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Assay Development as Critical for Drug Discovery
Against Human Intestinal Parasites

A Thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Arash Safavi

Committee in charge

Professor Raffi V. Aroian, Chair
Professor James T. Kadonaga
Professor Elizabeth A. Winzeler
Professor Elvira Tour

2013

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The Thesis of Arash Safavi is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2013

I dedicate this thesis to my grandmother, who taught me that no single act in life is more important than one which pursues betterment of the human state, and whose memory drives me to be the person I am. I miss you.

To my mother Bebe, and sister Azar, who fought to ensure I have everything I need to succeed in life, and whose resilience in the hardest of times has shown me the sheer strength of human will alone. My existence would be a void without your love and support. I thank you.

To my girlfriend Maryum, who once accused me of having a mistress for not believing I could possibly be in the laboratory so often or so late. You put a smile on my face when no one else can. “ooh ooh ahh ahh” <5

And finally, to caffeine, whose stimulation and mimicry of sympathetic activation is unparalleled by any compound not classified as a Schedule II controlled substance. Without you, I am lost.

TABLE OF CONTENTS

Signature Page	iii
Dedication	iv
Table of Contents	v
List of Figures	vi
List of Tables	vii
Acknowledgements	viii
Abstract	x
Chapter 1: Introduction	1
Chapter 2: Assay Development & Validation	7
Chapter 3: Library Screening	27
Chapter 4: Discussion	38
References	43

LIST OF FIGURES

Figure 1.	The hookworm life cycle	16
Figure 2.	Dosage response of <i>C. elegans</i> to a variety of known anthelmintics	17
Figure 3.	Dosage response of <i>H. bakeri</i> to a variety of known anthelmintics	18
Figure 4.	Dosage response of <i>A. ceylanicum</i> to a variety of known anthelmintics	19
Figure 5.	Hatch-rates of three nematodes in dose-response assays	20
Figure 6.	Dosage response of <i>T. muris</i> to a variety of known anthelmintics	26
Figure 7.	Comparison of overlapping hits in marine compound screen	30
Figure 8.	A proposed model to screen Compounds for anthelmintic activity	33
Figure 9.	Comparison of overlapping hits In LOPAC-1280 screen	34
Figure 10.	Examples of LOPAC hits on three nematodes	36

LIST OF TABLES

Table 1.	Summary of active marine metabolite fractions	31
Table 2.	Summary of LOPAC- 1280 hits on nematodes	35

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ABSTRACT OF THE THESIS

Assay Development as Critical for Drug Discovery Against
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by

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Master of Science in Biology

University of California, San Diego

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The soil-transmitted helminthes infect up to two billion people and are leading causes of morbidity in the developing world. The only subclass of drugs effective against these parasites in mass drug administration is facing the threat of resistance and is not efficacious against all parasites. With infection rates and demographics this

overwhelming, there is a growing need towards the discovery of new and more efficacious anthelmintics. Past anthelmintic discovery efforts have traditionally utilized veterinary (non-human) parasitic nematodes or non-parasitic nematodes altogether. We ask in the present study – are there superior alternatives to current anthelmintic discovery systems, specifically against human intestinal parasites? In answering this question, I develop and test a moderate-throughput 96-well format assay for anthelmintic screening against the free-living nematode *Caenorhabditis elegans*, and embryonic/larval stages of the parasitic nematodes *Heligmosomoides bakeri*, and *Ancylostoma ceylanicum*. This assay differs from existing larval assays in its capacity to look at a wider range of the parasite's life cycle and, in the case of *A. ceylanicum*, to incorporate a zoonotic human parasitic nematode. The assay was utilized in screening two small compound libraries to gauge the general overlap of hits between various nematodes and assess the feasibility of these methods. Significantly, compounds that hit on a human parasite would not necessarily be detected on a murine parasite or free-living nematode of the same clade. I discuss the implications of these results on future drug screening, which suggests that a human parasitic nematode could be successfully used for anthelmintic discovery.

