shows several times greater potency compared to dexamisole, the dextrorotatory isomer, yet with comparable toxicity levels (Raeymaekers, Roevens et al. 1967, Bullock, Hand et al.

1968). As a result, the D-isomer was separated from the racemic mixture, paving the way for the development of the L-isomer known as levamisole (Janssen 1976).

This anthelmintic exerts its action by paralyzing the worms, leading to their passive elimination from the host. This effect is linked to its ability to open nonselective cation ion channel receptors (AChRs) present on nematode nerve and body muscle (Harrow and Gration 1985, Martin 1997). When these channels open, they trigger depolarization, allowing calcium to enter the muscle cells and leading to contraction. These events result in spastic paralysis of the nematode, causing it to be expelled from its original location (Harrow and Gration 1985).

Levamisole is characterized by its rapid onset and short duration of action, being rapidly absorbed from the gastrointestinal tract or injection site (Renoux 1980). Plasma concentrations are higher, and the peak concentrations occur significantly earlier following a subcutaneous administration compared to oral or intraruminal routes in sheep (Marriner 1980). However, levamisole is found at higher concentrations in the abomasal fluid when the drug is administered orally. This is followed by the subcutaneous route and lastly, by intraruminal administration (Marriner 1980). Peak plasma concentrations typically occur between 10 and 80 minutes, following oral administration in small ruminants and cattle (Galtier, Escoula et al. 1981, Fernández, García et al. 1998, Myers, Howard et al. 2021). In ruminants, levamisole is used to treat nematode infections, though it has shown variable efficacy against the immature stages of some important GIN (Anderson 1977). However, it constitutes a key drug class in farms where resistance to the BZs and MLs is in the advanced stages. It is suggested that the subcutaneous route, which achieves higher plasma concentrations, and

therefore higher lung concentrations, would be the route of choice for treating lungworm (*Dictyocaulus*) infections (Marriner 1980). Conversely, the oral route, which achieves higher abomasal concentrations, would be the preferable route for treating infections with gastrointestinal nematodes (Marriner 1980). In the US, levamisole can be found as a single drug for oral delivery in small ruminants and cattle, and recently, also for cattle, in combination with doramectin to be administered subcutaneously.

Parasite control programs

The incorporation of anthelmintics into a parasite control program should be planned considering the species of nematode, the stage we are targeting, the presence of refugia at the time of treatment, the spectrum of action of the drug of choice, and the levels of anthelmintic resistance in the farm (Stromberg and Gasbarre 2006, Greer, Van Wyk et al. 2020).

The refugia-based strategy

In sheep, several novel approaches have proven effective for reducing the development of resistance and in managing resistant gastrointestinal nematodes. The common factor among these approaches is the preservation of refugia and the implementation of high efficacious treatments (Greer, Van Wyk et al. 2020, Kaplan 2020). By administering anthelmintics only to the animals that are suffering the consequences of parasitism at clinical and/or economically relevant levels, and thus will most likely benefit from treatment, herd-level parasite control can be achieved while a allowing a proportion of worms to escape drug selection (Van Wyk 2001). As this strategy is based on the principle of preserving and managing refugia, these are often referred to as refugia based strategies. Within the context of drug resistance, refugia

represent the portion of the worm population that have escaped drug selection, either in animals that did not receive treatment or by being in the environment (on pasture) at the time of treatment (Van Wyk 2001, Greer, Van Wyk et al. 2020). By escaping drug selection, this portion of the worm population will help maintain a pool of drug susceptible alleles that will dilute the resistant worms that survived treatment (Van Wyk 2001). Furthermore, the fewer the number of surviving resistant worms, the greater the dilution effect from the refugia. By maximizing the efficacy of all anthelmintic treatments that are administered, the number of surviving resistant worms will be minimized (Bartram, Leathwick et al. 2012, Lanusse, Canton et al. 2018). Achieving high efficacy therefore results in greater levels of dilution by existing refugia, thereby maximizing the benefits of refugia-based strategies (Bartram, Leathwick et al. 2012). Given the reductions in efficacy that can be expected due to drug resistance, the only practical means available to increase the efficacy of treatments is to use anthelmintics in combination (Kaplan 2020), whereby two or more different anthelmintics from different drug classes are administered at the same time.

There are 3 major benefits to using drugs in combination (Bartram, Leathwick et al. 2012, Geary, Hosking et al. 2012, Lanusse, Canton et al. 2018). (1) There is an additive effect with each drug used, thus the efficacy of the treatment increases with each additional drug given. (2) There is a return to broad-spectrum efficacy; since resistance is species and drug specific, a second (or third) drug may kill any species resistant to the first (or second) drug. This will then return the broad-spectrum efficacy that one aims to achieve (and that is specified on the product label). (3) By achieving a higher efficacy, there are fewer resistant survivors, thus there is a greater dilution of

resistant worms by the susceptible portion of the population (Kaplan 2020). But, for this strategy to achieve the desired goal, anthelmintics included in the combination need to have at least 60% efficacy or higher (Dobson, Barnes et al. 2011, Leathwick, Waghorn et al. 2012). If 2 anthelmintics each with 60% efficacy are included, then the treatment will yield an 84% overall efficacy. But if a 3rd drug with a 70% efficacy is included, then the efficacy increases to 95%. It is important to note that the sooner a combination treatment strategy is implemented, the greater the benefits since the greatest difference in the percent of resistant survivors is seen when efficacy of anthelmintics is very high. Thus, this strategy should be implemented immediately on farms, and not just used as a last-ditch salvage approach once resistance becomes a severe problem on the farm (Kaplan 2020).

Anthelmintic resistance

Anthelmintic resistance in a population of nematodes occurs when “a greater frequency of individuals within the population are able to tolerate doses of a compound than in a normal population of the same species, and is heritable” (Prichard, Hall et al. 1980). When anthelmintic drugs were first introduced, they were highly efficacious against most species of GIN. For example, when first registered in the US, fenbendazole 10% suspension (Safe-Guard®, Panacur®) showed efficacies greater than 96.9% against *Ostertagia ostertagi*, *T. axei, Bunostomum phlebotomum*, *Nematodirus helvetianus*, *C. punctata*, *C. oncophora*, *T. colubriformis*, and *Oesophagostomum radiatum* (F.D.A 1983). However, nematodes have shown remarkable adaptability and can develop resistance to anthelmintic drugs over time. This is favored by their short life cycles, high genetic diversity, high mutation rates and

large effective population sizes (Gilleard and Beech 2007). And among nematodes, the trichostrongylids are the most genetically diverse (Blouin 1998). For instance, the analysis of mitochondrial DNA (mtDNA) restriction site data in *O. ostertagi* revealed that 98% of the total genetic diversity in this species is distributed within populations (Blouin, Dame et al. 1992). This finding suggests that there is very high gene flow among different populations of *O. ostertagi*. High gene flow means that individuals from different populations are interbreeding frequently, and this could be happening due to the high levels of host movement (Blouin, Yowell et al. 1995). The high within-population diversity observed in trichostrongylid nematodes can primarily be attributed to large effective population sizes, potentially combined with an accelerated rate of nucleotide substitution (Blouin, Yowell et al. 1995). Furthermore, the average number of nucleotide substitutions per site among individuals within the same population was five to ten times greater than the typical estimates reported for species in other taxa (Blouin, Dame et al. 1992). Gilleard and Beech (Gilleard and Beech 2007), estimated that the variability between two sequences of DNA from different individual *H. contortus* haplotypes, can range from 0 - 5 nucleotide positions per kb in some individuals to over 200 in others. This along with other genetic changes such as insertions-deletions (indels), confers a genetic background that favors the occurrence of mutations (Gilleard and Beech 2007).

Origin of resistance mutations in nematodes

It is hypothesized that AR-conferring mutations could originate in parasitic nematode populations in a number of ways (Gilleard and Beech 2007). To begin with,

the resistance mutation may be ancient, meaning it existed in the parasite population long before anthelmintic use began (Coles, Rhodes et al. 2005, Gilleard and Beech 2007). In such instances, all resistance alleles trace back to a common origin. Secondly, a new resistance mutation might emerge either just before or during the period of anthelmintic use (Silvestre and Humbert 2002, Gilleard and Beech 2007). Again, all resistance alleles within a specific population would then share a common origin and be nearly identical because there has not been much time for further sequence variation. Initially, the frequency of these alleles could be extremely low, possibly even represented by a single individual in the population. Another possibility contemplates the appearance of recurrent resistant alleles, in a scenario in which mutation is common (Gilleard and Beech 2007, Redman, Whitelaw et al. 2015). In this case, the frequency of resistant alleles would likely be higher than if a single new mutation occurred but may not reach the frequency observed for pre-existing alleles. Lastly, resistance may have originated in a different location, with alleles introduced into the parasite population through migration (Gilleard and Beech 2007, Redman, Whitelaw et al. 2015).

Yet, for a long time the prevailing theory in the field has been that drug use exerts selection pressure on ancient or pre-existing alleles in the parasite population (Roos 1990, Silvestre and Humbert 2002). Initial studies on *H. contortus*, characterized three BZ-resistant and three BZ-susceptible populations from different geographical locations, based on egg hatch assay, beta-tubulin binding tests and restriction fragment length polymorphism (RFLP) after Southern blotting (Roos 1990). The results showed a greater number of hybridizing fragments in the susceptible populations than in the resistant, when probed with beta-tubulin isotype-1. Additionally, after selecting the

susceptible isolate with two rounds of thiabendazole, there was a reduction in the number of hybridizing fragments, denoting a reduction in the susceptibility of the population (Roos 1990). It was then shown that the RFLP fragment selected by treatment contained a substitution at position 200 of the beta-tubulin protein conferring the loss of BZ binding. The authors concluded that the genes conferring BZ resistance were present in *H. contortus* populations preceding the introduction of BZ drugs to the market. And this would explain the fast selection for BZ resistance on the field (Roos 1990). Another study that supported the theory of pre-existing alleles was conducted in isolated populations of *H. contortus*, *T. circumcincta* and *T. colubriformis* (Silvestre and Humbert 2002) from closed herds of dairy goats in France. The key finding was that some of the resistance alleles present on different farms, with no history of animal exchange, had 100% sequence identity over a 460 bp genomic fragment of the beta-tubulin isotype-1 gene. This led the authors to conclude that in those cases, the data were evidence of pre-existing BZ-resistance alleles that had been shared before the herds became closed (Silvestre and Humbert 2002).

However, this hypothesis has since been challenged by multiple studies that show alternative explanations for those results (Gilleard and Beech 2007, Silvestre, Sauve et al. 2009, Skuce, Stenhouse et al. 2010, Redman, Whitelaw et al. 2015). Silvestre and colleagues (Silvestre, Sauve et al. 2009) complemented the first study by looking at neutral genetic markers, along with beta-tubulin, with the aim to assess the genetic relatedness of the field populations of BZ-resistant *H. contortus* and *T. circumcincta.* A “neutral genetic marker” refers to a gene or a locus where variants are considered to confer no fitness advantage, such as microsatellites and mitochondrial

haplotypes, and are in Hardy-Weinberg equilibrium (HWE) (Hill 1975). However, when the population size is limited (e.g. bottleneck), genetic drift can occur and therefore by chance, certain alleles increase or decrease in frequency, resulting in a shift away from HWE. On the other hand, anthelmintic selection will affect the pattern of genetic variation of the locus under drug selection, i.e. beta-tubulin isotype-1 for BZ. This effect is largely determined by “genetic hitchhiking” and meiotic recombination (Gilleard and Beech 2007). For instance, many different sequences (haplotypes) of beta-tubulin isotype-1 are expected to be present in the nematode population before BZ use (Gilleard and Beech 2007). But when a new mutation appears in response to drug pressure, and provides a fitness advantage over “susceptible” haplotypes, this is expected to rise in frequency under positive selection (Gilleard and Beech 2007). Genes that are in proximity or associated to the loci under selection will also rise in frequency, despite not being themselves under drug selection pressure (Smith and Haigh 1974, Hermisson and Pennings 2005). This process is termed as “genetic hitchhiking” and can reduce the neutral diversity around the selected locus, resulting in non-random association of alleles, known as “linkage disequilibrium” (Hill 1975). The study conducted in France, found that genetic drift was responsible for high genetic differentiation between *H. contortus* populations, but found no genetic differentiation between the populations of *T. circumcincta* (Silvestre, Sauve et al. 2009). Authors concluded that these findings suggested severe bottlenecks in the populations of *H. contortus*, and that recurrent mutations generating new alleles in the isolated populations, or the introduction of existing alleles were equally likely hypotheses (Silvestre, Sauve et al. 2009).

A more recent study, conducted on seven commercial sheep farms in the United Kingdom (UK), investigated the population structure of *T. circumcincta* and *H. contortus* in relation to the genetics of BZ resistance (Redman, Whitelaw et al. 2015). Neutral microsatellite markers revealed that *T. circumcincta* has a higher genetic diversity but lower genetic differentiation between farms than *H. contortus*, being this mainly related to epidemiological factors (Redman, Whitelaw et al. 2015). The poor adaptation of *H. contortus* to the weather and climatic conditions in the UK results in seasonal bottlenecks. But in the case of *T. circumcincta*, a cold-tolerant nematode, larger population sizes can be expected to be maintained regardless of the season (Redman, Whitelaw et al. 2015). This is consistent with the higher levels of genetic diversity observed for *T. circumcincta* within farms, providing a greater supply of resistance mutations, with 28 resistant haplotypes detected on just seven farms (Redman, Whitelaw et al. 2015). In *H. contortus*, a higher genetic differentiation was observed between farms, with five resistant haplotypes on seven farms and a predominance of hard selective sweeps (Redman, Whitelaw et al. 2015). Under anthelmintic exposure, a single resistant mutation that provides a huge fitness advantage, may sweep through the population reaching fixation. This scenario is referred as “hard selective sweep”, reducing the diversity surrounding the resistant locus significantly (Doyle and Cotton 2019), and with the predominance of a single resistant haplotype in the population (Redman, Whitelaw et al. 2015). However, many AR-conferring mutations may exist in diverse genetic backgrounds within the same population, probably preceding the use of drug. In this case, we can expect a loss of diversity around the resistance locus with the use of anthelmintics, but still maintaining diverse genetic backgrounds to an extent.

These cases, are termed “soft selective sweeps” (Doyle and Cotton 2019). And in the UK study, these were more common in *T. circumcincta* due to the high levels of genetic diversity observed within a given population (Redman, Whitelaw et al. 2015). However, some farms showed evidence of hard selective sweeps with a single haplotype predominating in *T. circumcincta.* When looking at the haplotype analysis between farms, very different soft selective sweeps can be observed for both species of nematodes. For instance, two farms that had little genetic differentiation in *T. circumcincta* based on neutral markers, showed however, 14 different resistant haplotypes in total, with none in common for both farms (Redman, Whitelaw et al. 2015). This, along with the phylogenetic analysis, suggested multiple independent origins of BZ resistant mutations. In contrast, the same *H. contortus* resistance haplotype was observed predominating on five farms (Redman, Whitelaw et al. 2015). In this case, it is more likely that this resistant genotype originated and was spread due to animal movement. This study highlighted the complexity of anthelmintic resistance and the different ways in which resistance mutations can emerge in a population of nematodes. Furthermore, the epidemiology of AR will also be different for geographical regions that have distinctive climatic conditions. In the Southern US, *Haemonchus* can cycle year-round, and low levels of genetic differentiation have been seen in different *H. placei* and *H. contortus* populations (Chaudhry, Miller et al. 2014, Chaudhry, Redman et al. 2020), denoting high gene flow between different populations (Chaudhry, Miller et al. 2014). In this study, one resistant haplotype was observed in six populations of *H. placei* from three different Southern states. In the case of *H. contortus*, there were four different haplotypes in codon 200 and two different haplotypes for the mutations in

codon 167 (Chaudhry, Redman et al. 2020). This evidence provides additional support for the hypothesis that resistant mutations can originate from multiple independent soft and hard selective sweeps and then propagate regionally, through animal movement (Redman, Whitelaw et al. 2015, Chaudhry, Redman et al. 2020).

Benzimidazole resistance

Among all major anthelmintic classes, resistance to BZ is the most comprehensively understood. Early studies in nematodes showed that the loss of a specific gene encoding beta-tubulin, *ben-1*, resulted in a BZ resistant phenotype in *C. elegans* (Driscoll, Dean et al. 1989). This was followed by studies in *H. contortus*, demonstrating that a change from phenylalanine to tyrosine at position 200 (F200Y) of the beta-tubulin isotype-1 gene, was present at much higher frequency in resistant than susceptible parasite populations (Roos 1990, Kwa, Veenstra et al. 1994, Lubega, Klein et al. 1994). This was then further confirmed by the heterologous expression of the resistant gene from *H. contortus* in transgenic *C. elegans* (Kwa, Veenstra et al. 1995). A number of non-synonymous single nucleotide polymorphisms (SNPs) on the beta-tubulin isotype-1 gene have since been associated with BZ resistance in important GIN of livestock. These include phenylalanine to tyrosine (F167Y) (Silvestre and Cabaret 2002, Redman, Whitelaw et al. 2015), or to histidine at codon 167 (F167H) (Prichard 2001), and glutamic acid to alanine at codon 198 (E198A) (Ghisi, Kaminsky et al. 2007), or to leucine, valine, lysine, isoleucine and stop (E198L/E198V/E198K/E198I/E198stop) (Redman, Whitelaw et al. 2015, Mohammedsalih, Krücken et al. 2020). Structural modeling analyses indicate that SNPs at codons 167 and 200 might diminish the

binding affinity of BZs by increasing the polarity and hydrophilicity within the binding pocket (Aguayo-Ortiz, Méndez-Lucio et al. 2013). Similarly, polymorphisms at codon 198 are anticipated to interfere with the crucial hydrogen-bond interaction and shorten the aliphatic chain, thereby leading to the creation of an excessively large hydrophobic cavity within the binding site (Aguayo-Ortiz, Méndez-Lucio et al. 2013). A novel beta-tubulin mutation, Q134H (CAA>CAT), was recently identified in *Ancylostoma caninum* populations from domestic dogs across the US (Venkatesan, Jimenez Castro et al. 2023). These *A. caninum* isolates were displaying levels of BZ resistance that did not match the low levels of the F167Y mutation detected genetically in the beta-tubulin isotype-1 gene (Venkatesan, Jimenez Castro et al. 2023). However, deep amplicon sequencing of those samples showed that a change from glutamine to histidine at codon 134 of beta-tubulin isotype-1, was responsible for the observed discrepancies between phenotype and genotype (Venkatesan, Jimenez Castro et al. 2023). Functional significance was demonstrated by employing CRISPR-Cas9 editing, and introducing the Q134H substitution into the *C. elegans* beta-tubulin gene, *ben-1*. This genetic manipulation revealed that the mutation conferred resistance levels comparable to those observed with a *ben-1* null allele (Venkatesan, Jimenez Castro et al. 2023). Structural modeling suggests that the Q134 residue plays a direct role in BZ drug binding, and the substitution to 134H is predicted to significantly diminish binding affinity (Venkatesan, Jimenez Castro et al. 2023).

Beyond mutations at the previously known codons, numerous other mutations associated with BZ resistance have been detected in fungal species and the free-living nematode, *C. elegans* (Hawkins and Fraaije 2016, Hahnel, Zdraljevic et al. 2018). Field

and laboratory strains of fungi have revealed over 20 distinct beta-tubulin mutations associated to BZ resistance (Hawkins and Fraaije 2016). Genome-wide screenings of 249 wild isolates of *C. elegans* have identified multiple novel alleles specific to *ben-1* that are associated with resistance, including several nontraditional mutations such as deletions, splice variants, transposon insertions, insertions, and inversions (Hahnel, Zdraljevic et al. 2018).

However, the association between beta-tubulin mutations and BZ resistance is less clear in nematodes belonging to clade III phylogeny. For example, reduced BZ efficacy in *Ascaris lumbricoides*, has not been associated to changes in beta-tubulin, even when analyzing sequences from four beta-tubulin genes (Krücken, Fraundorfer et al. 2017). In another multi-state Brazilian study, over 600 individual eggs of *A. lumbricoides* were analyzed using a PCR-RFLP approach (Zuccherato, Furtado et al. 2018). No mutations were detected, though the history of anthelmintic treatments was not available to the authors. Interestingly, other studies have detected a polymorphism in position 167 but in albendazole-susceptible isolates of *A. lumbricoides* (Diawara, Halpenny et al. 2013). Furtado and colleagues (Furtado, Medeiros et al. 2019) also described a mutation at codon 200 at 0.5% allele frequency in a population of *A. lumbricoides* but the phenotypic resistance status of this isolate was unknown. In nematodes of veterinary importance, no mutations in the canonical codons have been associated to BZ resistance. For instance, whole genome sequencing of BZ-resistant isolates of *Ascaridia dissimilis* and *Heterakis gallinarum* did not reveal any of the SNPs in beta-tubulin associated to BZ resistance of clade V nematodes (Collins 2021). Likewise, these SNPs were not found in a BZ-resistant isolate of *Parascaris univalens*

from a Swedish farm stud, despite sequencing seven beta-tubulin genes (Martin, Halvarsson et al. 2021). A recent study conducted an extensive screening of two beta-tubulin isotypes in human and pig *Ascaris* samples from around the world. Among the 187 isolates analyzed, no mutations were found in the canonical codons (Jones, Kozel et al. 2024). This study further adds to the growing body of evidence that the canonical BZ resistance associated SNPs observed in other nematodes are absent in *Ascaris* and other ascarids (Jones, Kozel et al. 2024).

Additionally, the sole detection of polymorphisms within the beta-tubulin gene in resistant populations does not inherently imply that these alleles directly cause resistance (Dilks, Koury et al. 2021). Both proof of sufficiency and necessity are required to show a causal connection between the allele and the resistant phenotype (Dilks, Koury et al. 2021). This proof has been obtained for parasitic nematodes in 10 of these SNPs, demonstrating the causal connection between these mutations and BZ resistance (Kwa, Veenstra et al. 1995, Hahnel, Zdraljevic et al. 2018, Kitchen, Ratnappan et al. 2019, Dilks, Hahnel et al. 2020, Dilks, Koury et al. 2021, Venkatesan, Jimenez Castro et al. 2023).

While the primary focus of research on BZ resistance has centered on a candidate gene (beta-tubulin), recent genome-wide analyses using *C. elegans* laboratory strains and wild isolates have revealed beta-tubulin isotype-1 (*ben-1*)-independent quantitative trait loci (QTLs) associated with resistance (Zamanian, Cook et al. 2018). Genome-wide approaches represent more powerful tools to detect loci under drug selection, since no prior assumptions are made regarding the underlying

mechanisms of resistance (Gilleard and Beech 2007). In this regard, two populations of *H. contortus* with frequent use of BZ over 20 years, and two with no history of BZ use, were studied using genome-wide approaches (Wit, Workentine et al. 2022). The main locus found under selection was detected on chromosome I, corresponding to the beta-tubulin isotype-1 gene. Interestingly, two additional but weaker signatures of selection were evidenced near the middle of chromosome I, spanning 259 annotated genes, and on chromosome II spanning a region of 206 annotated genes (Wit, Workentine et al. 2022). This latter includes the beta-tubulin isotype-2 gene. However, when amplicon sequencing was performed in the beta-tubulin isotype-2 of all individual worms, no resistant mutations were found in codons 167, 198 or 200 (Wit, Workentine et al. 2022). Another study using a genome-wide approach generated a genetic cross between a susceptible and a multiple drug-resistant population (from the US) of *H. contortus*. Drug-selected pools of F3-generation progeny were then obtained from F2 adults treated *in vivo* with fenbendazole (Doyle, Laing et al. 2021). Again, a major QTL was found in chromosome I, containing the beta-tubulin isotype-1 locus, and within this gene, the polymorphism at codon 200 (TTC/ Phenylalanine → TAC/Tyrosine) was found increased in frequency post-treatment (Doyle, Laing et al. 2021). A slight increase in the frequency of the mutation F167Y was also observed post-treatment. Notably, another minor though not significant increase was observed in the frequency of a E198V variant at the beta-tubulin isotype-2 gene (Doyle, Laing et al. 2021). This mutation was also highly correlated to *in vitro* levels of BZ resistance (measured with Drenchrite®) and seemed to provide higher levels of resistance, given that F200Y was already fixed in the worm population from the US (Doyle, Laing et al. 2022). Interestingly, early studies had

associated the deletion of beta-tubulin isotype-2 gene with increased levels of resistance in *H. contortus* (Kwa, Kooyman et al. 1993). However, its implications on resistance levels in field populations remains unclear, since it has mainly been observed in *in vitro* studies (Rufener, Kaminsky et al. 2009, Doyle, Laing et al. 2022, Wit, Workentine et al. 2022).

Given the evidence provided in these two studies and *C. elegans* wide-genome studies (Hahnel, Zdraljevic et al. 2018, Zamanian, Cook et al. 2018), it would be unreasonable to assume that the mechanisms behind BZ resistance are fully elucidated. Moreover, the role of other mutations when a resistance allele becomes fixed in the population, still warrants further investigation (Doyle, Laing et al. 2021). For instance, Jimenez-Castro and colleagues (Jimenez Castro, Howell et al. 2019) demonstrated that when treating a resistant isolate of *A. caninum* with a single dose of fenbendazole, the phenotypic response increased dramatically, with no significant changes in the beta-tubulin SNP frequencies observed in the samples before and after treatment. The observed response in the resistance phenotype as measured by the EHA IC_{50}, suggested that the treatment may have triggered the induction of beta-tubulin-independent resistant mechanisms. Therefore, while the beta-tubulin isotype-1 locus is the primary determinant for BZ resistance in strongylid nematodes, it is plausible that additional mechanisms contribute to higher levels of resistance observed in field samples. Furthermore, the impact of noncanonical beta-tubulin mutations on BZ resistance in parasitic species, particularly among clade III phylogeny nematodes, remains unknown and warrants further investigation (Doyle and Cotton 2019).

Recent advances in resistance to other major drug classes

Levamisole exerts its mechanism of action by binding and opening nematode acetylcholine receptor (AChRs) ion channels (Aceves, Erlij et al. 1970, Martin 1997, Martin, Robertson et al. 2012). These receptors are composed of 5 identical homomeric, or related heteromeric, subunits (Duguet, Charvet et al. 2016). In *H. contortus* these comprise UNC-38, UNC-29, UNC-63, and ACR-8 subunits, with one subunit appearing twice (Duguet, Charvet et al. 2016, Blanchard, Guégnard et al. 2018). From these, the *acr-8* subunit has been mostly implied in mediating susceptibility to levamisole in *H. contortus* (Boulin, Fauvin et al. 2011, Blanchard, Guégnard et al. 2018). Different mechanisms of resistance have been proposed for *H. contortus* through the candidate gene approach; the expression of truncated transcripts of *acr-8* and *unc-63*, altered expression patterns of AChR subunits and a deletion present in the second intron of the *acr-8* gene (Fauvin, Charvet et al. 2010, Neveu, Charvet et al. 2010, Boulin, Fauvin et al. 2011, Williamson, Storey et al. 2011, Sarai, Kopp et al. 2013, Barrère, Beech et al. 2014, Sarai, Kopp et al. 2014, Raza, Kopp et al. 2016). Genome-wide studies were conducted on a genetic cross between a susceptible and a multiple drug resistant population of *H. contortus*, and drug selected pools of F3-generation progeny from F2 adults that were treated *in vivo* with levamisole (Doyle, Laing et al. 2022). A region on Chromosome V was identified under drug selection through whole-genome sequencing (Doyle, Laing et al. 2022). Within this region a single non-synonymous mutation was found in *acr-8* exon 4 encoding a serine-to-threonine substitution (S168T), and was highly associated with the resistant phenotype (Doyle, Laing et al. 2022). The role of S168T was further validated by conducting single PCR

worm assays in a controlled genetic cross between levamisole susceptible and resistant isolates of *H. contortus*. The presence of S168T was strongly associated with levamisole resistance in the parental isolates and F3 progeny of the genetic cross (Antonopoulos, Doyle et al. 2022).

The research into the genetic mechanisms underlying ivermectin resistance has also primarily focused on the candidate gene approach (Gilleard and Beech 2007, Kotze, Hunt et al. 2014). In *C. elegans* concurrent mutation of 3 genes encoding glutamate-gated chloride channel (GluCl) alpha-type subunits, *glc-1*, *avr-14*, *avr-15*, confers high-level resistance to ivermectin (Dent, Smith et al. 2000). However, there is still no evidence that these or other candidate genes are involved in resistance to ivermectin in parasitic nematodes (Doyle, Illingworth et al. 2019). Recent genome-wide studies have further emphasized the complex nature of resistance to this drug class. The genome-wide analysis of two genetic crosses between ivermectin-resistant and -susceptible *H. contortus* identified a ~5 Mb QTL under selection in chromosome V (Doyle, Illingworth et al. 2019). However, none of the previous candidate genes were identified among the 360 genes within this region (Doyle, Illingworth et al. 2019). Follow-up studies were conducted following the same approach to study resistance to levamisole and benzimidazoles; a genetic cross between a susceptible and a multiple drug resistant population of *H. contortus*, and drug selected pools of F3-generation progeny from F2 adults that were treated *in vivo* with ivermectin were whole-genome sequenced (Doyle, Laing et al. 2021). The authors found the same QTL in chromosome V under selection, and narrowed the region to approximately 300 kb wide, spanning approximately 25 genes (Doyle, Laing et al. 2021). Towards the middle of the QTL, a

transcription factor, *cky-1* was identified as a novel mediator of resistance to ivermectin. Additionally, *cky-1* was shown to be the only gene significantly upregulated in both males and females of a resistant isolate, when compared to a susceptible one (Laing, Doyle et al. 2022). In *C. elegans*, *cky-1* is found highly expressed in the embryo, restricted to pharyngeal cells (Packer, Zhu et al. 2019), however its role in *H. contortus* resistant and susceptible isolates needs further clarification (Laing, Doyle et al. 2022). The authors hypothesized that since *cky-1* is a transcriptional factor, the expression of several genes downstream from it, could be affected and thus, involved in ivermectin resistance in parasitic nematodes (Doyle, Laing et al. 2022).

Despite the recent advances in identifying loci involved with resistance to ivermectin and levamisole, achieved using genome-wide approaches, there are still many unknowns that require further research.

Diagnosis of anthelmintic resistance

Resistance in the field is usually suspected when reduced weight gains, and / or clinical PGE is observed, despite anthelmintic treatment (Gasbarre, Smith et al. 2009, Sauermann, Waghorn et al. 2024). However, the adoption of proper tests to confirm anthelmintic resistance on livestock farms remains uncommon (McArthur and Reinemeyer 2014). Additionally, there is a failure to rule out other potential causes for suboptimal efficacy, such as faulty dosing guns, inaccuracies in weight estimation leading to underdosing, or various other factors that could affect drug efficacy (Prichard, Hall et al. 1980, Prichard and Hennessy 1981, Morgan, Lanusse et al. 2022). Only after ruling out these alternative factors can anthelmintic resistance be reliably detected through a combination of *in vivo*, *in vitro*, and molecular-based diagnostic tests.

Detection of anthelmintic resistance using in vivo tests

There are two tests that determine the efficacy of anthelmintics *in vivo*: the fecal egg count reduction test (FECRT) and controlled efficacy studies. Both tests will yield a percent efficacy for all anthelmintic drug classes, across all animal species, and for multiple parasite species. The controlled efficacy test is considered the gold standard, and because animals must be euthanized, is exclusively used in research setting and by pharmaceutical companies when pursuing registration of anthelmintic drugs (Wood, Amaral et al. 1995, Geurden, Smith et al. 2022). This test relies on the comparison of at least two groups: a non-treated control and at least one treated group of experimental animals. Adequately parasitized animals are included in each treated and control group, and after a suitable period post-treatment, the animals are necropsied, and the parasites enumerated and identified. A statistically significant difference in parasite counts between treatments and control groups, and a calculated percent efficacy of at least 90% needs to be achieved for the registration of a new drug (Geurden, Smith et al. 2022).

In contrast, the FECRT allows the calculation of a percent efficacy without having to euthanize the animals for worm enumeration (Kaplan, Denwood et al. 2023). Additionally, the FECRT can be performed in the field by any veterinarian, without the requirement of expensive equipment or expertise knowledge for worm recovery and identification. Recently, the World Association for the Advancement of Veterinary Parasitology (WAAVP) released new guidelines for the FECRT, addressing multiple issues regarding the experimental design, choice of FEC method, statistical analysis, and interpretation of results (Kaplan, Denwood et al. 2023). These guidelines aim to

standardize the protocols for detecting AR in the field, and provide options for two types of studies: a clinical vs a more stringent research protocol. These new protocols include four major changes as compared to the previous recommendations by Coles et al (Coles, Bauer et al. 1992). It moves away from the requirement of a control (untreated) group, but rather into a paired study design using the same animals for pre- and post-treatment egg counts (Kaplan, Denwood et al. 2023). Therefore, the reduction is measured both individually, and collectively. Secondly, instead of having a minimum mean fecal egg count required for the treatment group, the new guidelines suggest a minimum total number of eggs counted under the microscope (prior to the application of the multiplication factor), that is set in relation to the statistical power to detect susceptibility or resistance (Denwood, Kaplan et al. 2023). Also, there is no longer a required sample size; the number of animals to be included depends on the expected egg counts and the size of the “grey zone” of uncertainty that is acceptable. Lastly, the expected efficacy for each major drug class was defined according to the host species, and parasite species (Kaplan, Denwood et al. 2023).

Detection of anthelmintic resistance using in vitro tests

These tests are laboratory-based measures of drug response, based on different phenotypes including growth, development, behavior and motility (Taylor, Hunt et al. 2002). Most frequently used are the egg hatch assay (EHA), larval development assay (LDA), and larval migration inhibition assay (LMIA) (Taylor, Hunt et al. 2002, Charlier, Bartley et al. 2022). The EHA was the first *in vitro* test developed to detect anthelmintic resistance. It was specifically designed for identifying resistance to BZ anthelmintics (Le Jambre 1976), leveraging the ovicidal properties inherent to this drug class (Hunt and

Taylor 1989). Undeveloped eggs are incubated for 48 hours under increasing concentrations of thiabendazole (TBZ), as this compound has the highest aqueous solubility within the class (McKellar and Scott 1990). After this period of time, development is stopped and the percentage of eggs that hatch at each concentration is determined. This is then corrected for natural mortality from control wells, and a dose-response curve is fitted. This is a fairly robust test, allowing the detection of BZ resistance in multiple GIN species of various livestock hosts, and internationally standardized protocols have been developed (von Samson-Himmelstjerna, Coles et al. 2009). In this test, the recommended threshold for the detection of BZ resistance has been set at >0.1 µg (0.5 µM) TBZ per ml for 50% egg hatch inhibition (Coles, Bauer et al. 1992).

On the other hand, the LDA constitutes a more resource-intensive and time-consuming test, requiring more expertise than EHA. For this test, eggs are isolated from the feces and incubated at increasing concentrations of the drugs to be tested for a period of 7 days. This is the time required for eggs to hatch and molt from L1s to L2s and finally to L3s. The advantage of this test over the EHA, is that it evaluates the development of nematode eggs to the L3 stage, and thus has the ability to detect the anthelmintic activity due to a multitude of effector mechanisms (Taylor 1990). Additionally, it can be used to detect AR against the major drug classes; BZ, imidazothiazoles (levamisole), AAD (monepantel) and MLs (ivermectin/moxidectin). However, it has only been validated against *Haemonchus* spp., *Trichostrongylus* spp. and *Teladorsagia* spp. in sheep (Kaplan, Vidyashankar et al. 2007, Ruffell, Raza et al.

2018) and one study showing its use for *C. oncophora* in cattle (Demeler, Küttler et al. 2010). Though, in this latter case results have not been replicated by other laboratories.

The LMIA was first described for testing BZ activity in the pig nematode, *O. dentatum* (Petersen, Friis et al. 1997). Later, the same authors described its use for discriminating pyrantel-resistant adult stages in the same nematode (Petersen, Craven et al. 2000). A slightly different method was successfully used for testing AR to the MLs in *H. contortus*, but with inconsistent results in other trichostrongylids from sheep and cattle (Kotze, Le Jambre et al. 2006, Demeler, Kleinschmidt et al. 2012, Areskog, Sollenberg et al. 2014, George, Lopez-Soberal et al. 2018). This assay is based on the ability of parasites to migrate through a mesh (Petersen, Friis et al. 1997, Petersen, Craven et al. 2000) or agar (Kotze, Le Jambre et al. 2006), following incubation with different concentration of anthelmintics. A newer platform has been developed to screen for anthelmintic activity in novel compounds (Harrington, Pyche et al. 2023) against *H. contortus*, but its ability to discriminate susceptible from resistant isolates of parasitic nematodes has not been demonstrated. In this newer assay, “the INVENesis Migration Trap Assay” (Rufener, Vernudachi et al. 2022), L3 exposed to drug or control treatments are transferred to a migration plate, that allows them to migrate from a deposit area to a trap area through a corridor. The motility of the worms in the trap area is monitored over a 21 min time window by an automated data acquisition system equipped with a camera that reads pixel displacement (Harrington, Pyche et al. 2023). This newer platform thus measures not only the ability of the worms to migrate, but also their motility response to treatment, thereby combining different phenotypes.

Moreover, LMIA has been rarely employed beyond a research setting, limiting its usefulness as a diagnostic tool for veterinarians / farmers. And it is further limited in the spectrum of GIN that it can be applied to, and the drugs that can be tested.

Initially, larval motility was measured visually, by counting the paralyzed and non-paralyzed larvae at each drug concentration (Martin and Le Jambre 1979, Gill, Redwin et al. 1995). These larval motility assays (LMA) were successfully used for detecting levamisole resistance in *Teladorsagia* spp., BZ resistance in *H. contortus*, *T. colubriformis*, and *T. circumcincta* and ML resistance in *H. contortus* (Martin and Le Jambre 1979, Sutherland and Lee 1990, Gill, Redwin et al. 1995). Automated micromotility readings were then introduced to measure the response to serial concentration of anthelmintics, in larval and adult stages from different parasitic nematodes (Bennett and Pax 1986). This test was based on the light deviation that parasites caused when moving in solution; this information was then processed by a computer generating a motility index. It successfully detected resistance to BZ in *H. contortus*, but was unsuccessful in the case of levamisole resistance (Coles, Folz et al. 1989). Additionally, the utilization of an LMA has also been explored in our laboratory, employing platform technologies for image analysis that can measure motility objectively (Storey, Marcellino et al. 2014). However, no consistent significant differences were found in the dose-response between ML-resistant and -susceptible *H. contortus* L3s and *Cooperia* spp. L3s and L4s, using motility as phenotype (George, Lopez-Soberal et al. 2018, Paras and Kaplan 2020). Furthermore, novel compounds were tested using the same platform, and despite identifying drugs with anthelmintic activity in the lower µM range against larval development, no effect was observed in

motility despite increasing the drug concentrations up to 500 µM (Zhang, Sicalo Gianechini et al. 2023). Therefore, inhibition of motility appears to be a much less sensitive phenotype than inhibition of migration for both the *in vitro* detection of AR and anthelmintic activity in general. Consequently, LMA is a sub-optimal method when used as the only phenotype for screening novel compounds for anthelmintic activity (Zhang, Sicalo Gianechini et al. 2023).

New high-throughput technologies have been developed to screen for anthelmintic activity in novel compounds (Wheeler, Gallo et al. 2022, Harrington, Pyche et al. 2023, Nunn, Juang et al. 2023, Wheeler, Ryan et al. 2023), or map the effects of commercially available anthelmintics in *C. elegans* (Wheeler, Gallo et al. 2022, Rehborg, Wheeler et al. 2023) and parasitic nematodes (Gallo, Wheeler et al. 2023, Ryan, Wheeler et al. 2023). wrmXpress is a comprehensive software package designed to analyze a diverse array of worm phenotypes, including neuromuscular activity, fecundity, mortality, development/size, and feeding behavior (Wheeler, Gallo et al. 2022). This new platform has been employed in drug screenings against *Brugia spp*. microfilariae (mf) and adults, *D. immitis* mf, and adult *Schistosoma* flatworms using a multivariate phenotypic approach (Gallo, Wheeler et al. 2023, Harrington, Pyche et al. 2023, Ryan, Wheeler et al. 2023, Wheeler, Ryan et al. 2023). Thus, different phenotypes can be measured in parallel using a customized imaging platform, significantly increasing the likelihood of detecting an effect through one of the analyzed phenotypes. Another advantage of this platform is the ability to efficiently analyze dozens of plates per day generated by high-throughput screens (Wheeler, Gallo et al.

2022). Despite being a promising platform, its application on the routine diagnosis of AR has not yet been tested.

In summary, the advantage of *in vitro* testing over FECRT is the requirement of a single pooled sample without having to run a clinical trial where animals are administered several different anthelmintic treatments, and two separate individual fecal collections are performed (Charlier, Bartley et al. 2022). In addition, for some assays, multiple drugs can be tested at the same time. However, there are a number of disadvantages. Samples need to be fresh, high-levels of labor input, expertise and laboratory resources are required, and some assays are drug class and or parasite genus-specific. Consequently, few labs offer these tests as a diagnostic service (Kotze, Gilleard et al. 2020, Charlier, Bartley et al. 2022). Therefore, the FECRT remains the most appropriate and practical method for the detection of AR on farms.

Detection of anthelmintic resistance using molecular based tests

The advancement of DNA-based tests for detecting AR has been constrained by the need to fully understand the mechanisms of resistance, and/or identify a specific marker associated with AR (Kotze, Gilleard et al. 2020). However, these tools are becoming increasingly available in the case of BZ (von Samson-Himmelstjerna 2006, Avramenko, Redman et al. 2019), and levamisole (Santos, Vasconcelos et al. 2019, Antonopoulos, Doyle et al. 2022, Antonopoulos, Higgins et al. 2023). For the BZs, molecular tests are based on the detection of non-synonymous mutations at codons 134, 167,198 and 200 of the beta-tubulin isotype-1 gene. These tests include pyrosequencing (von Samson-Himmelstjerna, Coles et al. 2009), digital droplet PCR-

technologies (Baltrušis, Halvarsson et al. 2020) and Loop-Mediated Isothermal Amplification (LAMP) (Tuersong, He et al. 2020) assays. One problem with all of these methods is that they require individual assays to be developed and fine-tuned for each genetic variation within each species of nematode, and reliable identification of AR-conferring alleles can be difficult when present at low frequencies (Avramenko, Redman et al. 2019). However, the recent development of deep amplicon next-generation sequencing technologies allows both the species composition and the frequency of BZ resistant alleles for each species to be determined from the same sample (Avramenko, Redman et al. 2015, Avramenko, Redman et al. 2019). In addition, this has enabled the identification of resistant GIN not only in livestock but also in wild ruminants (Avramenko, Redman et al. 2020). The test for BZ resistance relies on the amplification of a 300–350 bp fragment, encompassing codons 134, 167, 198 and 200 of the beta-tubulin isotype-1 gene, from all the trichostrongylid nematode species. Single nucleotide polymorphisms present in those codons, as well as their relative frequency are then determined bioinformatically (Avramenko, Redman et al. 2019). In the case of levamisole, recent studies have demonstrated the implications of a serine-to-threonine substitution (S168T) in the AChR subunit, *acr-8*, in strong association with resistance (Antonopoulos, Doyle et al. 2022, Doyle, Laing et al. 2022). Both an allele specific PCR, and a Loop-primer endonuclease cleavage-LAMP test have been developed for the detection of the S168T variant in *H. contortus* (Antonopoulos, Doyle et al. 2022, Antonopoulos, Higgins et al. 2023). However, they have not been tested outside the initial proof of concept studies.

The significance of not detecting SNPs through molecular tests warrants careful consideration. It becomes important to integrate molecular data with phenotype observations, as this is crucial for uncovering additional or novel genetic variants associated with resistance (Charlier, Bartley et al. 2022). Such is the case exemplified by the novel loci at codon 134 of the beta-tubulin isotype-1 gene, recently detected in *A. caninum* field populations (Venkatesan, Jimenez Castro et al. 2023). Moreover, the scalability of deep amplicon sequencing, along with its high sensitivity, makes it a powerful tool for the early detection of AR in GIN of veterinary importance (Avramenko, Redman et al. 2019).

Anthelmintic resistance on cattle and small ruminant farms

For many decades, parasite control in ruminants has relied primarily on the frequent and /or strategic use of anthelmintic drugs in order to maximize livestock health, productivity, and profitability (Kaplan 2020). Though this approach was initially highly successful, we are now experiencing ever-increasing levels of AR in all drug classes, involving virtually all of the most economically important parasites of livestock (Kaplan 2004, Kaplan and Vidyashankar 2012). Since the introduction of ivermectin in 1981 (Chabala, Mrozik et al. 1980), no new classes of anthelmintic have been marketed for ruminants in the US (Kaplan 2020). Currently, there is no evidence that new anthelmintic drugs are in the late phase pipeline (Nixon, Welz et al. 2020), nor that the new drugs available elsewhere (monepantel and derquantel) will be commercialized in the US anytime soon. Thus, it could be many years before a new anthelmintic drug class is introduced to the North American livestock market. Consequently, the continued development and spread of anthelmintic resistance will almost certainly outpace the

introduction of new anthelmintic classes (Kaplan 2020). Furthermore, in the future when a new anthelmintic is finally introduced for use in ruminants, it will almost certainly be considerably more expensive than the most expensive of the current products. Thus, the industry needs to change the way parasite control is practiced (McArthur and Reinemeyer 2014), bring back critical thinking to the process, and establish sustainable programs that help to preserve the efficacy of anthelmintics.