CHAPTER 1

INTRODUCTION

1. 1 The Parasites

Soil-transmitted helminths (STHs) are infectious roundworms of the human gastrointestinal tract, namely consisting of hookworm (*Ancylostoma duodenale* and *Necator americanus*), whipworm (*Trichuris trichiura*), large roundworms (*Ascaris lumbricoides*), and the threadworms (*Strongyloides stercoralis*). The World Health Organization predicts an overwhelming worldwide prevalence of up to 2 billion people are likely to be infected with at least one of these parasites (World Health Organization, 2013). It is reasonable to argue that the developing world is trapped in a cycle of poverty owing in some part to the burden delivered by intestinal worm infections (Hotez & American Society for Microbiology., 2013). For example, a study looking specifically at Kenyan children found that mass deworming decreased school absenteeism by more than 25% (Miguel & Kremer, 2004) and significantly increased the future wage earning capacity in individuals who were dewormed (Bleakley, 2007). This dramatic impact is largely accounted for when taking into consideration symptoms of intestinal worm infections. Upon penetration of the skin or ingestion, the worm larvae lodge themselves within the small-intestinal lining where they are able to feed on nutrients from the host's blood or intestinal tract. Here they develop into adult-stage worms capable of laying

thousands of eggs per day. These are then defecated and hatch in the environment where they can be allowed to repeat the cycle of infection (Bethony et al., 2006).

The parasites typically cause chronic infections that can last up to 10-20 years, but individuals are continuously reinfected as a result of living in conditions of poverty (Hall, Hewitt, Tuffrey, & de Silva, 2008b). While intestinal parasites are not directly associated with high mortality rates, they have subtle yet viscous side effects. For example, STH infections are known to trigger a dampening of the immune system which may render infected individuals more susceptible to contracting tuberculosis (Elias, Mengistu, Akuffo, & Britton, 2006), malaria (Druilhe, Tall, & Sokhna, 2005), or suffer from an increase in the severity of preexisting HIV infections (Borkow & Bentwich, 2006), (Alexander & De, 2009), (Sabatelli, Ghani, Rodrigues, Hotez, & Brooker, 2008), (Walson et al., 2008). Furthermore, heavy infections typically result in anemia and protein deficiency as a result of blood loss or more complex gastrointestinal problems. Malnutrition in turn can leave grown men lethargic, pregnant women at a higher risk for birth complications or maternal death, and small children vulnerable to retardation of both cognitive and physical developmental (Brooker, Hotez, & Bundy, 2008) (Hall, Hewitt, Tuffrey, & de Silva, 2008a).

1.2 Need for new drugs

Currently, only two existing classes of anti-nematode (or anthelmintic) drugs are approved by the World Health Organization to treat soil-transmitted helminths. These are the benzimidazoles (albendazole, mebendazole) and the L-type nicotinic acetylcholine receptor agonists (pyrantel, levamisole). The benzimidazoles are relied on most heavily due to their cost-effectiveness as an efficacious single-dose wide-spectrum treatment and

the convenience of not having to weigh patients during MDA of diseased populations (Keiser & Utzinger, 2008). Although albendazole has unmatched efficacy against *Ascaris* (the leading cause of STHs) (Lubis, Pasaribu, & Lubis, 2012), the single-dose cure rate (CR) against hookworm is much lower, averaging 72% but with rates as low as 36% CR (Soukhathammavong et al., 2012) and 21% CR cited in some studies (Keiser & Utzinger, 2008). Furthermore, none of the existing anthelmintic drugs are effective in a single dose against whipworms (Keiser & Utzinger, 2010). An even greater cause for concern, as with any scenario of an infectious agent and its chemotherapy, the inevitable signs of anthelmintic resistance are emerging in parasites of livestock and humans alike (Steppek, Buttle, Duce, & Behnke, 2006), (Soukhathammavong et al., 2012).

Also relevant to note, the current anthelmintics in wide use were entirely discovered and developed for utilization in the veterinary field as driven by demand for sustaining the health of cattle and livestock – not with human use in mind. For example the first benzimidazole, thiabendazole was discovered in 1961 by larval screens against nematodal parasites of ruminants in culture by Merck (Johnson, 1999). This finding elucidated the anthelmintic activity of the benzimidazoles and later led to the discoveries of mebendazole (Janssen Pharmaceuticals, 1968) and albendazole (SmithKline Animal Health Laboratories, 1972) (Theodorides, Gyurik, Kingsbury, & Parish, 1976). Tetramisole (and concurrently levamisole) was discovered in 1966 by anthelmintic screening in chickens by Janssen Pharmaceuticals (Greenwood, 2008). Pyrantel and ivermectin were both discovered by screening compounds against *H. polygyrus (bakeri)* in mice by Pfizer (1966) and Merck (1983), respectively (Johnson, 1999). The initial discovery of all of these drugs was motivated by treatment of parasitic worms in

veterinary animals, not humans (de Hostos, 2012). This begs the question as to whether a screen focused on anthelmintic discovery more relevant to human parasites would uncover a drug superior to those currently available for MDA.

1.3 Current Screening Methods

With these considerations noted, it is easy to see why anthelmintic drug discovery is of imminent importance. The pharmaceutical industry typically approaches drug discovery from a high-throughput perspective, looks at the activity of millions of compounds against a single target, of which only a handful of the best candidates will advance to hit-to-lead follow-up studies (Woods & Williams, 2007). Infecting murine models with parasites and treating them with compounds *in vivo* to analyze the resulting effect on worm-burden would crudely replicate this approach, for example as demonstrated with the discoveries of pyrantel and ivermectin. Such an approach to human intestinal parasites would likely be a multi-million dollar venture, and with no immediate market demand or foresight of financial returns, no companies are lining up to make the investment (Woods & Williams, 2007). Unfortunately, outside of the pharmaceutical industry this approach would be unacceptably slow and financially unfeasible. An ideal drug screening method would therefore be fast, cheap, reproducible, reliable, high content, and eventually bridge to *in vivo* and clinical studies. Subsequently, past anthelmintic screens have utilized whole-organism screening with *C. elegans* as it is a free-living nematode that can be easily maintained in laboratory settings (Brown, Jones, Buckingham, Mee, & Sattelle, 2006). Since this non-parasitic nematode propagates quickly and does not require a host to complete its life cycle, it became the ideal candidate for the pharmaceutical industry in the 1980s and 90s as a primary screening

tool for the discovery of novel anthelmintic compounds (Geary, Thompson, & Klein, 1999).

Anthelmintic screening in *C. elegans* has been questioned in its susceptibility to false-negatives, or the idea that some compounds that would kill parasites may not have detectable effects in *C. elegans* screening assays. Not only does the cuticle of *C. elegans* decrease the likelihood that some compounds reach adequate bioavailability in the organism to observe toxicity (Burns et al., 2010), but the nematode also differs genetically from parasites. For example, 34% of hookworm genes do not have a well-defined analog in *C. elegans*, even though the two worms are from the same genetic clade (Geary & Thompson, 2001). Thus, it is not hard to believe that after exhaustive screens for two decades, not a single new anthelmintic drug has come out of a primary *C. elegans* screen (Caffrey, 2012).

Existing *in vitro* assays that look at anthelmintic activity against parasitic worms most commonly look at worm motility. Often mechanical stimulation or hot water must be employed to induce movement (Tritten, Silbereisen, & Keiser, 2011). While this may be adequate for small screens against adult-stage worms or obtaining qualitative data for focused studies, it is counterintuitive to screening large libraries. Even then, individuals have to be trained for assessment of motility and the assay remains inherently subjective (Smout, Kotze, McCarthy, & Loukas, 2010). Some assays look at parasitic egg hatch-rates, but typically benzimidazoles are most known to inhibit hatching and these assays disregard that not all drugs with anthelmintic activity can be assumed to have ovicidal activity (von Samson-Himmelstjerna et al., 2009). Furthermore, a drug that inhibits hatching will not necessarily be useful in active deworming of individuals already

infected with established *adult* parasites in their intestines. Still other assays exist that look at feeding inhibition, colourimetric and enzymatic assessment of metabolic markers, and heat flow to assess worm health. But larval stages of parasites used in these assays are essentially in a dauer stage (Ogawa, Streit, Antebi, & Sommer, 2009) and have been found to be too small and metabolically inactive for meaningful conclusions (Tritten, Braissant, & Keiser, 2012). Using adult-stage worms extracted from sacrificed hosts is an effective workaround, but this would void the concept of high-throughput screening. In the present study, we ask if there are superior methods to drug screening, specifically against intestinal parasites relevant to humans. We seek to answer which nematodes are of greatest utility for anthelmintic discovery and demonstrate a novel system for drug screening against human intestinal parasites.

CHAPTER 2

ASSAY DEVELOPMENT AND VALIDATION

As the aim of this study was to compare the results of compound screening in three nematode systems, it was first essential to create a uniform template assay to harbor and maintain the health of the organisms for the course of the study. First, 96-well U-bottom microtiter plates (Falcon cat#35-3077) were chosen as the assay backbone since this format is compatible with most compound libraries, provides a large working area per well, and concentrates worms in the center of the round bottom which facilitates microscopic examination. Next, optimal parameters for culturing each nematode had to be identified such as incubation temperature, culture media, DMSO concentration (for drug solvation), and food source and concentration for the worms.