The development of AR is a threat on every farm that uses anthelmintics, though the rate at which resistance develops is affected by a large number of variables including the host, the parasite species, the drug, the frequency of anthelmintic administration, and the amount of refugia present at the time treatments are administered (Prichard 2005, Leathwick 2007). On sheep and goat farms, anthelmintic resistance originally developed and spread considerably more rapidly than on cattle farms, and the problem is now more severe on small ruminant farms. However, anthelmintic resistance is an increasingly serious problem in cattle GIN (Sutherland and Leathwick 2011, Kaplan and Vidyashankar 2012, Rose Vineer, Morgan et al. 2020), and is continually worsening (Sauermann, Waghorn et al. 2024). Therefore, it is important that the cattle industry make use of the lessons learned from the sheep and goat industry to slow the process of resistance in cattle parasites.

The development of anthelmintic resistance usually requires numerous generations of worms to be under drug selection (Gilleard and Beech 2007) taking many years to reach levels that cause a significant reduction in drug efficacy. However, anthelmintic resistance can appear very rapidly on a farm if new additions to the herd

harbor drug-resistant parasites (Gilleard and Beech 2007), or if treatments are conducted under low refugia situations (Leathwick, Pomroy et al. 2001). Depending upon how many animals are purchased that harbor resistant worms, the worm burdens of those animals, and other management and pasture factors such as amount of refugia, treatment failure due to drug resistance can occur practically instantly, or over a relatively short period (Gilleard and Beech 2007). This is why appropriate quarantine periods and treatments, along with regular testing for drug resistance, should be implemented on all ruminant farms (Kaplan 2020). Furthermore, “treat and move” constitutes another practice that can select for AR rapidly (Leathwick, Vlassoff et al. 1995, Waghorn, Miller et al. 2009). This is because when animals are relocated to a "clean" pasture after treatment, the surviving resistant parasites will be the main source of eggs to the pasture, with very little or no dilution (Leathwick, Vlassoff et al. 1995, Waghorn, Miller et al. 2009). Similarly, if treatments are conducted under low refugia, anthelmintic treatment results in a high selection pressure. The "summer-drench program in Australia" exemplifies this phenomenon (Woodgate and Besier 2010, Leathwick and Besier 2014). Treatment was administered during dry summer conditions when larval survival in pastures was minimal, thus minimizing reinfection. While initially effective for worm control, this strategy proved highly selective for resistance in just a few generations of the worm population (Woodgate and Besier 2010, Leathwick and Besier 2014).

Prevalence of AR on sheep farms

During the period from 2002-2009 two studies were performed investigating the prevalence of anthelmintic resistance on 80 sheep and goat farms in the Southern and

mid-Atlantic states of the US (Howell, Burke et al. 2008, Crook, O'Brien et al. 2016). In the Southern states (2002-2006) *H. contortus* from 45 (98%), 25 (54%), 35 (76%), and 11 (24%) farms were resistant to benzimidazoles, levamisole, ivermectin, and moxidectin, respectively (Howell, Burke et al. 2008). Resistance to all 3 classes of anthelmintics was detected on 22 (48%) farms, and resistance to all 3 classes plus moxidectin was detected on 8 farms (17%). Hence, on almost 20% of all farms tested, resistance was detected to all available anthelmintics; a situation referred to as "total anthelmintic failure". In the mid-Atlantic region (2007-2009), resistance to benzimidazoles, ivermectin, moxidectin, and levamisole was detected in *H. contortus* on 100%, 82%, 47%, and 24% of farms, respectively. Multiple-drug resistance to all 3 drug classes was detected on 18% of farms. Notably, in a study performed just a few years after Howell et al. (2008), the prevalence of moxidectin resistance on the mid-Atlantic farms was twice as high, at 47% (Crook, O'Brien et al. 2016). Other (unpublished) data from The Kaplan Laboratory collected on 34 goat farms in the Eastern US between 2011 and 2016 found prevalences of resistance in *H. contortus* of 100%, 44%, 94% and 56% for benzimidazoles, levamisole, ivermectin, and moxidectin, respectively (Kaplan 2020). Total anthelmintic failure was demonstrated in 30% of all the farms. The prevalences of resistance on sheep farms (n=58) in the eastern US were similar, though lower at 97%, 21%, 81% and 40% for benzimidazoles, levamisole, ivermectin, and moxidectin, respectively (Kaplan 2020). In contrast, resistance was less prevalent on sheep farms (n=32) in the western US, with prevalences of 91%, 13%, 38% and 3% for benzimidazoles, levamisole, ivermectin, and moxidectin, respectively (Kaplan unpublished data) (Kaplan 2020). These results are no different than the levels of

resistance observed in other regions of the world (Kaplan and Vidyashankar 2012). In Europe, a meta-analysis of the reports of resistance in 22 countries and spanning a period of 20 years, was recently published (Rose Vineer, Morgan et al. 2020). Aggregated data since 2010, showed prevalences of 86%, 48%, 52% and 21% to benzimidazoles, levamisole, macrocyclic lactones except moxidectin, and moxidectin, respectively on sheep and goat farms. In Australia, the compilation of data from 390 FECRT between 2009 - 2012, showed that 54%, 77% and 87% of sheep farms have resistance to moxidectin, abamectin and ivermectin, respectively (Playford, Smith et al. 2014). The parasite species mainly associated with resistance were *Teladorsagia* spp. in Tasmania and Western Australia, and *Haemonchus* spp. in New South Wales (Playford, Smith et al. 2014). In South America, the status is not different from other continents (Torres-Acosta, Mendoza-de-Gives et al. 2012). For instance, a recent study from Sao Paulo state, Brazil showed that 100% of the farms tested had efficacies lower than 90% for levamisole, benzimidazoles and macrocyclic lactones (Bassetto, Albuquerque et al. 2024). Further denoting the progression of AR in the state of Sao Paulo to total anthelmintic failure, and the need for sustainable parasite control.

Prevalence of AR on cattle farms

Resistance in parasites of cattle has developed at a slower pace than in the small ruminant and equine sectors, but over the past decade there has been a rapid increase in the levels and distribution of anthelmintic resistance in gastrointestinal worms of cattle worldwide (Ramos, Portella et al. 2016, Cristel, Fiel et al. 2017, Rose Vineer, Morgan et al. 2020, Sauermann, Waghorn et al. 2024). In the US, there are some published case reports of resistance in parasites of cattle, (Gasbarre, Smith et al.

2009, Edmonds, Johnson et al. 2010, Gasbarre, Ballweber et al. 2015) but no studies have been performed to establish the national prevalence of resistance. However, a study performed by the Kaplan Laboratory on twelve cow-calf farms in Georgia and on stocker cattle purchased at various stockyards in the Southern region suggest that anthelmintic resistance in the ML family (e.g., ivermectin) in cattle is both very common and widespread (Kaplan 2020). More than 90% of farms tested by our laboratory in the last 10 years show *Cooperia spp*. resistant to the ML class. Resistance in *Cooperia* spp. and *Haemonchus* spp. were the most common, but resistance in *Ostertagia* was also found at alarming levels. For instance, 40% of the cow-calf farms evaluated in Georgia exhibited resistance to the ML drugs in *Ostertagia* spp. (unpublished). Other recent data (unpublished) indicates that ML drugs have lost the ability to kill inhibited *Ostertagia* L4 in weaned beef calves in the Southern US (Kaplan 2020).

Overall, in the Southern US, it appears that resistance to the ML drugs is highly prevalent in *Cooperia* spp. and *Haemonchus* spp., and is in the emerging stages for *Ostertagia*. Further supporting these observations, the United States Department of Agriculture (USDA) National Animal Health Monitoring System (NAHMS) conducted a study in 2008 to examine the efficacy of anthelmintic treatment on 72 beef cow-calf operations across 19 states (Gasbarre, Ballweber et al. 2015). Notably, they found suboptimal fecal egg count reduction on 100% of the farms that had used a pour-on macrocyclic lactone as treatment 14-16 days prior. PCR analysis of the parasite populations surviving treatment indicated that the lack of efficacy was most likely due to anthelmintic resistance in *Cooperia spp*. and possibly *Haemonchus spp*. Therefore, even with limited information regarding the national prevalence of resistance in cattle

nematodes, the available evidence suggests that it is quite unlikely that there are major differences in the patterns of anthelmintic resistance in other regions of the US.

Other countries where data are available indicate that the problem is even worse than in the US. For instance, widespread anthelmintic resistance was confirmed in New Zealand in a survey conducted on 62 cattle farms in 2006 (Waghorn, Leathwick et al. 2006). At that time, resistance to ivermectin, albendazole and levamisole was reported on 92%, 76% and 6% of the farms, respectively. While only 7% of the farms showed susceptibility to all 3 drug classes. Furthermore, *Cooperia spp.* was resistant to ivermectin and albendazole on 74% of the farms. In contrast, the more pathogenic *Ostertagia* spp., was still susceptible to ivermectin and levamisole on 91% of the farms, whereas resistance to the benzimidazoles was observed on 35% of the farms. However, 10 years later, Waghorn conducted further testing on 6 commercial farms showing macrocyclic lactone failure to control *Ostertagia spp.* on all farms (Waghorn, Miller et al. 2016). In addition, inefficacy of albendazole and levamisole were also observed towards *Ostertagia spp.* on 50% and 100% of the farms, respectively. More recently, another study was conducted on four commercial farms following reports of poor animal growth rates and positive FEC post anthelmintic treatment (Sauermann, Waghorn et al. 2024). On all farms, levamisole was tested individually and then several combination products that contained levamisole. The individual use of levamisole achieved efficacies ranging between 44% and 71% against *Cooperia spp*. across all farms. The combination of abamectin and monepantel was the only efficacious treatment against *Cooperia spp*. (>95%). *Ostertagia spp.* was resistant to oxfendazole (BZ) on 3 farms, and on one farm this parasite showed resistance to all the drugs tested. These results clearly

demonstrate the advanced stages of multiple drug resistance in the most pathogenic GIN of cattle in New Zealand.

Similar results showing a high prevalence of resistance in multiple species of cattle nematodes including *Ostertagia* have been reported in other countries (Soutello, Seno et al. 2007, Suarez and Cristel 2007, Rendell 2010). Consequently, evidence strongly suggests that resistance in *Ostertagia* is already a problem on some farms, and will continue to worsen and become a serious threat to cattle health and productivity in the US in the near future, unless major changes in anthelmintic treatment strategies are implemented immediately.

Haemonchus contortus as a model for studying anthelmintic resistance and screening of novel compounds

Trichostrongylid nematodes that parasitize ruminants, stand as one of the primary targets for anthelmintic treatment due to their significant impact in livestock production (Geary 2016). Among them, *H. contortus* is the most pathogenic and most prevalent parasitic nematode of small ruminants. This species has emerged as a model in anthelmintic discovery (Geary 2016) and the study of anthelmintic resistance (Gilleard 2013). *Haemonchus* has developed resistance to all anthelmintic drug classes (Drudge, Szanto et al. 1964, Van Wyk and Gerber 1980, Green, Forsyth et al. 1981, Carmichael, Visser et al. 1987, van Wyk and Malan 1988, Mederos, Ramos et al. 2014), and multiple drug resistance has now emerged in many regions of the world (Kotze and Prichard 2016, Kaplan 2020). This remarkable adaptability to overcome drug treatment and other key biological features, have positioned *Haemonchus* as a model for studying AR and the pharmacology of drugs (Kotze and Prichard 2016, Lanusse, Alvarez et al. 2016).

As a clade V nematode (Blaxter, De Ley et al. 1998), *H. contortus* is closely related to the parasite species that have a huge economic impact on agriculture, as well as to the human hookworms (Diawara, Schwenkenbecher et al. 2013). Thus, studies in *Haemonchus* constitute a valuable resource for many parasites of human and veterinary importance. In addition, it shares many aspects of core biology with *C. elegans*, also a clade V nematode (Gilleard 2004), favoring the development of comparative studies and creating a “cycle of discovery” (Wit, Dilks et al. 2021). This was first exemplified by the experiments conducted to elucidate the molecular basis of resistance to BZ drugs in *H. contortus* (Kwa, Kooyman et al. 1993, Kwa, Veenstra et al. 1994, Kwa, Veenstra et al. 1995). Kwa and colleagues demonstrated that by introducing a beta-tubulin isotype-1 transcript from BZ-susceptible *H. contortus* into BZ-resistant *C. elegans* nematodes, susceptibility was reinstated in *C. elegans*. In contrast, the *H. contortus* constructs that carried tyrosine at position 200 did not alter the BZ drug phenotype (Kwa, Veenstra et al. 1995). This enabled the confirmation of the association between the beta-tubulin isotype-1 gene and resistance to BZ drugs.

However, in addition to studying sufficiency and necessity of alleles when conferring a resistant phenotype, it is also possible to study the impact of those alleles on the population of worms, when introduced in *C. elegans* (Wit, Dilks et al. 2021). Alleles associated with resistance can now be introduced in *C. elegans* using CRISPR-Cas9 genome-editing, and high-throughput assays can be performed to test for resistance, fitness effects, and, dominance (Dilks, Hahnel et al. 2020, Dilks, Koury et al. 2021). This “cycle of discovery” (Wit, Dilks et al. 2021), complements the strengths of each system in the study of the molecular aspects of AR, but also when predicting the

impact of this genetic changes on the phenotype, and hence understanding the observations on the field. Furthermore, *Haemonchus* is one of the few parasitic nematodes with a high-quality, highly contiguous reference genome (Laing, Kikuchi et al. 2013, Doyle, Tracey et al. 2020). This genomic resource can then be coupled with the utilization of genetic crosses and mapping experiments to elucidate the genomic loci and mechanisms of AR in parasitic nematodes (Doyle and Cotton 2019).

With a direct and short life cycle, each *Haemonchus* female can produce thousands of eggs per day, allowing the collection of large amounts of biological and genetic material to study (Laing, Kikuchi et al. 2013). The infective stage (L3) can be stored for months at room temperature or cryopreserved (Van Wyk, Gerber et al. 1977). These can then be used to infect live animals, with infections in young sheep/goats lasting for many weeks or months (Saccareau, Salle et al. 2017). Adults are relatively large, facilitating straightforward pharmacological studies both *in vivo* and *in vitro* (Geary 2016). *Haemonchus* can be used in drug discovery via *in vitro* screening of novel compounds on larval and adult stages, the use of lab animal models as initial *in vivo* screens, and to identify and validate anthelmintic targets (Geary 2016). For instance, the gerbil (*Meriones unguiculatus*) – *Haemonchus contortus* model, has aided in the screening of novel compounds since first described in 1990 (Conder, Jen et al. 1990). This model is based on the fact that *H. contortus* can infect gerbils and develop to the immature adult stage in immunosuppressed animals. Conder and colleagues demonstrated that commercially available anthelmintics clear >95% of *H. contortus*, and the doses needed are very similar to those used in ruminants (Conder, Jen et al. 1990).

Therefore, this is a highly predictive model for *in vivo* efficacy of novel compounds, and target validation (Conder, Jen et al. 1990).

An example of the value of using *H. contortus* paired with other systems for the screening of novel compounds, was the successful discovery of monepantel (Ducray, Gauvry et al. 2008, Kaminsky, Gauvry et al. 2008). Scientists with Novartis Animal Health screened 36 AAD analogs (Ducray, Gauvry et al. 2008) using a modified *H. contortus* and *T. colubriformis* larval development assay (Gill, Redwin et al. 1995). This was followed by *in vivo* testing using the gerbil-*Haemonchus contortus* model (Conder, Jen et al. 1990), and further testing of lead compounds on sheep parasitized with *H. contortus*. Through these screenings, the AAD-1566 (monepantel) compound was prioritized for development, ending as a commercially available anthelmintic (Zolvix, Novartis Animal Health Inc.) for sheep and recently, also for cattle in many parts of the world. Moreover, *Haemonchus* also played an important role in the discovery of the mode of action of monepantel in parasitic nematodes. A promising candidate gene target, *Ce-acr-23*, was initially identified in resistant mutants of *C. elegans* through forward genetic screenings (Kaminsky, Gauvry et al. 2008). The ACR-23 protein is a member of the DEG-3 group of nicotinic acetylcholine receptors (nAChRs), which is a subfamily specific to nematodes and not found in mammals. Subsequently, the gene exhibiting the closest resemblance to *Ce-acr-23*, was discovered in *H. contortus* and designated as *Hco-mptl-1*. Large batches of *Haemonchus* larvae were then exposed to monepantel and selected for resistance. The analysis of susceptible and resistant nematodes revealed that resistant individuals had lost at least part of the *H. contortus* homologue of *acr-23* (Kaminsky, Gauvry et al. 2008). These results suggested the

involvement of *Hco-mptl-1* as a target of monepantel, and highlight the value of complementary studies between *C. elegans* and *H. contortus* in drug discovery and, in the study of the mechanisms behind drug activity and anthelmintic resistance.

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CHAPTER 2

BROAD-SPECTRUM INHIBITORS FOR CONSERVED UNIQUE PHOSPHOETHANOLAMINE METHYLTRANSFERASES IN PARASITIC NEMATODES POSSESS ANTHELMINTIC EFFICACY [1]

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[1]Zhang X[#], Sicalo Gianechini L[#], Li K[#], Kaplan RM, Witola WH. Broad-Spectrum Inhibitors for Conserved Unique Phosphoethanolamine Methyltransferases in Parasitic Nematodes Possess Anthelmintic Efficacy. 2023. Antimicrob Agents Chemother 15;67(6): e0000823. doi: 10.1128/aac.00008-23. Reprinted here with permission of the publisher. [#] Contributed equally.

Abstract

In humans, nematode infections are prevalent in developing countries, causing long-term ill health, particularly in children. Worldwide, nematode infections are prevalent in livestock and pets, affecting productivity and health. Anthelmintic drugs are the primary means of controlling nematodes, but there is now high prevalence of anthelmintic resistance, requiring urgent identification of new molecular targets for anthelmintics with novel mechanisms of action. Here, we identified orthologous genes for phosphoethanolamine methyltransferases (PMTs) in nematodes within the families *Trichostrongylidae*, *Dictyocaulidae*, *Chabertiidae*, *Ancylostomatoidea*, and *Ascarididae*. We characterized these putative PMTs and found that they possess *bona fide* PMT catalytic activities. By complementing a mutant yeast strain lacking the ability to synthesize phosphatidylcholine, the PMTs were validated to catalyze the biosynthesis of phosphatidylcholine. Using an *in vitro* phosphoethanolamine methyltransferase assay with PMTs as enzymes, we identified compounds with cross-inhibitory effects against the PMTs. Corroboratively, treatment of PMT-complemented yeast with the PMT inhibitors blocked growth of the yeast, underscoring the essential role of the PMTs in phosphatidylcholine synthesis. Fifteen of the inhibitors with the highest activity against complemented yeast were tested against *Haemonchus contortus* using larval development and motility assays. Among them, four were found to possess potent anthelmintic activity against both multiple drug-resistant and susceptible isolates of *H. contortus*, with IC_{50} values (95% confidence interval) of 4.30 μM (2.15–8.28), 4.46 μM (3.22–6.16), 28.7 μM (17.3–49.5), and 0.65 μM (0.21–1.88). Taken together, we have

validated a molecular target conserved in a broad range of nematodes and identified its inhibitors that possess potent *in vitro* anthelmintic activity.

Keywords: conserved enzymes; helminths; molecular targets; novel anthelmintics; phosphatidylcholine biosynthesis.

Introduction

Parasitic nematode infections are common among humans, with over 300 species that are known to infect humans (Coombs and Crompton 1991, Vos, Allen et al. 2016). It is estimated that around 3.5 billion humans are infected by the most prevalent nematodes, including *Ascaris lumbricoides*, *Ancylostoma duodenale*, *Necator americanus* and *Trichuris trichiura* (Stepek, Buttle et al. 2006, Brooker 2010). In humans, nematode infections are highly prevalent in developing countries and are associated with low-income groups leading to health problems and thus require sustained control efforts (Anderson 2000). On the other hand, in animal health, nematode infections are highly prevalent worldwide and are among the most economically important factors affecting production in grazing livestock, costing the global livestock industry billions of dollars annually in lost production and treatment expenses (Morgan, Charlier et al. 2013, Leathwick and Besier 2014, Preston, Jiao et al. 2017).

The main mode of control of parasitic nematode infections in humans and animals is through the administration of anthelmintic drugs. At present, only a few main classes of anthelmintic drugs are commercially available, including benzimidazoles, imidazothiazoles, tetrahydropyrimidines, macrocyclic lactones, amino-acetonitrile

derivatives (AAD), salicylanilides, and cyclooctadepsipeptides (Kaplan 2004, Holden-Dye and Walker 2007, Ducray, Gauvry et al. 2008, Yadav and Singh 2011). However, anthelmintic resistance in livestock nematodes has become a serious global problem (Kaplan and Vidyashankar 2012). Although less prevalent than in livestock, anthelmintic treatment failures have also been reported in humans for most commonly used anthelmintics (Shoop 1993, De Clercq, Sacko et al. 1997, Reynoldson, Behnke et al. 1997, Geerts and Gryseels 2001). With the increasing prevalence of anthelmintic resistance, there is an urgent need for concerted efforts to identify novel strategies for developing new effective drugs (Sargison, Jackson et al. 2007).

Within the infected host, nematodes are prolific egg layers, requiring active biogenesis of nematode plasma membranes in which phospholipids, particularly phosphatidylcholine, are major constituents (Kent 1995, Vial 1998). Phosphatidylcholine is important for maintaining the plasma membrane architecture and functions, as well as for cellular signal transduction, thus indicating that it is indispensable for survival of parasitic nematodes (Lee and Jez 2014). Significant disparities in fundamental biochemical and metabolic pathways of phospholipids between parasitic nematodes and their animal hosts exist. A plant-like phosphobase methylation pathway involving the three-step S-adenosyl-l-methionine (SAM)-dependent methylation of phosphoethanolamine to phosphocholine for the biosynthesis of phosphatidylcholine has been characterized and found to be essential for the protozoan parasite, *Plasmodium falciparum* (Witola, El Bissati et al. 2008, Bobenchik, Witola et al. 2013), as well as for the free-living nematode *Caenorhabditis elegans* (Palavalli, Brendza et al.

2006) and the livestock parasitic nematode *Haemonchus contortus* (Lee, Haakenson et al. 2011, Witola, Cooks-Fagbodun et al. 2016, Witola, Matthews et al. 2016).

The phosphobase methylation step in *C. elegans* and *H. contortus* is catalyzed by two phosphoethanolamine methyltransferases, PMT1 and PMT2, that sequentially methylate phosphoethanolamine (PE) to phosphocholine (Palavalli, Brendza et al. 2006, Brendza, Haakenson et al. 2007, Lee, Haakenson et al. 2011). PMT1 possesses an N-terminal methyltransferase domain that catalyzes the first methylation of PE to phosphomonomethylethanolamine (PMME), while PMT2 possesses the C-terminal domain that catalyzes the subsequent methylation steps of converting PMME to phosphodimethylethanolamine (PDME) and then methylating PDME to phosphocholine, which enters the Kennedy pathway to be converted to phosphatidylcholine (Brendza, Haakenson et al. 2007, Lee and Jez 2013). Because this phosphobase methylation step does not exist in mammalian cells, it is considered an attractive nematode-specific molecular target for the development of a novel class of efficacious anthelmintic drugs (Elabbadi, Ancelin et al. 1997, McCarter 2004, Mitreva, Appleton et al. 2005, Witola, Cooks-Fagbodun et al. 2016, Witola, Matthews et al. 2016).

Here, we used genetic and biochemical approaches to functionally characterize putative phosphoethanolamine methyltransferases (PMTs) from various families of parasitic nematodes and identified their broad-spectrum inhibitors that we then tested for *in vitro* anthelmintic efficacy.

Materials And Methods

Identification of putative nematode PMTs

Mining of nematode protein and genome databases was performed by a BLAST search using amino acid sequences of the PMTs for the parasitic nematode *Haemonchus contortus* that have been previously cloned and characterized as H. contortus phosphoethanolamine methytransferase-1 (HcPMT1) and *H. contortus* phosphoethanolamine methytransferase-2 (HcPMT2) (Lee and Jez 2013, Witola, Matthews et al. 2016). The HcPMT1 N-terminal methyltransferase domains that catalyze the first step of methylating phosphoethanolamine (PE) to phosphomonomethylethanolamine (PMME) and the HcPMT2 C-terminal domains that functions to catalyze subsequent methylation steps to convert PMME to phosphodimethylethanolamine (PDME) and PDME to phosphocholine were targeted as the conserved sequences (Brendza, Haakenson et al. 2007, Lee and Jez 2013). The retrieved sequences were analyzed by multiple sequence alignment using TCoffee (http://tcoffee.crg.cat/apps/tcoffee/do:regular).

Cloning and expression of putative PMTs from different families of nematodes

The coding sequences for each putative PMT were retrieved from GenBank and submitted to Integrated DNA Technologies (IDT, USA) for synthesis. The submitted putative PMT coding sequences had the following GenBank accession numbers: *Ancylostoma duodenum* PMT1 (AcPMT1), KN730644; *Ancylostoma ceylanicum* PMT2 (AcPMT2), KE125104.1; *Ascaris suum* PMT (AsPMT), LK871972.1; *Dictyocaulus viviparus* PMT1 (DvPMT1), KN716207.1; *D. viviparus* PMT2 (DvPMT2), KN716960.1; *Oesophagostomum dentatum* PMT (OdPMT), KN550380.1; and *Toxocara canis* PMT (TcPMT2), JPKZ01000489.1. The synthesized gene fragments were supplied by IDT cloned in pUCIDT (AMP) vector and lyophilized. Before use, they were reconstituted in

sterile molecular-grade nuclease-free water. The primer pairs used for PCR-amplification of the PMT coding gene fragments for directional cloning in the pET15b expression vector (Novagen) in-frame with the N-terminal hexahistidine tag (His tag) are listed in Table S7. The recombinant expression vectors were sequenced to confirm identity of the PMT insert, amplified in the K-12 strain of *E. coli* cells (NEB Turbo; New England Biolabs) and transformed into protein expression BL21-CodonPlus (DE3)-RIL *E. coli* (Agilent Technologies). For *H. contortus* PMTs (HcPMT1 and HcPMT2), the protein expression *E. coli* cells generated previously were used (Witola, Matthews et al. 2016). Transformed *E. coli* were cultured at 37°C in Luria broth medium containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol to an absorbance of 0.6 to 0.8 at a wavelength of 600 nm, and protein expression induced by addition of 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG). For HcPMT1, after induction with IPTG, the culture was incubated at 16°C with shaking for 15 h. For the rest of the PMTs, bacteria were cultured at 37°C for a further 3 h after induction.

Bacterial cells were pelleted by centrifugation. For HcPMT1, 1 g of bacterial pellet was resuspended in 15 mL of lysis buffer (20 mM imidazole, 50 mM Tris base, 500 mM NaCl, 10% [vol/vol] glycerol [pH 8.0]) containing a 1× EDTA-free protease inhibitor cocktail (Pierce) and 75 kU of rLysozyme (Sigma). For the rest of the PMTs, 1 g of bacterial pellet was resuspended in 15 mL of lysis buffer composed of 50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 5% (vol/vol) glycerol, 0.5% (vol/vol) Sarkosyl, 1× EDTA-free protease inhibitor cocktail, and 60 kU of rLysozyme (pH 8.0). The resuspended pellets were lysed by sonication and clarified by centrifugation at 20,000 × g for 20 min at 4°C. The supernatant was collected and mixed with 2 mL of

prewashed Ni-NTA His Bind Resin suspension (Novagen) and incubated at 4°C for 2 h with agitation. The His-tagged recombinant proteins were purified under native conditions by nickel-affinity chromatography according to the manufacturer's instructions (Novagen). For HcPMT1, the wash buffer contained 20 mM imidazole, 50 mM Tris base, 500 mM NaCl, and 10% (vol/vol) glycerol (pH 8.0), while the elution buffer was composed of 250 mM imidazole, 50 mM Tris base, 500 mM NaCl, and 10% (vol/vol) glycerol (pH 8.0). For the rest of the PMTs, the wash buffer contained 50 mM NaH2PO4, 300 mM NaCl, and 20 mM imidazole (pH 8), while the elution buffer was composed of 50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 5% (vol/vol) glycerol, and 0.5% (vol/vol) Sarkosyl (pH 8). About 10 mL of the protein eluate was dialyzed in 2 L of a first buffer (50 mM Tris base, 100 mM NaCl, 10% [vol/vol] glycerol [pH 7.5]) at 4°C with stirring overnight, followed by dialysis in 2 L of a second buffer (25 mM HEPES, 100 mM NaCl, 10% [vol/vol] glycerol [pH 6.5]) at 4°C for 3 h. Further buffer exchange and concentration of the eluates was performed using protein concentrators (Fisher Scientific). The sample was first centrifuged for 20 min at 4,000 rpm at 12°C. The exchange buffer (5 mM HEPES-KOH, 0.5 mM dithiothreitol [pH 7.8]) was added to make up the volume to 20 mL, followed by centrifugation for 20 min at 1,500 × g at 12°C. Buffer exchange was repeated seven times, and the final sample volume was reduced to ~1 mL. The purity of the recombinant protein was analyzed by SDS-PAGE, and the concentration was measured using a Qubit 3.0 fluorometer (Life Technologies).

Generation of PMT monospecific antisera

The experimental animals (rats) procedures in this study were performed according to Tuskegee University Institutional Animal Care and Use Committee

approved protocol R1110-18-1. Blood was collected from five rats prior to immunization and used to extract preimmune sera. To raise polyclonal antibodies against PMT proteins, the purified recombinant proteins were emulsified with Freund complete adjuvant (Sigma-Aldrich) and injected into the five rats at 40 µg of protein per rat. Two subsequent booster immunizations were administered at 2-week intervals with 20 µg of the recombinant proteins emulsified in Freund incomplete adjuvant. The rats were sacrificed, blood was collected by cardiac puncture, and sera were isolated from the blood. Pooled preimmune and pooled immune sera were used in Western blotting assays to assess the specificity of the antisera against the recombinant proteins. Purification of the PMT antisera was performed as previously described (Witola, Cooks-Fagbodun et al. 2016). Briefly, 8 mL of slurry of Affi-Gel 15 (Bio-Rad) was washed twice with 50 mL of distilled water, followed by two washes in 50 mM $NaHCO_3$ buffer. The washed resin was mixed with 4 mL of solution containing 4 mg of the respective purified recombinant protein, followed by incubation at 4°C for 2 h with shaking. The mixture was centrifuged, the supernatant was removed, and the resin was washed with phosphate-buffered saline (PBS), followed by the addition of 800 µL of glycine (1 M, pH 12) and incubation at 4°C for 1 h. The mixture was washed twice in 50 mL of PBS, 4 mL of crude antiserum was added, and the mixture was incubated at 4°C with shaking overnight. The mixture was packed into a 15-mL column, and after four washes with PBS, the monospecific antibodies were eluted with 15 mL of 0.1 M glycine (pH 12.0). The eluate was collected in 1-mL fractions and neutralized with 150 µL of 1 M Tris (pH 6.8).

In vitro enzyme kinetics of putative nematode PMTs

We customized a commercial methyltransferase HT activity assay kit (ENZO) for use of recombinant PMT proteins as enzymes, S-adenosyl-l-methionine (SAM) as the methyl donor, and PE as the substrate, as reported previously (Witola, Matthews et al. 2016). To determine whether the recombinant PMT proteins possessed methyltransferase activity, we initially tested the activity of each protein at the conditions recommended by the kit manufacturer, with SAM and PE being used at final concentrations of 100 and 200 μM, respectively. Briefly, the reaction mixture contained 2.5 μL of the kit SAM-free methyltransferase reaction buffer, 3 μL of 10 mM PE solution (pH 7.5), 15 μL of 1 mM SAM solution (pH 7.8), and 1× transferase assay buffer to a final volume of 25 μL. Various concentrations (25-μL total volume) of recombinant PMT protein were added to reactions.

To determine the enzyme kinetic parameters on SAM, fixed concentrations of PMT recombinant proteins (between 15 and 100 ng/μL, depending on the derived optimal concentration) and PE (200 μM) were used in the reactions, with various concentrations of SAM (0 to 160 μM). On the other hand, to determine the enzyme kinetic parameters on PE, fixed concentrations of PMT recombinant proteins (between 15 and 100 ng/μL, depending on the derived optimal concentration) and SAM (100 μM) were used, with various concentrations of PE (0 to 250 μM).

For normalization, instead of purified protein, 25 μL of 100 μM S-(5′-adenosyl)-l-homocysteine (SAH) as a positive control, 25 μL of dialysis buffer as a negative control, and 25 μL of 1× transferase assay buffer as a blank control were added to the respective reaction mixtures. The reactions were prepared in triplicate wells using black flat-bottomed 96-well plates. Detection buffer (100 μL) was added into each reaction,

yielding a final reaction volume of 150 µL. The plates were covered in aluminum foil and incubated at room temperature with shaking for 30 min, and fluorescence read at 380EX/520EM using a SpectraMax Gemini EM microplate reader (Molecular Devices). Sample readouts were normalized with positive and negative controls.

PMT complementation of pem1Δ/pem2Δ S. cerevisiae

The *pem1Δpem2Δ S. cerevisiae* mutant strain (SKY010) used in this study was generously provided by Ryouichi Fukuda (Department of Technology, University of Tokyo, Tokyo, Japan). The strain can only grow in medium supplemented with choline, and as such, was propagated in 1069 YPAD medium (ATCC) supplemented with 1 mM choline chloride (Sigma) at 30°C (35). The PMT coding gene fragments were PCR amplified using the respective primer sets listed in Table S8 and T/A-cloned into the expression vector using a pYES2.1 TOPO TA yeast expression kit (Invitrogen). The recombinant plasmids were sequenced to confirm gene identity and correct orientation. Competent *pem1Δpem2Δ S. cerevisiae* was generated with an S.c. EasyComp transformation kit (Invitrogen) according to the manufacturer's instructions. The competent *pem1Δpem2Δ S. cerevisiae* was transformed with recombinant pYES2.1 plasmid harboring respective PMT-coding gene fragments or the negative-control LacZ-coding gene fragment following the instructions in the pYES2.1 TOPO TA yeast expression kit. For nematode PMTs having both PMT1 and PMT2, the yeast cells were transformed with a mixture of plasmids harboring both (HcPMT1+HcPMT2, AcPMT1+AcPMT2, or DvPMT1+DvPMT2), while those with only one PMT were transformed with a single plasmid harboring the respective PMT (AsPMT, OdPMT, or TcPMT). The pYES2.1 TOPO vector has a URA3 selection marker which enables the

uracil auxotrophic *pem1Δpem2Δ S. cerevisiae* to grow in uracil-deficient medium. Therefore, uracil-deficient selection plates containing 6.7 g/L yeast nitrogen base without amino acids (Sigma), 1.92 g/L yeast synthetic dropout medium supplement without uracil, 20 g/L agar, 2% glucose, and 1 mM choline chloride were used to grow the transformed yeast colonies. Ensuing colonies were analyzed by PCR to confirm presence of recombinant pYES2.1 plasmid bearing the respective PMT coding sequence. Positive colonies were inoculated and propagated in 15 mL of selection medium (6.7 g/L yeast nitrogen base without amino acids, 1.92 g/L yeast synthetic dropout medium supplement without uracil, 2% glucose, and 1 mM choline chloride) at 30°C with shaking at 275 rpm overnight.

To determine the ability of PMT- or LacZ-complemented p*em1Δpem2Δ S. cerevisiae* to grow in the absence of choline but in the presence of ethanolamine, 150 µL of the overnight culture was pelleted by centrifugation and resuspended in 10 mL of fresh minimal medium (containing 3.4 g/L yeast nitrogen base without amino acids, 2% glucose, 30 mg/L histidine, 100 mg/L leucine, 100 mg/L adenine and 50 mg/L tryptophan without choline) and cultured at 30°C with shaking for 24 h to starve the cells of choline. The cells were pelleted and resuspended in 1 mL of fresh minimal medium (containing 3.4 g/L yeast nitrogen base without amino acids, 2% galactose, 30 mg/L histidine, 100 mg/L leucine, 100 mg/L adenine, and 50 mg/L tryptophan) and 900 µL of the suspension further diluted 10 times with the same medium, with or without 1 mM ethanolamine. The diluted cultures were incubated at 30°C with shaking for 4 days, during which time the optical density at 600 nm (OD600) was measured every 24 h using a SpectraMax Plus 384 microplate reader (Molecular Devices) to monitor growth.

Western blotting

To determine the expression of PMTs in complemented yeast, 2 mL of the cultured yeast (with the opacity density adjusted to equal amounts) induced for transgene expression were pelleted and resuspended in 1 mL of PBS. The cells were lysed by repeated freeze-thaw, followed by sonication and the addition of an equal volume of Laemmli sample buffer. The samples were boiled for 5 min and snap-chilled on ice, followed by centrifugation. For each sample, 30 µL of the cleared lysate was fractionated by SDS-PAGE and transferred onto a nitrocellulose membrane. Immunoblotting was done using the respective monospecific-purified rat antisera at a dilution of 1:400 as primary antibodies, and horseradish peroxidase-conjugated chicken anti-rat (Thermo Fisher Scientific) as the secondary antibody at a 1:2,000 dilution. Signal generation was performed using Clarity Western ECL substrate (Bio-Rad), and imaging was performed using a FluoroChem R imager (Protein Simple).

Radioisotope incorporation, lipid extraction, and 2D-TLC

After choline starvation (as described above), the complemented *pem1Δ/pem2Δ S. cerevisiae* cultures were diluted 10× and cultured in minimal medium (3.4 g/L yeast nitrogen base without amino acids, 2% galactose, 30 mg/L histidine, 100 mg/L leucine, 100 mg/L adenine, and 50 mg/L tryptophan) containing 1 mM ethanolamine hydrochloride and 0.8 µCi/mL [^{14}C]ethanolamine hydrochloride (American Radiolabeled Chemicals, USA). Due to the inability of the LacZ-complemented *pem1Δ/pem2Δ S. cerevisiae* to grow in the absence of choline, 1 mM choline chloride was added to its culture. The cultures were incubated for 72 h at 30°C with shaking, after which the OD_{600} of cultures was adjusted to the same level by diluting with an appropriate volume

of medium. From each culture, 3 mL was harvested by centrifugation, and the pellet was washed in 10 mL of PBS twice. The lipids were extracted as reported previously (Folch, Lees et al. 1957), with some modifications. Briefly, the washed cell pellet was resuspended in 20 volumes of chloroform-methanol solvent (2:1, vol/vol), and the cells were lysed by sonication. The samples were then agitated in a shaker for 20 min at room temperature and the supernatant collected by centrifugation at 4,000 rpm for 15 min at 4°C. About 0.2 volumes of 0.9% NaCl solution was added to the supernatant and vortexed for 10 s, followed by centrifugation for 5 min at 2,000 rpm at room temperature to separate the aqueous (top clear layer) and organic (bottom layer) phases. The two layers were separately pipetted into sterile clean 10-mL clean glass vials and kept open in a clean bench drawer until completely evaporated. The evaporated organic phase was reconstituted in 200 µL of chloroform-methanol solvent (2:1, vol/vol). Glass chambers were conditioned by placing a 25 cm × 25 cm blotting paper vertically standing, with one end dipping in about 100 mL of first dimension solvent (chloroform-methanol-ammonium hydroxide [84.5/45/6.5, vol/vol/vol]), with the lid tightly sealed with a lining of grease overnight. The following day, Silica Gel-60 20 × 20 plates (Sigma-Aldrich) were baked at 325°C for 10 min. After cooling to room temperature, 50 µL of sample (reconstituted organic lipid phase) was spotted onto a premarked application point at one corner, about 4 cm from the edge and from the bottom. Once the spotted sample had air-dried, the plate was placed in the preconditioned chamber vertically to dip into the first-dimension solvent to a depth of about 1 cm (with the sample application just above the solvent line). The chamber was tightly sealed and the solvent left to migrate up to about 3 cm below the top edge of the plate, after which the plate was air

dried and placed in the chamber with the second solvent (chloroform-acetic acid-methanol-water [90/30/6/2.6, vol/vol/vol/vol]) at 90° angle to the first direction, with the sample application point just above the solvent line, but on the other end. The plate was left for the second solvent front to migrate up to about 4 cm from the top of the plates. After air-drying, the plate was placed in an imaging cassette (with the silica side facing up), and an X-ray film (Hyperfilm ECL; Cytiva) was placed on top. The cassette was closed tightly and kept at −80°C for 2 weeks; the film was then developed for signal visualization.

Screening of a natural compound library for PMT inhibitors

The chemical compounds were obtained from the National Cancer Institute/Developmental Therapeutics Program Open Chemical Repository. The library consisted of 419 compounds categorized as Natural Products Set IV (see Table S1). Compounds were individually reconstituted in molecular biology grade dimethyl sulfoxide (DMSO) from Sigma-Aldrich to a stock concentration of 10 mM and stored at −20°C until use. Initial screening was conducted using recombinant HcPMT1 protein as enzyme in the methyltransferase assay, with test compounds at a final concentration of 40 μM. The test reaction mixtures consisted of 40 μM test compound, 2.5 μL of SAM-free methyltransferase reaction buffer, 150 μM PE (pH 7.5), 120 μM SAM (pH 7.8), and 20 ng/μL of HcPMT1 protein, and the final volume made up to 50 μL with 1× transferase assay buffer. The volume of each test compound added did not exceed 1% of the total reaction volume. To the positive reaction mixture, instead of test compound, an equivalent volume of DMSO was added. In the negative-control reaction mixture, instead of HcPMT1 and test compound, equivalent volumes of 1× transferase buffer and

DMSO, respectively, were added. Detection buffer (100 µL) containing fluorescence substrate was added into each reaction mixture. The reactions were prepared in triplicate wells in 96-well plates. The plates were covered in aluminum foil and incubated at room temperature with agitation for 30 min, followed by reading the fluorescence at 380EX/520EM. The mean percent inhibition (MPI) of the test compound on enzymatic activity was calculated using the following formula:

$$\mathrm{MPI} = \frac{\Delta\mathrm{RFUpositive\ control} - \Delta\mathrm{RFUcompounds}}{\Delta\mathrm{RFUpositive\ control}} \times 100$$

where the positive control is a reaction without compound but with recombinant PMT protein and a volume of DMSO equivalent to that of compound used in test reactions; the negative control is the reaction without protein and compounds, but with volumes of dialysis buffer and DMSO equivalent to protein and compound added in the test reaction mixtures, respectively; $\Delta RFU_{positive\ control}$ represents the absolute relative fluorescence units (RFU) for the positive control, which equals $RFU_{positive\ control} - RFU_{negative\ control}$; and $\Delta RFU_{compounds}$ represents the absolute RFU value for the sample with the compounds, which equals $RFU_{compounds} - RFU_{negative\ control}$.

In vitro compound cytotoxicity assays

Compounds were tested for cytotoxicity in human ileocecal colorectal adenocarcinoma cells (HCT-8) (ATCC CCL-244; RRID:CVCL_2478) by using the cell proliferation reagent WST-1 (Roche) according to the manufacturer's protocol. The WST-1 assay is a quantitative colorimetric assay for measurement of metabolically active cells. This assay is based on the reduction of the tetrazolium salt (WST-1) by viable cells. About 5 × 104 HCT-8 cells were seeded per well in 96-well plates and

grown overnight in 200 µL of RPMI 1640 medium (without phenol red; Gibco) supplemented with 2.5 g/L of glucose, 1 mM sodium pyruvate, 1.5 g/L of sodium bicarbonate, 10% heat-inactivated FBS (Gibco), and 1× antibiotic-antimycotic (Gibco) at 37°C with 5% CO2 in a humidified incubator. Upon reaching 80 to 90% confluence, cells were treated with various concentrations of the test compounds for 24 h. Control wells received equivalent volumes of DMSO used in the reconstituted compounds. After 24 h of culture, 10 µL of the WST-1 reagent was added to each well, and the plates were incubated in the dark for 1 h at 37°C with 5% CO_2. The plates were well agitated, and 150 µL of the medium from each well was transferred to a new clear flat-bottomed black 96-well plate (Corning). The absorbance was read at a test wavelength of 440 nm and a reference wavelength of 690 nm using a multimode microplate reader (Spectra Max iD3; Molecular Devices, USA). All of the reactions were prepared in triplicate. The dose-response curves were generated by nonlinear regression analysis with GraphPad PRISM software using control-normalized data to determine the half-maximal cytotoxicity (IC_{50}) concentrations.

Screening HcPMT1 inhibitors for cross-inhibitory effect against AcPMT1, AsPMT, DvPMT1, OdPMT, and TcPMT

Compounds that had shown more than 50% inhibition on HcPMT1 activity and had no or very low cytotoxicity against HCT-8 cells were further tested for cross-inhibitory effect against the enzymatic activity of other recombinant PMT proteins (AcPMT1, AsPMT, DvPMT1, OdPMT, and TcPMT) at 40 µM. The enzymatic assay was set up as described above, with the respective PMT protein as enzyme. Compounds that depicted cross-inhibitory activity were further tested at various concentrations to

derive their half-maximal effective concentrations (EC_{50}) using nonlinear regression analysis by GraphPad Prism software.