2.1 *C. elegans* assays

N2 strain *C. elegans* was grown on nematode growth medium (NGM) plates seeded with OP50 and then gravid adult worms were bleached to release eggs according to standard protocol (Wood, 1988). Methods for growing *C. elegans* in S-medium liquid culture have been previously described (Stiernagle, 2006). As a food source for the *C. elegans*, *E. coli* (laboratory strain OP50) is the primary choice in most assays (Stiernagle, 2006). Multiple 96-well plates were set up with 2-30 worms per well (varied vertically

across the plate) in 100 μ L total culture. The variable range of egg numbers were tested against different concentrations of OP50 starting with 0 μ L and increasing in 2.5 μ L increments up to 20 μ L. For this and all future experiments discussed, a single OP50 colony was picked into LB media and cultured at 37°C overnight, then resuspended in culture media at a final optical density of 3.0 (λ = 600nm). It was found that 5-10 eggs prevented overcrowding yet offered enough animals per well to negate variance. Next, each of the variable OP50 test conditions was concurrently tested with a different DMSO concentration ranging from 0-5% v/v. Multiple parallel plates set up in this fashion were incubated at 20°C, 22°C and 25°C. The worms from each experimental condition were continuously monitored for development and general health over the course of 1 week.

The optimal parameters selected from this test were 10 μ L OP50, with up to 0.25% DMSO, incubated at 20°C for 72 hours. Under these conditions, at the end of a 72-hour incubation period, all eggs hatched and developed immaculately to gravid egg-laying adults. In wells containing less than 10 μ L OP50, food was visibly depleted and worm development would halt before reaching adult stage. In wells with more than 10 μ L, the dense bacterial aggregation reduced well visibility and even inhibited development of the worms. Worms cultured in media containing more than 1.00% DMSO sometimes displayed minor decomposition of the cuticle and generally ill health – 0.25% DMSO was thus chosen as a conservative value. When eggs were isolated from worms cultured with these optimal parameters and subject to culturing under the identical conditions, they hatched and were able to entirely repeat the life cycle.

2.2 *H. bakeri* assays

Heligmosomoides bakeri (also known as *Heligmosomoides polygyrus* or *Nematospiroides dubius*) is a mouse intestinal parasite commonly used as a laboratory model of nematode infections. Established adult-stage worms reside in the rodent's small intestine where they continuously lay eggs that are released into the environment upon defecation by the host. In the natural environment, eggs ideally hatch and develop from first-stage to infectious third-stage larvae between 4-6 days after defecation (Fahmy, 1956). At this point they can be ingested by a host and migrate to the small intestine where they develop into adult-stage worms and repeat the cycle. Because the maintenance and propagation of this worm is robust and cheap relative to other parasitic nematodes, it has been used for *in vivo* anthelmintic screening as previously mentioned with pyrantel and ivermectin. While others have described *in vitro* egg-hatch or larval assays with this worm in the past, usually to quantitate efficacy of various drugs, to our knowledge no one has described an assay that uses similar culture conditions or follows development of worms from embryonic stages to infectious larvae (Tritten, Nwosu, Vargas, & Keiser, 2012), (Wabo Pone et al., 2011), (Kone, Vargas, & Keiser, 2012).

H. bakeri eggs were isolated from overnight fecal collections of infected mice using a modified sucrose flotation adapted from a *C. elegans* protocol (Lewis & Fleming, 1995). Up to 20 grams of mouse feces was homogenized in 50ml H₂O by mechanical agitation with a spatula and passed through two layers of a gauze mesh (grade 40). The flow-through was distributed evenly into two 50mL conical tubes and filled with water, then pelleted by centrifugation at 600 x g for 2 minutes. The supernatant was carefully aspirated to 10mL and the pellet was resuspended in H₂O. The wash step was repeated until supernatant was nearly transparent. After the final wash, tubes were completely

aspirated and the pellets were re-combined in a total volume of 4mL ddH₂O. Of this, a 500uL aliquot was gently added to each of four 15mL conical tubes over 7.5mL of 30% sucrose and centrifuged at 600 x g for 5 minutes. From each tube, 1mL was extracted from the top where eggs were visible at interface between sucrose and water. These fractions were combined in a 15mL conical tube, raised to 15mL with water, pelleted by centrifugation at 600 x g, and aspirated down to the pellet. The eggs were sterilized by suspension in 5mL of a 10% bleach solution for 1 minute, neutralized by water, and washed five times as previously described, with sterile ddH₂O. The average yield for an overnight fecal collection from four mice infected with 200 worms at the peak of infection (about 3 weeks post-infection) ranged between 40-60 thousand eggs.