Effect of PMT inhibitors on the growth of PMT-complemented pem1Δ/pem2Δ S. cerevisiae

To determine the effect of PMT inhibitors on the growth of PMT- and LacZ-complemented *pem1Δpem2Δ S. cerevisiae*, the yeast cells were first starved of choline as described above, and the cells were diluted to an OD_{600} of ~0.6. The PMT-complemented yeast cells were cultured in SG medium (3.4g/L yeast nitrogen base without amino acids, 2% galactose, 30 mg/L histidine, 100 mg/L leucine, 100 mg/L adenine, and 50 mg/L tryptophan) supplemented with 1 mM ethanolamine hydrochloride, while the LacZ-complemented yeast cells were cultured in SG medium supplemented with 1 mM choline chloride. Compounds that depicted cross-inhibitory activity against PMT proteins *in vitro* were added at various concentrations to the cultures. As a negative control, DMSO (at a final concentration not exceeding 2% [vol/vol]) was added to yeast cultures designated control cultures. The cultures were incubated at 30°C with shaking at 275 rpm for 4 days. On a daily basis during the course of culture, 200-µL portions of culture aliquots were taken, and their OD_{600}s were measured using a microplate reader. In preliminary work, we had observed that the growth of PMT- and LacZ-complemented *pem1Δpem2Δ S. cerevisiae* plateaued at 3 to 4 days of culture. Therefore, to determine the inhibitory effect of nematode PMT inhibitors on the growth of PMT- and LacZ-complemented *pem1Δpem2Δ S. cerevisiae*, measurements taken on day 3 were used. The results were presented as percentage of growth rate using the following formula:

$$\text{PGR} = \frac{growth\ rate\ (plus\ inhibitor)}{growth\ rate\ (minus\ inhibitor)} \text{ x } 100$$

where the percent growth rate (PGR) is the growth rate of inhibitor-treated PMT- or LacZ- complemented *Pem1Δpem2Δ S. cerevisiae*, considering the growth rate of untreated PMT- or LacZ- complemented *Pem1Δpem2Δ S. cerevisiae* is 100%; the growth rate = ΔA600Day 3/ΔA600Day 0; ΔA600Day 0 is the absolute value of A600 of PMT- or LacZ- complemented *pem1Δ/pem2Δ S. cerevisiae* at day 0, which equals the A600 of the culture minus the A600 of the growth medium only; ΔA600Day 3 is the absolute value of the A600 of PMT- or LacZ complemented *pem1Δ/pem2Δ S. cerevisiae* at day 3, which equals the A600 of the culture at day 3 minus the A600 of growth medium only; the growth rate (plus inhibitor) is the growth rate of PMT- or -LacZ complemented *pem1Δpem2Δ S. cerevisiae* in the presence of test compound; and the growth rate (minus inhibitor) is the growth rate of the PMT- or LacZ complemented *pem1Δ/pem2Δ S. cerevisiae* in the absence of test compound, but with a volume of DMSO equal to that of compound added in the treated culture. The dose-response curves were generated with GraphPad Prism software and IC_{50} concentrations of the compounds in PMT- and LacZ-complemented yeast cells derived.

In vitro testing of anthelmintic activity of broad-spectrum PMT inhibitors

The *H. contortus* isolates used were TxPh-2011-S (Texas A&M University, USA) and UGA MDR 2020 (University of Georgia Athens, USA). TxPh-2011-S (Texas A&M University, USA) was originally isolated from Pronghorn antelope in West Texas in 2011 and is fully drug susceptible, phenotypically determined by the DrenchRite larval development assay (LDA; Microbial Screening Technologies, Kemps Creek, Australia).

To establish this as a laboratory isolate, it was initially passaged in goats at Texas A&M University and then at The University of Georgia using both goats and sheep, and it is currently maintained in lambs at Louisiana State University. A nemabiome assay (Avramenko, Redman et al. 2015) was conducted at the University of Calgary to confirm that the isolate is 100% *H. contortus*. The UGA MDR 2020 (University of Georgia Athens, USA) isolate is resistant to ivermectin, benzimidazoles, levamisole, and moxidectin, as determined by the DrenchRite LDA. To establish this as a laboratory isolate, feces collected from a sheep farm in Georgia with known history of multiple anthelmintic resistance were cultured. Two donor goats were infected with the larvae recovered, which were morphologically (van Wyk and Mayhew 2013) identified as 89% *H. contortus*. A fecal egg count reduction test (FECRT) was then performed in the infected goats. First, a combination treatment with albendazole at 15 mg/kg and ivermectin at 0.4 mg/kg was performed, followed by a treatment with levamisole at 12 mg/kg. Later, and to confirm resistance to moxidectin, another treatment was administered using a dose of 0.4 mg/kg of moxidectin. Posttreatment feces were recovered, coprocultures were established, and L3 larvae were identified as 100% *H. contortus*. Resistance to all drugs was subsequently confirmed using a DrenchRite assay.

Nematode eggs were isolated as previously described (Howell, Burke et al. 2008). After the eggs were resuspended in water, a cleaning protocol (Kotze, O'Grady et al. 2009) was followed to minimize contamination in the assay plates. Eggs were agitated gently in a solution of 8.4-mg/L sodium hypochlorite for 12 min and then washed three times with water. The eggs were diluted in distilled water at a

concentration of 50 to 100 eggs per 20 µL after the addition of amphotericin B (final concentration, 90 µL/mL) and used immediately for LDAs.

Testing with the LDA was done in two phases. In the first phase, all compounds were tested against the susceptible isolate. In the second phase, all compounds that demonstrated activity in the first phase were retested against both susceptible and the resistant isolates, yielding between 3 and 4 biological replicate assays for each hit compound. Technical replicates were defined as replicate wells within the same assay plate with eggs derived from the same pool of feces and tested with drug solutions made from the same stock solution. Biological replicates were represented by different fecal collections and respective egg isolation on a different day from other biological replicates. Compounds dissolved in 100% DMSO were diluted in the assay plate using water as an initial diluent to 5% DMSO and then in agar to a final concentration of 1% DMSO. No precipitation was observed in any of the diluted concentrations. Eight drug concentrations were tested—125.000, 31.250, 7.810, 1.950, 0.490, 0.120, 0.031, and 0.008 µM—with three replicate wells for each concentration per plate. In the case of compound NSC99791, the original stock concentration differed from the other compounds, yielding final concentrations of 100.000, 25.000, 6.250, 1.560, 0.390, 0.098, 0.024, and 0.006 µM. Three compounds were tested in each assay plate, and 24 wells served as controls (i.e., no drug and 1% DMSO in 2% agar). Plates were prepared in advance and stored at 4°C for periods no longer than 5 days. Prior to use, plates were warmed to room temperature, and 20 µL of water was added to each well to fully hydrate the agar. Between 50 and 100 eggs contained in 20 µL of water were then added, depending on the number of eggs isolated. Plates were sealed with Parafilm to

reduce moisture loss and placed in an incubator at 25°C. After 24 h, 20 µL of nutritive media composed of 0.87% Earle's balanced salts (Sigma-Aldrich, St. Louis, MO), 1% yeast extract (BD Difco, VWR; Becton Dickinson, Sparks, MD), 0.76% NaCl (Sigma-Aldrich), and 1% E. coli OP50 was added to each well. The plates were resealed and incubated for 6 additional days, and the assays were ended with the addition of 20 µL of 50% Lugol iodine to each well. The contents of every well were then transferred to a clean 96-flat well plate, and all eggs and larvae in each well were counted under an inverted microscope as previously described (Tandon and Kaplan 2004). Development to the L3 stage was corrected for all of the treatment wells based on the average development in the control wells. The percent larval development (to the L3 stage) was calculated for each well by dividing the number of L3 by the total count of larvae (L1+L2+L3) for that well and then multiplying that value by 100. The percent inhibition in larval development (%ILD) for each individual drug well was then calculated compared to the percent development in control wells by dividing the percent larval development of each drug well by the mean percent larval development of the control wells (for that plate) according to the following formula:

$$\%\text{ILD} = \frac{\text{mean \% development control} - \text{mean \% development treated}}{\text{mean \% development control}} \text{ x } 100$$

Data from wells were discarded before Prism analyses if there was evidence of excessive dryness or fungal or bacterial overgrowth. Outliers were identified by using the Z-score outlier detection method. Moreover, Z-score of zero represents a value that equals the mean while a Z-score of 2 indicates that an observation is two standard deviations above or below the mean. Any value outside the Z-score of ±2 for each given

concentration was classified as outlier and discarded from further analysis. The statistical analysis of the data was done using GraphPad Prism version 9.4.0. A nonlinear regression model with a variable slope (GraphPad Software, La Jolla, CA) was the method applied. Between 9 and 12 replicate values were obtained for each drug concentration, previously corrected for control. Drug concentrations were log10 transformed, and then IC_{50} concentrations and dose-response curves were obtained from the log[inhibitor] versus the normalized response variable slope logistic equation for each drug.

For LMAs, the first phase testing using compounds NSC133100 and NSC177383 was done using the Worminator system in parallel with the LDA. Initial stock solutions of 12.5 mM were prepared for each compound using 100% DMSO as a solvent. Initial stock solutions (25 µL) were then diluted in 475 µL of culture media to yield working stock solutions of 625.00, 156.30, 39.10, 9.80, 2.40, 0.60, 0.20, 0.04, and 0.00 µM in 5% DMSO for each drug. In plate, 200 µL of working stock solutions were then diluted in 800 µL of culture media, yielding concentrations of 125.00, 31.25, 7.81, 1.95, 0.49, 0.12, and 0.00 µM in 1% DMSO. For the second phase of testing, compounds showing anthelmintic activity with the LDA were tested in a second round at higher concentrations than in the first phase of testing. Initial stocks of 50 mM were prepared for compounds NSC133100, NSC177383, NSC145612, and NSC56410 using 100% DMSO as a solvent. Initial stock solutions (25-µL portions) were then diluted in 475 µL of culture media consisting of 65% Lauria-Bertani (LB) (Thermo Fisher Scientific, Branchburg, NJ), 25% NCTC (Sigma-Aldrich), 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), Pen/Strep (Sigma-Aldrich) at a final concentration of

100 mg/mL, and amphotericin B (Sigma-Aldrich) at a final concentration of 2.5 mg/mL. The working stock solutions yielded concentrations of 2,500.00, 625.00, 156.30, 39.10, 9.80, 2.40, and 0.00 μM in 5% DMSO for each compound. In each well in the plate, 200 μL of working stock solutions were then diluted in 800 μL of culture media, yielding final concentrations of 500.00, 125.00, 31.30, 7.81, 1.95, 0.49, and 0.00 μM in 1% DMSO.

For the motility assays, 24-well plates with inserts were used (Costar, catalog no. 3379; Corning, Inc., Corning, NY). Portions (640 μL) of culture media were added to each well, and approximately 50 exsheathed larvae contained in 160 μL of culture media in each insert. Once pretreatment readings were taken, 160 μL of the drug solution was added to the well, and another 40 μL was added to the insert, reaching the final concentrations described in the protocol above. All concentrations were tested in triplicate. After addition of the drugs, plates were incubated at 5% CO2 and 37°C. Motility readings were taken at 1, 4, 24, 48, 72, and 96 h. A medium change was conducted at 48 h, with new drug added. Prior to each reading, plates were shaken for 15 min at ambient temperature using a benchtop shaker to stimulate motility of the L3 larvae (previously tested in our lab). The Worminator assay was performed as previously described (George, Lopez-Soberal et al. 2018). The percent inhibition in motility for each drug well was calculated by comparing the mean motility to the mean motility of the control wells. Statistical analyses were performed using Prism 9.4.0 (GraphPad). Drug concentrations were log10 transformed, and a variable slope nonlinear regression algorithm was applied to the data. IC_{50} values were generated using log[inhibitor] versus response variable slope (four parameters).

Statistical analysis

Data were analyzed using two-way analysis of variance with Tukey's multiple-comparison post hoc test using GraphPad Prism v9.4.0. In addition, Student t tests were performed to evaluate differences between groups. P values of ≤0.05 were considered significant.

Results

Orthologous putative PMTs in parasitic nematodes of different families

Despite the limited genome databases for parasitic nematodes, we performed BLAST searches and identified orthologues of *H. contortus* PMT 1 and 2 (HcPMT1 and HcPMT2) in other parasitic nematodes within the families *Trichostrongylidae*, *Dictyocaulidae*, *Chabertidae*, *Ancylostomatoidea*, and *Ascarididae*. *H. contortus* HcPMT1 possesses N-terminal methyltransferase domains that catalyze the first step of methylating phosphoethanolamine (PE) to phosphomonomethylethanolamine (PMME) (Lee and Jez 2013). We identified gene orthologues containing these domains in nematode genera, including *Oesophagostomum*, *Ancylostoma*, *Dictyocaulus*, and *Ascaris* (Fig. 1). In some of these same genera and others, we also identified genes that are orthologous to HcPMT2 (Fig. 2). HcPMT2 possesses C-terminal domains that function to catalyze the subsequent methylation steps of converting PMME to PDME and methylating PDME to phosphocholine (Brendza, Haakenson et al. 2007, Lee and Jez 2013). A coding gene sequence (orthologous to HcPMT1) from *Ancylostoma duodenale* is 1,026 bp long and encodes a 341-amino-acid polypeptide (GenBank accession number KIH60772.1) with an estimated molecular weight of 37.6 kDa and is

here named AcPMT1 (Fig. 1). In addition, from *Ancylostoma ceylanicum*, we found a 1,296-bp coding gene sequence that is orthologous to HcPMT2 that encodes a 431-amino-acid polypeptide (GenBank accession number EPB71549.1) with an estimated molecular weight of 47.5 kDa and is here called AcPMT2 (Fig. 2). The lungworm (*Dictyocaulus viviparus*) genome contained coding sequences of both HcPMT1 and HcPMT2 orthologues, namely, DvPMT1 (1,452 bp) and DvPMT2 (645 bp). DvPMT1 and DvPMT2 code for 483- and 214-amino-acid polypeptides (GenBank accession numbers KJH50371.1 and KJH40940.1), with calculated molecular weights of 53.3 and 23.7 kDa, respectively (Fig. 1 and 2).

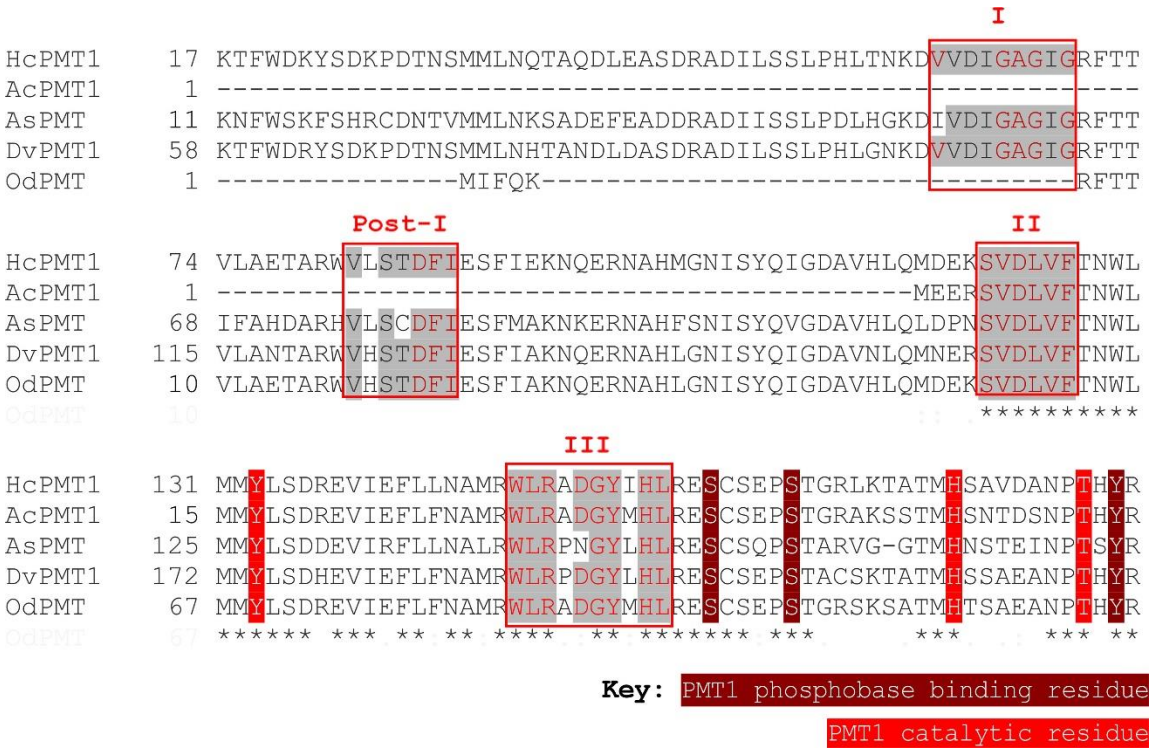

Figure 1. Multiple sequence alignment of *Haemonchus contortus* PMT1 (HcPMT1, 4KRG_A), *Ancylostoma duodenale* PMT1 (AcPMT1, KIH60772.1), *Ascaris suum* PMT1 (AsPMT1, ERG79882.1), *Dictyocaulus viviparus* PMT1

(DvPMT1, KJH50371.1), and *Oesophagostomum dentatum* PMT1 (OdPMT1, KHJ94304.1) by using TCoffee (http://tcoffee.crg.cat/apps/tcoffee/do:regular). Four *S*-adenosyl methionine (SAM)-binding motifs that define the methyltransferases domains are labeled I, Post-I, II, and III and are boxed as previously described (Lee and Jez 2013, Lee and Jez 2014). Asterisks (*) indicate positions with a single, fully conserved residue.

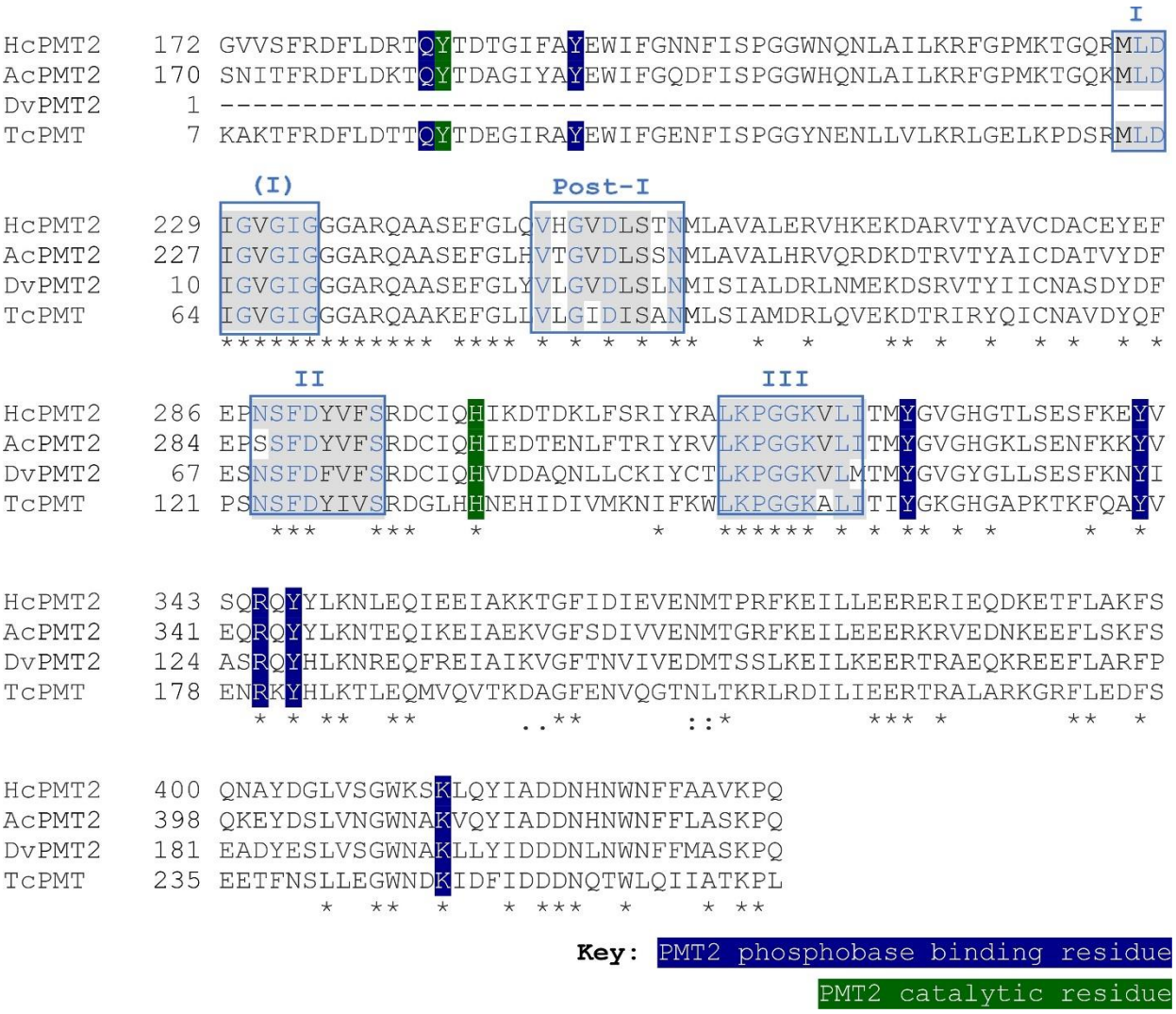

Figure 2. Multiple sequence alignment of *Haemonchus contortus* PMT2 (HcPMT2, 4KRI_A), *Ancylostoma ceylanicum* PMT2

(AcPMT2, EPB71549.1), *Dictyocaulus viviparous* PMT2 (DvPMT2, KJH40940.1), and *Toxocara canis* PMT2 (TcPMT2, KHN87001.1) by using Tcoffee (http://tcoffee.crg.cat/apps/tcoffee/do:regular). Four *S*-adenosyl methionine (SAM)-binding motifs that define the methyltransferases domains are labeled I, Post-I, II, and III and boxed as previously described (Lee and Jez 2013, Lee and Jez 2014). Asterisks (*) indicate positions with a single, fully conserved residue.

The available genomes of three nematodes, namely, *Ascaris suum*, *Oesophagostomum dentatum*, and *Toxocara canis*, each yielded a single PMT orthologue. The *A. suum* genome contained a coding sequence (orthologous to HcPMT1) that is 1,383 bp long, encodes a 460-amino-acid polypeptide (WormBase Parasite ID: LK871972.1) with a calculated molecular weight of 47.5 kDa, and is here named AsPMT (Fig. 1). The genome of *O. dentatum* possessed a coding sequence which is 1,119 bp long and encodes a 372-amino-acid polypeptide orthologous to HcPMT1 (GenBank accession number KHJ94304.1) with a calculated molecular weight of 41 kDa and is here called OdPMT (Fig. 1). On the other hand, the *T. canis* genome contained a HcPMT2 orthologue with a 807-bp coding sequence for a 268-amino-acid polypeptide (GenBank accession number KHN87001.1) with a calculated molecular weight of 29.6 kDa and is here named TcPMT (Fig. 2).

The amino acid sequences of putative AcPMT1, AsPMT1, DvPMT1, and OdPMT share 77.13, 53.59, 76.87, and 77.42% identity with HcPMT1, respectively, and <20% identity with HcPMT2. On the other hand, the putative AcPMT2, DvPMT2, and TcPMT share about 66.82, 66.82, and 51.87% identity with HcPMT2, respectively, but only 20%

identity with HcPMT1. The putative AcPMT1, DvPMT1, AsPMT, and OdPMT were found to possess the conserved methyltransferase catalytic domains as well as residues that are important for substrate (phosphoethanolamine) and cosubstrate (SAM) binding (Fig. 1). On the other hand, the putative AcPMT2, DvPMT2, and TcPMT possessed catalytic domains with the substrate and cosubstrate binding residues (Fig. 2) that have been reported to be conserved in HcPMT2 (29).

Nematode-specific PMTs catalyze SAM-dependent methylation of phosphoethanolamine and depict Michaelis-Menten kinetics

The recombinant PMTs were expressed in *Escherichia coli* as N-terminal His6-tagged proteins and purified using nickel affinity column chromatography. Analysis of the purified proteins by SDS-PAGE depicted the molecular weights of AcPMT1, AcPMT2, AsPMT, DvPMT1, DvPMT2, OdPMT, and TcPMT to be approximately 38, 48, 51, 53, 24, 41, and 30 kDa, respectively, consistent with their predicated molecular weights (see Fig. S1 in the supplemental material). To analyze the *in vitro* SAM-dependent methyltransferase activities of the recombinant proteins, we used a fluorescence-based assay with PE as the substrate, SAM as the methyl donor, and individual purified recombinant nematode PMT1 proteins as enzymes. The concentrations of PE and SAM were fixed at 200 and 100 μM, respectively, while the catalytic activity of each protein was measured at various concentrations. All the putative PMT1 proteins that were orthologous to HcPMT1 (AcPMT1, AsPMT, DvPMT1, and OdPMT) depicted concentration-dependent increase in catalytic activity (see Fig. S2), consistent with our previous observations using HcPMT1 as enzyme in the assay (Witola, Matthews et al. 2016). Because the genome database of *T. canis* only yielded a single PMT (TcPMT)

that was orthologous to HcPMT2 instead of HcPMT1, we tested this protein for catalytic activity of methylating PE. Interestingly, we found that unlike previous observations with HcPMT2 in which no significant catalytic activity was observed using PE as the substrate (Witola, Matthews et al. 2016), TcPMT showed concentration-dependent methyltransferase catalytic activity of methylating PE. The derived optimal concentrations of AcPMT1, AsPMT, DvPMT1, OdPMT, and TcPMT were 28.5, 22.5, 99, 28.5, and 50 ng/μL, respectively.

Subsequently, steady-state kinetic parameters of the putative PMTs on SAM and PE were determined by varying the SAM concentration, while maintaining a fixed concentration of PE and vice versa. The catalytic activities of the recombinant proteins (AcPMT1, AsPMT, DvPMT1, OdPMT, and TcPMT) were consistent with Michaelis-Menten kinetics on the substrate, PE (Table 1), and the cosubstrate, SAM (Table 2).

Table 1. Comparison of different PMT proteins' enzymatic kinetic parameters on Phosphoethanolamine

Enzyme	**Mean ± SEM**			k_{cat}/K_m (s^{-1} M^{-1})
	K_m (μM)	V_{max} ($nmol^{-1}$ mg^{-1} min^{-1})	k_{cat} (min^{-1})	
HcPMT1	73.37 ± 54.91	629.10 ± 160.90	1,066.30 ± 72.70	2.42×10^5
AcPMT1	1,079.00 ± 65.11	4,738.00 ± 764.50	7,071.00 ± 1,141.00	1.09×10^5
AsPMT	99.93 ± 36.63	184.60 ± 27.93	194.30 ± 29.40	3.20×10^4
DvPMT1	308.00 ± 41.68	2,378.00 ± 237.60	966.80 ± 96.59	5.23×10^4
OdPMT	80.97 ± 12.83	231.10 ± 14.92	335.00 ± 21.62	6.90×10^4
TcPMT	85.00 ± 77.76	1,031.00 ± 366.30	624.70 ± 221.90	1.22×10^5

Table 2. Comparison of different PMT proteins' enzymatic kinetic parameters on *S*-adenosyl-l-methionine

Enzyme	Mean ± SEM			k_{cat}/K_m (s^{-1} M^{-1})
	K_m (μM)	V_{max} ($nmol^{-1}$ mg^{-1} min^{-1})	k_{cat} (min^{-1})	
HcPMT1	18.34 ± 3.80	2,544.00 ± 122.20	4,312.00 ± 203.70	3.91×10^6
AcPMT1	27.33 ± 3.58	2,172.00 ± 86.63	3,241.00 ± 120.90	1.98×10^6
AsPMT	13.66 ± 0.62	3,207.00 ± 34.95	8,668.00 ± 94.47	1.06×10^7
DvPMT1	35.02 ± 5.05	444.60 ± 50.05	237.70 ± 12.87	1.13×10^5
OdPMT	48.95 ± 7.07	3,131.00 ± 204.30	2,723.00 ± 177.70	9.27×10^5
TcPMT	20.31 ± 2.23	1,083.00 ± 32.36	656.50 ± 19.61	5.39×10^5

Expression of nematode PMTs in pem1Δpem2Δ Saccharomyces cerevisiae mutant yeast cells

Yeast cells inherently lack PMT activity but express phosphatidylethanolamine N-methyltransferase (PEMT1 and PEMT2) proteins that are required for the three-step methylation of phosphatidylethanolamine (PtdEtn) to phosphatidylcholine (PtdCho). Deletion of both PMETs is lethal for yeast unless exogenous choline is provided in growth medium (Kodaki and Yamashita 1987, Summers, Letts et al. 1988). Therefore, to determine the *bona fide* role of nematode PMTs, we analyzed their ability to complement the loss of PtdCho biosynthesis in pem1Δpem2Δ *S. cerevisiae* mutant

strain (Kobayashi, Mizuike et al. 2014). The coding sequences of nematode PMTs (HcPMT1, HcPMT2, AcPMT1, AcPMT2, AsPMT, DvPMT1, DvPMT2, OdPMT, and TcPMT) and that of LacZ (as a negative control) were transformed and expressed in *pem1Δ/pem2Δ S. cerevisiae* under the control of the GAL1-inducible promoter. Western blot analysis using affinity-purified polyclonal antibodies raised in rats against corresponding nematode PMT recombinant proteins showed that the antisera recognized their respective protein bands (with predicted molecular weights) in whole-cell lysates of PMT-complemented *pem1Δ/pem2Δ S. cerevisiae* grown under induction conditions (minimal medium with 2% galactose supplemented with 1 mM ethanolamine) (Fig. 3). In yeast transformed with two PMTs (HcPMT1+HcPMT2, AcPMT1+AcPMT2, or DvPMT1+DvPMT2), the expression of both proteins in the same yeast was detected (Fig. 3). No PMT-specific bands were detected in the lysate of LacZ-complemented yeast (Fig. 3).

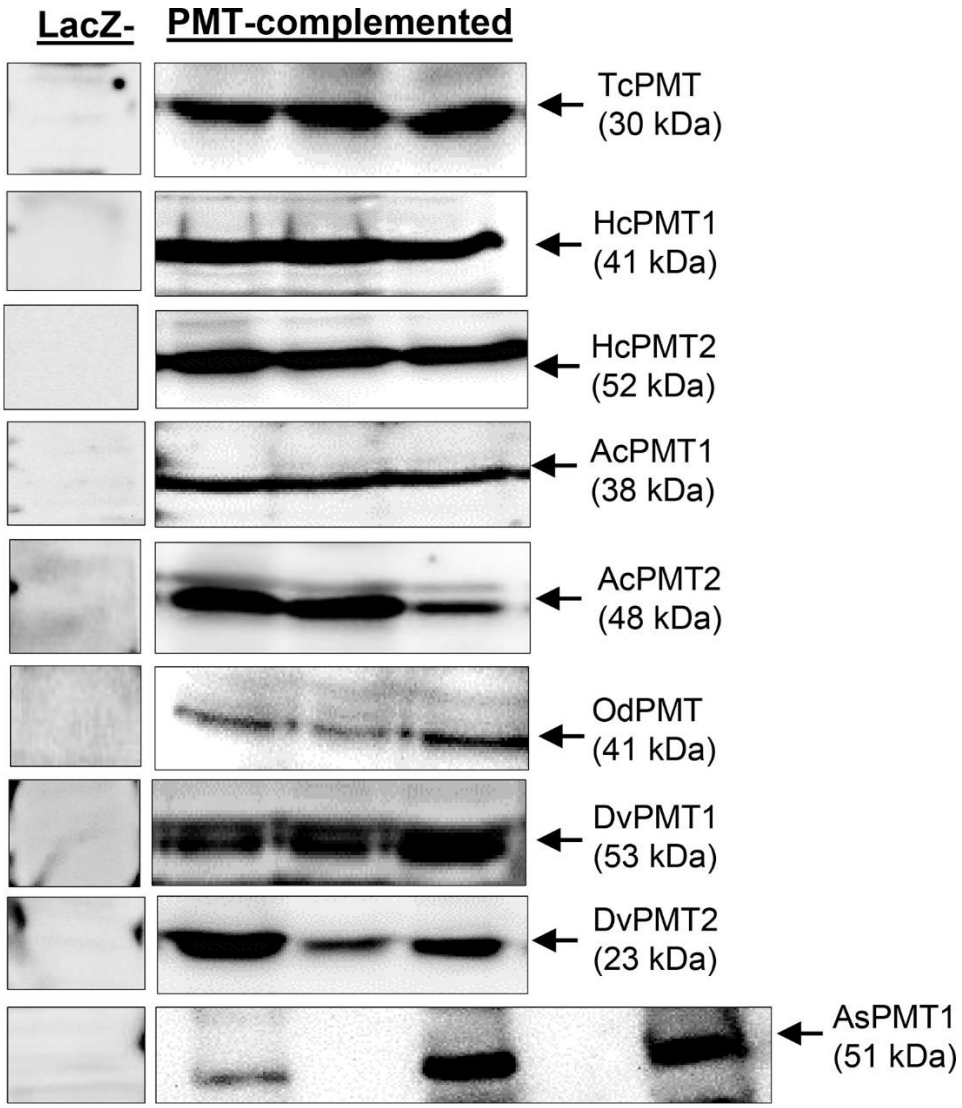

Figure 3. Western blotting of the expression of nematode PMT proteins in complemented yeast. Lysates of cultured complemented yeast cells induced to express PMT transgenes were fractionated by SDS-PAGE, followed by immunoblotting using affinity-purified antisera against respective nematode PMTs. For each complemented yeast strain, lysates generated from three individual yeast colonies were analyzed, and the expressed PMT protein band is shown in three separate lanes (PMT-complemented). The control lane was loaded with yeast lysate from LacZ-complemented yeast (LacZ-). The expected molecular weight is indicated in kDa in

parenthesis below the name of the PMT. TcPMT, *Toxocara canis* PMT; HcPMT, *Haemonchus contortus* PMT; AcPMT, *Ancylostoma ceylanicum* PMT; OdPMT, *Oesophagostomum dentatum* PMT; DvPMT, *Dictyocaulus viviparus* PMT; AsPMT, *Ascaris suum* PMT.

Complementation of pem1Δ/pem2Δ S. cerevisiae with nematode PMTs rescues growth in the absence of choline

The choline auxotroph *pem1Δ/pem2Δ S. cerevisiae* is unable to grow in medium lacking exogenous choline. Therefore, we cultured the nematode PMT-complemented *pem1Δ/pem2Δ S. cerevisiae* mutant yeast in medium lacking choline but supplemented with 1 mM exogenous ethanolamine in order to determine whether nematode PMT-catalyzed methylation of PE to phosphocholine would lead to de novo biosynthesis of PtdCho and rescue yeast growth. By measuring the growth of the yeast cells over 3 days, we found that the LacZ-complemented *pem1Δ/pem2Δ S. cerevisiae* strain did not show significant growth over time (Fig. 4), consistent with previous findings that lack of both PEM1 and PEM2 is lethal for *S. cerevisiae* unless choline is provided in medium (Kodaki and Yamashita 1987, Summers, Letts et al. 1988). Intriguingly, we found that all the PMT-complemented *pem1Δ/pem2Δ S. cerevisiae*, depicted significant progressive growth overtime (Fig. 4). Notably, there were some variations in the magnitude of growth conferred by different nematode PMTs, with AcPMT1+AcPMT2 complementation depicting the highest growth, followed by AsPMT, TcPMT, HcPMT1+HcPMT2, DvPMT1+DvPMT2, and TcPMT, in that order (Fig. 4). Variations in magnitude of growth

can be attributed to the variations in levels of expression of the respective PMTs and the corresponding amount of PtdCho synthesized in the yeast.

To confirm that nematode PMT-complemented *pem1Δ/pem2Δ S. cerevisiae* was able to synthesize PtdCho, we performed radioisotope incorporation assays by supplementing the yeast with [^{14}C]ethanolamine hydrochloride, along with cold ethanolamine, in medium lacking choline. Two-dimensional thin-layer chromatography (2D-TLC) analysis of the organic-phase lipid extracts from the yeast showed the presence of both PtdEtn and PtdCho with incorporated radioisotope in the PMT-complemented *pem1Δ/pem2Δ S. cerevisiae* strains (Fig. 5). To allow growth of the LacZ-complemented yeast, in addition to hot and cold ethanolamine, the medium was also supplemented with choline. As expected, unlike the PMT-completed yeast, LacZ-complemented yeast had radioisotope incorporated only in PtdEtn, synthesized through the CDP-ethanolamine pathway (Fig. 5). The positions and patterns of the detected bands of PtdEtn and PtdCho were consistent with previous observations in studies using *Plasmodium* PMT (Witola, El Bissati et al. 2008). These results indicate that the yeast mutant was able to use ethanolamine to synthesize PtdEtn which cannot be converted to PtdCho because of the lack of PEMT1 and PEMT2 enzyme. However, the expression of the exogenous nematode PMTs catalyzed the methylation of ethanolamine to phosphocholine, which was then converted to PtdCho via the Kennedy Pathway, as evidenced by the presence of a band of radiolabeled PtdCho in the complemented mutant yeast (Fig. 5). Noteworthy, the intensity of the PtdCho band varied among the different PMT-complemented strains, which can be attributed to the differences in the expression levels of the nematode PMTs and their activity in the yeast

cells. Together, these results validate the role of nematode PMTs in the biosynthesis of PtdCho.

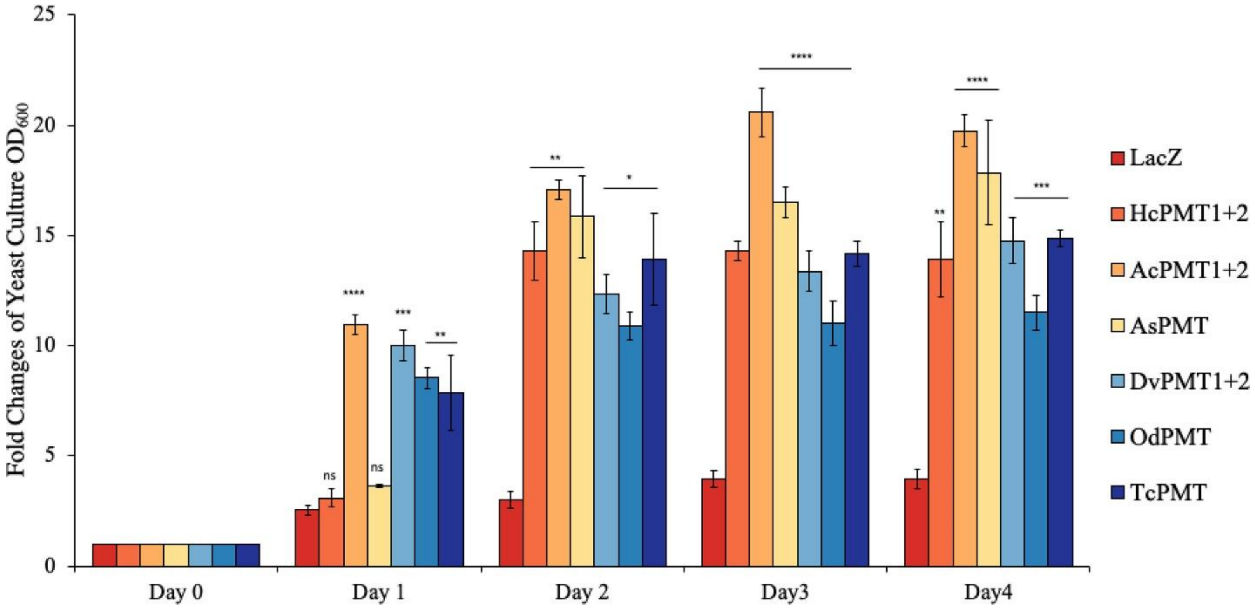

Figure 4. Analysis of the growth of nematode PMT-complemented *pem1Δpem2Δ S. cerevisiae* in culture over time. The growth of *pem1Δpem2Δ S. cerevisiae* expressing the respective nematode PMTs or LacZ growth medium supplemented with ethanolamine and galactose was determined over a 4-day culture period. TcPMT, yeast expressing *Toxocara canis* PMT; HcPMT1+2, yeast expressing *Haemonchus contortus* PMT1 and PMT2; AcPMT1+2, yeast expressing *Ancylostoma ceylanicum* PMT1 and PMT2; OdPMT, yeast expressing *Oesophagostomum dentatum* PMT; DvPMT1+2, yeast expressing *Dictyocaulus viviparus* PMT1 and PMT2; AsPMT, yeast expressing *Ascaris suum* PM; LacZ, yeast expressing *lacZ* gene (control). The data shown represent means from three independent experiments with standard error bars.

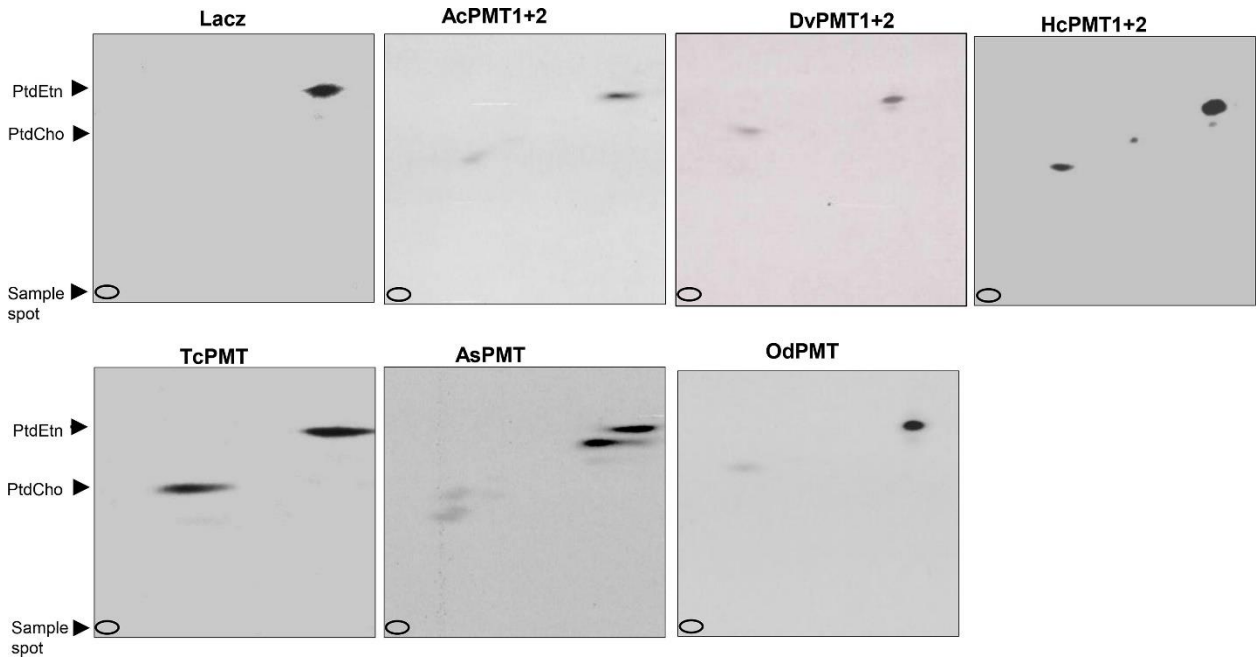

Figure 5. 2D-TLC analysis of the synthesis of phosphatidylcholine and phosphatidylethanolamine using [^{14}C]ethanolamine as the substrate in *pem1Δ/pem2Δ* yeast complemented with nematode PMT(s). The complemented yeast strains were grown in medium with [^{14}C]ethanolamine hydrochloride and galactose for 3 days, and lipids were extracted by the Folch method. The organic phase of the lipid extracts was resolved by 2D-TLC, and signals were generated by autoradiography. PtdEtn and PtdCho, positions of phosphatidylethanolamine and phosphatidylcholine, respectively; LacZ, yeast expressing LacZ gene (control); AcPMT1+2, yeast expressing *Ancylostoma ceylanicum* PMT1 and PMT2; DvPMT1+2, yeast expressing *Dictyocaulus viviparus* PMT1 and PMT2; HcPMT1+2: yeast expressing *Haemonchus contortus* PMT1 and PMT2; TcPMT, yeast expressing *Toxocara canis* PMT; AsPMT, yeast expressing *Ascaris suum* PMT; OdPMT, yeast expressing *Oesophagostomum dentatum* PMT. The data shown are representative of three independent experiments.

Natural compounds with broad-spectrum inhibitory activity against nematode PMTs

To identify chemical compounds that have inhibitory effect against the methyltransferase activity of nematodes PMTs, we screened a Natural Products Set IV library consisting of 419 compounds (see Table S1) from the National Cancer Institute Open Chemical Repository. Because HcPMT1 had earlier been characterized (Lee, Haakenson et al. 2011, Lee and Jez 2013, Witola, Matthews et al. 2016), we used it as an enzyme in the methyltransferase assay for the initial screening of the compound library. Of 419 compounds screened, we identified 45 that showed a ≥50% inhibitory effect ($P < 0.05$) against the catalytic activity of recombinant HcPMT1 at 40 μM (see Fig. S3 and S4). The rest of the compounds either had <50% inhibitory effect or augmented the catalytic activity of recombinant HcPMT1 and were thus not pursued further. Prior to evaluation of their inhibitory effect on the rest of the nematode PMTs, the identified HcPMT1 inhibitors were tested for mammalian cell cytotoxicity at and above the concentrations that inhibited HcPMT1. Among the 45 HcPMT1 inhibitors, 20 compounds showed no cytotoxicity in HCT-8 cells (American Type Culture Collection CCL244) at the highest concentration (160 μM) tested. Those compounds included NSC12097, NSC87511, NSC133100, NSC56410, NSC177858, NSC333856, NSC620709, NSC35676, NSC24872, NSC99791, NSC156219, NSC186301, NSC145612, NSC177383, NSC335989, NSC113497, NSC227186, NSC328426, and NSC320301. The rest of the compounds displayed significant cytotoxicity against HCT-8 cells at ≤40 μM and were thus not pursued further. The nontoxic 20 HcPMT1 inhibitors were next tested for cross-inhibitory activity against other nematode PMTs from *A. duodenale* (AcPMT1), *A. suum* (AsPMT), *D. viviparus* (DvPMT1), *O. dentatum* (OdPMT1), and *T.*

canis (TcPMT). For initial screening, all compounds were tested at 40 μM, and seven compounds (NSC87511, NSC133100, NSC56410, NSC62709, NSC35676, NSC145612, and NSC177383) showed significant cross-inhibitory activity against all the five PMTs (see Fig. S5). Subsequently, for each PMT protein, various concentrations of each of the seven compounds were tested, and the enzyme half-maximal inhibitory concentrations (IC_{50}Enzyme) against the various PMTs' catalytic activities were derived (Table 3). Alongside that, the seven compounds were also tested for mammalian cell cytotoxicity at various concentrations and their cytotoxicity half-maximal inhibitory concentrations (IC_{50}Cytotoxicity) derived (Table 3). From these data, the selectivity indexes (SI) for the compounds were calculated (Table 4), and compounds NSC35676, NSC62709, NSC145612, NSC177383, and NSC133100 displayed SI values that were >1 based on the IC_{50}Enzyme values of the PMT proteins tested (Table 4), indicating that they were effective PMT cross-inhibitors at nontoxic concentrations. Compounds NSC56410 and NSC87511 had SI values of >1 for four and two of the six PMTs tested, respectively (Table 4).