Eggs isolated in this manner were tested for optimal growth parameters identically to *C. elegans* as previously described. It was found that hatching and development were most optimal with 15-30 eggs per well, in the presence of 5μL OP50, and up to 0.25% DMSO in a total volume of 100μL Special S-medium (SS medium, pH = 7.3, no CaCl₂) incubated at 20°C. After 7 days of incubation under these conditions, approximately 75% of worms had developed fully to third-stage larvae and seemed entirely healthy in appearance and motility. *H. bakeri* larvae cultured in this fashion were used in multiple instances to infect mice, and resulting eggs were isolated and cultured as above to yield healthy larvae, ensuring the assay replicates the natural course of larval development without deleterious effects.

2.3 *A. ceylanicum* assays

Ancylostoma ceylanicum is a zoonotic hookworm species that is primarily endemic in Southeast Asia. It largely infects dogs and cats, but also plays a significant

role in human infections as it is harbored by a quarter to a third of human hookworm carries in endemic areas (Ngui, Ching, Kai, Roslan, & Lim, 2012), (Conlan, Sripa, Attwood, & Newton, 2011). *A. ceylanicum* is genetically a close relative of one of the primary human hookworms, *A. duodenale*, but is unique in that an infection can be transiently maintained in hamsters – rendering *A. ceylanicum* a potentially useful tool for studying human hookworm infections in laboratory settings (Ray & Bhopale, 1972). To date, experiments conducted with these worms have been done either by culturing eggs in feces followed by isolation of L3 larvae for assaying, or by culturing isolated eggs with an agar plate method (Tritten et al., 2011), (Reiss, Harrison, Bungiro, & Cappello, 2007), (Tritten, Braissant, et al., 2012). Figure 1 demonstrates the hookworm life cycle. To my knowledge, there is no reference in scientific literature to any method whereby isolated *A. ceylanicum* eggs are cultured in a microtiter plate and followed through development from eggs to infectious L3 larvae, making this assay the first of its kind.

A. ceylanicum eggs were isolated from infected hamsters and purified by a slightly modified NaCl flotation method (Mes, Eysker, & Ploeger, 2007). Up to 20 grams of an overnight fecal collection was soaked in 30mL 13%NaCl for 30 minutes with mechanical agitation with a spatula to homogenize the solution. This mixture was filtered through a metal sieve (grade 20). The flow through was transferred to a 50mL conical tube and centrifuged for 5 minutes at 1800 x g. Following isolation, eggs were washed with 10% bleach for 1 minute and re-washed 5 times with sterile ddH₂O. Hookworm Culture Media (HCM) consisting of RPMI-1640 culture medium supplemented with 25mM HEPES (pH 7.0), 100 IU/ml of penicillin, 100 mg/ml of streptomycin, 1ug/mL amphotericin B, and 50% FBS has been used in the past to culture adult hookworms, and

has been shown to help L3 hookworm larvae transition to the infectious L3i stage *in vitro* (Cappello et al., 2006), (Datu et al., 2008). HCM was selected as the culture medium and 15-30 isolated *A. ceylanicum* eggs per well were tested for optimal assay conditions as with the previous two nematodes. It was found that worms cultured in HCM with 5 μ L OP50, and 0.25% DMSO at 25°C for 7 days developed immaculately to infectious L3i larvae which could successfully be used to infect hamsters and propagate the hookworm life cycle.

2.3 Assay Validation: Three Drugs, Three Nematodes

With the establishment of a uniform assay to successfully culture *C. elegans*, *H. bakeri*, and *A. ceylanicum* through development of all of their free-living stages *in vitro*, it was next critical to validate the assay by showing that it was capable of detecting the effects of existing anthelmintics in a somewhat meaningful manner. For this proof-of-concept, three well-known anthelmintics from unique drug-classes were selected for dosage responses assays against the three nematodes. In addition to acting as a proof-of-concept, dosage response assays would prove useful in determining what concentration compound libraries should or could be screened at in the future. These drugs were albendazole, ivermectin, and pyrantel. While the mode of action of albendazole has not been identified with certainty, it most likely functions as an inhibitor of β -tubulin polymerization. It is believed that diminished microtubule polymerization in albendazole treated worms leads to an impairment in their ability to uptake glucose, eventually killing them by energy depletion. Ivermectin may function as an agonist of glutamate-gated chloride channels leading to CNS paralysis and death. Pyrantel also functions by CNS

modulation, but by acting as a nicotinic acetylcholine receptor agonist, which causes intense muscle contraction in the worms and eventually death.

Albendazole, ivermectin, pyrantel pamoate and dimethyl sulfoxide were purchased from Sigma-Aldrich (cat# A4673-10G, P6210-5G, I8898-250MG, and D8418-250ML, respectively). For these dosage response assays, 10mg of drug was serially diluted initially in 100% DMSO, and the final volume was raised to achieve desired dose with S-medium for *C. elegans* and *H. bakeri* assays or RPMI for *A. ceylanicum* assays. 10 μ L of drug solution was pipetted into 90 μ L of culture solution in each well, for a total of 100 μ L with a final concentration of 0.20% DMSO in all working conditions. Drugs were assayed at 0.2, 2, 20, and 200 μ g/mL. Nine wells per assay contained eggs cultured with 0.20% DMSO in the absence of drug as negative control. Upon experiment completion, if control worms failed to develop nominally or displayed ill health (low motility, discoloration, degradation, or death), the assay was discarded. This was a rare occurrence that could always be attributed either to external contamination or very old host animals in the case of the parasites (more than 1.5 months post-infection). Each experimental condition was done in triplicate per assay, and each assay was repeated at least three times. The assay plates were stored in a plastic container containing a 500mL beaker filled with sterile water, 100 IU/ml of penicillin, 100 mg/ml of streptomycin, and 1 μ g/mL amphotericin B. An electric pump external to the container provided a pressurized air supply directly into this reservoir. This served to increase humidity (and thus decrease evaporation from the plates) and prevent contamination of the cultures. Pictures of each experimental condition were taken by carefully pipetting well contents

into spot plates and using an eyelash pick to center the worms and remove any excessive debris.

At 72 hours post-incubation, 100% of *C. elegans* eggs in liquid media containing 0.2% DMSO have hatched and developed immaculately to L4/ young-adult stage worms (fig. 2A). Albendazole (fig. 2B) and pyrantel pamoate (fig. 2D) both display a dose-dependent response at the selected drug concentrations, with a visible reduction in normal development size of the worms correlating to an increase in drug concentration. Ivermectin shows no dosage response – it is equally potent at all of the selected doses, causing larval arrest at the L1 stage and paralysis (fig. 2C). None of the drugs in this assay exhibited lethality on *C. elegans* as the worms were visibly motile, or twitched in place when challenged with hot water (as with ivermectin treated worms). The soil-dwelling nematode responds most discernibly to ivermectin. Albendazole and pyrantel differ in terms of greater efficacy at different doses. For example at 2.0ug/mL, albendazole appears more efficacious than pyrantel, while at 200ug/mL pyrantel seems superior. None of the anthelmintics had any effects on the hatch rate of *C. elegans* (fig. 5A).

After 7 days post-incubation, greater than 75% of *H. polygyrus bakeri* eggs cultured in 0.2% DMSO successfully hatched and developed to L3 stage larvae (fig. 3A). At all of the selected doses, albendazole visibly inhibits hatch-rate, and any worms that do hatch in the lower dose wells display defective development and no motility (annihilation of all motility is assumed to be synonymous with worm death) (fig. 3B). Ivermectin acts identically at all doses, displaying developmental arrest and decreased motility, but worms are not entirely immobilized and there is no lethality (fig. 3C).

Pyrantel has a visible dosage response, with some worms slightly smaller than control visible at 0.2 and 2.0ug/mL, developmental arrest at 20ug/mL, and high lethality (diminished motility) at 200ug/mL (fig. 3D). It also inhibits hatching in a dose response fashion (fig. 5B). Note: pictures are not consistent with hatch-rate values in figure 3 as hatched and ill worms were preferably photographed where available.