Table 3. PMT inhibition IC_{50}^{Enyme} and mammalian cell cytotoxicity $IC_{50}^{Cytotoxicity}$ values for tested compounds

NSC	Mean IC_{50} (μM) ± SEM						
	Cytotoxicity	Enzyme					
		HcPMT1	AcPMT1	AsPMT	DvPMT1	OdPMT	TcPMT
87511	171.80 ± 8.68	156.70 ± 26.02	65.80 ± 9.78	177.20 ± 15.62	503.50 ± 188.20	487.80 ± 161.70	496.50 ± 184.00
133100	101.80 ± 16.58	27.81 ± 1.71	31.49 ± 2.25	34.05 ± 2.85	88.17 ± 4.14	94.73 ± 5.03	99.10 ± 9.00

56410	188.40 ± 4.19	53.49 ± 5.85	68.41 ± 5.39	58.12 ± 12.95	1,916.00 ± 112.00	210.50 ± 73.54	84.48 ± 6.53
62709	298.90 ± 6.27	27.53 ± 1.97	13.40 ± 1.30	44.31 ± 1.35	29.20 ± 2.13	31.82 ± 1.80	27.59 ± 1.26
35676	566.20 ± 55.94	20.25 ± 1.64	11.55 ± 0.94	12.42 ± 1.10	42.12 ± 3.16	16.75 ± 0.41	45.86 ± 2.90
145612	302.40 ± 9.33	27.91 ± 1.57	27.37 ± 2.92	45.33 ± 1.71	55.12 ± 4.46	33.20 ± 2.26	109.00 ± 12.58
177383	85.47 ± 2.77	21.74 ± 2.05	14.84 ± 2.06	24.60 ± 1.79	47.57 ± 7.41	26.71 ± 2.00	41.27 ± 2.80

Table 4. Selectivity index values for PMT inhibitors

Compound NSC	**SI[a]**					
	HcPMT1	**AcPMT1**	**AsPMT**	**DvPMT1**	**OdPMT**	**TcPMT**
87511	1.10	2.61	0.97	0.34	0.35	0.35
133100	3.67	3.23	3.00	1.15	1.07	1.03
56410	3.52	2.75	3.24	0.10	0.90	2.23
62709	10.86	22.31	6.75	10.24	9.39	10.83
35676	27.96	49.02	45.59	13.44	33.80	12.35
145612	10.83	11.05	6.67	5.49	9.11	2.77
177383	3.93	5.76	3.47	1.80	3.20	2.07

[a]The selectivity index (SI) was calculated as $IC_{50}^{Cytotoxicity}/IC_{50}^{Enzyme}$.

Broad-spectrum PMT inhibitors depict concentration-dependent growth-inhibitory effect on PMT-complemented pem1Δ/pem2Δ S. cerevisiae

To determine the de novo efficacy of nematode PMT inhibitors, we performed growth kinetic studies by treating LacZ (control)- and nematode PMT-complemented yeast with various concentrations of the broad-spectrum PMTs inhibitors. We found that compounds NSC56410, NSC62709, NSC177858, NSC133100, NSC35676, NSC87511, and NSC145612 (seven compounds that possess *in vitro* cross-inhibitory activity

against nematode PMTs' catalytic activity) depicted concentration-dependent inhibitory effect on the growth of PMT-complemented mutant yeast (Table 5), while having no or negligible inhibitory effect against LacZ-expressing mutant yeast. The PMT-complemented yeast cells were grown in medium lacking choline but supplemented with ethanolamine which facilitated synthesis of phosphatidylcholine using ethanolamine as precursor via the catalytic activity of nematode PMTs. On the other hand, the LacZ-complemented yeast was grown in medium supplemented with choline, which necessitated the synthesis of phosphatidylcholine using choline as precursor. Therefore, by inhibiting the growth of nematode PMTs-complemented yeast only, it indicates that these compounds were shutting down the biosynthesis of phosphatidylcholine (which is essential for yeast growth) via inhibition of the enzymatic activity of the nematode PMTs. Importantly, all the seven compounds had no effect against the LacZ-complemented yeast when used at concentrations that were effective against PMT-complemented yeast, indicating that they were nontoxic to the yeast cells. Notably, the compounds' inhibitory effects against the growth of the various PMT-complemented yeast varied, which can be attributed to various levels of nematode PMT proteins expression and differences in the actual physiological activity of the PMT proteins in the yeast cells.

Table 5. Growth inhibition EC_{50} concentrations of PMT inhibitors against PMT- and LacZ-complemented *pem1*Δ/*pem2*Δ *S. cerevisiae*

NSC	Mean growth inhibition EC_{50} (μM) ± SEM on complemented yeast						
	LacZ	HcPMT1+2	AcPMT1+2	DvPMT1+2	AsPMT	OdPMT	TcPMT
87511	NA[a]	2.08 ± 0.29	26.40 ± 3.45	17.05 ± 1.15	25.87 ± 1.24	57.88 ± 5.18	58.24 ± 7.00
133100	NA	18.74	35.08	35.73	23.39	36.56	60.13

		± 2.07	± 5.01	± 3.67	± 1.85	± 8.4	± 5.85
56410	50.44 ± 13.21	1.48 ± 0.21	40.26 ± 9.42	45.64 ± 7.61	50.74 ± 3.87	37.65 ± 4.87	45.14 ± 3.97
62709	NA	3.03 ± 0.26	26.88 ± 7.63	25.56 ± 5.07	15.39 ± 2.13	19.54 ± 2.56	29.76 ± 7.61
35676	6.02 ± 0.25	5.45 ± 0.95	17.59 ± 3.72	12.93 ± 1.58	15.10 ± 0.70	3.98 ± 1.08	22.88 ± 5.00
145612	NA	8.28 ± 0.99	29.31 ± 2.54	23.00 ± 2.15	28.88 ± 4.38	32.78 ± 2.64	27.17 ± 7.70
177383	NA	6.11 ± 1.84	21.93 ± 7.31	15.36 ± 1.91	12.30 ± 1.11	19.83 ± 3.90	11.76 ± 1.43

[a]NA, not applicable.

Nematode PMT inhibitors possess in vitro anthelmintic activity against both drug-susceptible and MDR nematodes

Fifteen compounds that showed the highest inhibitory activity against complemented yeast (NSC133100, NSC177383, NSC87511, NSC145612, NSC35676, NSC62709, NSC12097, NSC24872, NSC99791, NSC186301, NSC320301, NSC335989, NSC56410, NSC156219, and NSC177858) were screened for *in vitro* anthelmintic activity using both a fully susceptible (TxPh-2011-S) and multiple drug-resistant (MDR; UGA MDR 2020) isolate of *H. contortus*. Two assays that measure the effects of increasing concentrations of drug on two distinct phenotypes were employed: a larval development assay (LDA) that assesses the development of nematode eggs from L1 to L3 stages (Taylor 1990, Gill, Redwin et al. 1995), and a larval motility assay (LMA) that assesses the movement of exsheathed L3 larvae (Storey, Marcellino et al. 2014).

Using the LDA, 4 of the 15 compounds tested demonstrated fairly potent anthelmintic activity, with no differences in response between the MDR and drug-susceptible isolates. Dose-response curves for these compounds are shown in Fig. 6. The derived IC_{50} values with 96% confidence intervals, goodness of fit (R^2) values, and hill slope values for the four compounds are shown in Table 6. One assay plate was discarded for compound NSC56410 due to poor larval development, resulting in fewer data points. Despite a high level of interassay variability, all compounds exhibited a dose response with IC_{50} values in the low micromolar range, indicating they possessed potent anthelmintic efficacy. The small differences in IC_{50} values between compounds NSC133100 and NSC177383 may not be very meaningful with regard to predictions of compound potency given the variability in the Hill slope of the dose response, but these two compounds were clearly more potent than NSC145612 by ~7-fold. On the other hand, compound NSC56410 showed the highest potency among the four compounds, with ~5-fold, 6-fold, and 39-fold higher potency than NSC133100, NSC177383, and NSC145612, respectively. Between the compared compounds, the IC_{50} values with nonoverlapping 95% CI indicated that they were statistically significantly different and vice versa.

Table 6. Anthelmintic IC_{50}s for PMT inhibitors against both susceptible and MDR isolates of *H. contortus* determined by LDA

PMT inhibitor	IC_{50} (μM)[a]	R^2	Hill slope
NSC133100	4.29 (2.15–8.28)	0.60	0.60
NSC177383	4.76 (3.47–6.53)	0.78	1.23
NSC145612	31.49 (19.41–52.75)	0.55	0.79
NSC56410	0.80 (0.27–2.20)	0.45	0.45

[a]The IC_{50} was derived from dose-response curves of the percent inhibition in larval development exerted by each compound. The 95% confidence interval in indicated in parentheses.

The LMA was also used to screen for compound activity. This assay measures motility of *H. contortus* L3 as a phenotype for detecting anthelmintic activity in candidate compounds. The LMA was performed using the Worminator system (Storey, Marcellino et al. 2014), a high-definition digital video imaging system that provides real-time quantitative measurement of nematode motility. LMAs were performed in parallel with the LDAs for the first two compounds tested (NSC133100 and NSC177383). Though both compounds demonstrated potent activity with the LDA (Fig. 6A and and B), no anthelmintic activity was observed at any of the drug concentrations or time points tested for the LMA (Fig. 7A). To further investigate the discrepancy in results between the two assays, we performed a second round of LMAs on the four compounds (that had anthelmintic activity in the LDA assay) using 4-fold higher drug concentrations (up to 500 µM). Even with the higher concentrations, no measurable activity was seen using motility as screening phenotype (see Tables S2 to S6). Figure 7 shows the results for the 24-h reading for both rounds of testing.

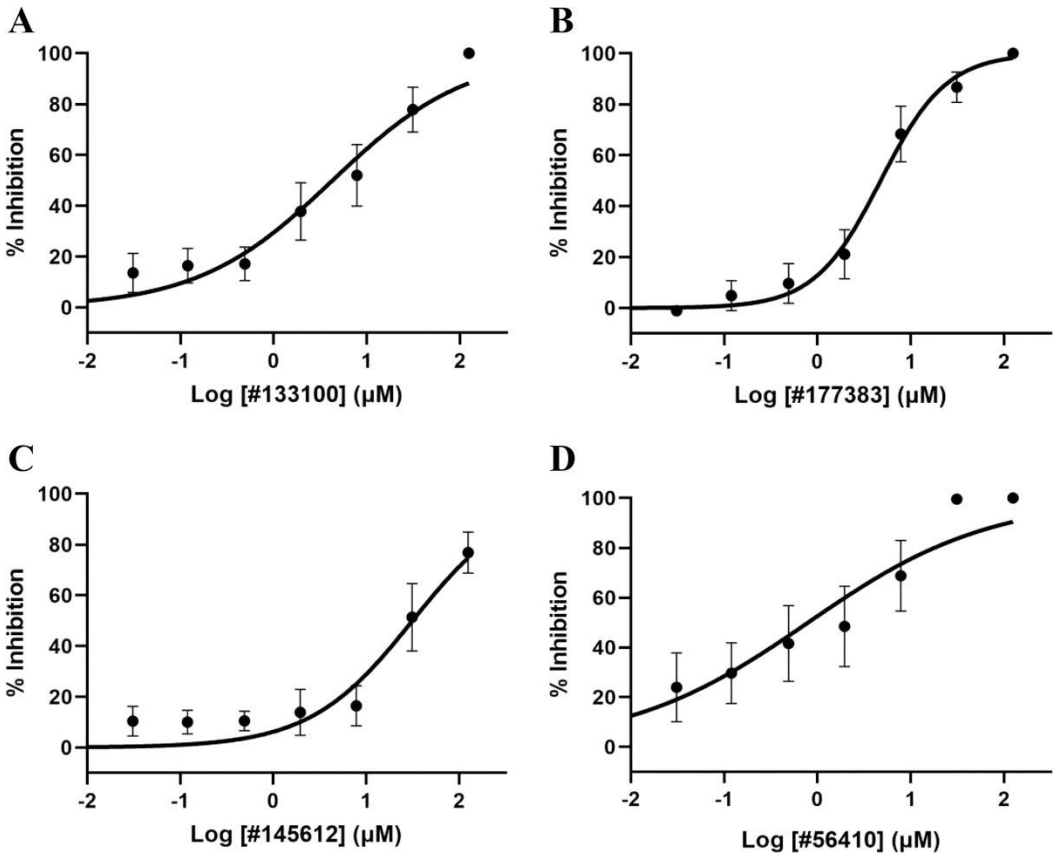

Figure 6. Effect of PMT inhibitors on the larval development of *Haemonchus contortus*. The percent inhibitions of larval development at various concentrations of NSC133100 (A), NSC177383 (B), NSC145612 (C), and NSC56410 (D) are depicted as dose-response curves. The data presented are averages for the compounds' effects on both drug-susceptible and MDR isolates of *H. contortus.* Curves were fitted using a log[inhibitor] versus normalized response variable slope model in Prism 9.4.0 (GraphPad). Error bars represent the standard errors of the mean (SEM) for the mean response.

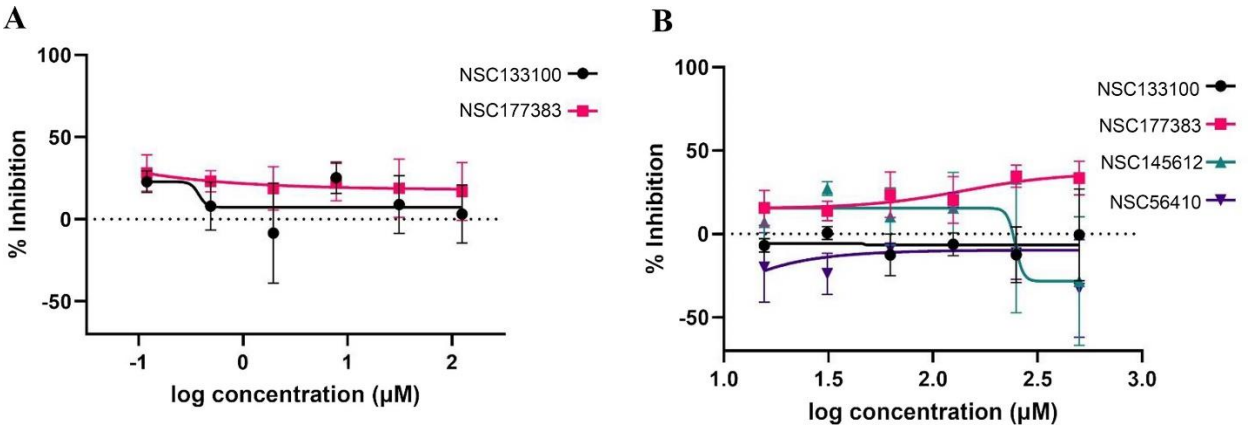

Figure 7. Effect of PMT inhibitors on the larval migration of *Haemonchus contortus*. The percent inhibitions of larval migration for initial lower concentrations for NSC133100 and NSC177383 (A) and extended concentrations for NSC133100, NSC177383, NSC145612, and NSC56410 (B) are depicted as dose-response curves after 24 h of treatment. Curves were fitted using a log[inhibitor] versus normalized response variable slope model in Prism 9.4.0 (GraphPad). Error bars represent the SEM for the mean response.

Discussion

The nematode families *Trichostrongylidae*, *Dictyocaulidae*, *Chabertiidae*, *Ancylostomatoidea*, and *Ascarididae* include parasitic species of health and economic importance in humans and animals (Vos, Allen et al. 2016). For over 5 decades, the use of anthelmintic drugs has been the primary method of control for nematode infections in both humans and animals, but there is now an alarming worldwide prevalence of drug-resistant parasitic nematodes, especially in livestock (Kaplan and Vidyashankar 2012). Furthermore, many reports have recently documented resistance to all major classes of anthelmintics (benzimidazoles, macrocyclic lactones, and tetrahydropyrimidines/imidazoles) (Kaplan and Vidyashankar 2012). Thus, there is an

urgent need to identify novel molecular targets for developing new effective anthelmintics.

Within the infected host, nematodes are prolific egg layers, requiring active biogenesis of nematode plasma membranes in which phospholipids, particularly phosphatidylcholine (PtdCho) are major constituents (Kent 1995, Vial 1998). Significant disparities in fundamental biochemical and metabolic pathways of phospholipids between parasitic nematodes and their animal hosts exist. The plant-like phosphobase methylation pathway involving the three-step SAM-dependent methylation of phosphoethanolamine (PE) to phosphocholine (PCho) (catalyzed by PMT proteins) for the biosynthesis of PtdCho is unique to nematodes and essential, but it does not exist in mammals (Elabbadi, Ancelin et al. 1997, McCarter 2004, Mitreva, Appleton et al. 2005, Palavalli, Brendza et al. 2006, Brendza, Haakenson et al. 2007, Witola, Matthews et al. 2016).

In the present study, we identified, cloned, expressed, and functionally characterized the putative PMTs of parasitic nematodes from five different families of medical and veterinary importance namely, *Ancylostomatoidea*, *Ascarididae*, *Chabertiidae*, *Dictyocaulidae*, and *Trichostrongylidae.* Four PMT1 proteins containing a putative N-terminal methyltransferase domain were AcPMT1 from *A. duodenale*, a hookworm that infects humans, AsPMT from *A. suum*, a common swine parasitic nematode of economic importance, DvPMT1 from *D. viviparus*, a lungworm of cattle, and OdPMT from *O. dentatum*, a common swine nematode. Those PMT1 proteins were found to catalyze the methylation of PE to phosphomonomethylethanolamine (PMME), consistent with the enzymatic activity of previously reported orthologous PMT1 proteins

in *C. elegans* and *H. contortus* (Lee, Haakenson et al. 2011, Lee and Jez 2013). Three putative PMT2 proteins containing the C-terminal methyltransferase domain—AcPMT2 from *A. ceylanicum*, a hookworm that infects both humans and animals; DvPMT2 from *D. viviparus*; and TcPMT2 from *T. canis*, a common nematode that infects dogs and humans—were also identified and characterized. Those PMT2 proteins were orthologous to the *C. elegans* and *H. contortus* PMT2 proteins known to catalyze the subsequent two-step methylation of PMME to phosphodimethylethanolamine (PDME), and eventually to PCho (Lee, Haakenson et al. 2011, Lee and Jez 2013, Lee and Jez 2014).

Consistent with our previous observations, the identified PMT2 proteins (AcPMT2 and DvPMT2) orthologous to the *H. contortus* HcPMT2 did not depict significant catalytic activity for the methylation of PE to PMME *in vitro* (Witola, Matthews et al. 2016). However, TcPMT from *T. canis*, which is orthologous to HcPMT2, was found to possess significant catalytic activity for the methylation of PE to PMME. Notably, unlike other nematodes whose genomes contained both putative PMT1 and PMT2, *T. canis* genome yielded only a putative PMT2. X-ray crystal structures of HcPMT1 and HcPMT2 have illustrated the conserved nature of the SAM-binding fold at the catalytic domains and also revealed the structural differences at the phosphobase binding site that can be associated with the differences in the substrate preferences of the two PMT proteins (Lee and Jez 2013). At the catalytic domain of HcPMT1, the phosphobase binding site is constrained by surrounding amino acids (Trp14, Met26, Met27, and Trp123) that facilitate methylation of PE to PMME but do not favor additional methylation (Lee and Jez 2013). In contrast, HcPMT2 has a different collection of residues at the active site

that are responsible for widening the substrate binding site so that it accommodates the larger PMME and PDME molecules (Lee and Jez 2013). It is likely that TcPMT has a rearranged configuration of amino acids at the phosphobase binding site which allows both PE and the larger PMME and PDME molecules to bind and, therefore, functions like the tripartite *P. falciparum* PMT which has the ability to catalyze all three methylation steps (Pessi, Choi et al. 2005, Reynolds, Takebe et al. 2008, Lee and Jez 2013). By enzyme kinetic analysis, all the identified putative PMTs, with activity for the methylation of PE, depicted Michaelis constant (Km) values for SAM that were relatively lower than those reported for *C. elegans* and *H. contortus* PMT1 proteins (Palavalli, Brendza et al. 2006, Brendza, Haakenson et al. 2007, Lee, Haakenson et al. 2011), suggesting that they possessed relatively higher affinity for the methyl-donor/cosubstrate SAM.

By complementing a mutant *S. cerevisiae* strain lacking the ability to endogenously synthesize PtdCho (that is essential for yeast growth in medium without exogenous choline), we validated that nematode PMTs are critical *bona fide* enzymes in the biosynthesis of PtdCho. Wild-type *S. cerevisiae* mainly uses the Bremer-Greenberg pathway in which phosphatidylethanolamine (PtdEA) is converted to PtdCho by two SAM-dependent phosphatidylethanolamine methyltransferases, PEMT1 and PEMT2 (Bremer and Greenberg 1959, Greenberg, Klig et al. 1983, Kodaki and Yamashita 1987, Summers, Letts et al. 1988). PEMT1 only catalyzes the conversion of PtdEA to phosphatidyl-N-monomethylethanolamine (PtdMME), which can then be methylated by PEMT2 to phosphatidyl-N,N-dimethylethanolamine (PtdDME), and eventually to PtdCho (Kodaki and Yamashita 1987, Summers, Letts et al. 1988, Carman and Henry 1989). Yeast uses the Kennedy pathway as an alternative route for PtdCho biosynthesis, in

which exogenous choline is phosphorylated to PCho, and subsequently converted to PtdCho (Kennedy and Weiss 1956, Sundler and Akesson 1975, Kent 1995). In the present study, by complementing the mutant yeast (lacking PEM1 and PEM2) with the nematode PMTs, we were able to rescue the growth of the mutant through restoring the *de novo* biosynthesis of PtdCho in the absence of exogenous choline, but with ethanolamine supplementation. These findings indicate that expression of the nematode PMTs in mutant *S. cerevisiae* introduced the nematode phosphobase pathway through which PtdCho could be synthesized via the nematode PMT-based methylation of PEA to PCho, which then feeds into the Kennedy Pathway. Consistent with the rescued growth of the complemented mutant yeast, by using radioisotope incorporation assays, we found that radiolabeled substrate, [^{14}C]ethanolamine, was incorporated into the PtdCho synthesized in the yeast.

Using an *in vitro* PMT assay, with the recombinant nematode PMT proteins as enzymes, we screened a natural compound library and identified compounds with cross-inhibitory activity against the catalytic activity of the various nematode PMTs at nontoxic concentrations. Corroboratively, we found that those PMT inhibitors blocked the growth of PMT-complemented mutant yeast by inhibiting the enzymatic activity of the nematode-derived PMTs and thereby shutting down the de novo biosynthesis of PtdCho via the phosphobase pathway. Collectively, these findings provide genetic and biochemical evidence for the role of the putative nematode PMTs as *bona fide* PMTs for the biosynthesis of PtdCho.

Among the identified broad-spectrum PMT inhibitors that also blocked growth of nematode PMT-complemented mutant yeast (see Fig. S6), NSC62709 is structurally

similar to streptonigrin, which is an antimicrobial and antineoplastic agent isolated from *Streptomyces flocculus* (Bolzán and Bianchi 2001). The difference between the two is that streptonigrin has a dioxo-amino-quinoline, while NSC62709 has a dimethyl-imidazole-quinoline, which likely makes NSC62709 more tolerable in mammalian cells than streptonigrin is (McBride, Oleson et al. 1966). NSC35676 (see Fig. S6) is a benzotropolone-containing natural product that is contained in the nutgall of *Quercus* spp. and certain oak barks (Wu, Zeng et al. 1996, Abou-Karam and Shier 1999). NSC35676 is also known as purpurogallin, and has been found to have antimicrobial and antioxidant properties (Inamori, Muro et al. 1997, Abou-Karam and Shier 1999, Zhen, Piao et al. 2019). It is noteworthy that purpurogallin (NSC35676) has previously been reported to inhibit the catalytic activity of another SAM-dependent methyltransferase, catechol O-methyltransferase (COMT), isolated from both human and the yeast, *Candida tropicalis* (Lambert, Chen et al. 2005, Gosslau, Li et al. 2018). These findings are consistent with the inhibitory effect of purpurogallin (NSC35676) against diverse PMT proteins in the present study. NSC145612, NSC133100, and NSC177383 (see Fig. S6) are structurally similar to rifampin and its derivatives with activity against mycobacteria (Floss and Yu 2005, Htoo, Brumage et al. 2019).

The anthelmintic activity of 15 PMT inhibitors was evaluated against *H. contortus* using two different screening phenotypes. The LDA evaluates the development of nematode eggs to the L3 stage and thus has the ability to detect the anthelmintic activity due to a multitude of effector mechanisms (Taylor 1990). In contrast, the motility of L3s may not be an appropriate phenotype to detect anthelmintic activity depending on the tissue- and stage-specific expression of the main molecular targets of the drugs

assessed (George, Lopez-Soberal et al. 2018). Potent commercially available anthelmintic drugs that induce paralysis of the body wall muscle often exert a dose-response on motility (Martin 1997, Várady and Corba 1999, George, Lopez-Soberal et al. 2018). However, these responses on motility often are highly variable and only occur at high concentrations. Consequently, the motility phenotype often provides less reliable data, particularly when attempting to discriminate drug-resistant from drug-susceptible isolates (Várady and Corba 1999, George, Lopez-Soberal et al. 2018). In addition, drugs that do not directly induce paralysis, such as benzimidazoles, do not provide a useful dose-response for motility (Várady and Corba 1999). Our results clearly demonstrate that the motility of the L3 stage does not constitute a useful predictor of anthelmintic activity for PMT inhibitors. This is relevant, since motility is the most common phenotype used in high-throughput screening systems (Mackenzie and Geary 2013, Partridge, Forman et al. 2020). These and other recently published data strongly indicate that the screening of experimental compounds for anthelmintic activity can be considerably improved by using *in vitro* assays targeting more than one phenotype.

Here, we show that PMT enzymes are functional and druggable targets in *H. contortus* and likely in many other nematodes that possess PMT orthologs and catalytic activity, such as *O. dentatum*, *A. suum*, and *D. viviparus*, among others. Four PMT inhibitors (NSC133100, NSC177383, NSC145612, and NSC56410) showed activity in the lower micromolar range when tested against both drug-susceptible and MDR isolates of *H. contortus*. Since targeting the enzymes in the phosphobase methylation pathway constitutes a novel mechanism of action, activity against the MDR isolate was expected to be similar to the susceptible. As expected, no differences were observed

between the two isolates, further confirming this premise. LDA wells with 100% inhibition contained only larvae at the L1 stage when development was stopped at day 7. This suggests that these compounds do not possess ovicidal effect but have a mechanism of action that prevents the development of larvae once they hatch. Nematodes are a phylum of molting animals (Blaxter, De Ley et al. 1998). Thus, by affecting membrane biogenesis, these PMT inhibitors could potentially have deleterious effects on molting, egg production by females, establishment of L4 in host tissues, host-parasite interactions, and any other biological process that requires active synthesis of phosphatidylcholine (Lee and Jez 2014, Wang, Nie et al. 2018, Wang, Ma et al. 2020). While the effect observed in our experiments is limited to larval stages of nematodes, it is likely that PMT inhibitors would have effects against adult nematodes as well. Since phosphatidylcholine accounts for 40 to 60% of cell membrane phospholipid content (Kent 1995), it influences cell membrane curvature and vesicle formation (van Meer, Voelker et al. 2008), which in turn is critical for overall cell functionality and survival. Further, in nematodes, changes in cell membrane composition are crucial for the parasite to efficiently adapt to variations in host animal physiological dynamics (Cipollo, Awad et al. 2005). This suggests that depletion of phosphatidylcholine levels in nematodes through inhibition of PMTs would lead to loss of viability and eventual death of adult worms, underscoring the potential efficacy of PMTs inhibitors against adult nematodes, in addition to their lethal effect on larval stages. Moreover, activity of commercially available anthelmintic classes in the LDA is a good predictor of drug efficacy *in vivo* (Gill, Redwin et al. 1995, Kaminsky, Gauvry et al. 2008, Ruffell, Raza et

al. 2018). Therefore, it is reasonable to expect that the efficacy observed in the free-living stages of *Haemonchus* will translate to efficacy against adult stages.

The data presented here constitute a proof of concept and further validation of PMTs as a novel drug target in parasitic nematodes. PMT inhibitors have the potential to serve as a new generation of molecular target-specific broad-spectrum anthelmintic drugs for the treatment of nematode infections in livestock and humans.

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CHAPTER 3

IN VIVO EFFICACY OF NOVEL PHOSPHOETHANOLAMINE METHYLTRANSFERASE INHIBITORS AGAINST *HAEMONCHUS CONTORTUS* IN JIRDS (*MERIONES UNGUICULATUS*)

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Abstract

Anthelmintic resistance (AR) poses a significant challenge to the treatment of parasites in livestock and pets. Anthelmintic failure due to AR is increasingly prevalent among gastrointestinal nematodes in animals, and resistance is becoming a growing concern in human medicine. Despite the pressing demand for compounds with novel mechanisms of action, the introduction of new anthelmintics to the market has been scarce. In this study, we investigated the in *vivo* efficacy of compounds previously demonstrated to be inhibitors of phosphoethanolamine methyltransferases (PMTs), enzymes that are highly conserved among parasitic nematodes and represent a novel drug target. Inhibition of PMT enzymes disrupts phosphocholine biosynthesis, which is crucial for parasite viability. This pathway is essential to nematodes and is not present in mammals, making it an attractive and highly selective drug target. Based on previous results obtained from *in vitro* screenings against both drug-susceptible and multidrug-resistant (MDR) *Haemonchus contortus*, three candidate compounds with potent *in vitro* activity were selected for use in a pilot *in vivo* study. Here, we employed the jird (*Meriones unguiculatus*) – *H. contortus* model, using an MDR isolate of *H. contortus*. The dosing regimens used were selected based on available toxicological data, but since pharmacokinetic-pharmacodynamic (PKPD) profiles of these compounds are not known, dosing regimens were not optimized. Furthermore, as this was a pilot study, small groups of animals were used. Nevertheless, relatively high reductions in worm counts were achieved, ranging from 53.5 to 72.6%, and for two of the three compounds this reduction was statistically significant ($P = 0.008$, $P = 0.025$). These findings provide further support for the potential of PMT enzymes as viable drug targets in parasitic nematodes. Future studies should investigate the PKPD of these compounds so that

dosing regimens can be further optimized. Additionally, combinations of these compounds should be tested to determine if targeting more than one PMT simultaneously can yield synergistic activity.

Introduction

Parasitic nematodes that infect humans, animals, and plants constitute a major health and economic burden on a global scale. In humans, it is estimated that between 1.5 and 3.5 billion people suffer from soil-transmitted helminthiasis, representing approximately a quarter of the world's population (Stepek, Buttle et al. 2006, Garcia-Bustos, Sleebs et al. 2019, W.H.O 2023). In agriculture, nematodes impact the health of livestock and have an important negative impact on animal productivity (Bernard, Egnin et al. 2017, Strydom, Lavan et al. 2023). Consequently, nematode parasites pose a critical risk to animal health and farm profitability and, consequently, to human food security (Fitzpatrick 2013). The administration of anthelmintic drugs is the primary means used for the control of nematode infections (Geary 2012, Selzer and Epe 2021). However, chemotherapy relies on relatively few main classes of anthelmintics, with many of these classes only registered for a few animal species, and only in some countries. These include benzimidazoles and pro-benzimidazoles, salicylanilides and substituted phenols, imidazothiazoles, tetrahydropyrimidines, macrocyclic lactones and, more recently, amino-acetonitrile derivatives, cyclooctadepsipeptides, and spiroindoles (Harder and von Samson-Himmelstjerna 2002, Holden-Dye and Walker 2007, Ducray, Gauvry et al. 2008, Yadav and Singh 2011, Panda, Jones et al. 2016).

Unfortunately, the regular and/or strategic administration of anthelmintic drugs (Prichard, Hall et al. 1980, Martin 1987, Waller 1997), has led to ever-increasing levels

of anthelmintic resistance (AR) in nematodes to all drug classes, involving the most economically important parasites of livestock and the most prevalent and clinically important parasites of pet dogs (Kaplan and Vidyashankar 2012, Jimenez Castro, Howell et al. 2019, Kaplan 2020). Cases of AR have been extensively reported in sheep and goats (Waghorn, Leathwick et al. 2006, Howell, Burke et al. 2008, Veríssimo, Niciura et al. 2012, Crook, O'Brien et al. 2016, Rose Vineer, Morgan et al. 2020), cattle (Gasbarre, Smith et al. 2009, Edmonds, Johnson et al. 2010, Ramos, Portella et al. 2016, Cristel, Fiel et al. 2017, Rose Vineer, Morgan et al. 2020, Sauermann, Waghorn et al. 2024), horses (Kaplan 2002, Kaplan, Klei et al. 2004, Nielsen 2022) and more recently also in pets (Hampshire 2005, Kopp, Kotze et al. 2007, Blagburn, Dillon et al. 2011, Pulaski, Malone et al. 2014, Jimenez Castro, Howell et al. 2019, Kitchen, Ratnappan et al. 2019, D'ambroso Fernandes, Rojas Guerra et al. 2022, Jimenez Castro, Durrence et al. 2023). In humans, mass drug administration campaigns pose a risk for the development of AR (Vercruysse, Albonico et al. 2011, Geary 2012), and reduced efficacies have been reported in a few occasions (Shoop 1993, De Clercq, Sacko et al. 1997, Reynoldson, Behnke et al. 1997, Geerts and Gryseels 2001).

Despite the pressing demand for compounds with novel mechanisms of action, the process of developing and introducing new anthelmintics to the market has been slow (Nixon, Welz et al. 2020). Consequently, there is a critical need for enhanced collaboration between academia and industry in the pursuit of anthelmintic discovery (Nixon, Welz et al. 2020). Additionally, anthelmintics discovered in veterinary medicine have been historically adapted for human use (Geary and Gauvry 2011, Geary 2016),

constituting a critical resource for the treatment of neglected tropical diseases that involve helminths.

We previously identified a panel of compounds that target phosphoethanolamine methyltransferases (PMT) enzymes and block the production of phosphocholine in nematodes (Zhang, Sicalo Gianechini et al. 2023). Nematodes use a plant-like phosphobase methylation pathway for the biosynthesis of phosphatidylcholine, involving the three-step sulfur-adenosylmethionine (SAM)-dependent methylation of phosphoethanolamine to phosphocholine, and that are catalyzed by PMT proteins (Palavalli, Brendza et al. 2006, Brendza, Haakenson et al. 2007, Witola, Matthews et al. 2016). This pathway is essential to nematodes and is not present in mammals (Palavalli, Brendza et al. 2006, Brendza, Haakenson et al. 2007), making it an attractive and highly selective drug target. Fifteen PMT inhibitor compounds were previously tested *in vitro* against susceptible and multiple-drug resistant (MDR) *Haemonchus contortus*, of which 4 showed activity in the lower μM range (Zhang, Sicalo Gianechini et al. 2023). Here, we tested the activity of 3 of those compounds *in vivo,* using the jird - *H. contortus* model (Conder, Jen et al. 1990).

Materials and methods

Parasite isolate

Compounds were tested against an MDR *H. contortus* isolate that we have previously used for the *in vitro* screening of these drugs (Zhang, Sicalo Gianechini et al. 2023). The UGA MDR 2020 (University of Georgia Athens, USA) *H. contortus* isolate is highly resistant to ivermectin, benzimidazoles, levamisole, and moxidectin, as determined by the DrenchRite® larval development assay (Table 1).

Table 1. Half maximal inhibitory concentration (IC_{50}) for the drug classes tested using the Drenchrite® larval development assay, against the UGA MDR 2020 *Haemonchus contortus* isolate. The IC_{50} was derived from dose-response curves of the percent inhibition in larval development exerted by each compound. Curves were fitted using a log[inhibitor] versus normalized response variable slope model in Prism 10.2.0 (GraphPad). The Resistance Ratio (RR) were calculated as the IC_{50} of the resistant isolate divided by the IC_{50} of the susceptible isolate (TxPh-2011-S) (Zhang, Sicalo Gianechini et al. 2023).

Drug tested	IC_{50}	95% CI	R^2	RR
Thiabendazole	16.28 µM	11.92 to 21.07	92.02	139
Ivermectin aglycone	263.90 nM	230.90 to 302.20	98.86	34
Levamisole	6.06 µM	5.41 to 6.76	98.60	11

Preparation of larvae for infections

Feces were collected from donor goats and coprocultures (Great Britain Ministry of Agriculture and Food 1986) set in order to obtain the larvae at stage 3 (L3). The L3s were then stored in water at 10°C, and used within two months of storing. The day prior to infections, the L3s were cleaned using the Baermann technique (Dinaburg 1942), to eliminate dead larvae. Up to 50,000 larvae were then placed into a 50-ml centrifuge tube containing 10-15 ml of EBSS. A glass Pasteur pipette connected to 100% CO_2 was placed in contact with the bottom of the tube and the tube was sealed with parafilm. CO_2 was gently bubbled into the tube for 10 minutes. The pipette and parafilm were removed from the tube and the tube lid closed quickly. The tube was then placed

horizontally in a shaking incubator at 37°C for 1 to 1.5 hours, checking larval exsheathment every 30 minutes by taking an aliquot from the tube for examination under a microscope. For purposes of this assay, 70% of exsheathment was considered satisfactory, since further exsheathment is expected to occur over time, and probably also *in vivo.* The larval preparation was then centrifuged at 275 x *g* for 10 minutes, the supernatant was removed, and sufficient EBSS was added to re-suspend the larvae. A count of the larvae was performed, and sufficient EBSS was then added to yield a concentration 5000 exsheathed L3 per ml.

Compound preparation

Compounds were prepared as previously described (Zhang, Sicalo Gianechini et al. 2023). Briefly, the chemical compounds were obtained from the National Cancer Institute / Developmental Therapeutics Program Open Chemical Repository. These were individually reconstituted in molecular biology grade dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) to a stock concentration of 10 mM and stored at −20°C until use. Three compounds that had previously shown *in vitro* activity against *H. contortus* (Zhang, Sicalo Gianechini et al. 2023) were tested in this study. Stock concentrations of the compounds were thawed at room temperature and a “fresh” batch of diluted drug was prepared in the delivery vehicle the day prior to each treatment. Drugs were serially diluted in 100% DMSO until achieving the desired concentration to be introduced in the vehicle, which was made up of 17% DMSO and 83% of an aqueous solution of carboxymethylcellulose (CMC) at 1%. After dilution the dissolution of the compounds was confirmed by visual examination, and immediately prior to administration, the solution was thoroughly mixed using a vortex mixer. Dosing

regimens were determined empirically based on toxicological data available for other rodent species (data not shown) to use a dose on the higher end of the range that was likely to be safe.

In vivo testing in Meriones unguiculatus

An *H. contortus* - jird (*Meriones unguiculatus*) model (Conder, Jen et al. 1990) was used to evaluate the efficacy of the experimental compounds (Fig. 1). All protocols were approved by the IACUC and conducted in compliance with AUP A2020 11-006-Y1-A1. After acclimation for 3 days, jirds were fed an immunosuppressive diet containing 200ppm (0.02%) of hydrocortisone acetate. On day 7 after arrival, all animals were infected by oral gavage with approximately 1,000 exsheathed *H. contortus* L3, suspended in 0.2 ml of Earle's Balanced Salt Solution (EBSS; pH 7.2 - 7.6). The compounds were then administered on days 8 and 9 post-infection, using different doses and regimens for the various compounds (Table 2). Compounds were administered orally and delivered in a total volume of 0.5 ml. For this, the stock concentrations were diluted in 100% DMSO until achieving the desired concentration to be delivered in a vehicle that consisted of 17% DMSO and 83% of a 1% aqueous solution of CMC. Control groups were delivered 0.5 ml of a placebo that consisted of a mixture of 17% DMSO and 83% of a 1% aqueous solution of CMC.

Table 2. Regimen for compound delivery in jirds receiving one of four treatments.

Experiment 1	Dose (mg/kg)	Schedule	Route of administration	Total volume (ml) / administration
Control	Placebo	Twice daily for 2 days	Oral gavage	0.5
NSC133100	200	Twice daily for 2 days	Oral gavage	0.5

NSC56410	10	Once daily for 2 days	Oral gavage	0.5
Experiment 2				
Control	Placebo	Twice daily for 2 days	Oral gavage	0.5
NSC145612	100	Twice daily for 2 days	Oral gavage	0.5

On the day prior to initiating the treatment regimens, all animals including the non-treated controls, were weighed to determine the appropriate amount of compound to deliver. Animals were dosed according to the weight of the heaviest animal in the group. Four days after the last treatment on day 13 post-infection, jirds were fasted for 2-4 hours and then euthanized. The stomachs were removed, opened longitudinally, and contents were rinsed from the mucosa with water. The organs were then soaked in water for 3 hours at 37°C in an incubator to facilitate the detachment of worms from the mucosa. After incubation, the worms, which were at L4 stage, were collected in water, stained with iodine, and counted using a stereomicroscope. Worm enumeration was conducted in a blind manner; individuals performing the counting of worms were unaware of the corresponding experimental group.

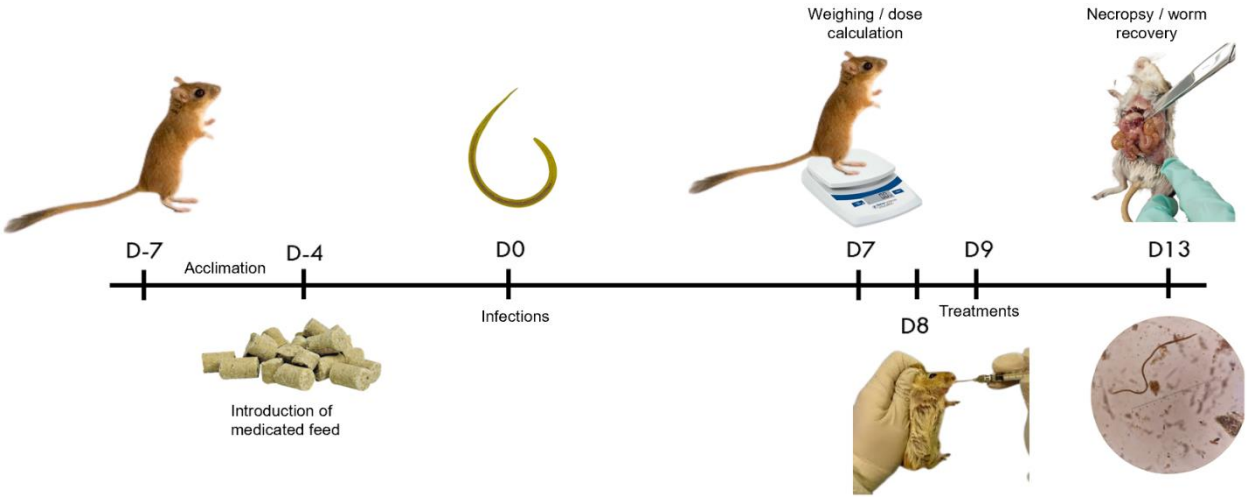

Figure 1. Study design for the jird – *Haemonchus contortus* model.

Experiments

Forty jirds were infected and allocated randomly to control or treatment groups. We conducted two independent experiments; in the first we tested compounds NSC133100 (n=7), NSC56410 (n=8), and a control group (n=8). A second experiment was performed to test compound NSC145612 (n=8), and a control group (n=9).

Experiment 1:

Group 1 (n=8): control group received a placebo twice daily for two consecutive days.

Group 2 (n=7): compound NSC133100 was delivered at 200 mg/kg twice daily for two consecutive days.

Group 3 (n=8): compound NSC56410 was delivered at 10 mg/kg, once daily for two consecutive days.

Experiment 2:

Group 1 (n=9): control group received a placebo twice daily for two consecutive days.

Group 2 (n=8): compound NSC145612 was delivered at a dose of 100 mg/kg twice daily for two consecutive days.

Statistical analysis

Efficacy against *H. contortus* was calculated as percent reduction in worm counts using the following formula:

$$\%\ \text{reduction} = \frac{\text{Control} - \text{Treatment}}{\text{Control}}\ \text{x}\ 100$$

where *Control* is the arithmetic mean number of worms in an untreated control group and *Treatment* is the arithmetic mean number of worms in a treatment group. The 95% confidence intervals were calculated using a Bayesian hierarchical model to assess anthelmintic efficacy available at https://shiny.math.uzh.ch/user/furrer/shinyas/shiny-eggCounts/. Groups were then compared using nonparametric tests, Mann-Whitney or Kruskal-Wallis followed by Dunn's multiple comparison procedure for multiple comparisons. Analyses were performed using GraphPad Prism version 10.2.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com.

Results

Good establishment of *Haemonchus* larvae were obtained for the control groups in both experiments (Table 3 and 4). Reductions of 62.23% (95% CI; 22.40, 80.30), 72.58% (95% CI; 12.40, 89.00) and 53.51% (95% CI; 10.90, 76.90), in worm counts were obtained for compounds NSC133100, NSC56410, and NSC145612, respectively (Table 4). This reduction was significantly different from the controls in the case of

compounds NSC56410 (P = 0.008), and NSC145612 (P = 0.025) but not for compound NSC133100 (P = 0.127) (Fig. 2). There was no significant difference between the efficacies achieved by compounds NSC133100 and NSC56410 (P >0.999, Fig. 2). The wide confidence intervals are a consequence of the overdispersion of worm numbers between animals and the relatively small group sizes. The lack of significance for NSC133100 despite demonstrating a similar level of efficacy to the other two compounds is a reflection of the high variability and small group size, with the NSC133100 treatment group having one less animal than the other two treatment groups.

Table 3. Individual worm counts in jirds receiving one of four treatments. SEM= standard Error of the Mean. Mean and SEM were obtained from GraphPad Prism version 10.2.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com.

	Compound			Controls	
	NSC133100 (N=7)	**NSC56410 (N=8)**	**NSC145612 (N=8)**	**Experiment 1 (N=8)**	**Experiment 2 (N=9)**
	25	3	7	18	51
	66	12	75	147	109
	28	3	36	263	99
	35	1	41	34	51
	55	85	29	79	14
	31	33	21	104	93
	30	72	5	91	87
	-	15	48	81	102
	-	-	-	-	28
Mean	38.57	28.00	32.75	102.13	70.44
SEM	5.90	11.66	8.11	26.97	11.68

Table 4. Summary of worm counts in jirds receiving one of four treatments. Descriptive statistics were obtained using GraphPad Prism version 10.2.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com.

Experiment 1	Sample size	Mean worm count (± SEM)	% Reduction in worm counts (95% CI)	Dose (mg/kg)
Control	8	102.13 (± 26.97)[a]	NA	Placebo
NSC133100	7	38.57 (± 5.90)[ab]	62.23 (22.40, 80.30)	200
NSC56410	8	28.00 (± 11.66)[b]	72.58 (12.40, 89.00)	10
Experiment 2				
Control	9	70.44 (± 11.68)[a]	NA	Placebo
NSC145612	8	32.75 (± 8.11)[b]	53.51 (10.90, 76.90)	100

Mean worm counts with a superscript in common do not differ with a level of significance of 5% over all comparisons. NA= not applicable

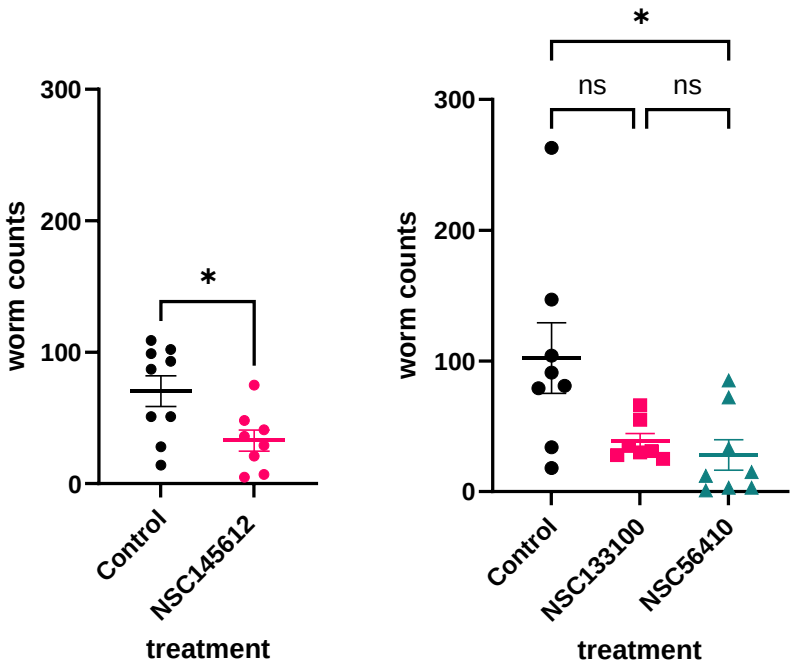

Figure 2. Kruskal-Wallis (NSC133100 and NSC56410) followed by Dunn's multiple comparison procedure for comparisons between groups, and Mann-Whitney test (NSC145612). Analyses were performed using GraphPad Prism version 10.2.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com. *P (NSC145612) = 0.025; P (NSC56410) = 0.008; P (NSC133100) = 0.127.

Discussion

The three PMT inhibitors tested here demonstrated relatively potent *in vitro* activities, with IC_{50}s ranging from 0.80 to 31.49 μM in previous studies (Zhang, Sicalo Gianechini et al. 2023). The jird model used here allows testing of the *in vivo* response of experimental compounds against parasitic stages of *H. contortus* in a laboratory setting (Conder, Jen et al. 1990). This model has demonstrated a clearance of over 95% in worm counts during trials with commercially available anthelmintics, and achieved such efficacy at doses (mg/kg) that align with those typically effective against

H. contortus in ruminants (Conder, Jen et al. 1990). These data strongly suggest that this represents a highly predictive model for assessing the *in vivo* activity of novel compounds (Conder, Jen et al. 1990).

Average worm establishment in the control animals was relatively high for both experiments, however, as is common with nematode infections, the distribution of worms was over-dispersed (Barger 1985, Torgerson, Schnyder et al. 2005). The compounds evaluated here demonstrated reductions in worm counts ranging from 53.5 to 72.6 %. This reduction was significant in the case of compounds NSC56410 and NSC145612. One animal was lost in the case of compound NSC133100 and so the power to detect a significant reduction when compared to the controls may have been affected. A limited number of experimental units could affect the statistical power leading to non-acceptable rates of type II error (Festing 2006), especially given the variability between animals. A small sample size is acceptable when the biological variability between experimental units is negligible, and a large effect size is expected (Nakagawa and Cuthill 2007). However, given the high variability in worm counts between animals, the unexpected loss of an experimental unit can have a significant effect in the overall reduction. For instance, the control group in experiment 1 had a worm establishment that ranged from 18 to 263 worms, with a SEM of 26.97 (Table 3). This high biological variability within the untreated group exemplifies the challenges associated with parasite studies.

Furthermore, this study served as a pilot trial to determine whether PMT enzymes are a viable drug target. As such, animal numbers were deliberately kept low in the interest of the animal research principle of reduction (Tannenbaum and Bennett

2015). The doses used and the regimens selected for this study were based on toxicological data available for other rodent species (data not shown), and were selected to ensure a high likelihood that toxic effects would not be observed. This goal was achieved, as we did not observe any adverse effects in jirds at the doses administered. However, the regimen employed here serves only as an initial exploration, and may not represent the optimal approach for achieving maximal efficacy. Furthermore, a Pharmacokinetic-Pharmacodynamic (PKPD) study, is essential to predict the therapeutic index accurately and formulate an optimal pharmacokinetic design for developing an effective dose regimen during clinical proof of concept (Tuntland, Ethell et al. 2014). However, such studies would be conducted at a later stage (Nixon, Welz et al. 2020), after gathering sufficient preliminary data, such as that provided by this study. The promising initial efficacy achieved in this study using an MDR *H. contortus* isolate further support PMT enzymes as novel drug targets in parasitic nematodes.

The phosphobase methylation step in *H. contortus* is catalyzed by two phosphoethanolamine methyltransferases, PMT1 and PMT2, that sequentially methylate phosphoethanolamine to phosphocholine (Witola, Cooks-Fagbodun et al. 2016, Witola, Matthews et al. 2016). These compounds were verified to inhibit the enzymatic activity of PMT-1 (Zhang, Sicalo Gianechini et al. 2023), by binding the catalytic domains of the enzyme. Therefore, future studies should focus on optimizing the drug regimen through PK/PD studies and could also explore the combination of compounds with activity against both PMT-1 and PMT-2.

Conclusion

These data further support PMT enzymes as a viable drug target, and PMT inhibitors as a potential new drug class for treating parasitic nematodes. The compounds tested here produced reductions in worm counts using an MDR *H. contortus* isolate that ranged from 53.5 to 72.6%. Based on these and previous data reported by our group (Zhang, Sicalo Gianechini et al. 2023), PMT inhibitors have the potential to become a new class of broad-spectrum anthelmintics.

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CHAPTER 4

INVESTIGATING PHENOTYPIC, GENOTYPIC AND GENOMIC CHANGES IN A DRUG-SUSCEPTIBLE ISOLATE OF *HAEMONCHUS CONTORTUS* UNDER FIELD SELECTION WITH FENBENDAZOLE

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Abstract

Benzimidazole (BZ) anthelmintics are important drugs for the control of parasites in pets, livestock, and humans. Benzimidazoles bind to beta-tubulin inhibiting microtubule formation, and missense mutations in this gene at codons 134, 167, 198, and 200 are associated with BZ-resistance in strongylid nematodes. However, evidence suggests that other mechanisms may also be involved. The aim of this study was to investigate changes in drug susceptibility phenotype, beta-tubulin allele frequencies and haplotype diversity in a population of *Haemonchus contortus* under field selection with a BZ drug. Twenty goats were infected with a drug-susceptible isolate of *H. contortus*, assigned to treatment (n=14) or control group (n=6), and grazed on two paddocks. Subtherapeutic dosages (0.25-0.75 mg/kg) of fenbendazole were administered at intervals of 4-8 weeks to the treatment group. Pre- and post-treatment fecal samples were collected for fecal egg count reduction testing, egg hatch assays (EHA), and larval development assays (LDA). Simultaneously, eggs were obtained from control animals for EHA and LDA. Samples from both groups were archived for genomic analysis. GLOWORM-FL simulations were performed to predict levels of egg excretion and larval contamination of pastures. Ten treatments were administered throughout the study, with efficacies (95% CI) ranging from 30.1% (43.7,91.9) to 70.4% (16.0,76.1). Half-maximal inhibitory concentration (IC_{50}) shifted >18-fold from 0.14 to 2.67μM in the EHA, and >5-fold from 0.04 to 0.22μM in the LDA. Resistance was confirmed by a final treatment with fenbendazole at 5mg/kg, yielding an efficacy of 58.8% (90% CI; 24.8-83.7). The primary beta-tubulin isotype-1 mutation selected was F200Y, increasing from 1.9% to fixation, following the full-dose treatment. Low levels of F167Y (<1.56%) were also observed on

3 occasions. The relationship between EHA / LDA IC_{50} and F200Y% followed a quadratic model, demonstrating a good fit (R^2=64%/89%, respectively). Analysis of beta-tubulin isotype-1 gene revealed high haplotype diversity throughout the study (n=16), with a marked reduction after full-dose treatment (n=3). GLOWORM-FL simulations showed that a small proportion of the herd was responsible for most of the pasture contamination and potentially, the development of resistance. Further analyses of DNA samples will be performed to detect novel genomic loci under BZ selection.

Keywords: Benzimidazole resistance, anthelmintic resistance, *Haemonchus contortus*, beta-tubulin mutations, GLOWORM-FL, EHA, LDA, goats

Introduction

Chemical treatments using anthelmintic drugs remain the primary means for controlling and treating infections caused by parasitic nematodes, prevalent in both human and veterinary medicine (Vercruysse, Behnke et al. 2011). Initially introduced to the veterinary market in the 1960's, benzimidazole drugs (BZ) were mainly used for treating infections with gastrointestinal nematodes in livestock and companion animals (Campbell 1990, Lanusse and Prichard 1993). Over time, BZs have also become one of the most widely used drugs for controlling soil-transmitted helminths in humans (Vercruysse, Behnke et al. 2011, Pilotte, Manuel et al. 2022). Additionally, BZs have been extensively used in the agriculture sector due to their remarkably broad spectrum against plant pathogenic fungi (Davidse 1986). The widespread adoption of BZ drugs across agricultural, veterinary, and human domains is largely attributed to broad spectrum of activity, high efficacy, and wide margins of safety (Lanusse and Prichard 1993).

However, the frequent and / or strategic use of anthelmintic drugs has led to increasing levels of anthelmintic resistance (AR) across all drug classes, involving virtually all the most important GIN in livestock and pets (Kaplan 2004, Kaplan and Vidyashankar 2012, Jimenez Castro, Howell et al. 2019). In human helminths, the escalation of mass drug administration campaigns in Africa, Asia, and South America is anticipated to intensify drug selection pressure (Vercruysse, Behnke et al. 2011), thereby promoting the emergence of drug resistance (Vercruysse, Albonico et al. 2011). Consequently, AR is becoming a growing concern in human medicine (Vercruysse, Albonico et al. 2011, Geary 2012, Pilotte, Manuel et al. 2022).

Early detection of AR and continuous monitoring of treatment efficacy are fundamental for establishing sustainable parasite control programs (Kotze, Gilleard et al. 2020) in both human and veterinary medicine. Molecular-based tests provide the opportunity to identify and track changes in genetic markers associated with resistance (Kotze, Gilleard et al. 2020), eliminating the need to perform logistically challenging and expensive *in vivo* trials. However, successful implementation of this approach depends on the comprehensive identification of all relevant genetic markers for each drug class, and understanding their relative effect on the phenotypic response to treatment (Gilleard and Beech 2007).

Early studies in *Caenorhabditis elegans* nematodes showed that the loss of a specific gene encoding beta-tubulin, *ben-1*, resulted in a BZ resistant phenotype (Driscoll, Dean et al. 1989). This was followed by studies in the sheep nematode *Haemonchus contortus*, demonstrating that a change from phenylalanine to tyrosine at position 200 (F200Y) of the beta-tubulin isotype-1 gene, was present at much higher

frequency in resistant than susceptible parasite populations (Roos 1990, Kwa, Veenstra et al. 1994, Lubega, Klein et al. 1994). Further confirmation was obtained by the heterologous expression of the resistant gene from *H. contortus* in transgenic *C. elegans* (Kwa, Veenstra et al. 1995). Since then, a number of non-synonymous single nucleotide polymorphisms (SNPs) on the beta-tubulin gene isotype-1 have been associated with BZ resistance in important GIN of domestic animals. These include phenylalanine to tyrosine (F167Y) (Silvestre and Cabaret 2002, Redman, Whitelaw et al. 2015), or to histidine at codon 167 (F167H) (Prichard 2001), and glutamic acid to alanine at codon 198 (E198A) (Ghisi, Kaminsky et al. 2007), or to leucine, valine, lysine, isoleucine and stop (E198L/E198V/E198K/E198I/E198stop) (Redman, Whitelaw et al. 2015, Mohammedsalih, Krücken et al. 2020). More recently, a change from glutamine to histidine at codon 134 (Q134H) was shown to confer a resistant phenotype in the dog hookworm, *Ancylostoma caninum* (Venkatesan, Jimenez Castro et al. 2023). Both proof of sufficiency and necessity that show a causal connection between the allele and the resistant phenotype (Dilks, Koury et al. 2021), have been obtained for parasitic nematodes in these 10 SNPs (Kwa, Veenstra et al. 1995, Hahnel, Zdraljevic et al. 2018, Kitchen, Ratnappan et al. 2019, Dilks, Hahnel et al. 2020, Dilks, Koury et al. 2021, Venkatesan, Jimenez Castro et al. 2023). Genome-wide studies have confirmed that the beta-tubulin isotype-1 gene is the most important locus under selection in BZ resistant populations of *H. contortus* (Doyle, Laing et al. 2022, Wit, Workentine et al. 2022). However, one study also identified two additional but weaker signatures of selection, including the beta-tubulin isotype-2 gene (Wit, Workentine et al. 2022). Furthermore, Doyle and colleagues (Doyle, Laing et al. 2022) found that, a E198V variant in the beta-

tubulin isotype-2 gene was highly correlated to *in vitro* levels of BZ resistance and seemed to provide higher levels of resistance, given that F200Y was already fixed in the *H. contortus* population. In an early study, it was reported that the deletion of beta-tubulin isotype-2 gene was associated with resistance in *H. contortus* (Kwa, Kooyman et al. 1993), and this was also seen in an *in vitro* study (Rufener, Kaminsky et al. 2009). However, this finding has only rarely been reported, thus its importance in regard to resistance in field populations remains unclear.

Recent genome-wide analyses using *C. elegans* laboratory strains and wild isolates have revealed *ben-1* independent quantitative trait loci (QTLs) associated with resistance (Zamanian, Cook et al. 2018). Additionally, multiple novel alleles specific to *ben-1* (Q131L, S145F, A185P, M257I, D404N, G104S, G142E, G142R, E198K, and R241H), that are associated with resistance in *C. elegans*, were also found (Hahnel, Zdraljevic et al. 2018, Pallotto, Dilks et al. 2022). The impact of beta-tubulin-independent resistant mechanisms on BZ resistance in parasitic species remains unknown and requires further investigation (Doyle and Cotton 2019). For instance, a recent study reported that treatment of a dog infected with a BZ-resistant isolate of *A. caninum* with a single dose of fenbendazole produced more than a 10-fold increase in the *in vitro* levels of resistance measured 13 days after treatment. However, the beta-tubulin SNP frequencies observed in those samples before and after the treatment with fenbendazole did not significantly differ (Jimenez Castro, Howell et al. 2019). The observed response in the resistance phenotype as measured by the EHA IC_{50}, suggested that the treatment may have triggered the induction of beta-tubulin-independent resistant mechanisms. Therefore, while the beta-tubulin isotype-1 locus is

the primary determinant for BZ resistance in strongylid nematodes, it is plausible that beta-tubulin independent mechanisms contribute to higher levels of resistance observed in field samples. Some studies have shown that variation in the *C. elegans* gene *cyp-35D1* affects the response to thiabendazole (TBZ). Specifically, two naturally occurring alleles at position 267 of this gene directly alter the organismal response and metabolism of TBZ within *C. elegans* (Jones, Flemming et al. 2015, Dilks 2021). There is also evidence for *cyp* involvement in mediating susceptibility to TBZ in the parasitic nematodes, *Cooperia oncophora* and *Ostertagia ostertagi* (AlGusbi, Krücken et al. 2014). In *H. contortus*, while none of the cytochrome P450 monooxygenases were inducible by TBZ, the expression of the CYP34/35 family member “HCOI100383400” was significantly higher, ranging from 2.4 to 3.7 times, in a multi-drug resistant isolate compared to a susceptible and intermediate-level BZ-resistant isolates (Yilmaz, Ramünke et al. 2017). Therefore, it is plausible that CYPs could mediate higher levels of resistance in a field population that has reached fixation of a resistance-associated SNP at the beta-tubulin isotype-1 locus.

Additionally, the association between beta-tubulin mutations and BZ resistance is less clear in nematodes belonging to clade III phylogeny. A growing body of evidence indicates that the canonical BZ resistance associated SNPs observed in clade V nematodes are absent in *Ascaris* spp.*, Parascaris* spp.*, Ascaridia* spp., and *Heterakis* spp. (Diawara, Halpenny et al. 2013, Krücken, Fraundorfer et al. 2017, Zuccherato, Furtado et al. 2018, Furtado, Medeiros et al. 2019, Collins 2021, Martin, Halvarsson et al. 2021, Jones, Kozel et al. 2024). Therefore, other mechanisms of BZ resistance need to be elucidated.

Here, we conducted an *in vivo* trial to progressively apply drug selection pressure in a susceptible isolate of *H. contortus.* The aim of this study was to investigate changes in drug susceptibility phenotype, beta-tubulin alleles frequency and haplotype diversity in a population of *H. contortus* under field selection with a BZ drug. To accomplish this objective, two groups of goats designated as treatment and control, were artificially infected and placed on pastures with no recent history of livestock use in Georgia, United States. Over a period of 13 months, 10 subtherapeutic treatments of fenbendazole were administered to the treatment group, followed by a final full therapeutic dose to confirm the resistance status. No treatments were given to the control group. Pre- and post-treatment samples were collected for conducting fecal egg counts (FEC), egg hatch (EHA) and larval development (LDA) assays to track the change in phenotypic susceptibility to BZ in the treatment group. The control group was sampled on dates corresponding to either the pre- or post-treatment sampling of the treatment group, and FEC, EHA, and LDA were performed. The genomic changes were evaluated for both groups, using deep amplicon sequencing of beta-tubulin genes, and haplotype analysis of beta-tubulin isotype-1 gene. Additionally, the GLOWORM-FL model (Rose, Wang et al. 2015) was used to predict the levels of egg excretion and larval contamination across both control and treatment pastures. This enabled a comparative analysis of pasture contamination levels between the treated and untreated group, and allowed us to assess the impact of subtherapeutic treatments on parasite transmission dynamics.

To our knowledge, this is the first study that applied drug selection pressure progressively in a field trial, allowing for natural re-infection of animals between

treatments, and in which changes in the drug susceptibility phenotype, beta-tubulin mutation allele frequencies and haplotype diversity were tracked throughout the entire study. This study design aimed to mimic the drug selection process that occurs in a typical pasture system, when resistance is in its early stages before major canonical beta-tubulin mutations are present at high frequencies in the nematode population.

Material and methods

Parasite isolate used for initial infections

The TxPh-2011-S is a drug-susceptible *H. contortus* population, originally isolated from pronghorn antelope in West Texas by Dr. Tom Craig at Texas A&M University, USA, in 2011 and established in goats. This isolate was then received by our laboratory in 2017, where it was passaged in both goats and sheep and the drug susceptibility phenotype was characterized using the DrenchRite® LDA (Zhang, Sicalo Gianechini et al. 2023). To further genetically characterize the isolate prior to infecting the herd of goats, a nemabiome assay (Avramenko, Redman et al. 2015) followed by deep amplicon sequencing of the beta-tubulin isotype-1 gene was conducted at the University of Calgary. Briefly, DNA was isolated from 4 replicates of a single sample, each containing approximately 1250 stage 3 larvae (L3) of TxPh-2011-S, stored in 70% ETOH. Results of all 4 replicates confirmed that the isolate was 100% *H. contortus*, and eight amplicon sequence variants (ASVs) were identified and mapped to the *H. contortus* beta tubulin isotype-1 reference sequence. A low frequency of the resistance-associated F200Y SNP (mean= 1.9%, table 1) was detected, but codons 167 and 198 showed the wildtype BZ-susceptible sequence in 100% of the reads.

Table 1. Deep amplicon sequencing, targeting the internal transcribed spacer 2 (ITS2) rDNA locus and beta-tubulin isotype-1 gene of *H. contortus* TxPh-2011-S. Four samples, each containing approximately 1250 L3 of TxPh-2011-S, were sequenced. F= phenylalanine, Y= tyrosine.

Sample	Total reads for ITS2	*H. contortus* reads (% of total)	Susceptible 200F reads	Resistant 200Y reads	Total reads	% Resistant F200Y
1	16821	16821 (100%)	25285	530	25815	2.05
2	13014	13014 (100%)	20851	309	21160	1.46
3	14331	14331 (100%)	15291	294	15585	1.89
4	12317	12317 (100%)	18903	428	19331	2.21

Experimental design

The field trial was conducted between June 2022 and November 2023 in compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines under the Animal Use Protocol (AUP) A2022 06-017-Y1-A3 (supplementary table 1). In June 2022, twelve Saanen and four Nigerian dwarf 3-month-old castrated male goats were purchased and housed on concrete. After three days of acclimation, fecal samples were collected and animals received a combination treatment consisting of levamisole (12 mg/kg), fenbendazole (10 mg/kg) and ivermectin (0.4 mg/kg). Fecal egg counts (FEC) were checked on each goat individually `two weeks post-treatment by reading three Mini-FLOTAC® discs (5 EPG each) per animal. No eggs were seen confirming the absence of strongyle egg excretion. Some animals also received ponazuril at 15 mg/kg to treat *Eimeria* spp. infections. Goats were then moved to the pasture, and FEC (one mini-FOLATC disc read; 5 EPG) were checked once per week for two weeks. Fecals remained negative confirming the worm free status. Two weeks after being moved to

the pasture, animals were infected orally with 2500 L3 of *H. contortus* (TxPh2011-S). Fourteen days post administration of L3s, the infections became patent in two animals, and feces were collected for the isolation of eggs and larvae, which were saved in 70% ETOH for further analyses. The goats were then left to graze the pasture for a period of seven more weeks in order to contaminate the pasture with *H. contortus* larvae prior to initiating the selection protocol with fenbendazole. Just prior to the initial treatment, goats were blocked by FEC and breed and randomly assigned to either control or treatment groups, keeping the breed proportions equal between groups. The control group, consisted of three Saanen goats and one Nigerian dwarf goat, while the treatment group included nine Saanen and three Nigerian dwarf goats. The pasture was then divided into 3 paddocks (Fig. 1). The paddock for the treatment group measured approximately 6400 m² (0.053 hectares/goat), while the paddock for the control group measured approximately 2000 m² (0.050 hectares/goat). Between those two paddocks, an area of approximately 2000 m^2 was left ungrazed to create a buffer zone that prevented contact between the two groups, and minimized the chances of cross-pasture contamination with *H. contortus* larvae.

Figure 1: Google maps image denoting the area of study, created with https://www.google.com/maps/. The treatment pen is delimited in green while the control pen is delimited in blue.

Treatment schedule

The treatment protocol was designed to apply selection pressure with fenbendazole on the population of worms without producing rapid fixation of the beta-tubulin F200Y SNP, which was present at a low frequency (Table 1). To achieve this, pastures were allowed to become contaminated with infective L3 to provide refugia prior to initiating treatments, subtherapeutic doses that would kill approximately 50% of the worms in the animals were used, and re-infection from the pasture was allowed to occur

between treatments (Fig. 2). We expected that this strategy would result in an infrapopulation of worms at each treatment that would include both worms that survived the previous treatment, and newly established worms acquired from the pasture since the previous treatment. The protocol had a target of an 8-week interval between treatments to allow adequate time for re-infection, however, in the interest of animal welfare the protocol was left flexible so that at times of high transmission treatments could be given more frequently to particular animals. Thus, depending on the time of year, and clinical need as determined by FAMACHA scores and FEC, individual animals could receive treatments at any point between four to eight weeks since the previous treatment. Likewise, at times of the year of low transmission (winter), treatment interval occasionally exceeded 8 weeks due to low FEC.

In the interest of animal welfare, animals suffering from anemia as determined by FAMACHA score of 4 or 5 and a hematocrit of 15% or lower, were administered a salvage treatment with levamisole (12 mg/kg) and were temporarily removed from the pasture. Throughout the study, three animals from the treatment group and one animal from the control group required salvage treatments. Fecal egg counts were checked seven days post-treatment with the Mini-FLOTAC® technique and if eggs were still being excreted, a second treatment was performed. Once the animal was “worm free” and the hematocrit levels returned to a baseline of 19%, affected animals were placed back to the pasture and were allowed to become reinfected naturally from the pasture.

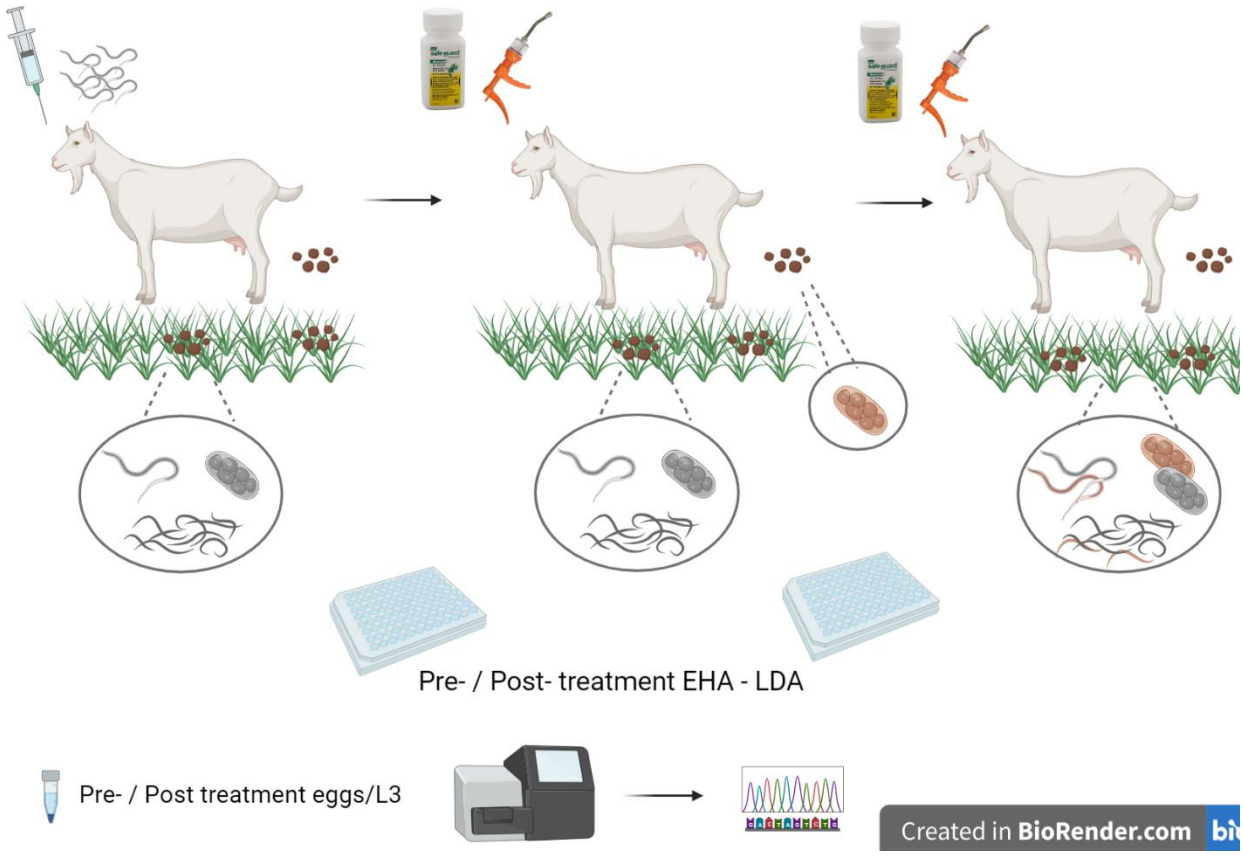

Figure 2: Experimental design. Goats were infected orally with a susceptible isolate of *Haemonchus contortus*, and selection pressure was applied with subtherapeutic doses of fenbendazole. Phenotypic, genotypic and genomic analyses were performed using pre- and post-treatment eggs and larvae. EHA= egg hatch assay, LDA: larval development assay, L3=larvae at stage 3.

Doses of fenbendazole used

The initial dose of fenbendazole was selected based on data from a previously published study that used an *in vivo* selection protocol in sheep infected with a susceptible isolate of *H. contortus* (Düwel 1977). Based on these previous data and having a target efficacy of 50%, we performed a test on half of the treatment group

goats using 0.1 mg/kg. However, no significant reduction in egg counts was observed as compared to the untreated goats from the same group (data not shown). Consequently, we increased the dose to 0.25 mg/kg for the initial treatment. This dose was then adjusted throughout the study in response to changes in the percent reduction in egg counts as compared to prior treatments. This approach resulted in administering four treatments at 0.25 mg/kg, four treatments at 0.5 mg/kg, and two treatments at 0.75 mg/kg (supplementary table 1).

Drug preparation and administration

Fenbendazole was prepared from the commercially available product for goats (Safe-guard® 10% suspension, Merck Animal Health, Rahway, NJ, USA). Briefly, on treatment days, after thoroughly shaking the bottle of Safe-guard®, 10 ml of 10% fenbendazole were mixed with 190 ml of 1% CMC yielding a final drug concentration of 5 mg/ml, which was then left on a stirring plate until used. Goat weight was assessed one or two days before each treatment using a weight estimation tape (Coburn®) for the first 12 months of the study, and for the final five months of the study, a scale was used (A and A scales, VS-660 Goat Scale). The dose for each animal was calculated, and then rounded up to the nearest 0.2 ml. For instance, a 100 lb. (45.45 kg) goat receiving 0.25 mg/kg, would be administered 2.4 ml (2.27 ml rounded up) of the 5 mg/ml fenbendazole suspension in 1% CMC. The drug was then delivered to each goat using a plastic syringe attached to a metal drenching extension (Tondiamo®, 12 cm in length).

Animal replacements

Animals were removed from the study if they became resistant to infection with *H. contortus* or developed health issues that prevented their continuation in the study. One goat from the control pen became resistant to infection with *H. contortus*, as evidenced by FEC <50 EPG for more than 8 weeks during the high transmission season. This goat was removed from the study in August 2023, and two recently weaned goats were added to both the control and treatment pens. These extra young susceptible goats were added as a precaution in case other animals became resistant. Thus, in the control pen, one Saanen goat was replaced with two 3-month-old Nigerian dwarf castrated males, and on the same day in the treatment pen, two 3-month-old Nigerian dwarf castrated males were introduced. These animals received the same treatments and testing protocol as the original cohort of goats, so were worm-free when added to the pastures. These goats were then allowed to become naturally infected from their respective pastures. Following this adjustment, the treatment and control groups ended up with fourteen (0.046 hectares / goat) and five goats (0.040 hectares / goat), respectively.

Fecal egg counts and fecal egg count reductions

Fecal egg counts were performed using a modified McMaster technique (Gordon and Whitlock 1939) for purposes of monitoring egg excretion and for fecal egg count reduction tests (FECRT). However, the Mini-FLOTAC® technique (Cringoli, Maurelli et al. 2017) was used for checking if animals were excreting eggs after re-introduction to the pasture following a salvage treatment with levamisole, and to confirm “worm-free” status prior to initial infections. For modified McMaster, 2 grams of feces were thoroughly mixed with 28 ml of sodium nitrate solution (Feca-Med®, Vedco Inc., St.

Jospeh, MO), and strained using a double layer of cheesecloth. An aliquot was then loaded into the McMaster chamber (0.3 ml reading surface), and read under a compound microscope. The eggs were counted and then multiplied by a correction factor of 50. For FECRT, three McMaster chambers were read pre- and post-treatment for each animal using a slightly modified procedure. In these cases, 2 grams of feces and 25 ml of solution were used, yielding a 45-correction factor. Hence, when using http://shiny.math.uzh.ch/user/furrer/shinyas/shiny-eggCounts/ analysis procedure, the data were entered as the average of the 3 chambers and using a correction factor of 15 to account for the total numbers of eggs counted under the microscope.

Coprocultures and archiving of parasite material

Both eggs and L3 were saved from all pre- and post-treatment samples, as well as from other opportunistic samplings between treatments. Eggs were isolated following the standard procedures described in the "*in vitro* assays" section, and stored in 70% ethanol at -80°C. For coprocultures, feces were mixed with vermiculite and water, and cultured for 14 days at room temperature. The coprocultures were stirred once daily and water was adjusted based on visual evaluation of moisture content. After 14 days, the cultures were harvested using the Baermann technique (Dinaburg 1942) and the larvae collected in a 15 ml centrifuge tube. Larvae at stage 3 were morphologically identified (van Wyk and Mayhew 2013), counted, and stored in 70% ethanol at -80°C.

In vitro assays

Egg hatch and larval development plate preparation

Susceptibility of the *H. contortus* population to BZ drugs was phenotypically characterized using two different assays: the larval development assay (LDA) and the egg hatch assay (EHA). The aim was to perform two replicates of each assay both before and after treatment for the treatment group, and two replicates for the control group, aligned with one of the two treatment group time points. The pre-treatment assays were conducted with eggs isolated from the entire group and post-treatment assays with eggs obtained only from the treated animals. The same procedure was used to prepare both the EHA and LDA plates, with the only difference being the concentrations of thiabendazole (TBZ) that were used.

Ten TBZ concentrations and four replicates per concentration were included on each plate. The TBZ concentrations in the assay plates for the treatment group were adjusted throughout the study to provide an optimal range of concentrations for measuring an accurate dose response given the level of IC_{50}s measured in the previous set of assays. The treatment group EHA concentrations of TBZ ranged from 0.017-0.667 µM in the first assays, and from 0.13-8.0 µM in the last set of plates. The concentrations of TBZ used in the control group EHA plates remained stable, ranging from 0.016 to 1.0 µM. The concentrations of TBZ used in the LDA ranged from 0.017-0.667 µM in the first treatment group assays, and from 0.019-150 µM in the last set of plates. In the latter, these concentrations correspond to the Drenchrite® assay, which was used to evaluate the full-dose treatment response. The concentrations used for the control group LDA remained unchanged, ranging from 0.017 to 0.667 µM, throughout the study. However, the Drenchrite® assay was also conducted on the control group at the time when the treatment group received the full-dose treatment.

Thiabendazole was first dissolved in 100% dimethyl sulfoxide (DMSO) to create a stock concentration of 100 µM. This stock solution was subsequently diluted in 100% DMSO to produce a series of working stock solutions. All stock solutions were stored at -20°C for a maximum of one month. Both EHA and LDA plates were prepared from the same stock solutions generated in each month. On the day that assay plates were made, working solutions of TBZ were prepared by diluting the stock solutions in deionized (DI) water, to yield a DMSO concentration of 5%. Thirty ul of each working TBZ or control solution was placed in the wells of a flat-bottomed black 96-well plate (Corning®), followed by the addition of 120 ml of 2.5% agar. This yielded a final in-plate concentration of 1% DMSO in 2% agar. Sixteen control wells (no drug) containing 1% DMSO in 2% agar were set per plate. Plates were prepared in advance and stored at 4°C for no longer than 5 days. A few hours prior to use, plates were warmed to room temperature, and 20 µl of water was added to each well to fully hydrate the agar.

Nematode eggs were isolated from feces as previously described (Howell, Burke et al. 2008, Zhang, Sicalo Gianechini et al. 2023). Once isolated, the eggs were resuspended in water and a cleaning protocol (Kotze, O'Grady et al. 2009) was used to minimize microbial contamination in the assay wells. For cleaning, the eggs were mixed gently in a solution of 8.4-mg/l sodium hypochlorite for 12 min and then washed three times with DI water. The eggs were diluted in DI water to achieve a concentration of 50 to 100 eggs per 20 µl, followed by the addition of amphotericin B (90 µl/ml).

LDA

Immediately after the cleaning procedure, 20 µl of nematode egg solution was added to each well, plates were sealed with Parafilm to reduce moisture loss, and then

placed in an incubator at 25°C. After 24 h, 20 µl of nutritive media composed of 0.87% Earle's balanced salts (Sigma-Aldrich, St. Louis, MO), 1% yeast extract (BD Difco, VWR; Becton Dickinson, Sparks, MD), 0.76% NaCl (Sigma-Aldrich), and 0.75% *Escherichia coli* OP50, was added to each well. The plates were resealed and incubated for 6 additional days, and the assays were ended with the addition of 20 ul of 50% Lugol's iodine to each well. The contents of each well were then transferred to a clean 96-flat well plate, and all eggs and larvae in each well were counted under an inverted microscope as previously described (Tandon and Kaplan 2004). The percent larval development (to the L3 stage) was calculated for each well by dividing the number of L3 by the total count of larvae (L1-L2-L3) for that well and then multiplying that value by 100. The percent inhibition in larval development (%ILD) for each individual drug well was then calculated compared to the percent development in control wells by dividing the percent larval development of each drug well by the mean percent larval development of the control wells (for that plate) according to the following formula:

$$\%\ \mathrm{ILD} = \frac{\text{mean \% development control} - \text{mean \% development treatment}}{\text{mean \% development control}}\ \mathrm{x}\ 100$$

EHA

Immediately after the cleaning procedure, 20 ul of nematode egg solution was added to each well, plates were sealed with Parafilm® wrapping film to reduce moisture loss, and placed in an incubator at 25°C. After 40-48 hours the development/hatching of the eggs was stopped by adding 20 µl of 50% Lugol's iodine to each well. The contents of each well were then transferred to a clean 96-flat well plate, and all eggs and larvae in each well were counted under an inverted microscope. To calculate the percentage

hatching for each well, the total larvae count was divided by the total number of larvae and eggs and then multiplied by 100. The percent inhibition in egg hatching (%IEH) for each individual well was then calculated as per the LDA, using the following formula:

$$\%\ \mathrm{IEH} = \frac{\text{mean \% development control} - \text{mean \% development treatment}}{\text{mean \% development control}}\ \mathrm{x}\ 100$$

Simulation of the pasture contamination with free-living stages and population dynamics of H. contortus.

The GLOWORM-FL model framework (Rose, Wang et al. 2015) was used to simulate pasture contamination with free-living stages of *H. contortus* and to model climate-dependent population dynamics. The model was implemented in R version 4.2.0 “Vigorous Calisthenics” (R Core Team 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/), using the lsoda function in the deSolve package (Soetaert, Petzoldt et al. 2010). The simulation period ran from July 12, 2022, one week after the animals were infected, to December 31, 2023, which was 44 days after the goats were removed from the pasture. This permitted the prediction of the population dynamics throughout the entire study period.

Model input required estimation of the total daily number of *H. contortus* eggs deposited on each paddock, for the control and treatment study animals. This was achieved by multiplying the estimated daily FEC by estimated daily feces production for each animal. Daily feces production per animal was determined by using a rate of 20

grams per kilogram of live weight (Singleton, Stear et al. 2011). Fecal egg counts and live weight measurements were taken at different time points during the study. The *approxfun* R function was therefore used to linearly interpolate data points to create a continuous set of values between FEC and live weight observations separated by missing values. A value for FEC and liveweight was thus assigned to each day of the simulation period, for each of the study animals. To reflect the grazing history of each paddock, the initial numbers of eggs and L3 on pasture were set to zero at the start (7/12/2022) of each simulation. The number of eggs being put onto the pasture was set to zero for the day after the animals were removed from the pasture (11/18/2023) and for the end of the simulation period (12/31/23). This provided an opportunity to assess the short-term changes in the predicted number of L3 on pasture for study animals following the end of grazing, without further egg input. Mean daily air temperature (°C) and total daily precipitation (mm) were employed as climatic inputs for model simulation. These were obtained from a weather station located approximately 8 km away from the study location available at: http://www.weather.uga.edu/. Date and time data were converted and manipulated using *chron* (James, D., Hornik, K., 2023. chron: Chronological objects which can handle dates and times. R package version '2.3.61') package in R. The geosphere package (Robert J. Hijmans (2022), geosphere: Spherical Trigonometry. R package version 1.5.18. https://CRAN. R-project.org/package=geosphere) was employed for functions relevant to geographic coordinates. Final model inputs consisted of daily total precipitation (mm), daily mean temperature (°C) and predicted total number of *H. contortus* eggs deposited by each study animal, per day. The final model outputs for each individual animal were exported

to an Excel file for further analysis. Model outputs were adjusted to predict daily L3 per m^2 for fields grazed by control and treatment animals. Data visualizations were created in GraphPad prism version 10.2.2 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com.

Genotype characterization: nemabiome and deep amplicon sequencing of beta-tubulin isotype-1 / Haplotype analysis

DNA preparation

Bulk DNA lysates were prepared from pools of 2000 - 5000 eggs or L3s using a modified commercial kit (QIAamp® PowerFecal® Pro DNA Kit). Briefly, aliquots of the specimens preserved in 70% ethanol were obtained and washed 3 times by centrifugation at 2500g with lysis buffer (50 mM KCl, 10 mM Tris (pH8.3), 2.5 mM MgCl2, 0.45% Nonidet P-40, 0.45% Tween 20, 0.01% (w/v) gelatin). The pelleted samples were re-suspended in a final volume of <250 µl lysis buffer. After transferring the samples to the PowerBead Pro tubes with 800 µl of solution CD1, they were placed in a thermomixer set at 65°C and shaking to 1000 RPM for 10 minutes. The protocol provided with the DNA kit user's manual was then followed without modifications. DNA was eluted to a final volume of 100 ul, and preserved at -80°C until further processing.

Determination of gastrointestinal nematode species identity and relative abundance

The pools of L3 were morphologically identified (van Wyk and Mayhew 2013) and as determined by using ITS2 rDNA nemabiome metabarcoding (Avramenko, Redman et al. 2015). Nemabiome was conducted in 3 replicates of the DNA lysate

obtained from the eggs and L3s. Amplification of the ITS2 marker using NC1 and NC2 primers and metabarcoding library preparation was conducted as previously described (Avramenko, Redman et al. 2015). Further details can be obtained at https://www.nemabiome.ca/sequencing.html.

Bioinformatic analysis for Nemabiome analysis

All bioinformatic analysis was performed using R v3.6 (Core team, 2020). The fastq files were passed through a bioinformatic pipeline based on the DADA2 R package (Callahan, McMurdie et al. 2016). Specific details can be found at https://www.nemabiome.ca. In brief, Cutadapt (Martin 2011) is used to remove primers from the sequence reads and the data was then filtered on the basis of length and quality. A dynamic error learning algorithm was then employed, called denoising that is able to identify and remove sequencing errors from the dataset. The forward and reverse sequence reads are then merged and/or catenated. Chimera's are removed and the resulting list of amplicon sequence variants (or ASVs) are reported. These ASVs are assigned against the Nematode ITS2 database version 1.3 (https://www.nemabiome.ca) using IDTaxa (Murali, Bhargava et al. 2018) at a confidence threshold of 60%. The percentage species composition of samples was calculated by dividing the total reads assigned to each species in a sample by the total number of reads per sample to obtain the relative percentage of each species. Detailed ITS2 rDNA nemabiome sequencing and bioinformatic analysis information are available at https://www.nemabiome.ca/analysis.html.