At 7 days post-incubation, greater than 75% of *A. ceylanicum* eggs cultured in 0.2% DMSO successfully developed to L3 stage larvae (fig. 4A). Generally, *A. ceylanicum* responds to the anthelmintics in the same pattern as *H. bakeri*. Albendazole clearly has the strongest effect at all doses, with greater than 50% inhibition of hatching and complete lethality at the lowest dose, and complete inhibition of hatching at the highest dose (fig. 4B). Next, pyrantel causes developmental arrest and lethality at the intermediate doses and some inhibition of hatching at the highest dose (fig. 4D). Lastly, ivermectin is not lethal at any dose, but causes a stark L1 developmental arrest in the hookworm, as was the case with *C. elegans*. Both *H. bakeri* (fig. 5B), and *A. ceylanicum* (fig. 5C) display similar respective hatch-rate curves in response to the three anthelmintics, while the anthelmintics have no effect on the hatch rate of *C. elegans* (fig. 5A).

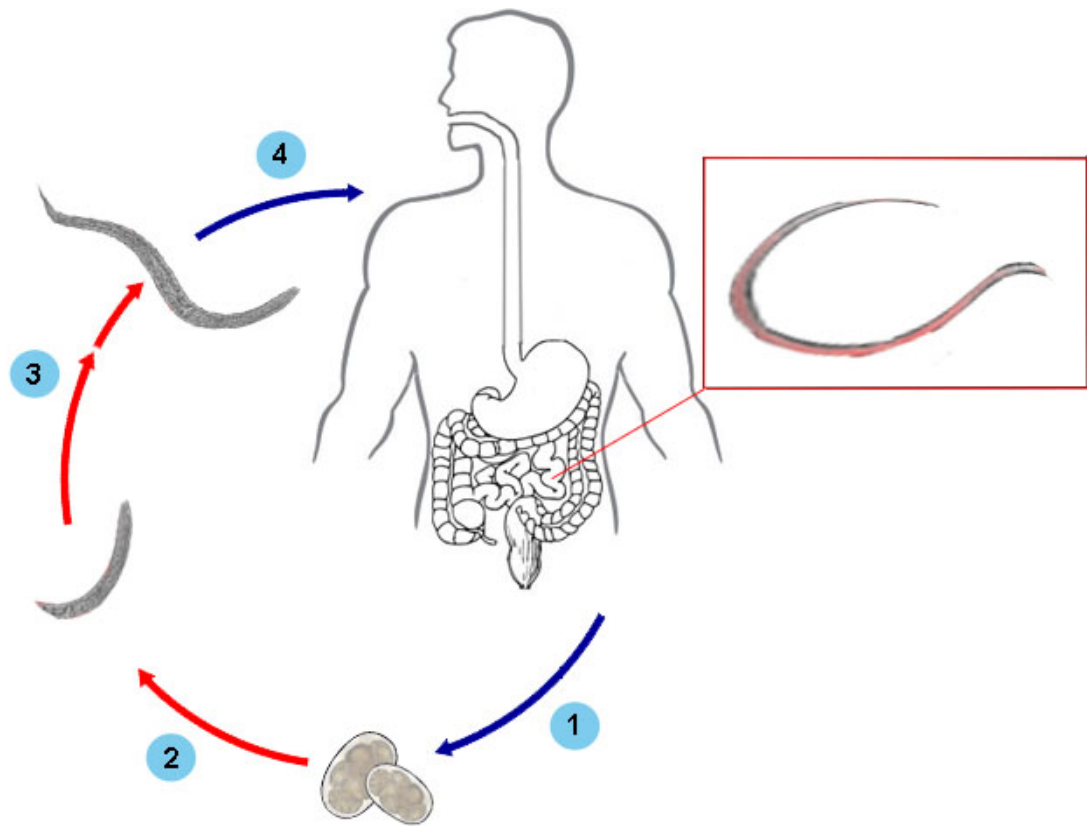


Figure 1: The hookworm life cycle. Adult stage hookworms residing in the host's intestinal tract lay eggs (1) which are passed into the environment upon defecation. Eggs hatch into rhabditiform larvae (2) after a few days. Within a week, these larvae can develop from L1, to L2, and finally to the final free-living stage, the infectious third-stage filariform larvae (3). Worms at this stage may enter a host either through the oral route from contaminated food/ water or by penetration of the skin (4). If entry is through the skin, worms migrate to bronchial cavities where they are coughed up and swallowed, eventually finding their way to the small intestine. Here, they complete their development to the adult stage and begin laying eggs to complete the cycle (1).