Deep amplicon sequencing PCR amplification of the beta-tubulin isotype-1 fragment

The DNA isolated for nemabiome metabarcoding was also used for deep amplicon sequencing of the beta-tubulin isotype-1 fragment, and run in 3 replicates. At least 3 negative controls (water in master mix) were included per plate. Briefly, a mix of 7 forward primers and 11 reverse primers were used, as described before (Avramenko, Redman et al. 2019). Five µl of DNA template were mixed with 20 µl of a Q5 hot start high-fidelity master mix (11.65 µl molecular-grade H_2O, 5 µl of 5× NEB Q5 Reaction Buffer (New England Biolabs Ltd, USA), 0.5 µl dNTPs (10mM), 1.25 µl forward Primer mix (10mM), 1.25 µl reverse Primer mix (10mM), 0.25 µl NEB Q5 Polymerase, 0.1 µl bovine serum albumin (Thermo Fisher Scientific). The thermocycling conditions were set to 98°C for 2 minutes, followed by 40 cycles of 98°C for 10 seconds, 60°C for 15 seconds and 72°C for 25 seconds, and 5 minutes at 72°C after the 40 cycles were run. All PCR steps were carried out with best practices to reduce aerosol formation, including the use of filter pipette tips, working in a PCR cabinet, and the use of the easy release Microseal 'A' Film (Bio-Rad, USA MSA5001) PCR plate cover.

The PCR products were then purified with AgenCourt AMPure XP Magnetic Beads (Beckman Coulter, Inc USA), following the manufacturer's recommended protocol. Thirty µl of the purified products were saved at -80°C until further processing.

Illumina Barcoded regions, referred to as Unique Dual Indexes or UDIs and P5/P7 sequencing regions were added to the purified PCR products in a second limited cycle PCR. A mastermix (8.75 µl molecular-grade H_2O, 5 µl KAPA HiFi HotStart Fidelity Buffer (KAPA Biosystems, USA), 0.75 µl dNTPs (10mM), 0.5 µl KAPA HiFi HotStart Polymerase) was prepared. Fifteen µl of this mastermix were dispensed to plates that contained a combination of 2 Illumina PCR UD Indexes (Oligonucleotide sequences

2023 Illumina, Inc. USA), and 5 µl of the initial PCR products. These index primer plates had been generated in-house to make up to 384 unique forward/reverse sample unique index combinations per miseq run. The thermocycling conditions were set to 98°C for 45 seconds, followed by 7 cycles of 98°C for 20 seconds, 63°C for 20 seconds, 72°C for 2 minutes, and kept at 10°C until stopped. The amplicons obtained were purified using the AgenCourt AMPure XP Magnetic Beads protocol as described above.

The quantity of the purified products resulting from the PCR were estimated using absorbance on a BioTek Synergy HTX multi-mode reader. To generate a normalized library 50 ng of DNA per sample were combined in a pool, ensuring equal concentrations in the library mix. The final concentration of the pooled library was assessed with the KAPA qPCR Library Quantification Kit (KAPA Biosystems, USA, KK4824), following the manufacturer's recommended protocol. We then diluted the library to 4 nM with molecular-grade H2O, based on the qPCR results.

The pooled library was run on an Illumina Desktop Sequencer using a 500-cycle paired-end reagent kit (MiSeq Reagent Kit, v2, MS-103–2003) at a final concentration of 12 pM, with the addition of 20% PhiX control v3 (Illumina, FC-110-3001). The MiSeq was set to generate only FASTQ files, with no post-run analysis. Samples were automatically demultiplexed with the MiSeq, based on the supplied index combinations. All protocols were carried out using Illumina's standard MiSeq operating protocol (Illumina Inc.)

Bioinformatic analysis for amplicon sequencing of Isotype-1 beta tubulin gene

Cutadapt (Martin 2011) was used to remove forward and reverse primers from the sequence reads. Following adapter trimming and quality filtering, all the forward and reverse reads were processed using the DADA2 bioinformatic pipeline to obtain Amplicon Sequence Variants (ASVs) (Callahan, McMurdie et al. 2016). Overlapping forward and reverse reads were merged, allowing a maximum mismatch of 4 bp in the overlap region. The ASVs generated using the DADA2 pipeline were then aligned to the isotype-1 beta-tubulin reference sequence (GenBank # EF198865) using a global (Needleman-Wunsch) pairwise alignment algorithm without end gap penalties. Following alignment, the ASVs were discarded if they were <180 bp or >350 bp long, or if they had a percentage identity <70% to the reference sequence. A data frame summarizing the frequency distribution of these ASVs across individual samples was also generated by the pipeline and can be used to study the within-species diversity at the isotype-1 beta tubulin locus.

The codons 167, 198 and 200 were then analyzed for the presence of any variants resulting in non-synonymous changes. The frequency of mutations at positions 167, 198 and 200 were calculated by dividing the number of reads carrying the resistant mutation by the total number of reads that are assigned to *H. contortus* in a given sample.

Statistical analysis

Percent fecal egg count reductions were calculated using the University of Zurich “eggCounts” package. This program was designed to address statistical problems inherent with FECRT including individual efficacy, between-animal egg aggregation and Poisson errors (Wang, Torgerson et al. 2018). A Bayesian framework is used for the

computation of anthelmintic resistance probability given an arbitrary threshold, and provides summary statistics (Wang, Torgerson et al. 2018).

The statistical analysis of the *in vitro* data for EHA and LDA, was done using GraphPad Prism version 10.2.2. A nonlinear regression model with a variable slope (GraphPad Software, La Jolla, CA) was the method applied. Drug concentrations were log10 transformed, and then half-maximal inhibitory concentration (IC_{50}) concentrations and dose-response curves were obtained from the log[inhibitor] versus the normalized response variable slope logistic equation.

Pre vs post-treatment EHA IC_{50} and F200Y% were analyzed using a Wilcoxon matched pairs signed rank test, and a paired Student *t* test, respectively. The significance level (α) was set at 5% for both tests, and *p* values of ≤0.05 were considered significant. The relationship between EHA IC_{50} and F200Y% was modeled using a quadratic regression. This choice was made to better capture the non-linear nature of the data, as a preliminary analysis indicated that a linear model did not adequately describe the observed relationship. The analyses were conducted in GraphPad Prism version 10.2.2 (GraphPad Software, La Jolla, CA).

Results

Parasite infections

The infection of young, highly susceptible goats ensured that continuous year-round cycling of *Haemonchus* would occur in the subtropical climate of Georgia, USA. (Fig. 11). Two animals became patent by 14 days post-infection, with the rest of the herd becoming patent in the subsequent week. A seven-week period without treatment was

then provided to allow for the contamination of the pasture with *H. contortus* L3, so that adequate refugia would be present before initiating subtherapeutic treatments. The usefulness of this strategy was supported by the results of the simulation model GLOWORM-FL (Rose, Wang et al. 2015). Using FEC data from the goats and weather data from a local weather station, the model output showed that by the time the first treatment with fenbendazole was administered, high levels of free-living stages were present on the pasture (Fig. 11). Subtherapeutic treatments with fenbendazole were then administered to the treatment group at intervals varying from four to eight weeks (Table 2). Despite the relatively frequent treatments, the model output confirmed the clinical data, indicating that parasite transmission was maintained successfully throughout the entirety of the study (Fig. 11).

To ensure animal welfare, the herd's health was monitored bi-weekly using FAMACHA scores and monthly body condition scores (BCS). Salvage treatment was administered to three animals in the treatment group and one animal in the control group due to high FAMACHA scores and hematocrit levels below 15%. These animals recovered quickly after treatment with levamisole and were returned to their respective pastures. Both the control and treatment groups maintained consistent nutritional status, as indicated by steady BCS values, with no significant differences between the groups (Supplementary Fig. 1A). Additionally, no notable differences in FAMACHA scores were observed between the groups at any time (Supplementary Fig. 1B).

Fecal egg count reductions

Ten subtherapeutic treatments were administered from September 9, 2022, to October 19, 2023, with a full-dose treatment at the end of the study to confirm

resistance to fenbendazole (Table 2). The initial dose aimed to remove approximately 50% of the adult resident worms in the animals. To achieve this targeted level of efficacy throughout the study, the dosage had to be gradually increased from 0.25 mg/kg to 0.75 mg/kg in the final subtherapeutic treatment (Table 2). At the conclusion of the study, full clinical resistance to a label therapeutic dosage was confirmed by conducting a FECRT, which yielded a mean reduction of 58.8% with a 90% CI of 24.8-83.7% (Table 2). All FECR tests were conducted following the latest guidelines from the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Kaplan, Denwood et al. 2023). For all subtherapeutic treatments, the percent reduction of the subtherapeutic treatments was calculated using the eggCount modeler (Wang, Torgerson et al. 2018), which provides 95% CIs and a modeled mean reduction, accounting for individual treatment efficacy and egg aggregation between animals. The subtherapeutic treatments resulted in reductions ranging from 30.1% (95% CI: 12.3% to 58.9%) to 71.8% (95% CI: 45.0% to 87.6%; table 2). The wide 95% confidence intervals indicate a high degree of uncertainty of the true % reduction, which is due to the high variability in individual responses to treatment. High variability between animals is expected given the target efficacy of 50% because there are many factors that influence both the true biological reduction and the measurement of that reduction when efficacy is in this range (Torgerson, Paul et al. 2012, Morgan, Lanusse et al. 2022). Despite this, the mean of the FECRT performed was 52.8%, which is quite close to the target efficacy of 50% in the experimental design.

Table 2. Fecal egg count reductions and associated 95% confidence intervals (CI), obtained from eggCount modeler (https://shiny.math.uzh.ch/user/furrer/shinyas/shiny-

eggCounts/). The therapeutic treatment, conducted at the full label dose for goats (5 mg/kg) was analyzed using https://mdenwood.shinyapps.io/data_analysis/, where 90% CI were obtained.

Date	Animals (n)	Dose (mg/kg)	% Reduction	Lower 95% CI	Upper 95% CI
Subtherapeutic treatments					
9-Sep-2022	12	0.25	70.4	43.7	91.9
14-Oct-2022	3	0.25	44.7	15.6	72.7
4-Nov-2022	12	0.25	37.6	16.2	59.4
6-Dec-2022	5	0.25	30.1	12.3	58.9
3-Mar-2023	3	0.50	46.2	16.0	76.1
21-Apr-2023	11	0.50	71.8	45.0	87.6
16-Jun-2023	12	0.50	63.9	40.0	78.0
25-Jul-2023	12	0.50	50.9	24.3	72.2
14-Sep-2023	13	0.75	69.2	54.7	78.9
19-Oct-2023	14	0.75	43.3	19.8	64.1
Mean FECR	-	-	52.8	-	-
Therapeutic treatment					
2-Nov-2023	14	5.00	58.8	24.8*	83.7*

*90% CI.

In vitro assays

EHA

Treatment group: Paired pre- and post-treatment assays were conducted for all treatments except for the second treatment. Egg hatch assay IC_{50} shifted 5.3-fold, from 0.14 µM (95% CI; 0.136-0.150 µM) in the first pre-treatment to 0.77 µM (95% CI; 0.676 to 0.887 µM) in the last pre-treatment, with no overlap in the 95% CI. Post-treatment IC_{50} increased greater than 15-fold, from 0.17 (95% CI; 0.163 to 0.182 µM) in the first post-treatment assay to 2.67 µM (95% CI; 2.421 to 2.953 µM) in the last one (Table 3). The shift in IC_{50} was significant when comparing pre- vs post-treatment assays (P =

0.0391). However, an accurate IC_{50} could not be calculated in treatment 10, both pre- and post-treatment, since the highest concentrations tested were too low to capture the full dose-response curve (Fig. 3). Despite this limitation, all the R^2 were very high (>91%; Table 3), indicating a good fit to the model.

Egg hatch assay IC_{99} demonstrated a huge shift of 86.7-fold, from 0.82 µM (95% CI; 0.670 to 1.017 µM) in the first pre-treatment to 71.15 µM (95% CI; 39.370 to 142.700 µM) in the last one. Post-treatment IC_{99} increased even further to over 255.8-fold, from 0.89 (95% CI; 0.720 to 1.124 µM) in the first post-treatment assay to 228.4 µM (95% CI; 147.600 to 371.800 µM) in the last one (Table 3).

Control group: Nine EHA tests were conducted using samples from the control group (Table 4). The first test was performed on a pooled sample from all of the goats, establishing a baseline before the goats were assigned to separate treatment and control groups. The IC_{50}s were fairly stable throughout the study, but did demonstrate a shift of 2.8-fold, ranging from 0.14 µM (95% CI; 0.136 to 0.150 µM) to 0.39 µM (95%CI; 0.383 to 0.405). The IC_{99} demonstrated a similar trend, with a shift of 2.0-fold, ranging from 0.82 µM (95% CI; 0.670 to 1.017 µM) to 1.67 µM (95% CI; 1.370 to 2.055 µM).

A resistant ratio (RR) was calculated based on the ratios of the IC_{50} / IC_{99} from the treatment as compared to the control group, when data were available for the same dates. The IC_{50} ratio ranged from 1 to 3.8, and from 1.2 to 47.8 for the IC_{99} RR (Table 4).

Table 3. Egg hatch assay results for the treatment group. Dose-response was calculated using a nonlinear regression model in GraphPad Prism version 10.2.2

(GraphPad Software, La Jolla, CA). Half maximal inhibitory concentration= IC_{50} (μM), 99% inhibitory concentration=IC_{99} (μM), 95% Confidence intervals= 95%CI, Coefficient of determination= R^2.

Treatment	Type of Assay	Date	IC_{50} (μM)	95% CI	R^2	IC_{99} (μM)	95% CI
1	Pre-Tx	9-Sep-22	0.14	0.136 to 0.150	0.97	0.82	0.670 to 1.017
	Post-Tx	16-Sep-22	0.17	0.163 to 0.182	0.98	0.89	0.720 to 1.124
2	Pre-Tx	14-Oct-22	x	x	x	x	x
	Post-Tx	21-Oct-22	x	x	x	x	x
3	Pre-Tx	4-Nov-22	0.19	0.182 to 0.193	0.99	0.74	0.650 to 0.837
	Post-Tx	18-Nov-22	0.18	0.172 to 0.191	0.96	1.47	1.186 to 1.850
4	Pre-Tx	6-Dec-22	0.23	0.218 to 0.233	0.98	0.89	0.770 to 1.024
	Post-Tx	13-Dec-22	0.49	0.463 to 0.524	0.91	5.40	3.995 to 7.645
5	Pre-Tx	3-Mar-23	0.32	0.309 to 0.340	0.97	2.18	1.800 to 2.654
	Post-Tx	15-Mar-23	1.18	1.090 to 1.289	0.93	17.10	12.33 to 24.94
6	Pre-Tx	21-Apr-23	0.25	0.238 to 0.255	0.98	0.86	0.744 to 0.986
	Post-Tx	1-May-23	0.45	0.423 to 0.485	0.93	6.34	4.532 to 9.284
7	Pre-Tx	16-Jun-23	0.23	0.217 to 0.244	0.97	3.05	2.447 to 3.876
	Post-Tx	23-Jun-23	0.27	0.257 to 0.292	0.96	4.69	3.529 to 6.252
8	Pre-Tx	25-Jul-23	0.59	0.553 to 0.633	0.94	15.92	11.060 to 23.700
	Post-Tx	1-Aug-23	0.54	0.513 to 0.573	0.97	23.47	17.100 to 32.970
9	Pre-Tx	14-Sep-23	0.53	0.503 to 0.554	0.96	7.14	5.439 to 9.577
	Post-Tx	25-Sep-23	1.05	0.982 to 1.129	0.96	83.21	59.330 to 119.800

10	Pre-Tx	19-Oct-23	0.77	0.676 to 0.887	0.93	71.15	39.370 to 142.700
	Post-Tx	26-Oct-23	2.67	2.421 to 2.953	0.96	228.40	147.600 to 371.800

Table 4. Egg hatch assay results for the control group. Dose-response was calculated using a nonlinear regression model in GraphPad Prism version 10.2.2 (GraphPad Software, La Jolla, CA). Half maximal inhibitory concentration= IC_{50} (µM), 99% inhibitory concentration=IC_{99} (µM), 95% Confidence intervals= 95%CI, Coefficient of determination= R^2. Resistance Ratio (RR) was calculated as IC_{50} of the treatment group divided by IC_{50} of the control group.

Type of Assay	Date	IC_{50} (µM)	95% CI	R^2	IC_{99} (µM)	95% CI	RR IC_{50}	RR IC_{99}
EHA	9-Sep-22	0.14	0.136 to 0.150	0.97	0.82	0.670 to 1.017	-	-
EHA	5-Dec-22	0.15	0.141 to 0.161	0.94	0.76	0.583 to 1.007	1.5	1.2
EHA	24-Jan-23	0.11	0.107 to 0.116	0.98	0.41	0.347 to 0.483	-	-
EHA	3-Mar-23	0.29	0.276 to 0.301	0.96	1.67	1.370 to 2.055	1.1	1.3
EHA	26-Apr-23	0.17	0.163 to 0.173	0.99	0.70	0.616 to 0.801	1.5	1.2
EHA	27-Jun-23	0.26	0.251 to 0.270	0.97	0.96	0.815 to 1.132	1	4.9
EHA	18-Jul-23	0.26	0.250 to 0.268	0.97	0.83	0.712 to 0.966	2.3	19.1
EHA	26-Sep-23	0.28	0.267 to 0.288	0.99	1.57	1.356 to 1.829	3.8	21.9
EHA	19-Oct-23	0.39	0.383 to 0.405	0.97	1.49	1.299 to 1.742	2	47.8

LDA

Treatment group: LDA IC_{50} shifted 4.4-fold, from 0.04 µM (95% CI; 0.035 to 0.038 µM) in the first pre-treatment to 0.16 µM (95%CI; 0.139 to 0.193 µM) in the last pre-treatment assay (Table 5). Post-treatment LDA IC_{50} increased 4.3-fold, from 0.05 µM (95%CI; 0.045 to 0.052) to 0.22 µM (95%CI; 0.186 to 0.261 µM) in the last post-treatment. Many assays had poor larval development that prevented the measurement

of an accurate dose-response, so the dose-response both before and after treatment could only be measured on three occasions: at the beginning (September 2022), middle (March 2023), and end (October 2023) of the selection protocol. Dose response curves are presented for the first and last treatments, along with the corresponding assays for the EHA (Fig. 3). Other assays were performed to monitor the IC_{50} throughout the study (Table 5). Pre- and post-treatment assays were also conducted for the full therapeutic treatment to confirm the resistance status. The pre- vs post-treatment assays demonstrated a shift in the IC_{50} of 2.4-fold in this last treatment, increasing from 0.21 µM (95% CI; 0.194 to 0.229 µM) to 0.50 µM (95% CI; 0.482 to 0.527 µM, Fig. 4).

IC_{99} shifted 48-fold from 0.062 µM (95% CI; - to 0.120 µM) in the first pre-treatment, to 3.02 µM (95% CI; 1.565 to 6.729 µM) in the last pre-treatment (Table 5). Post-treatment IC_{99} increased 55.7-fold going from 0.09 µM (95% CI; 0.087 to 0.104) in the first post-treatment to 5.25 µM (95% CI; 2.530 to 11.820 µM) after the full therapeutic treatment.

Control group: Six assays were performed for the control group (Table 6). The IC_{50} shifted 1.8-fold, ranging from 0.04 µM (95% CI; 0.035 to 0.038 µM) to 0.07 µM (95% CI; 0.067 to 0.079 µM), while the last assay conducted displayed an IC_{50} of 0.05 µM (95% CI; - to 0.051 µM). The IC_{99} shifted 3.6-fold, ranging from 0.06 µM (95% CI; - to 0.120 µM) to 0.22 µM (95% CI; 0.154 to 0.313 µM, Table 6).

A resistance ratio (RR) was calculated by dividing the IC_{50} and IC_{99} values of the treatment group by those of the control group, respectively, when data were available for the same dates. The IC_{50} RR ranged from 0.9 to 4.3, with the latter value corresponding to the full dose treatment (Table 6). The IC_{99} RR ranged from 1.1 to 58.3,

indicating that a small proportion of the treatment population was highly resistant to fenbendazole.

Table 5. Larval development assay results for the treatment group. Dose-response was calculated using a nonlinear regression model in GraphPad Prism version 10.2.2 (GraphPad Software, La Jolla, CA). Half maximal inhibitory concentration= IC_{50} (µM), 99% inhibitory concentration=IC_{99} (µM), 95% Confidence intervals= 95%CI, Coefficient of determination= R^2.

Treatment	Type of Assay	Date	IC_{50}	95% CI	R^2	IC_{99}	95% CI
1	Pre-Tx	9-Sep-22	0.04	0.035 to 0.038	0.96	0.06	- to 0.1202
	Post Tx	16-Sep-22	0.05	0.050 to 0.052	0.99	0.09	0.086 to 0.104
2	Pre-Tx	11-Oct-22	0.04	0.041 to 0.045	0.91	0.09	0.073 to 0.111
3	Pre-Tx	4-Nov-22	0.05	0.045 to 0.048	0.98	0.11	0.090 to 0.131
4	-	24-Jan-23	0.05	0.048 to 0.052	0.92	0.17	0.135 to 0.233
5	Pre-Tx	3-Mar-23	0.06	0.056 to 0.062	0.91	0.17	0.137 to 0.232
	Post Tx	3-Apr-23	0.10	0.095 to 0.106	0.94	0.48	0.383 to 0.618
6	Post Tx	11-May-23	0.08	0.074 to 0.080	0.96	0.19	0.159 to 0.238
7	Pre-Tx	8-Jun-23	0.07	0.072 to 0.076	0.97	0.19	0.157 to 0.224
9	Pre-Tx	14-Sep-23	0.10	0.086 to 0.111	0.91	0.57	0.341 to 0.995
10	Pre-Tx	19-Oct-23	0.16	0.139 to 0.193	0.90	3.02	1.565 to 6.729
	Post Tx	27-Oct-23	0.22	0.186 to 0.261	0.97	5.25	2.530 to 11.820

Table 6. Larval development assay results for the control group. Dose-response was calculated using a nonlinear regression model in GraphPad Prism version 10.2.2 (GraphPad Software, La Jolla, CA). Half maximal inhibitory concentration= IC_{50} (µM), 99% inhibitory concentration=IC_{99} (µM), 95% Confidence intervals= 95%CI, Coefficient of determination= R^2. Resistance Ratio (RR) was calculated as IC_{50} of the treatment group divided by IC_{50} of the control group.

Type of Assay	Date	IC_{50}	95% CI	R^2	IC_{99}	95% CI	RR IC_{50}	RR IC_{99}
LDA	9-Sep-23	0.04	0.035 to 0.038	0.96	0.06	- to 0.120	-	-
LDA	3-Mar-23	0.07	0.064 to 0.066	0.97	0.08	- to 0.086	0.9	2.1
LDA	15-May-23	0.05	0.05 to 0.056	0.91	0.17	0.131 to 0.235	1.5	1.1
LDA	9-Jun-23	0.06	0.062 to 0.063	0.97	0.08	0.079 to 0.092	1.2	2.4
LDA	25-Jul-23	0.07	0.067 to 0.079	0.94	0.22	0.154 to 0.314	-	-
LDA	1-Nov-23	0.05	- to 0.0590	1.00	0.09	- to 0.098	4.4	58.3

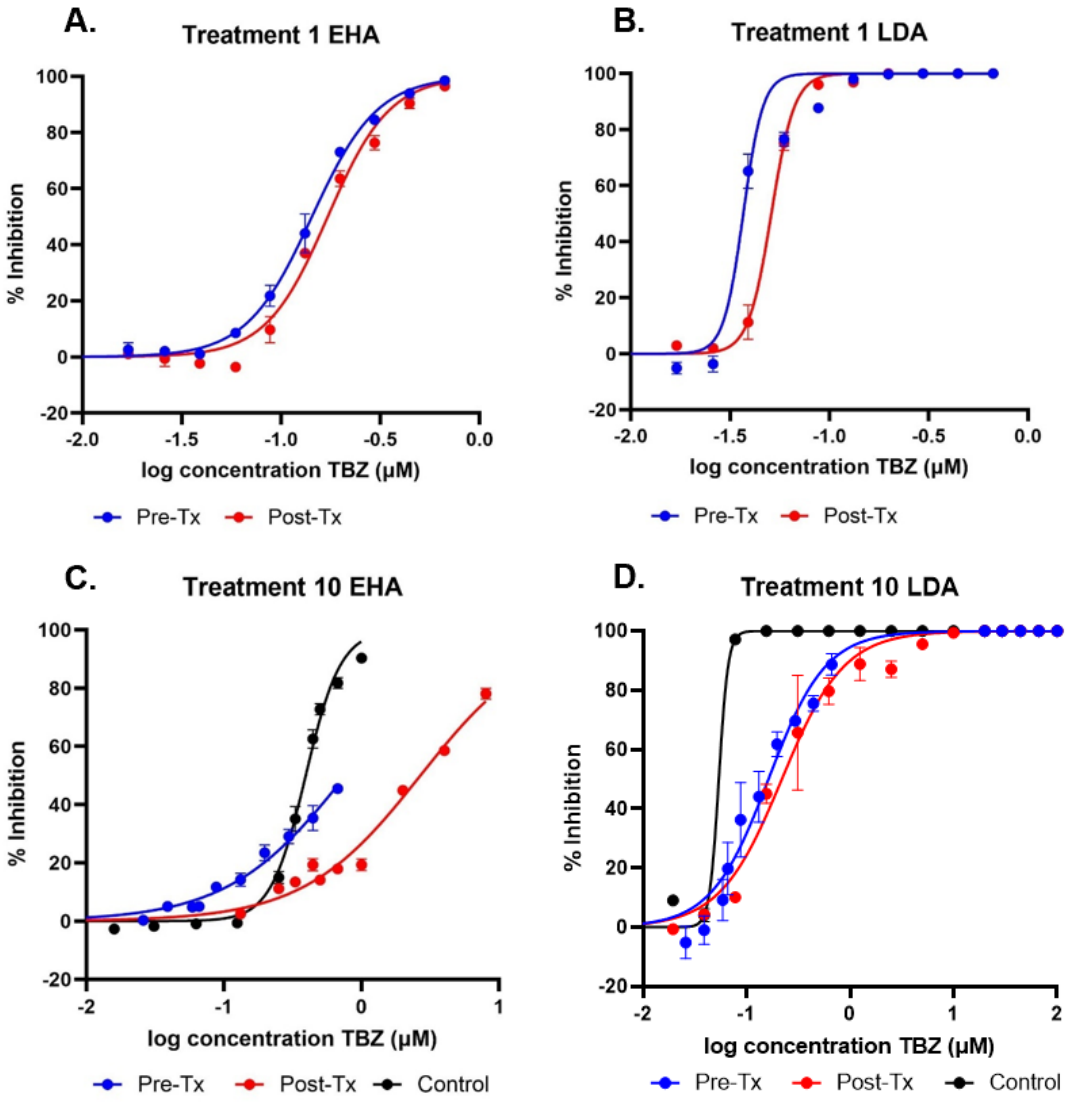

Figure 3. A. Dose-response curves for Egg Hatch Assay of *Haemonchus contortus* corresponding to pre- and post-treatment 1**. B.** Dose-response curves for Larval Development assay of *Haemonchus contortus* corresponding to pre- and post-treatment 1. **C.** Dose-response curves for Egg Hatch Assay of *Haemonchus contortus* corresponding to pre- and post-treatment 10**. D.** Dose-response curves for Larval Development Assay of *Haemonchus contortus* corresponding to pre- and post-treatment 10**.**

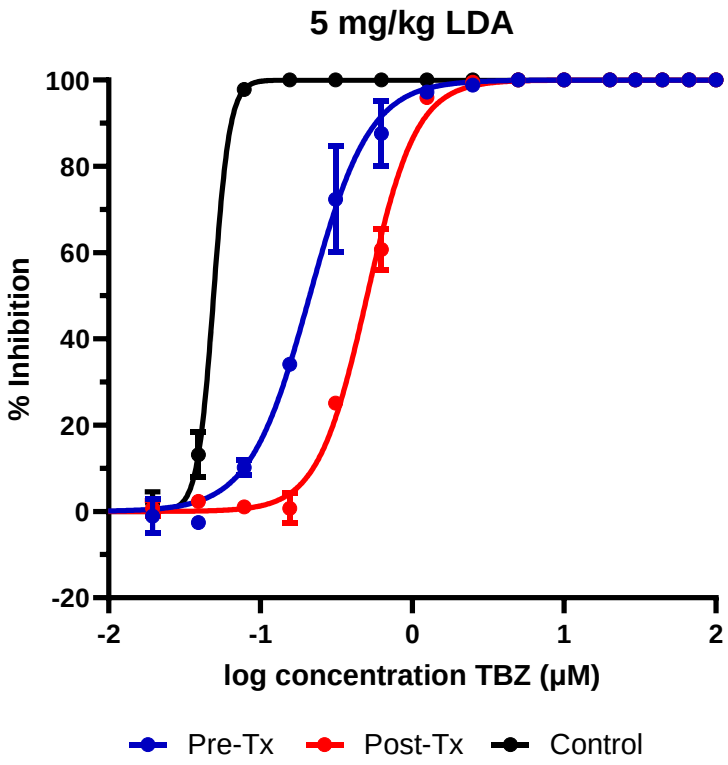

Figure 4. Dose-response curves for Larval Development assay of *Haemonchus contortus* corresponding to the full dose treatment (5 mg/kg) pre- and post-treatment, and control group dose response for a larval development performed at the same timepoint.

Allele frequency of beta-tubulin isotype-1 gene single nucleotide polymorphisms

The isolate used in this study exhibited a low frequency of the resistance-associated F200Y SNP (mean = 1.9%, Table 1), while codons 167 and 198 displayed

the wild-type BZ-susceptible sequence. The frequency of the F200Y SNP then gradually increased over the course of the study in response to the selection protocol we employed (figure 5, table 8). Towards the end of the selection process, the F167Y resistance-associated mutation was detected at low frequencies on three different sampling dates, but the frequency of this allele never exceeded 2% and did not increase over time. A small percentage of worms were detected with both the mutation at codons 167 and 200 (<0.2%) on those same 3 sampling dates. The frequencies of the F200Y SNP in the pre-treatment samples increased over the first eight months of the study, but remained relatively low, and varied up and down over time with a mean of 6.29%. However, once the population exceeded 20% F200Y SNP in June 2023, the frequency steadily increased over the next five months. Over this period, the resistant allele frequency in the pre-treatment samples increased markedly, from <10% to >90%. And following the full-dose treatment at the end of the study, the F200Y mutation frequency increased to 100%, reaching fixation in the *H. contortus* population (Table 8, Fig. 5).

In the control group, F200Y frequencies remained low throughout the study, ranging from 0.0 to 4.4% across time with no apparent trend over time (Table 7, Fig. 6). This was anticipated, as no anthelmintic treatment was administered to these animals, and consequently, resistance-associated mutations in beta-tubulin were not selected by treatment.

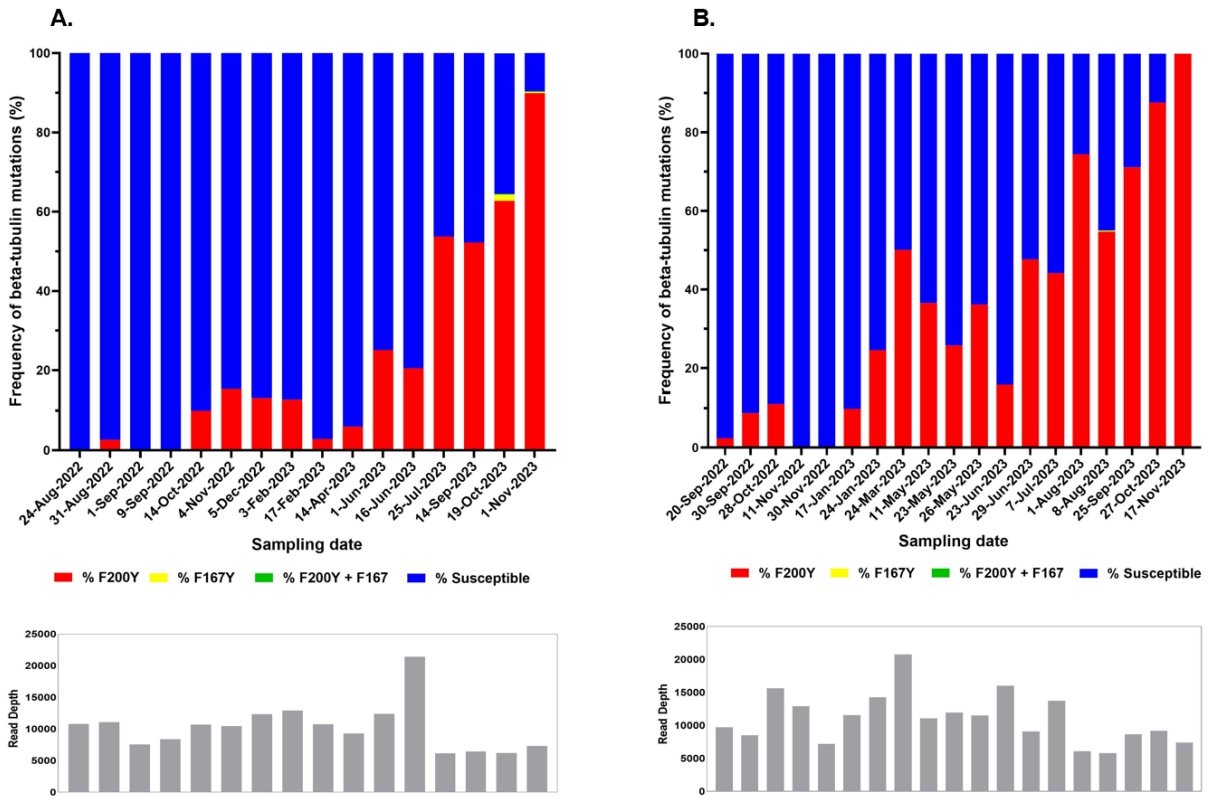

Figure 5. A. Relative allele frequency of single nucleotide polymorphisms associated with benzimidazole resistance in the beta-tubulin isotype-1 gene in eggs and larvae from *Haemonchus contortus* in the pre-treatment samples of the treatment group. **B.** Relative allele frequency of single nucleotide polymorphisms associated with benzimidazole resistance in the beta-tubulin isotype-1 gene in eggs and larvae from *Haemonchus contortus* in the post-treatment samples of the treatment group.
Note: the frequency of mutations at positions 167, 198, and 200 was calculated by dividing the number of reads carrying the resistant mutation by the total number of reads assigned to *H. contortus* in a given sample.

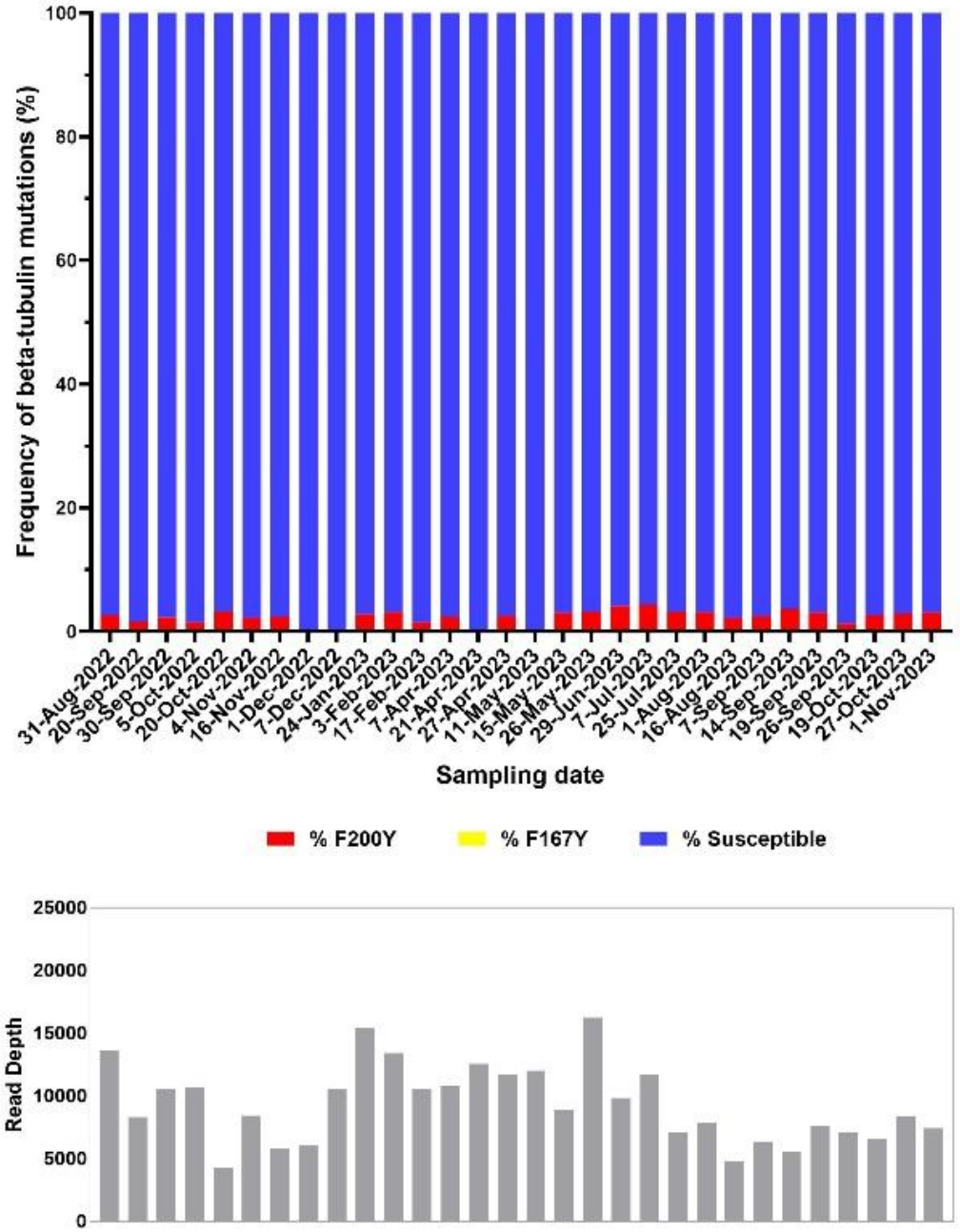

Figure 6. Relative allele frequency of single nucleotide polymorphisms associated with benzimidazole resistance in the beta-tubulin isotype-1 gene in eggs and larvae from *Haemonchus contortus* in the control group. Note: the frequency of mutations at positions 167, 198, and 200 was calculated by dividing the number of reads carrying the resistant mutation by the total number of reads assigned to *H. contortus* in a given sample.

Table 7. Relative frequency of beta-tubulin isotype-1 gene mutations in the control group population of *Haemonchus contortus*. SEM=standard error of the mean. Note: the

frequency of mutations at positions 167, 198, and 200 was calculated by dividing the number of reads carrying the resistant mutation by the total number of reads assigned to *H. contortus* in a given sample.

Dates	% F200Y± SEM	% Susceptible
31-Aug-2022	2.6 ± 0.11	97.4
20-Sep-2022	1.6 ± 0.21	98.4
30-Sep-2022	2.3 ± 0.40	97.7
5-Oct-2022	1.5 ± 0.29	98.5
20-Oct-2022	3.2 ± 0.51	96.8
4-Nov-2022	2.2 ± 0.06	97.8
16-Nov-2022	2.4 ± 0.65	97.6
1-Dec-2022	0.0 ± 0.00	100.0
7-Dec-2022	0.0 ± 0.00	100.0
24-Jan-2023	2.8 ± 0.06	97.2
3-Feb-2023	3.1 ± 0.17	96.9
17-Feb-2023	1.5 ± 0.44	98.5
7-Apr-2023	2.4 ± 0.27	97.6
21-Apr-2023	0.0 ± 0.00	100.0
27-Apr-2023	2.5 ± 0.31	97.5
11-May-2023	0.0 ± 0.00	100.0
15-May-2023	3.0 ± 0.14	97.0
26-May-2023	3.2 ± 0.09	96.8
29-Jun-2023	4.1 ± 0.34	95.9
7-Jul-2023	4.4 ± 0.25	95.6
25-Jul-2023	3.2 ± 0.01	96.8
1-Aug-2023	3.1 ± 0.03	96.9
16-Aug-2023	2.2 ± 0.33	97.8
7-Sep-2023	2.5 ± 0.06	97.5
14-Sep-2023	3.7 ± 0.26	96.3
19-Sep-2023	3.1 ± 0.33	96.9
26-Sep-2023	1.3 ± 1.25	98.8
19-Oct-2023	2.7 ± 2.67	97.3
27-Oct-2023	2.9 ± 1.07	97.1
1-Nov-2023	3.0 ± 1.52	97.0

Table 8. Relative frequency of beta-tubulin isotype-1 gene mutations in the treatment group population of *Haemonchus contortus.* SEM=standard error of the mean. Note: the frequency of mutations at positions 167, 198, and 200 was calculated by dividing the

number of reads carrying the resistant mutation by the total number of reads assigned to *H. contortus* in a given sample.

Pre-treatment samples				
Dates	**% F200Y ± SEM**	**% F167Y ± SEM**	**% F200Y + F167 ± SEM**	**% Susceptible**
24-Aug-2022	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	100.0
31-Aug-2022	2.7 ± 0.70	0.0 ± 0.00	0.0 ± 0.00	97.4
1-Sep-2022	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	100.0
9-Sep-2022	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	100.0
14-Oct-2022	10.0 ± 0.23	0.0 ± 0.00	0.0 ± 0.00	90.0
4-Nov-2022	15.3 ± 11.40	0.0 ± 0.00	0.0 ± 0.00	84.7
5-Dec-2022	13.2 ± 0.17	0.0 ± 0.00	0.0 ± 0.00	86.8
3-Feb-2023	12.8 ± 0.24	0.0 ± 0.00	0.0 ± 0.00	87.2
17-Feb-2023	2.9 ± 1.20	0.0 ± 0.00	0.0 ± 0.00	97.1
14-Apr-2023	6.0 ± 0.17	0.0 ± 0.00	0.0 ± 0.00	94.0
1-Jun-2023	25.1 ± 0.38	0.0 ± 0.00	0.0 ± 0.00	75.0
16-Jun-2023	20.5 ± 1.91	0.0 ± 0.00	0.0 ± 0.00	79.5
25-Jul-2023	53.7 ± 0.92	0.0 ± 0.00	0.0 ± 0.00	46.3
14-Sep-2023	52.3 ± 0.27	0.0 ± 0.00	0.0 ± 0.00	47.7
19-Oct-2023	62.8 ± 5.07	1.6 ± 1.28	0.2 ± 0.13	35.5
1-Nov-2023	89.9 ± 0.37	0.3 ± 0.02	0.2 ± 0.04	9.6
Post-treatment samples				
Dates	**% F200Y ± SEM**	**% F167Y ± SEM**	**% F200Y + F167 ± SEM**	**% Susceptible**
20-Sep-2022	2.4 ± 0.08	0.0 ± 0.00	0.0 ± 0.00	97.6
30-Sep-2022	8.8 ± 0.79	0.0 ± 0.00	0.0 ± 0.00	91.2
28-Oct-2022	11.1 ± 0.20	0.0 ± 0.00	0.0 ± 0.00	88.9
11-Nov-2022	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	100.0
30-Nov-2022	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	100.0
17-Jan-2023	9.9 ± 0.30	0.0 ± 0.00	0.0 ± 0.00	90.1
24-Jan-2023	24.6 ± 1.16	0.0 ± 0.00	0.0 ± 0.00	75.4
24-Mar-2023	50.1 ± 0.47	0.0 ± 0.00	0.0 ± 0.00	49.9
11-May-2023	36.6 ± 0.47	0.0 ± 0.00	0.0 ± 0.00	63.4
23-May-2023	25.8 ± 0.63	0.0 ± 0.00	0.0 ± 0.00	74.3
26-May-2023	36.2 ± 0.31	0.0 ± 0.00	0.0 ± 0.00	63.8
23-Jun-2023	15.8 ± 9.36	0.0 ± 0.00	0.0 ± 0.00	84.2
29-Jun-2023	47.7 ± 0.17	0.0 ± 0.00	0.0 ± 0.00	52.3
7-Jul-2023	44.2 ± 0.40	0.0 ± 0.00	0.0 ± 0.00	55.8

1-Aug-2023	74.4 ± 7.26	0.0 ± 0.00	0.0 ± 0.00	25.6
8-Aug-2023	54.7 ± 1.14	0.26 ± 0.18	0.07 ± 0.05	45.0
25-Sep-2023	71.1 ± 1.80	0.0 ± 0.00	0.0 ± 0.00	28.9
27-Oct-2023	87.5 ± 6.50	0.0 ± 0.00	0.0 ± 0.00	12.5
17-Nov-2023	100.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0

Frequency of beta-tubulin isotype-1 haplotypes

Eight haplotypes of the beta-tubulin isotype-1 gene were identified (Fig. 7) in the *H. contortus* isolate used for initial infections. One of the haplotypes contained the TAC resistant genotype at position 200 (Fig. 7), but the susceptible TTC genotype at positions 198 and 167. The other seven haplotypes were susceptible and contained the TTC genotype at position 200, 167 and 198. Four samples thought to belong to deer, were collected from the area outside of the fenced pasture between June and July 2022, prior to placing the goats out in the pasture. Interestingly, these samples showed haplotypes very similar to those of our *H. contortus* isolate (Fig. 7). However, 14 Amplicon Sequence Variants (ASV) were detected in these samples, with 2 different resistant haplotypes for position 200. Some of these haplotypes were unique to the “deer” and not present in the isolate we used for the initial infections. More specifically, ASV-5, which had the TAC-resistant genotype at position 200, and ASVs 10, 285, 489, 525, and 549, which had the susceptible genotype at position 167, 198 and 200, were only present in the deer samples. On the other hand, ASV-12, which was susceptible at codons 167, 198 and 200 was only present in the laboratory isolate.

The samples sequenced from the goats that first became patent show a mixture of haplotypes from both the infection isolate and the deer (Fig. 7). A total of twelve

haplotypes were identified in these samples. Among these, ASV-5, which was found exclusively in the deer, ASV-12 which was unique to our laboratory isolate, and ASV-19 which was not present in either the deer or our laboratory isolate, suggesting it originated from other *Haemonchus* larvae in the pasture. These findings indicate that deer had crossed into the goat pasture, causing low-level larval contamination before the study began. Consequently, the goats ingested some *Haemonchus* larvae of deer origin that were already present on the pasture.

Despite this minor contamination, several factors limited its impact on our selection protocol. First, the goats received a large inoculum of 2500 L3 PO, so that small numbers of deer-origin larvae ingested initially by the goats would have been heavily diluted by our *H. contortus* isolate. Subsequently, the eggs produced and shed from this mixed infection would be predominantly of the goat isolate, leading to a further dilution of the deer-origin worms on the pasture. Ultimately, small numbers of fecal pellets from deer with low FEC would be rapidly overwhelmed in numbers by the constant grazing and fecal egg deposition from the goats. Secondly, both the treatment and control populations of *H. contortus* consisted predominantly of the haplotypes that were shared by deer and our laboratory isolate. Among the haplotypes unique to deer, only ASV-5 persisted throughout the entire study period.

Six months post-infection, new susceptible haplotypes sporadically emerged in the control group but at very low levels (<0.4%). The treatment population showed less diversity for the susceptible haplotypes, maintaining almost exclusively those present in the isolate used for infections. However, new resistant sequences emerged at loci 200 and 167, with four different haplotypes observed at position 200 and two at position 167

(Fig. 8). Among the haplotypes unique to deer, only ASV-5 persisted in the treatment population, which also increased in response to fenbendazole treatment. Although other resistant haplotypes emerged during the study, the predominant resistant haplotype, reaching 76.8% after the full-dose treatment, was the one present in the isolate used to infect our goats (Fig. 8).

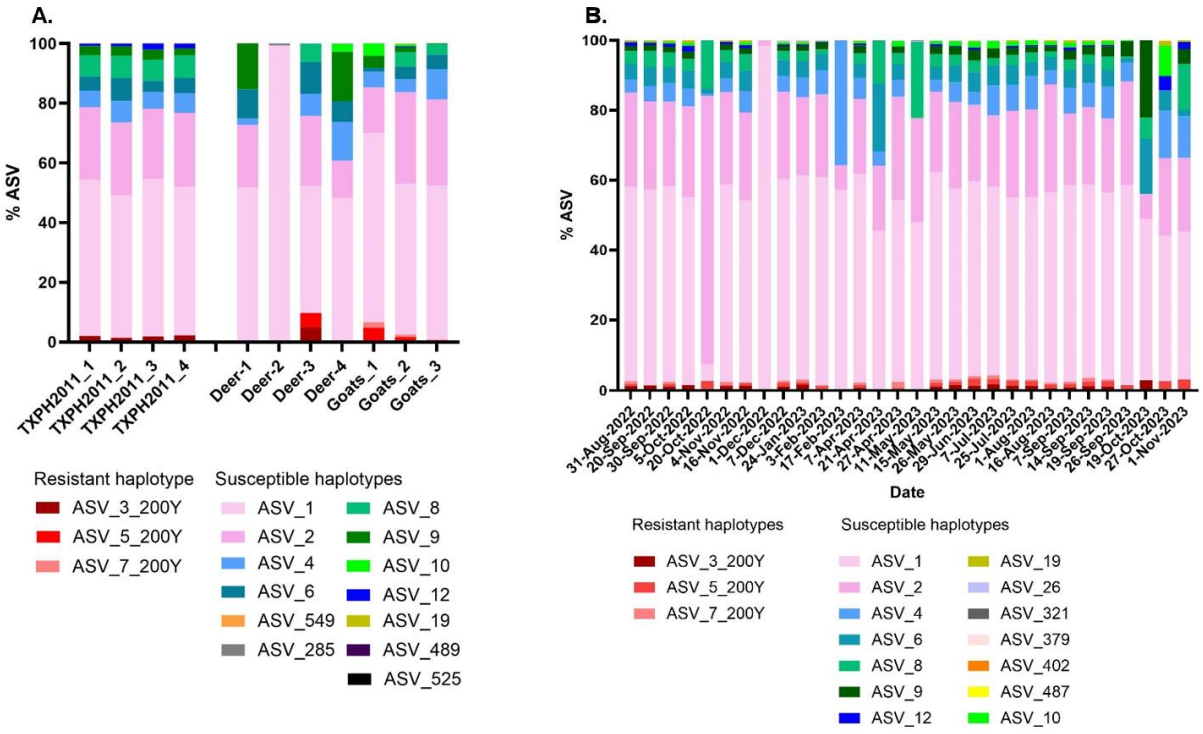

Figure 7. Relative frequency of beta-tubulin isotype-1 haplotypes in **A.** the original *Haemonchus contortus isolate* (TxPh2011-S; four replicates) used for infections, deer samples collected from the pasture (Deer -1, -2, -3, -4), and samples from three goats that first became patent (Goats_1, Goats_2, and Goats_3). **B**. Relative frequency of beta-tubulin isotype-1 haplotypes in *H. contortus* samples from the control group.

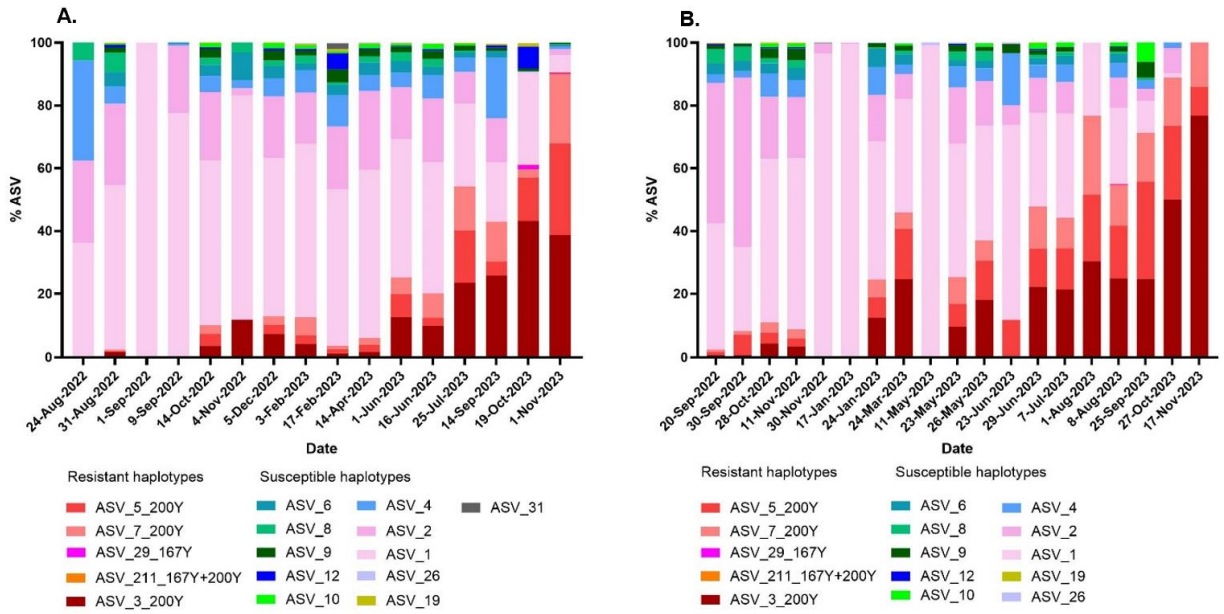

Figure 8. A. Relative frequency of beta-tubulin isotype-1 haplotypes in *Haemonchus contortus* samples in the pre-treatment samples from the treatment group. **B.** Relative frequency of beta-tubulin isotype-1 haplotypes in *H. contortus* samples in the post-treatment samples from the treatment group.

Relationship between beta-tubulin mutations and EHA/LDA IC_{50}

The relationship between EHA / LDA IC_{50} and the frequency of F200Y was analyzed using a quadratic model (Fig. 9). For the EHA we analyzed 27 points, each representing the mean IC_{50} value from two *in vitro* assays compared to the mean frequency of F200Y mutation from three replicates. For the LDA, we analyzed 18 data points. The regression equation for EHA IC_{50} and F200Y was $\hat{Y}=0.2462-0.006295x+0.0002819x^2$, and for LDA a IC_{50} and F200Y was $\hat{Y}=0.05161+0.0001552x+2.177\times10^{-5}x^2$. The quadratic model explained 67.77% of the variability in the frequency of the F200Y mutation for the EHA IC_{50} values (R^2 = 0.6777, standard error = 0.3095, n = 27). In comparison, the model for LDA IC_{50} values

explained 88.89% of the variability (R^2= 0.8889, standard error = 0.01665, n = 18). These results indicate that the LDA assay provides a more accurate estimate of the F200Y mutation frequency than the EHA assay.

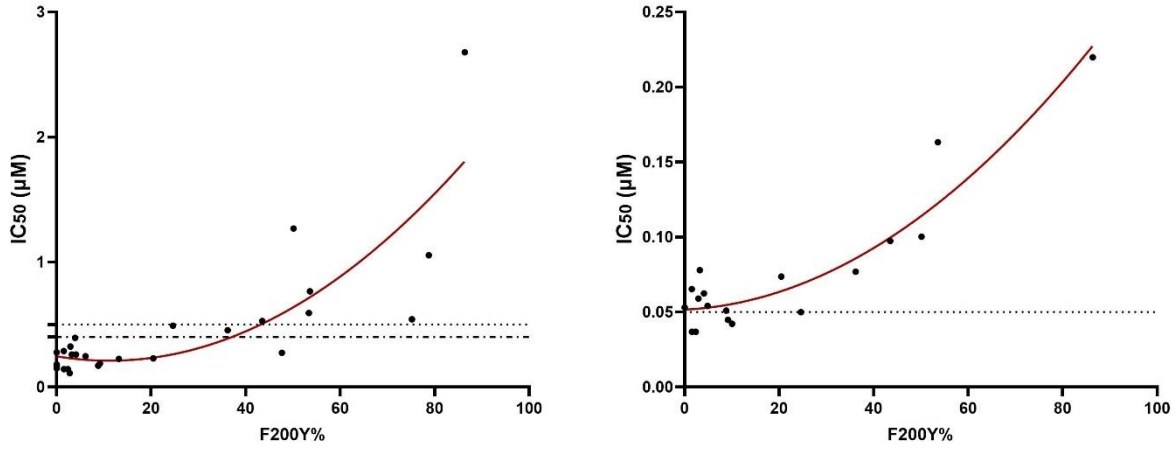

Figure 9. A. Comparison of TAC allele frequency at codon 200 of beta-tubulin isotype 1 with Egg Hatch Assay IC_{50} values in *Haemonchus contortus* isolates, from control and treatment groups (n=27). **B.** Comparison of TAC allele frequency at codon 200 of beta-tubulin isotype 1 with Larval Development Assay IC_{50} values in *Haemonchus contortus* isolates, from control and treatment groups (n=18). A polynomial quadratic model was the method applied in GraphPad Prism version 10.2.2 (GraphPad Software, La Jolla, CA). Dotted line in EHA denotes a 0.5 µM IC_{50}, which corresponds to the discriminating concentration for this assay, along with a dotted-dashed line at 0.4 µM which corresponds to the proposed discriminating concentration for our isolate. Dotted line in LDA denotes a concentration of 0.05 µM IC_{50}, corresponds to the discriminating concentration for this assay.

Simulation of the pasture contamination with free-living stages and population dynamics of H. contortus

Based on the model output, parasite transmission was maintained successfully throughout the study duration (Fig 10. A). This was aligned with our clinical observations during the study, which were based on fecal egg counts in the animals (data not shown). The model also showed that a substantial population of larval stages had been

established on the pasture by the time treatment began. Thus, the period that was provided to permit the pasture to become contaminated with larvae prior to administering the first treatment was adequate given the goals of the study (Fig. 10A). Weather and climatic conditions greatly influenced transmission dynamics during the winter (Fig. 10), however since we had anticipated this, we did not conduct any treatments during the cold-weather months. Through selective treatment of a subset of animals before and after winter, we effectively preserved a substantial proportion of worms in refugia during this critical time of reduced transmission of the cold-intolerant *H. contortus* (Fig. 10A). On a per square meter (m^2) basis, the control group exhibited similar levels of pasture contamination (Fig. 10B). This was achieved by adjusting the stocking rate to ensure comparable contamination levels. Additionally, both groups showed similar transmission patterns when accounting for surface area. The similarities in parasite transmission patterns between the control and treatment groups suggest that environmental factors had a greater influence on parasite transmission than anthelmintic treatments (Fig. 10B). The suboptimal efficacy of treatments applied on the treatment group may have not been enough to influence the pattern of parasite transmission, however, enough to select for anthelmintic resistance.

As anticipated, animals exhibited a disproportionate contribution to pasture contamination, with 20-30% of individuals responsible for over 50% of all pasture contamination (Fig. 10-11; Supplementary Fig. 2) (Barger 1985, Wilson and Grenfell 1997). This was accentuated after four months of infection, likely attributed to the development of immunity in certain animals (Supplementary Fig. 2A, C). Thus, a few animals in the herd contributed to the majority of pasture contamination, in both groups.

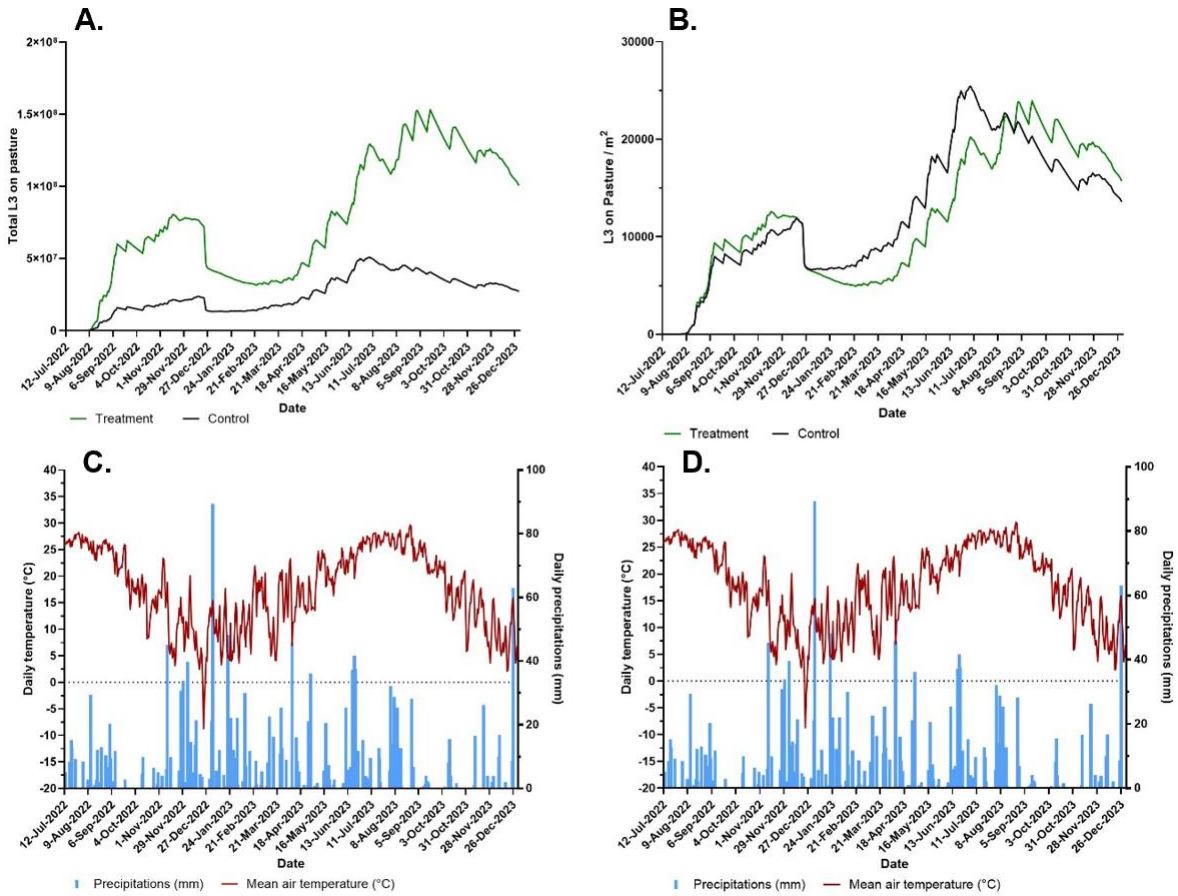

Figure 10. A. Simulations of the overall contamination of pasture with *Haemonchus contortus* L3 in the control (black line) and treatment group (green line). **B.** Simulations of the overall contamination of the pasture by m^2 with *Haemonchus contortus* L3 in the control (black line) and treatment group (green line). **C, D.** Daily precipitations (mm) (blue bars) and average daily temperature (red line) for the period of study in Athens, Georgia, US. Simulations were done using GLOWORM-FL model. Precipitations and temperature were obtained from http://georgiaweather.net/

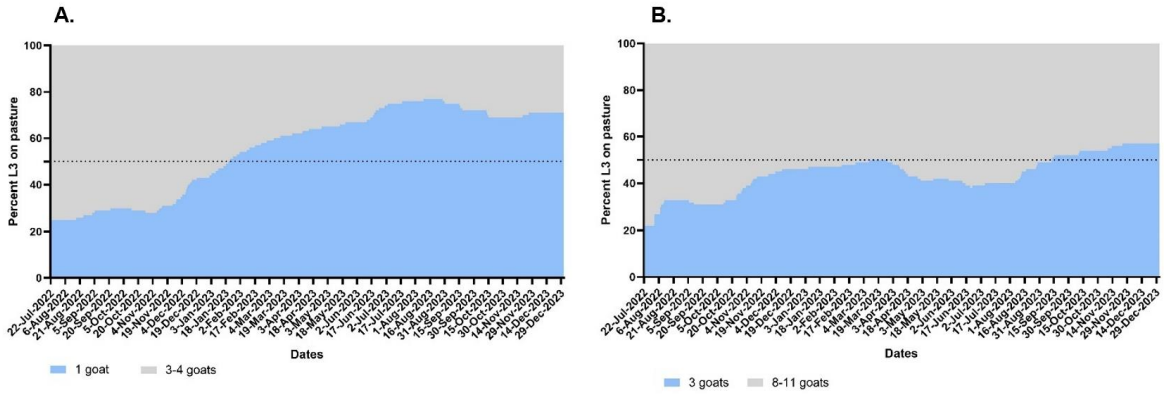

Figure 11. **A**. Percent collaboration of L3 in the pasture by 25-33% of the animals in the control group. The dotted line represents 50% of L3s in pasture. **B.** Percent collaboration of L3 in the pasture by 21-27% of the animals in the treatment group. The dotted line represents 50% of L3s in pasture.
The production of eggs by animals, and the development to L3 was simulated with GLOWORM-FL model.

Discussion and Conclusions

The findings of this study provide a comprehensive analysis of the development of BZ resistance in a population of *H. contortus*. Given that the beta-tubulin isotype-1 locus is the primary determinant of benzimidazole resistance in strongylid nematodes (Kwa, Veenstra et al. 1994), we focused on monitoring the frequency of beta-tubulin isotype-1 mutations throughout our study. Additionally, we conducted *in vitro* tests to observe the effects of these mutations on the drug susceptibility phenotype. We employed a drug selection protocol that gradually selected for a pre-existing beta-tubulin isotype-1 resistance F200Y (TTC>TAC) allele in our laboratory isolate, which is the most common BZ resistance-associated SNP for trichostrongylid species (von Samson-Himmelstjerna, Walsh et al. 2009, Redman, Whitelaw et al. 2015, Avramenko, Redman et al. 2019). The F200Y (TTC>TAC) allele frequency increased slowly but not progressively over the first 8-9 months, and then rapidly escalated over the final 5-6 months eventually reaching fixation. We also detected F167 (TTC>TAC), on three occasions after a year of field selection. However, the low frequencies of this polymorphism were not present on the three replicates conducted for the samples obtained in October 2023, and only appeared consistently replicated in the other two sampling opportunities (Aug and Nov 2023). Consequently, these results should be interpreted with caution, as the data presented were not filtered for read depth or

replication, making it challenging to reliably interpret polymorphisms with frequencies lower than 0.5% (Avramenko, Redman et al. 2019) and correlate their presence with the drug susceptibility phenotype. Further analysis that takes into account the read depth, the variation within the replicates and stricter filtering may modify these results.

Haplotype analysis of the beta-tubulin isotype-1 gene suggested that the goats likely ingested some *Haemonchus* larvae of deer origin. Our paddocks were built using normal livestock fencing that are not high enough to prevent deer from entering. Thus, it seems likely that deer jumped the fence and contaminated the goat pasture with feces prior to the start of our study. We identified eight beta-tubulin haplotypes in our laboratory isolate that was used for infecting the goats. However, *H. contortus* collected from the first goats that became patent following the experimental inoculation showed haplotype diversity that included 12 haplotypes. Some of these were unique to our laboratory isolate (e.g., ASV-12), one was only present in the deer samples (ASV-5), and a few others (e.g., ASV-7, ASV-19) were absent in samples from both origins. This suggests that the goats also ingested other *Haemonchus* larvae present in the pasture that we did not sample, and/or some of these ASVs are invalid and more stringent filtering may show this. Despite this, the predominant haplotypes throughout the study were those from our susceptible isolate. Additionally, new susceptible haplotypes sporadically emerged in the control group at very low levels (<0.4%), which were not filtered for read depth or replication. Consequently, these results may also change once a more rigorous filtering and analysis is conducted.

Previous studies in *H. contortus* populations from sheep and goats from Southeastern US, demonstrated a haplotype diversity for the beta-tubulin isotype 1

gene, ranging from two to eight haplotypes (Chaudhry, Redman et al. 2020, George, Vatta et al. 2021) However, our starting isolate was initially already diverse (n=8) and showed an increased haplotype diversity when cycled in the goat herds. The subtropical climatic conditions in Georgia, US allow the winter development and survival of larval stages on the pasture (Terrill, Miller et al. 2012). Whereas the spring and summer conditions with high rainfall and humidity, and warm temperatures promote high levels of transmission of *H. contortus*. This allows *H. contortus* to maintain large population sizes, that along with the high mutation rates displayed by trichostrongylid nematodes provide the opportunity for mutations to frequently arise in the worm population (Blouin 1998, Gilleard and Beech 2007). This could explain why the control group population exhibited up to 17 different haplotypes for the beta-tubulin isotype-1 gene throughout the study. High haplotype diversity for beta-tubulin isotype-1 has been previously reported in trichostrongylid nematodes in the United Kingdom, where 28 different resistant haplotypes were identified for *Teladorsagia circumcincta* across only seven farms (Redman, Whitelaw et al. 2015).

The treatment group also displayed a high haplotype diversity along the study, with 16 different haplotypes displayed in total. Eleven of which were susceptible at positions 167, 198 and 200. Notably, up to nine different susceptible haplotypes were maintained until the last pre-treatment. However, after the full dose-treatment, only three haplotypes that carried the resistant genotype in position 200 persisted. This rapid adaptation in response to selection are termed “selective sweeps” at the loci under selection, and can be either “soft” or “hard” in nature (Hermisson and Pennings 2005, Karasov, Messer et al. 2010, Redman, Whitelaw et al. 2015). The difference lies in that,

in hard sweeps, a single resistance haplotype resulting from a single mutation arises, sweeps through the population, and eventually reaches fixation. But in soft sweeps, multiple resistance haplotypes in the population can arise, either from recurrent *de novo* mutations appearing on different susceptible haplotype backgrounds after the onset of the selection, or from polymorphisms already present in the standing genetic variation before the onset of drug selection (Redman, Whitelaw et al. 2015). In our study, even though there was a predominant resistant haplotype at position 200, three different resistant haplotypes were selected by the final full dose, and up to five were observed throughout the study. The predominant resistant mutation was already present in the population used for infections, and the other two surviving treatment were likely brought through migration, due to the deer coming into the pastures. Migratory ungulates can influence parasite transmission patterns and serve as a source for the dispersion of parasitic nematodes (Khanyari, Milner-Gulland et al. 2022). Their seasonal movements can lead to infections from livestock farms, thereby facilitating the spread of parasites between farms. This host movement can also result in high gene flow among populations of parasitic nematodes (Blouin, Yowell et al. 1995), which has been observed in *H. contortus* populations from the Southeastern US (Chaudhry, Redman et al. 2020).

Our laboratory isolate (TxPh2011-S) was obtained from pronghorn antelopes in Texas. Wild populations of pronghorn antelopes migrate 150 miles each year, one of the last long-distance animal migrations in the world (Sawyer, Lindzey et al. 2005). The wide range of land, covered each year by these animals, provides the opportunity to act as vectors and geographical dispersion of parasites (Morgan, Lundervold et al. 2006,

Morgan, Medley et al. 2007). Therefore, it could be possible that many of the haplotypes present in our laboratory isolate, are maintained and cycled in wild ruminants. Thus, the similar haplotypes found between the deer samples and our laboratory isolate.

Simulations of *H. contortus* dynamics on pasture using the GLOWORM-FL model provided valuable insights. This model has been validated for *H. contortus*, *Teladorsagia circumcincta*, *Ostertagia ostertagi* and *Cooperia oncophora* (Rose, Wang et al. 2015, Wang, Vineer et al. 2022). GLOWORM-FL has been employed to predict the seasonal dynamics of the free-living stages of trichostrongylid nematodes on pasture (Rose, Wang et al. 2015, Filipe, Kyriazakis et al. 2022, Wang, Vineer et al. 2022). The simulations indicated that year-round cycling of *H. contortus* occurred on the pasture. The decision to avoid treating the entire herd at certain timepoints was done to maintain adequate levels of refugia and prevent a bottleneck event in the population of *H. contortus*. Preventing bottleneck effects is crucial to avoid genome-wide changes in genetic markers (Gilleard and Beech 2007). Although we did not test independent loci to control for this effect (Gilleard and Beech 2007), sequencing of neutral markers is however, planned for a later stage. Though, the gradual increase in the F200Y mutation frequency also suggests the absence of bottleneck effects, which could have rapidly fixed this allele in the population of *H. contortus*.

The simulation also showed that a few goats were primarily responsible for the transmission of *H. contortus* in the herd; 3 of the 14 goats contributed over 50% of pasture L3 contamination. The aggregation of worms in a subset of the herd may also play a key role in the development of AR. However, we did not conduct sequencing of beta-tubulin in samples from individual animals to determine if the frequency of F200Y

was higher in the few animals contributing most to pasture contamination. But these animals received treatment on the selective treatment dates (Table 2). Therefore, it is highly likely that higher selection pressure was exerted on the worms harbored by these subset of goats.

It is noteworthy that the simulation suggested that our treatments with ~50% efficacy had little impact on parasite transmission. However, treatments conducted with subtherapeutic doses applied drug selection pressure leading to the development of full clinical resistance. The use of drugs with suboptimal efficacy is common in production systems, and contributes to the widespread occurrence of AR, worldwide (Prichard, Hall et al. 1980, Martin 1987, Prichard 1990). These results also highlight the importance of administering highly efficacious treatments at the appropriate dosage to prevent suboptimal drug concentrations reaching the worms.

The development of full clinical resistance was confirmed by a full label dose treatment that yielded a FEC reduction of 59% (90% CI; 24.8 – 83.7%). It is not surprising that the strategy used here promoted the relatively quick development of anthelmintic resistance, as subtherapeutic treatments allowed worms that could tolerate increasing concentrations of fenbendazole to survive over time. Intervals of four to eight weeks between treatments allowed susceptible genotypes in refugia to dilute the surviving genotypes. However, once the F200Y mutation frequency exceeded 20% in the pre-treatment samples, the frequency of this mutation escalated rapidly in the treatment population (Fig. 5). At this point, the EHA IC_{50} from pre-treatment samples reached the discriminating concentration for resistance (0.5 μM) (Coles, Bauer et al. 1992).

In this study, frequencies of F200Y over 20% translated in general, into EHA IC_{50}s greater than 0.4 µM. A previous study suggested that an EHA IC_{50} discriminating concentration of 0.1µg/mL (0.5 µM) correlated with F200Y levels of 30% or less (von Samson-Himmelstjerna, Walsh et al. 2009). And that lowering the cut-off of the EHA to 0.05 µg/mL (0.25 µM), could increase the sensitivity to detect isolates of *H. contortus* that have ≥ 10% of F200Y (von Samson-Himmelstjerna, Walsh et al. 2009). However, the control group, which was not under drug selection, and maintained a F200Y below 5%, displayed EHA IC_{50} that were generally above 0.25 µM. Therefore, taken together, these results suggest that an EHA IC_{50} of 0.4 µM would be a more accurate threshold phenotypic response of this isolate (Fig 9). Using this threshold, 9/10 samples that showed frequencies of F200Y >20% would be classified as resistant with only one sample being misclassified. But it is important to note that the relationship between EHA/LDA IC_{50} and F200Y% in our study does not follow a linear model but rather a parabolic pattern. For instance, within the range of 0-20% F200Y, we observe minimal changes in EHA IC_{50} (Fig. 9). Hence, the influence of F200Y% on the drug response seems to be non-linear and depends both on the mutation frequency and the quadratic term. This suggests that there is a complex interplay between F200Y and IC_{50} on a biological level.

Furthermore, the EHA was more sensitive in detecting changes in IC_{50} than LDA, shifting 5-fold and 15-fold when comparing pre-treatment and post-treatment samples of the first and last treatments, respectively. In comparison, the LDA IC_{50} shifted 4-fold for both pre- and post-treatment assays. The IC_{99} showed much larger shifts in

susceptibility for both assays; the EHA increased 87- and 256-fold, and the LDA shifted 48- and 56-fold for pre- and post-treatment assays, respectively.

Previous studies have indicated higher sensitivity in the LDA assay for detecting resistance (Várady, Cudeková et al. 2007). However, if we apply the discriminating concentration for LDA (0.05 μM), seven samples with F200Y <10% would be classified as resistant. The highest IC_{50} among these samples was 0.08 μM, with a corresponding F200Y% of 3.2. If we move the threshold to 0.8 μM, three samples with F200Y >20% fall between 0.05 and 0.08 μM for their IC_{50}. Therefore, there was no clear IC_{50} threshold to be used for the LDA in our study, that would allow us to distinguish between samples with low and high frequencies of F200Y. Despite these caveats, our data and previous studies show that both EHA and LDA are suitable for the clinical detection of BZ resistance in *H. contortus* (Várady, Cudeková et al. 2007, von Samson-Himmelstjerna, Coles et al. 2009, von Samson-Himmelstjerna, Walsh et al. 2009, Čudeková, Várady et al. 2010).

The remaining 11-33% of unexplained variability in EHA/LDA IC_{50} could be due to factors not accounted for in this model, such as other genetic changes or unknown variables. Variations in IC_{50} occur naturally over the course of an infection due to the age of the worms and dynamics of the infection, irrespective of drug selection (Borgsteede and Couwenberg 1987, Kerbceuf and Hubert 1987). By maintaining a group of animals as controls we could control for natural changes in the IC_{50} of our susceptible isolate, that were unrelated to drug-selection; IC_{50}s ranged from 0.11 to 0.39 μM and 0.04 to 0.07 μM for EHA / LDA, respectively. We also observed variation in the frequency of the F200Y allele in the control group, with values that ranged from 0 to

4.4%, but without a distinct trend. Overall, across the study, the mean F200Y allele frequency in the control group was 2.35% (SEM=0.21), which is quite similar to that measured in the starting population (1.9%).

Overall, this study reinforces that F200Y is a major determinant of BZ resistance in *H. contortus*, and that both EHA and LDA are reliable predictors of its presence. However, it is notable that 100% of F200Y was reached at relatively low levels of phenotypic resistance, as measured by egg hatch (2.7 μM) and larval development (0.5 μM). Historically, our laboratory has surveyed numerous (n>500) *H. contortus* populations with LDA IC_{50} values as high as 50 μM (data not shown). Achieving 100-fold increase in drug tolerance likely involves additional resistance mechanisms, beyond F200Y. We have not yet conducted sequencing of beta-tubulin isotype-2 or whole genome sequencing to identify other loci under selection, however, these analyses are planned for the future. The protocol used here that gradually selected for resistance with low doses in a field situation with natural reinfection, tends to favor genes that can contribute to resistance even if the effects are minimal (Gilleard and Beech 2007). Therefore, by performing whole genome sequencing at different timepoints in this study, we may detect additional loci under selection that play a role in resistance to BZ, even if they are not the major loci under drug selection.

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CHAPTER 5

CONCLUSIONS

Anthelmintic drugs constitute the primary means for treating and controlling infections with parasitic nematodes both in human and animals. However, the frequent and /or strategic use of anthelmintic drugs has led to ever-increasing levels of anthelmintic resistance in all drug classes, involving virtually all of the most economically important parasites of domestic animals. In humans, the escalation of mass drug administration campaigns in Africa, Asia, and South America is anticipated to intensify drug selection pressure, thereby promoting the emergence of drug resistance. As a result, anthelmintic resistance is becoming a significant concern in human medicine, especially with benzimidazole drugs, which are essential components of current therapeutic strategies.

Consequently, it is important to deepen our understanding of the mechanisms driving anthelmintic resistance in parasitic nematodes, as well as to develop new drugs with novel modes of action. This dissertation addresses both objectives. We investigated the phenotypic, genotypic, and genomic responses of a model parasitic nematode to field selection with a benzimidazole drug and validated a novel drug target using the same parasitic nematode. The outcomes of these specific aims are discussed in the following paragraphs.

***Specific aim 1*:** The data presented in Chapters 2 and 3 provide proof of concept and further validation of phosphoethanolamine methyltransferases (PMTs) as novel drug targets in parasitic nematodes. We assessed the *in vitro* activity of a panel of 15 candidate PMT inhibitor compounds using two phenotypic assays: the larval development assay and the larval motility assay. Compounds demonstrating reasonably potent anthelmintic efficacy *in vitro* were then tested *in vivo* using the jird (*Meriones unguiculatus*) – *Haemonchus contortus* model. Four PMT inhibitors showed *in vitro* activity in the lower micromolar range against both drug-susceptible and multidrug-resistant isolates of *H. contortus*. When tested *in vivo*, three of these compounds achieved relatively high reductions in worm counts, with efficacy ranging from 53.5% to 72.6%. These findings further support the potential of PMT enzymes as viable drug targets in parasitic nematodes and indicate that PMT inhibitors could serve as a new generation of broad-spectrum anthelmintic drugs for the treatment of nematode infections in animals and humans.

***Specific aim 2*:** This study investigated benzimidazole resistance development in a field population of *H. contortus*, focusing on beta-tubulin isotype-1 mutations and their correlation with drug susceptibility. Through a 13-month on-field drug selection protocol involving 20 goats, we monitored the development of resistance through *in vitro* tests, and correlated these findings with the genotypic response. This study design was specifically planned to identify non-beta tubulin genomic loci under selection that contribute to benzimidazole resistance. We implemented a gradual selection protocol with low doses of fenbendazole, in a field situation with natural reinfection, to favor genes that can contribute to resistance even if their effects are small. This approach can

help us uncover mechanisms that potentially cause worms to reach levels of resistance much higher than what is seen when canonical mutations in beta-tubulin reach fixation. Future studies will focus on performing whole-genome sequencing using DNA from the eggs and larvae archived in 70% ETOH throughout the study. Those results will then be paired with the phenotypic and genotypic data already collected to produce deeper insights into the full genomic basis of benzimidazole resistance.

Overall, the aims of this dissertation are interconnected in their goal of addressing anthelmintic resistance in parasitic nematodes. By validating PMT inhibitors as novel drug targets (Specific Aim 1), we are laying the groundwork for the development of new therapeutic strategies that can overcome existing resistance mechanisms. However, the development of new anthelmintics is a lengthy, resource-intensive, very costly and challenging process. *In vitro* efficacy is just the first step; a compound must also demonstrate appropriate safety, stability, solubility, and desirable pharmacokinetic and pharmacodynamic properties, as well as *in vivo* efficacy, to progress to clinical development. Our *in vivo* study used small groups of jirds with only one drug concentration tested, constituting a pilot study. Thus, future research should investigate the pharmacokinetics and pharmacodynamics of PMT inhibitor compounds to optimize dosing strategies, as well as testing in target animals. Based on the promising results obtained in our studies, funding will be pursued to support further studies of novel PMT inhibitor compounds in our laboratory. Compounds that show substantial efficacy will be tested in sheep infected with naturally acquired mixed-species parasite infections. This will establish the efficacy of the compounds against livestock parasitic nematodes other than *H. contortus*, in a clinically relevant host.

Moreover, developing sustainable approaches for using new drugs with novel mechanisms of action relies on our capacity to monitor anthelmintic resistance in nematode populations. Genomic markers of resistance present a promising avenue for efficiently assessing resistance levels to multiple drugs simultaneously, potentially eliminating the need for extensive *in vivo* trials. However, this approach requires a comprehensive knowledge of the underlying genetic mechanisms of anthelmintic resistance and their relative impact on drug susceptibility phenotype.

By identifying the role that specific beta-tubulin mutations play in benzimidazole resistance, we can improve diagnostic tools and treatment approaches, monitor resistance patterns and contribute to elucidating additional resistance mechanisms. With our field trials (Specific Aim 2), we have contributed to the existing knowledge of benzimidazole resistance by precisely measuring levels of the F200Y beta-tubulin isotype-1 mutation in a population of *H. contortus*., while simultaneously measuring the *in vitro* and *in vivo* susceptibility phenotype over time. We then assessed the correlation of the F200Y beta-tubulin isotype-1 mutation with phenotypic resistance. The archived samples collected throughout our drug-selection field study will serve as the basis for the next phase of this research, which will investigate which non-beta tubulin genomic loci are under selection from benzimidazole drugs. To achieve this, we will conduct whole genome sequencing of samples collected throughout the study to identify additional loci under selection. This will allow us to comprehensively examine genome-wide changes at the onset of selection and throughout the study period, correlating these changes with changes in the level of drug susceptibility. Additional loci detected

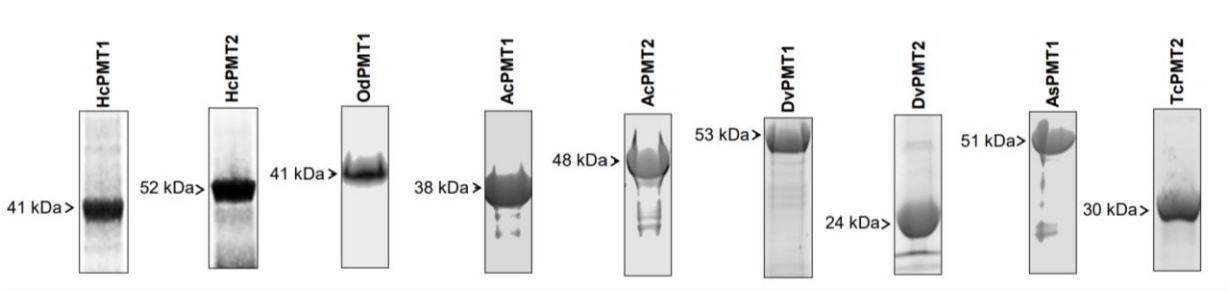
through this strategy could then be studied in *C. elegans* through the heterologous expression of associated alleles, and the use of CRISPR.

Identifying all molecular resistance markers associated with anthelmintic resistance is crucial for the development of more accessible and practical field tests. Such advancements would revolutionize parasite control by enabling the efficient tracking of resistance development in parasitic nematodes affecting both animals and humans, particularly during drug administration campaigns in human populations. Access to this technology would transform current practices. Instead of relying on traditional methods, such as conducting pre- and post-treatment fecal egg counts (FEC) to measure the efficacy of treatments, DNA from individual or pooled samples could be amplified and analyzed. This approach would eliminate the need for labor-intensive individual FECs, and a potentially less expensive alternative. In the future, integrating new drugs with novel mechanisms of action must be approached sustainably. With the advancement of wide genome scans in parasitic nematodes, researchers in the industry could conduct drug selection experiments to detect genomic loci under selection. This proactive approach would enable us to identify and monitor specific markers when launching new drugs, thereby avoiding the need for extensive trials when drug resistance is already at advanced field stages. Such foresight would extend the effectiveness of newly introduced products, benefiting both the animal industry and human medicine.

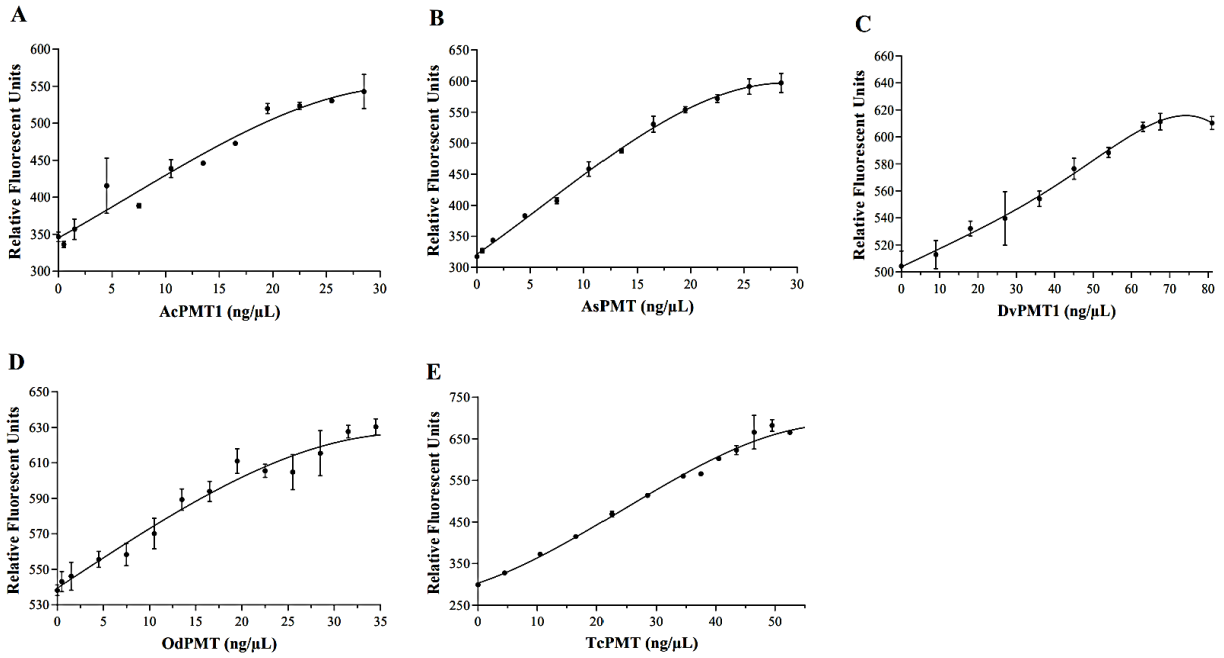
APPENDICES

APENDIX A

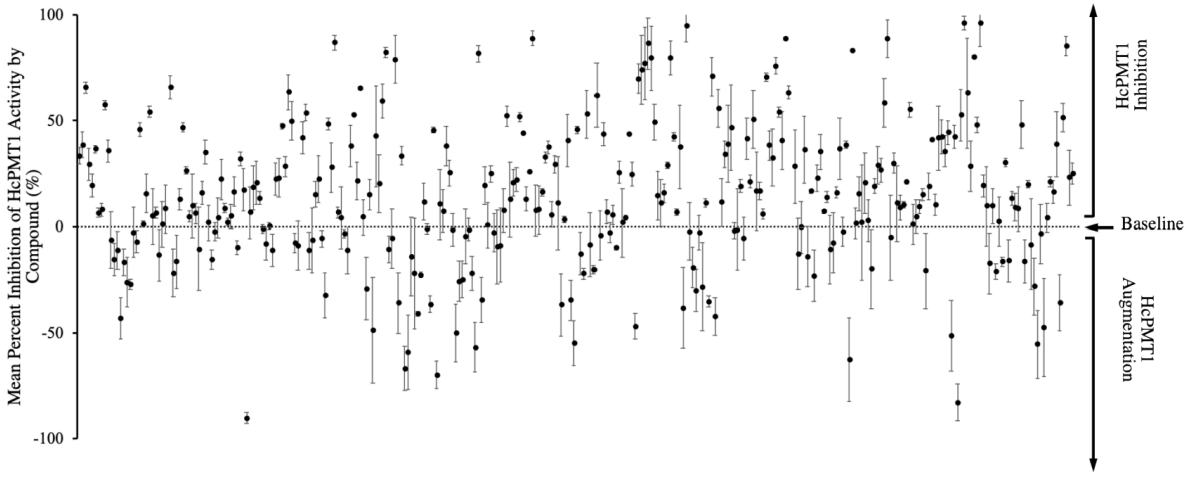
CHAPTER 2 SUPPLEMENTARY INFORMATION



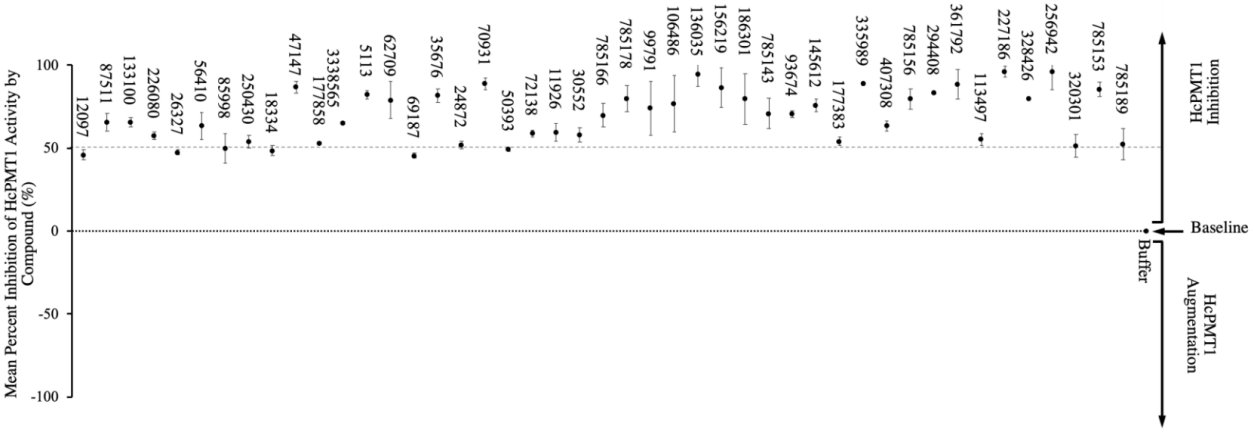
S1 Figure. SDS-PAGE analysis of the nickel affinity-purified recombinant PMT proteins. The names of the respective PMTs are indicated and their molecular weight in kDa based on the protein ladder marker. HcPMT: *Haemonchus contortus* PMT; OdPMT: *Oesophagostomum dentatum* PMT; AcPMT: *Ancylostoma ceylanicum* PMT; DvPMT: *Dictyocaulus viviparus* PMT; AsPMT: *Ascaris suum* PMT; TcPMT: *Toxocara canis* PMT.



S2 Figure. Analysis of the concentration-dependent enzymatic activity of natively purified recombinant PMT proteins in the methyltransferase assay with S-adenosylmethionine and phosphoethanolamine each maintained at 100 µM and 200 µM, respectively. Titration curves for (A) AcPMT1, (B) AsPMT, (C) DvPMT1, (D) OdPMT, and (E) TcPMT. The data shown represent means of three independent experiments with standard error bars.

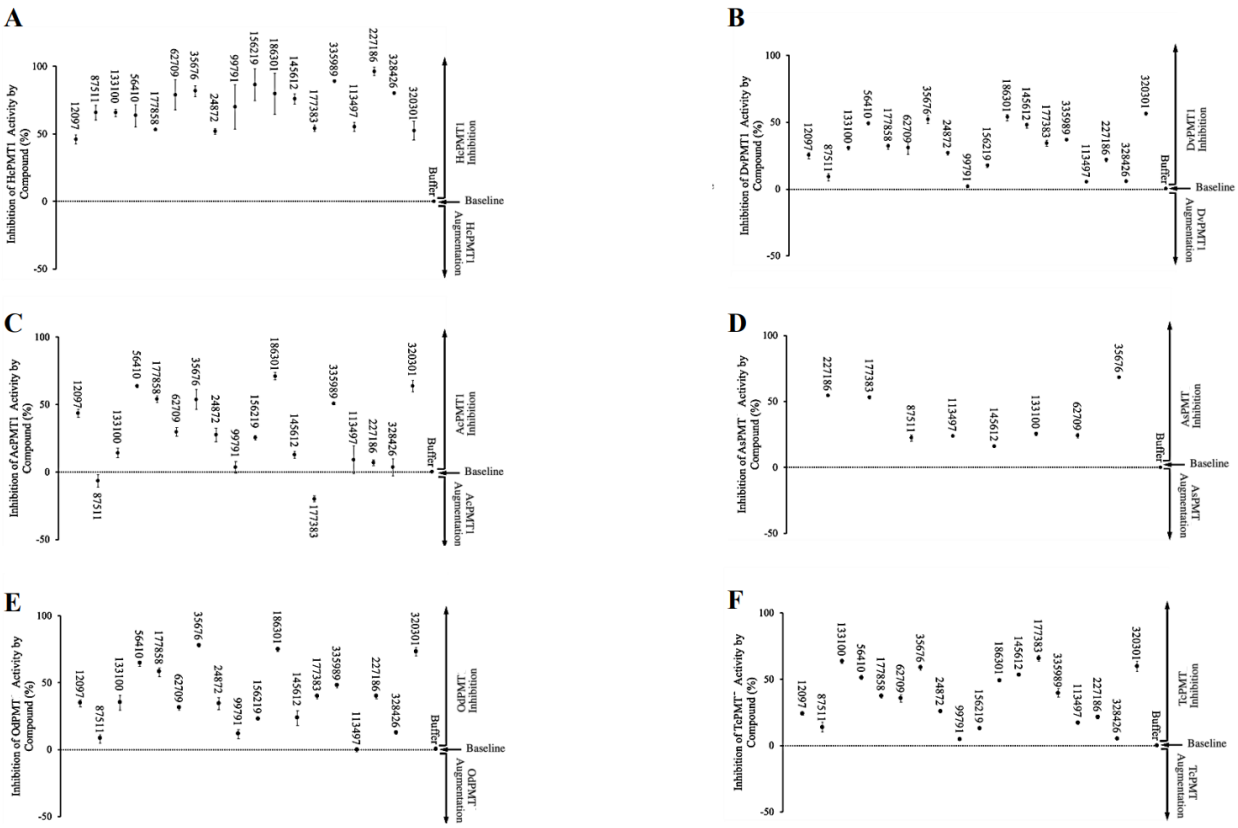


S3 Figure. Effect of compounds from the NCI Natural Product Set IV library on the enzymatic activity of *Haemonchus contortus* PMT (HcPMT1) recombinant protein. Individually reconstituted compounds were used at a final concentration of 40 μM in the HcPMT1-catalyzed reaction for the methylation of phosphoethanolamine. The mean percent inhibition of HcPMT1 activity by each compound was derived by dividing the difference in fluorescence between the compound-treated wells and the DMSOtreated wells by the fluorescence of the DMSO-treated wells and multiplying the product by 100. The baseline mean percent inhibition of 0 was for the reaction without compound, but with an equivalent volume of DMSO used to reconstitute the compounds. Compounds with mean percent inhibition values greater than 0 were designated as inhibitors of the activity of HcPMT1, while those with mean percent inhibition values less than 0 were classified as augmenters. Each reaction was performed in triplicate, and the data shown represent the mean of three independent experiments. Bars represent standard errors of the mean (SEM).



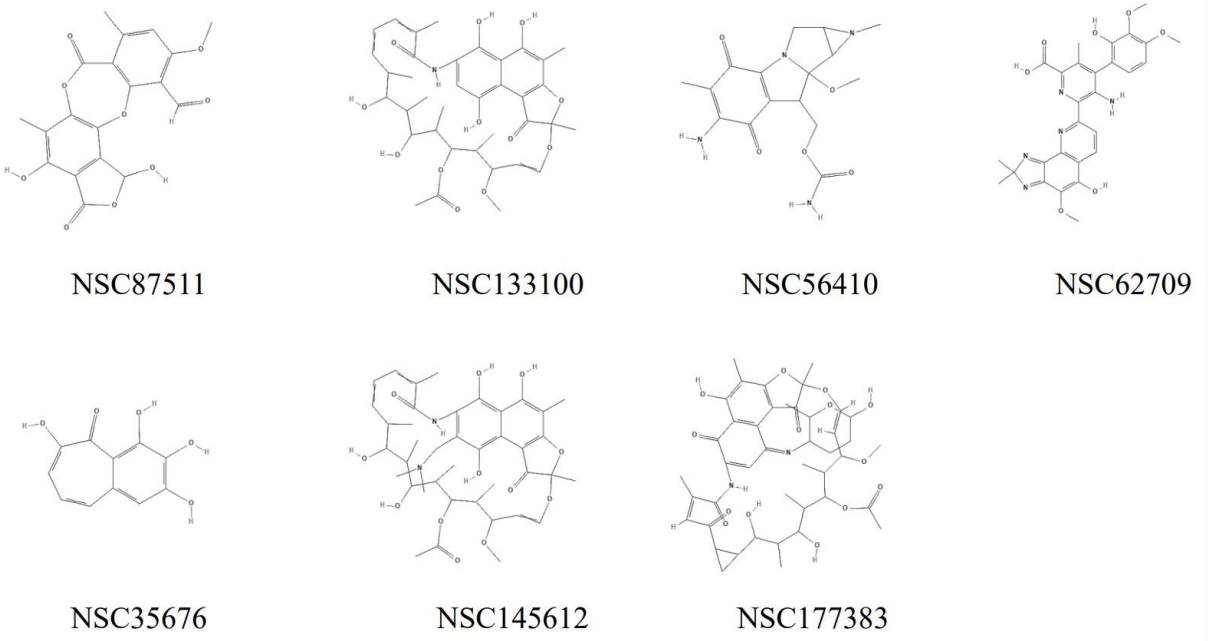
S4 Figure. Compounds from the NCI Natural Product Set IV library with >50% inhibitory activity against HcPMT1 enzymatic activity. Individually reconstituted compounds

(indicated by their NSC numbers) were used at a final concentration of 40 μM in the HcPMT1-catalyzed reaction for the methylation of phosphoethanolamine. The mean percent inhibition of HcPMT1 activity by each compound was derived by dividing the difference in fluorescence between the compound-treated wells and the DMSO-treated wells by the fluorescence of the DMSO-treated wells and multiplying the product by 100. The baseline mean percent inhibition of 0 was for the reaction without compound, but with an equivalent volume of DMSO used to reconstitute the compounds. Each reaction was performed in triplicate, and the data shown represent the mean of three independent experiments. Bars represent standard errors of the mean (SEM).



S5 Figure. Compounds from the NCI Natural Product Set IV library depicting cross-inhibitory effective against the enzymatic activity of various nematode PMTs

recombinant proteins in vitro. Individually reconstituted compounds (indicated by their NSC numbers) were used at a final concentration of 40 μM in (A) HcPMT1, (B) DvPMT1, (C) AcPMT1, (D) AsPMT, (E) OdPMT or (F) TcPMT catalyzed reactions for the methylation of phosphoethanolamine. The mean percent inhibition of enzyme activity by each compound was derived by dividing the difference in fluorescence between the compound-treated wells and the DMSO-treated wells by the fluorescence of the DMSO-treated wells and multiplying the product by 100. The baseline mean percent inhibition of 0 was for the reaction without compound, but with an equivalent volume of DMSO used to reconstitute the compounds. Each reaction was performed in triplicate, and the data shown represent the mean of three independent experiments. Bars represent standard errors of the mean (SEM).



S6 Figure. Chemical structures of broad-spectrum nematode PMT inhibitors.

S1 Table. Natural Products Set IV compound library.

Plate number	Well number	NSC number	Molecular weight	Molecular formula
13160330	C03	757	399	C22H25NO6
13160330	C04	12097	213	C12H7NO3
13160330	C05	31048	611	C28H34O15
13160330	C06	56464	646	C34H47NO11
13160330	C07	87511	386	C19H14O9
13160330	C08	133100	721	C37H47NO12.Na
13160330	C09	226080	914	C51H79NO13
13160330	C10	302289	232	C15H20O2
13160330	C11	349438	168	C9H12O3
13160330	C12	637086	278	C13H18N4O3
13160330	C13	3716	116	C5H8O3
13160330	C14	7533	985	C49H76O20
13160330	C15	9699	270	C18H22O2
13160330	C16	15624	382	C22H26N2O4
13160330	C17	22939	224	C12H20N2O2
13160330	C18	31754	256	C14H8O5
13160330	C19	36407	414	C22H22O8
13160330	C20	46709	131	C6H13NO2
13160330	C21	62786	183	C9H13NO3
13160330	C22	76627	912	C49H85NO14
13160330	D03	7524	674	C36H51NO11
13160330	D04	23969	346	C22H35NO2
13160330	D05	36351	339	C20H21NO4
13160330	D06	71795	246	C17H14N2
13160330	D07	118343	236	C12H12O5
13160330	D08	176503	362	C20H26O6
13160330	D09	284200	345	C21H44O3
13160330	D10	332598	626	C35H47NO9
13160330	D11	382796	552	C31H25N3O7
13160330	D12	2150	152	C8H8O3
13160330	D13	5897	322	C19H14O5
13160330	D14	8661	286	C16H14O5
13160330	D15	13123	113	C4H7N3O
13160330	D16	19509	391	C21H26N2O3.ClH
13160330	D17	26254	194	C7H14O6
13160330	D18	34552	185	C10H19NO2
13160330	D19	43338	164	C10H12O2
13160330	D20	51351	284	C17H16O4
13160330	D21	72715	605	C23H28N2O5.C6H8O7
13160330	D22	85235	246	C15H18O3

13160330	E03	2952	300	C20H28O2
13160330	E04	13252	515	C22H23ClN2O8.ClH
13160330	E05	31867	398	C23H26O6
13160330	E06	58368	640	C26H34O7.C12H23N
13160330	E07	89671	280	C16H24O4
13160330	E08	145118	460	C25H33NO7
13160330	E09	250429	364	C20H28O6
13160330	E10	305222	494	C30H39NO5
13160330	E11	350085	270	C16H14O4
13160330	E12	661755	877	C46H48N2O8.2C2H4O2
13160330	E13	4143	433	C9H9I2NO3
13160330	E14	7535	985	C49H76O20
13160330	E15	10105	355	C21H25NO4
13160330	E16	16631	154	C7H6O4
13160330	E17	23615	228	C9H12N2O5
13160330	E18	32743	339	C17H25NO6
13160330	E19	36437	376	C22H32O5
13160330	E20	46728	376	C22H32O5
13160330	E21	63946	858	C45H79NO14
13160330	E22	79404	380	C20H28O7
13160330	F03	7668	304	C16H32O5
13160330	F04	26258	394	C23H22O6
13160330	F05	36398	304	C15H12O7
13160330	F06	72116	354	C21H26N2O3
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13160330	F08	177406	591	C34H54O8
13160330	F09	284437	248	C15H20O3
13160330	F10	332876	318	C20H30O3
13160330	F11	400978	248	C15H20O3
13160330	F12	2347	176	C6H8O6
13160330	F13	6435	302	C20H30O2
13160330	F14	8751	86	C4H6O2
13160330	F15	14135	248	C14H16O4
13160330	F16	19990	770	C40H51NO14
13160330	F17	26327	242	C15H14O3
13160330	F18	34758	313	C17H15NO5
13160330	F19	43339	232	C15H20O2
13160330	F20	56410	348	C16H20N4O5
13160330	F21	72861	305	C17H23NO4
13160330	F22	85998	265	C8H15N3O7
13160330	G03	3053	1255	C62H86N12O16
13160330	G04	14975	497	C25H36O10
13160330	G05	32192	367	C20H17NO6
13160330	G06	60387	393	C24H27NO4

13160330	G07	94600	348	C20H16N2O4
13160330	G08	150817	942	C47H75NO18
13160330	G09	250430	421	C26H28O5
13160330	G10	307981	434	C26H26O6
13160330	G11	361902	264	C15H20O4
13160330	G12	719655	379	C22H25N3O3
13160330	G13	4586	356	C20H20O6
13160330	G14	7606	158	C4H6N4O3
13160330	G15	11866	238	C16H14O2
13160330	G16	18334	828	C42H53NO16
13160330	G17	23878	276	C15H16O5
13160330	G18	32944	540	C28H38N2O4.2ClH
13160330	G19	36508	471	C26H30O8
13160330	G20	47147	323	C20H25N3O
13160330	G21	67392	152	C10H16O
13160330	G22	81463	400	C22H28N2O5
13160330	H03	8519	260	C14H12O5
13160330	H04	26271	261	C7H15Cl2N2O2P
13160330	H05	42038	261	C15H19NO3
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13160330	H08	177858	824	C43H49N7O10
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13160330	H10	333856	1337	C67H96N2O24.Na
13160330	H11	401005	354	C21H22O5
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13160330	H13	6832	150	C10H14O
13160330	H14	8797	393	C24H40O4
13160330	H15	14664	152	C5H4N4O2
13160330	H16	20103	170	C7H6O5
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13160330	H18	35550	431	C20H23NO4.C2H2O4
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13160330	H20	59258	192	C7H12O6
13160330	H21	72862	364	C22H24N2O3
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13160330	I03	5113	420	C25H24O6
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13160330	I06	62709	546	C28H27N5O7
13160330	I07	96911	242	C12H10N4O2
13160330	I08	153858	692	C34H46ClN3O10
13160330	I09	255109	546	C28H39N3O8
13160330	I10	325014	383	C14H20Cl2N2O6

13160330	I11	369397	282	C15H22O5
13160330	I12	824	200	C10H16O4
13160330	I13	5036	194	C8H10N4O2
13160330	I14	7616	174	C6H6O6
13160330	I15	11905	242	C15H14O3
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13160330	J06	82151	564	C27H29NO10.ClH
13160330	J07	122750	561	C29H40N2O9
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13160330	J09	287088	511	C28H30O9
13160330	J10	337783	362	C21H30O5
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13160330	J12	2835	321	C18H34O3.Na
13160330	J13	7521	531	C30H42O8
13160330	J14	8973	170	C10H18O2
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13160330	J16	20264	347	C10H14N5O7P
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13160330	K06	63701	291	C12H13N5O4
13160330	K07	105388	671	C35H58O12
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13160330	K11	375294	234	C15H22O2
13160330	K12	1115	192	C7H12O6
13160330	K13	5379	271	C18H38O

13160330	K14	7652	267	C10H13N5O4
13160330	K15	12444	182	C9H10O4
13160330	K16	19028	222	C15H10O2
13160330	K17	24872	258	C15H14O4
13160330	K18	33410	462	C27H27NO6
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13160330	K20	50132	446	C23H26O9
13160330	K21	70931	451	C29H38O4
13160330	K22	83436	344	C20H24O5
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13160330	L04	29854	637	C35H44N2O9
13160330	L05	45923	216	C12H8O4
13160330	L06	85236	262	C15H18O4
13160330	L07	122819	657	C32H32O13S
13160330	L08	209870	547	C28H50O10
13160330	L09	292567	748	C40H68O11.Na
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13160330	L11	407306	354	C21H26N2O3
13160330	L12	3071	152	C8H8O3
13160330	L13	7525	693	C36H52O13
13160330	L14	9170	373	C20H23NO4S
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13160330	L16	21725	244	C15H20N2O
13160330	L17	30238	148	C4H4O6
13160330	L18	36294	248	C14H16O4
13160330	L19	44175	292	C18H12O4
13160330	L20	59729	361	C13H19N3O5S2
13160330	L21	72942	295	C8H15N5O.H2O4S
13160330	L22	89937	299	C15H25NO5
13160330	M03	5366	413	C22H23NO7
13160330	M04	22842	268	C15H8O5
13160330	M05	35611	905	C45H73NO15.ClH
13160330	M06	67574	923	C46H56N4O10.H2O4S
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13160330	M09	270914	447	C24H30O8
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13160330	M12	2080	504	C18H32O16
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13160330	M14	8625	174	C10H6O3
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13160330	M16	19038	344	C19H20O6
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13160330	M18	34202	503	C30H46O6
13160330	M19	38270	1197	C58H84O26
13160330	M20	50393	224	C15H12O2
13160330	M21	72138	579	C32H38N2O8
13160330	M22	83439	334	C20H30O4
13160330	N03	11926	341	C17H11NO7
13160330	N04	30552	410	C24H26O6
13160330	N05	51001	842	C42H67NO16
13160330	N06	85239	262	C15H18O4
13160330	N07	129536	310	C15H18O7
13160330	N08	210236	334	C20H30O4
13160330	N09	301683	262	C13H10O6
13160330	N10	345647	547	C30H26O10
13160330	N11	614552	189	C8H15NO4
13160330	N12	3590	514	C20H23N7O7.Ca
13160330	N13	7532	969	C49H76O19
13160330	N14	9248	194	C6H10O7
13160330	N15	15307	322	C20H22N2O2
13160330	N16	21728	335	C19H16N2.HNO3
13160330	N17	30625	411	C21H33NO7
13160330	N18	36354	507	C20H23NO5.C4H6O6
13160330	N19	45384	521	C26H24N4O8
13160330	N20	61809	398	C18H24NO4.Br
13160330	N21	75527	358	C20H22O6
13160330	N22	90636	907	C46H56N4O9.H2O4S
13160331	C03	93047	229	C9H11NO6
13160331	C04	100290	606	C30H35NO10.ClH
13160331	C05	111041	189	C9H7N3O2
13160331	C06	123977	551	C30H46O9
13160331	C07	143648	346	C12H15N5O5.ClH
13160331	C08	172924	521	C26H32O11
13160331	C09	237671	1330	C63H87N13O19
13160331	C10	266535	307	C14H13NO7
13160331	C11	291312	519	C27H34O10
13160331	C12	330917	237	C9H11N5O3
13160331	C13	403148	398	C22H22O7
13160331	C14	526417	1101	C51H64N12O12S2
13160331	C15	785154	334	C21H22N2O2
13160331	C16	785166	268	C10H12N4O5
13160331	C17	785178	425	C24H28N2O5
13160331	D03	99791	221	C5H12N2O3.2ClH
13160331	D04	106486	786	C40H51NO15
13160331	D05	121849	416	C22H25NO5S
13160331	D06	136035	673	C34H48N4O10

13160331	D07	156219	563	C29H38O11
13160331	D08	186301	621	C31H41ClN2O9
13160331	D09	255112	509	C28H28O9
13160331	D10	269756	623	C33H38N4O6.ClH
13160331	D11	314018	420	C23H33NO6
13160331	D12	354844	379	C14H25N3O9
13160331	D13	616348	182	C10H14O3
13160331	D14	785148	240	C12H20N2O3
13160331	D15	785160	260	C15H16O4
13160331	D16	785172	385	C21H23NO6
13160331	D17	785184	328	C17H28O6
13160331	E03	93373	292	C17H24O4
13160331	E04	100858	635	C32H43ClN2O9
13160331	E05	112906	433	C19H26N2.C4H6O6
13160331	E06	127445	321	C20H19NO3
13160331	E07	145150	350	C21H22N2O3
13160331	E08	172946	360	C18H16O8
13160331	E09	244387	272	C11H13NO2.BrH
13160331	E10	267033	701	C36H48N2O12
13160331	E11	292222	246	C15H18O3
13160331	E12	332294	812	C42H53NO15
13160331	E13	403169	773	C40H48N6O10
13160331	E14	785143	563	C29H38O11
13160331	E15	785155	263	C11H13N5O3
13160331	E16	785167	429	C23H27NO7
13160331	E17	785179	406	C22H30O7
13160331	F03	99792	595	C36H38N2O6
13160331	F04	106969	389	C20H18N2O5.Na
13160331	F05	121859	248	C9H16N2O6
13160331	F06	136044	242	C10H14N2O3S
13160331	F07	156236	755	C40H54N2O12
13160331	F08	208734	823	C43H54N2O14
13160331	F09	259968	428	C25H33N3O.ClH
13160331	F10	269760	1085	C52H76O24
13160331	F11	316458	561	C29H36O11
13160331	F12	355637	439	C21H20F3NO6
13160331	F13	642099	448	C22H24O10
13160331	F14	785149	410	C23H26N2O5
13160331	F15	785161	314	C15H10N2O6
13160331	F16	785173	352	C21H24N2O3
13160331	F17	785185	236	C12H12O5
13160331	G03	93674	490	C28H43NO6
13160331	G04	100880	846	C42H55NO17
13160331	G05	112907	1572	C61H88N18O21S2.H2O4S

13160331	G06	127473	565	C29H40O11
13160331	G07	145612	570	C27H21Cl2N3O7
13160331	G08	177383	495	C25H34O10
13160331	G09	247562	781	C41H64O14
13160331	G10	269146	244	C8H12N4O5
13160331	G11	292463	341	C16H23NO7
13160331	G12	335989	315	C18H21NO4
13160331	G13	407308	379	C22H21NO5
13160331	G14	785144	487	C28H38O7
13160331	G15	785156	715	C38H34O14
13160331	G16	785168	542	C28H31NO10
13160331	G17	785180	487	C20H26N2O8S2
13160331	H03	99794	696	C40H45N3O6S
13160331	H04	106995	375	C23H34O4
13160331	H05	121860	364	C16H13NO4.BrH
13160331	H07	159632	236.22	C12H12O5
13160331	H08	216128	412	C23H28N2O5
13160331	H09	265211	867	C47H78O14
13160331	H10	276382	549	C28H36O11
13160331	H11	327993	268	C11H16N4O4
13160331	H12	359079	730	C37H47NO14
13160331	H13	132791	256	C8H17N3O4.ClH
13160331	H14	785150	535	C27H34O11
13160331	H15	785162	401	C21H23NO5S
13160331	H16	785174	354	C19H14O7
13160331	H17	785186	456	C29H45NO3
13160331	I03	95099	418	C22H26O8
13160331	I04	102816	483	C28H34O7
13160331	I05	113087	349	C18H23NO6
13160331	I06	128487	302	C14H19NO4.ClH
13160331	I07	146396	302	C12H18N2O7
13160331	I08	179834	307	C17H25NO4
13160331	I09	248605	549	C29H40O10
13160331	I10	269148	350	C19H26O6
13160331	I11	294408	478	C29H35NO5
13160331	I12	337851	523	C18H22N2O4.C6H8O7
13160331	I13	407806	510	C27H43NO8
13160331	I14	785145	333	C16H19N3O3S
13160331	I15	785157	298	C17H14O5
13160331	I16	785169	296	C16H24O5
13160331	I17	785181	316	C16H12O7
13160331	J03	99799	697	C35H37ClN2O11
13160331	J04	107041	156	C7H8O4
13160331	J05	121865	391	C23H34O5

13160331	J06	138320	805	C40H52O17
13160331	J07	165563	248	C15H20O3
13160331	J08	218321	580	C27H29NO11.ClH
13160331	J09	265450	381	C22H23NO5
13160331	J10	278619	325	C12H15N5O4S
13160331	J11	328166	229	C12H11N3O2
13160331	J12	361792	482	C25H43N3O6
13160331	J13	178249	433	C25H36O6
13160331	J14	785151	486	C25H26O10
13160331	J15	785163	364	C20H28O6
13160331	J16	785175	549	C29H40O10
13160331	J17	785187	374	C22H30O5
13160331	K03	96021	291	C14H13NO6
13160331	K04	104943	357	C19H23N3O4
13160331	K05	113497	291	C12H13N5O4
13160331	K06	129230	354	C21H26N2O3
13160331	K07	147340	325	C19H19NO4
13160331	K08	180516	326	C19H18O5
13160331	K09	248958	1665	C72H85N19O18S5
13160331	K10	269753	594	C30H51N5O7
13160331	K11	295426	410	C21H30O8
13160331	K12	343256	519	C27H34O10
13160331	K13	601422	561	C30H44N2O8
13160331	K14	785146	785	C42H68N6O6S
13160331	K15	785158	469	C22H22F3NO5S
13160331	K16	785170	293	C19H19NO2
13160331	K17	785182	297	C18H19NO3
13160331	L03	99804	325	C19H19NO4
13160331	L04	107453	311	C18H17NO4
13160331	L05	123383	311	C18H17NO4
13160331	L06	141538	313	C19H23NO3
13160331	L07	169517	596	C36H40N2O6
13160331	L08	227186	337	C19H15NO5
13160331	L09	266032	367	C21H21NO5
13160331	L10	281245	297	C18H19NO3
13160331	L11	328426	355	C20H21NO5
13160331	L12	365793	321	C19H15NO4
13160331	L13	256942	339	C20H21NO4
13160331	L14	785152	608	C37H40N2O6
13160331	L15	785164	313	C19H23NO3
13160331	L16	785176	351	C20H17NO5
13160331	L17	785188	263	C17H13NO2
13160331	M03	98542	279	C18H17NO2

13160331	M04	105827	338	C21H26N2O2
13160331	M05	114341	351	C20H17NO5
13160331	M06	135962	339	C20H21NO4
13160331	M07	148790	622	C38H42N2O6
13160331	M08	184398	297	C18H19NO3
13160331	M09	250682	295	C19H21NO2
13160331	M10	269754	309	C18H15NO4
13160331	M11	302979	325	C20H23NO3
13160331	M12	349155	325	C19H19NO4
13160331	M13	607097	337	C19H15NO5
13160331	M14	785147	385	C21H23NO6
13160331	M15	785159	329	C19H23NO4
13160331	M16	785171	341	C20H23NO4
13160331	M17	785183	355	C21H25NO4
13160331	N03	99843	327	C19H21NO4
13160331	N04	108088	281	C18H19NO2
13160331	N05	123389	325	C19H19NO4
13160331	N06	142227	281	C18H19NO2
13160331	N07	170365	279	C18H17NO2
13160331	N08	236580	476	C32H32N2O2
13160331	N09	266071	325	C19H19NO4
13160331	N10	283445	325	C19H19NO4
13160331	N11	330500	281	C18H19NO2
13160331	N12	376128	413	C26H23NO4
13160331	N13	320301	610	C37H42N2O6
13160331	N14	785153	295	C19H21NO2
13160331	N15	785165	476	C32H32N2O2
13160331	N16	785177	281	C18H19NO2
13160331	N17	785189	341	C20H23NO4

S2 Table. Percent inhibition of larval motility for the different timepoints tested for compound NSC133100, against the susceptible isolate of *H. contortus.* Data for 3 technical replicates were averaged for analysis.

Concentration (µM)	Initial	1 hour	4 hours	24 hours	48 hours	72 hours	96 hours
125	-8.9%	4.3%	-12.0%	3.2%	23.2%	3.1%	19.2%
31.25	-64.8%	10.6%	7.1%	9.0%	35.2%	19.9%	28.7%
7.81	-74.0%	27.5%	9.1%	25.3%	35.3%	32.1%	38.0%

1.95	-159.9%	-57.6%	-16.4%	-8.4%	-37.5%	-49.0%	-50.7%
0.49	-118.5%	-29.1%	4.3%	8.0%	-9.5%	-14.9%	-9.0%
0.12	-93.9%	-7.4%	14.9%	22.9%	8.7%	26.1%	7.4%

S3 Table. Percent inhibition of larval motility for the different timepoints tested for Compound NSC177383, against the susceptible isolate of *H. contortus.* Data for 3 technical replicates were averaged for analysis.

Concentration (µM)	Initial	1 hour	4 hours	24 hours	48 hours	72 hours	96 hours
125	22.02%	42.55%	10.81%	16.94%	42.97%	27.79%	41.45%
31.25	28.34%	14.47%	38.95%	18.83%	26.89%	16.58%	-23.43%
7.81	22.03%	26.94%	26.72%	22.63%	34.44%	14.00%	-53.76%
1.95	21.82%	30.56%	33.37%	18.80%	14.16%	-3.70%	-23.78%
0.49	-11.69%	25.94%	18.27%	23.09%	12.98%	-5.94%	-72.91%
0.12	-1.62%	29.41%	4.95%	28.20%	2.41%	4.89%	-16.32%

S4 Table. Percent inhibition of larval motility for the different timepoints tested for Compound NSC133100, against the MDR isolate of *H. contortus.* Data for 3 technical replicates were averaged for analysis.

Concentration (µM)	Initial	1 hour	4 hours	24 hours	48 hours	72 hours	96 hours
500	5.8%	33.9%	-2.0%	-0.5%	22.2%	12.1%	18.5%
250	-11.5%	38.9%	-5.6%	-12.4%	28.6%	-1.2%	0.9%
125	-12.3%	33.8%	3.0%	-6.2%	18.4%	14.0%	26.6%
62.5	-25.7%	13.5%	-11.1%	-12.6%	-2.3%	-9.5%	-1.6%
31.25	-21.7%	6.3%	1.6%	0.6%	11.9%	3.1%	5.5%
15.625	-7.4%	6.1%	6.9%	-6.8%	-11.1%	-11.7%	-3.4%

S5 Table. Percent inhibition of larval motility for the different timepoints tested for Compound NSC177383, against the MDR isolate of *H. contortus.* Data for 3 technical replicates were averaged for analysis.

Concentration (µM)	initial	1 hour	4 hours	24 hours	48 hours	72 hours	96 hours
500	36.4%	27.1%	-12.9%	26.6%	49.1%	32.3%	21.4%
250	43.9%	28.8%	-42.2%	30.2%	44.8%	46.1%	31.2%
125	22.5%	26.8%	-34.1%	20.5%	31.1%	4.1%	5.7%
62.5	16.5%	3.9%	-28.6%	23.5%	26.0%	19.1%	14.7%
31.25	23.2%	2.3%	5.2%	13.8%	41.2%	25.5%	17.1%
15.625	19.9%	-22.8%	-24.6%	15.6%	3.0%	-5.8%	-22.4%

S6 Table. Percent inhibition of larval motility for the different timepoints tested for Compound NSC145612, against the MDR isolate of *H. contortus.* Data for 3 technical replicates were averaged for analysis.

Concentration (µM)	initial	1 hour	4 hours	24 hours	48 hours	72 hours	96 hours
500	9.3%	-19.2%	0.4%	-12.0%	14.1%	9.6%	-5.1%
250	25.9%	13.0%	47.0%	-8.6%	13.8%	9.2%	12.8%
125	21.6%	31.7%	34.3%	15.7%	8.1%	3.0%	-6.9%
62.5	10.1%	16.7%	32.0%	10.4%	14.1%	18.3%	3.8%
31.25	21.0%	20.1%	-11.2%	27.2%	21.9%	43.2%	3.9%
15.625	20.3%	-10.3%	7.3%	7.0%	8.1%	23.0%	17.4%

APPENDIX B

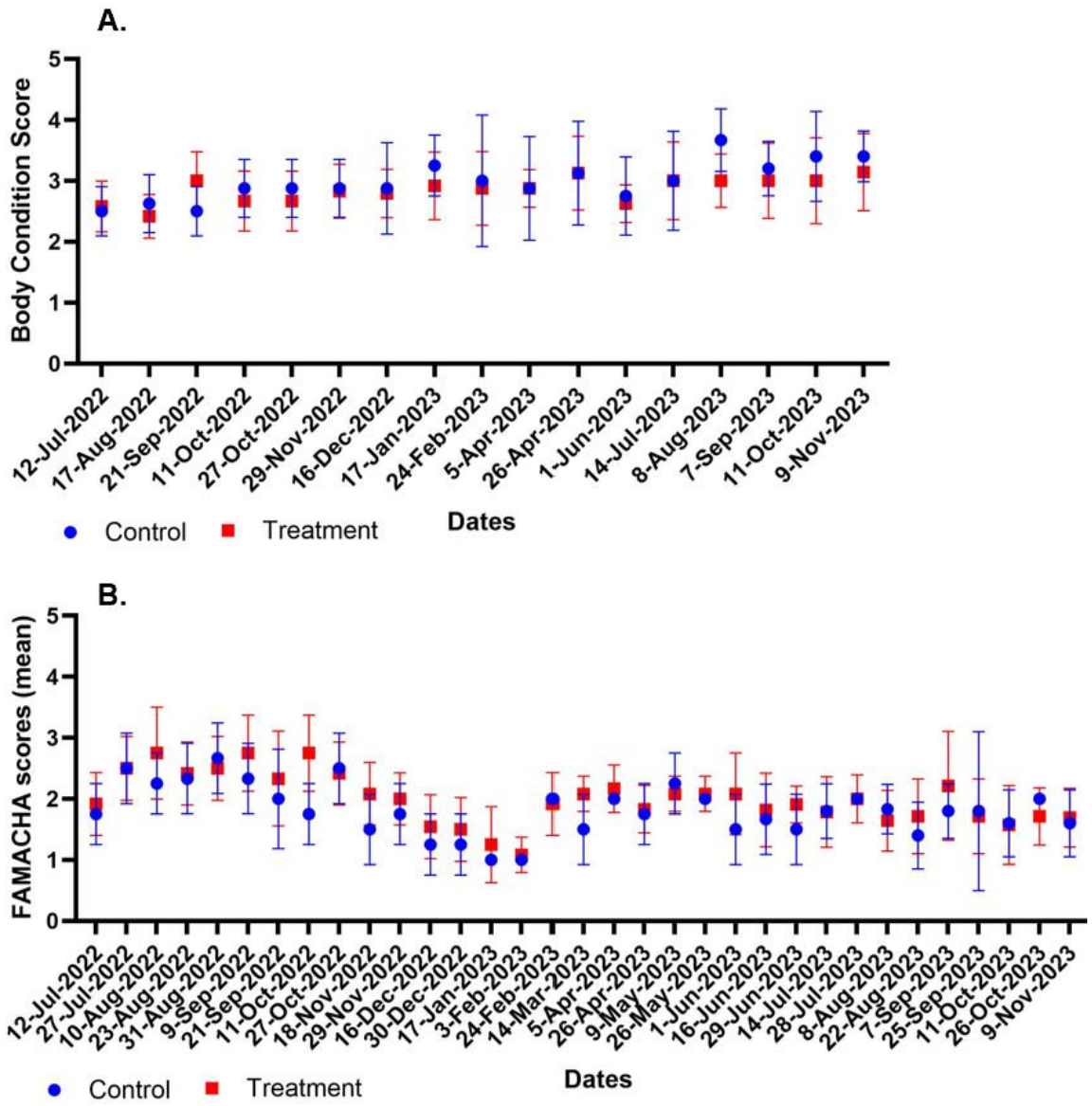
CHAPTER 4 SUPPLEMENTARY INFORMATION

S1 Table. Timeline of experiment with dates of fecal egg counts (FEC), fecal egg count reduction tests (FECRT), larval development assays (LDA), egg hatch assays (EHA), and deep amplicon sequencing of beta-tubulin isotype-1.

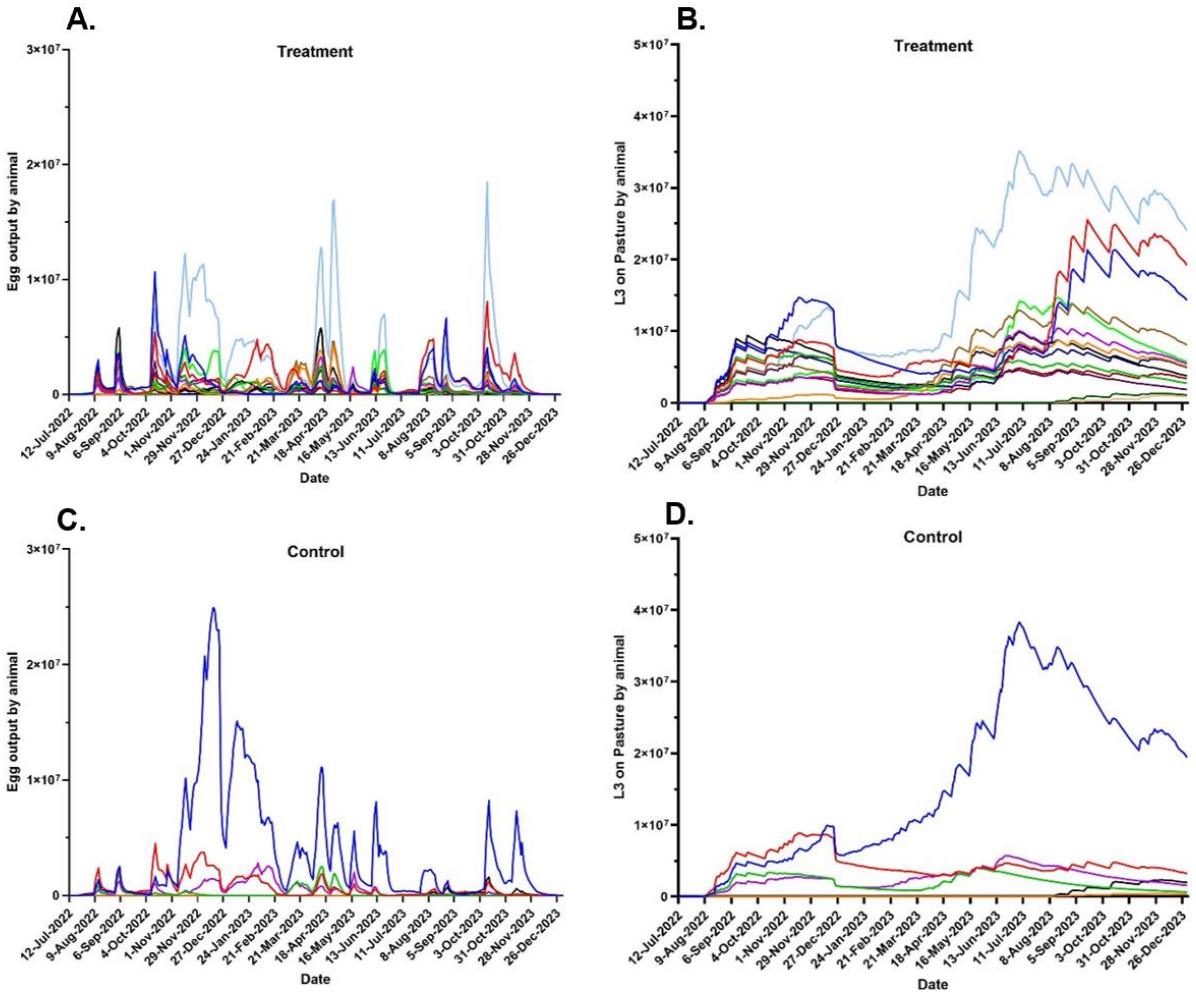
Date	Description	FEC	FECRT	EHA	LDA	Beta-tubulin sequencing
2/20/22	Laboratory *H. contortus* checked					x
6/7/22	Received goats	x				
6/10/22	Goats treated with LVM+ALB+IVM	x				
6/22/22	Goats introduced to pasture	x				
6/30/22	Fecal collection	x				
7/6/22	Infected with *Haemonchus contortus*	x				
7/12/22	Fecal collection	x				
7/20/22	Fecal collection. Infections patent in 2 animals	x				
7/27/22	FAMACHA					
8/10/22	FAMACHA					
8/17/22	BCS					
8/23/22	FAMACHA+Fecal collection					x
8/31/22	FAMACHA+Fecal collection	x				x
9/9/22	Treatment of 12 goats 0.25 mg/kg+fecal collection+FAMACHA	x	x	x	x	x
9/16/22	Fecal collection	x		x	x	
9/21/22	FAMACHA+BCS					x
9/30/22	Fecal collection					x
10/4/22	Fecal collection	x				x
10/11/22	FAMACHA+BCS+Fecal collection				x	
10/14/22	Treatment of 3 goats 0.25 mg/kg+Fecal collection	x	x			x
10/21/22	Fecal collection	x				
10/27/22	FAMACHA+BCS+Fecal collection					x
11/2/22	Fecal collection controls + FAMACHA	x				

11/4/22	Treatment of 12 goats 0.25 mg/kg+Fecal collection	x	x	x	x	x
11/11/22	Fecal collection	x				x
11/18/22	FAMACHA			x		
11/29/22	Fecal collection + BCS+FAMACHA	x				x
12/6/22	Treatment of 5 goats 0.25 mg/kg+Fecal collection	x	x	x		x
12/13/22	Fecal collection	x		x		
12/16/22	FAMACHA+BCS					
12/30/22	FAMACHA					
1/17/23	Fecal collection+FAMACHA+BCS	x		x	x	x
1/24/23	Fecal collection					x
2/3/23	Fecal collection+FAMACHA	x				x
2/17/23	Fecal collection	x				x
2/24/23	Fecal collection+FAMACHA+BCS	x				
3/1/23	Fecal collection	x				
3/3/23	Treatment of 3 goats 0.5 mg/kg+Fecal collection	x	x	x	x	
3/14/23	FAMACHA+Fecal collection			x		
3/26/23	Fecal collection	x				x
4/5/23	FAMACHA+BCS+Fecal collection				x	
4/12/23	Fecal collection	x				
4/21/23	Treatment of 11 goats 0.5 mg/kg+Fecal collection+FAMACHA+BCS	x	x	x		x
4/27/23	Fecal collection					x
5/1/23	Fecal collection	x		x		
5/9/23	FAMACHA+Fecal collection				x	x
5/23/23	Fecal collection					x
5/26/23	Fecal collection+FAMACHA	x				x
6/1/23	FAMACHA+BCS					x
6/8/23	Fecal collection				x	
6/14/23	Fecal collection	x				
6/16/23	Treatment of 12 goats 0.5 mg/kg+Fecal collection+FAMACHA	x	x	x		x
6/23/23	Fecall collection					x
6/29/23	Fecal collection+FAMACHA	x		x		
7/7/23	Fecal collection	x				x
7/14/23	FAMACHA+BCS					
7/18/23	Fecal collection	x				
7/25/23	Treatment of 12 goats 0.5 mg/kg+Fecal collection	x	x	x		x
7/28/23	FAMACHA					
8/1/23	Fecal collection	x		x		x
8/8/23	FAMACHA+BCS					x
8/22/23	FAMACHA					
9/7/23	FAMACHA+BCS					

9/14/23	Treatment of 13 goats 0.75 mg/kg+Fecal collection	x	x	x		x
9/25/23	Fecal collection+FAMACHA	x		x	x	x
10/11/23	FAMACHA+BCS					
10/19/23	Treatment of 14 goats 0.75 mg/kg+Fecal collection	x	x	x	x	x
10/26/23	Fecal collection+FAMACHA	x		x	x	x
11/2/23	Treatment of 14 goats 5 mg/kg+Fecal collection	x	x		x	x
11/9/23	FAMACHA+BCS					
11/14/23	Fecal collection	x			x	
11/17/23	Animals removed from pasture+fecal collection					x
11/21/23	Animals treated with MOX+ALB+LVM+Fecal collection	x	x			
12/1/23	Fecal collections	x				



S1 Figure. A. Mean Body Condition Score (scale 1-5), by group throughout the study period. BCS were conducted monthly in both treatment groups. **B**. Mean FAMACHA scores (scale 1-5), by group throughout the study period. FAMACHA scores were obtained biweekly for both treatment groups.



S2 Figure. A. Simulations of egg output by animal, for the treatment group throughout the entire duration of the study. **B.** Simulations of the total L3 in the pasture deposited by animal, for the treatment group throughout the entire duration of the study. **C.** Simulations of egg output by animal, for the control group throughout the entire duration of the study. **D.** Simulations of the total L3 in the pasture deposited by animal, for the control group throughout the entire duration of the study. The simulations were done using the GLOWORM-FL model.