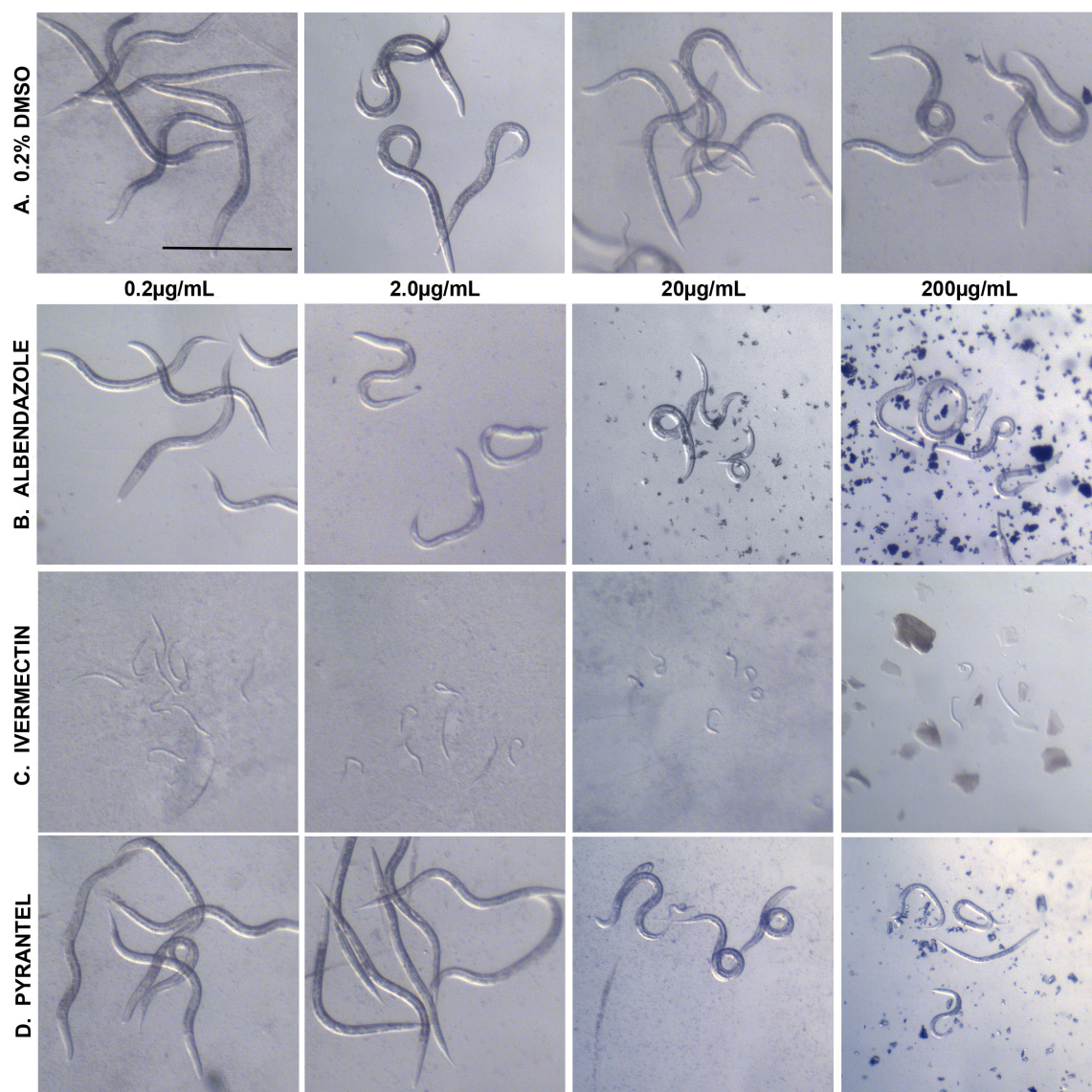


Figure 2: Dose-response of *C. elegans* to a variety of known anthelmintics. *C. elegans* eggs were cultured in the presence of B) albendazole, C) ivermectin, and D) pyrantel pamoate in a tenfold dose-response fashion: 0.2-200 µg/mL from left to right. A) Worms cultured in 0.2% DMSO in the absence of drug served as negative control. The scale bar in top left panel is 0.5mm. Note that particulate matter in 200 µg/mL panels consists of drug that has precipitated out of solution.

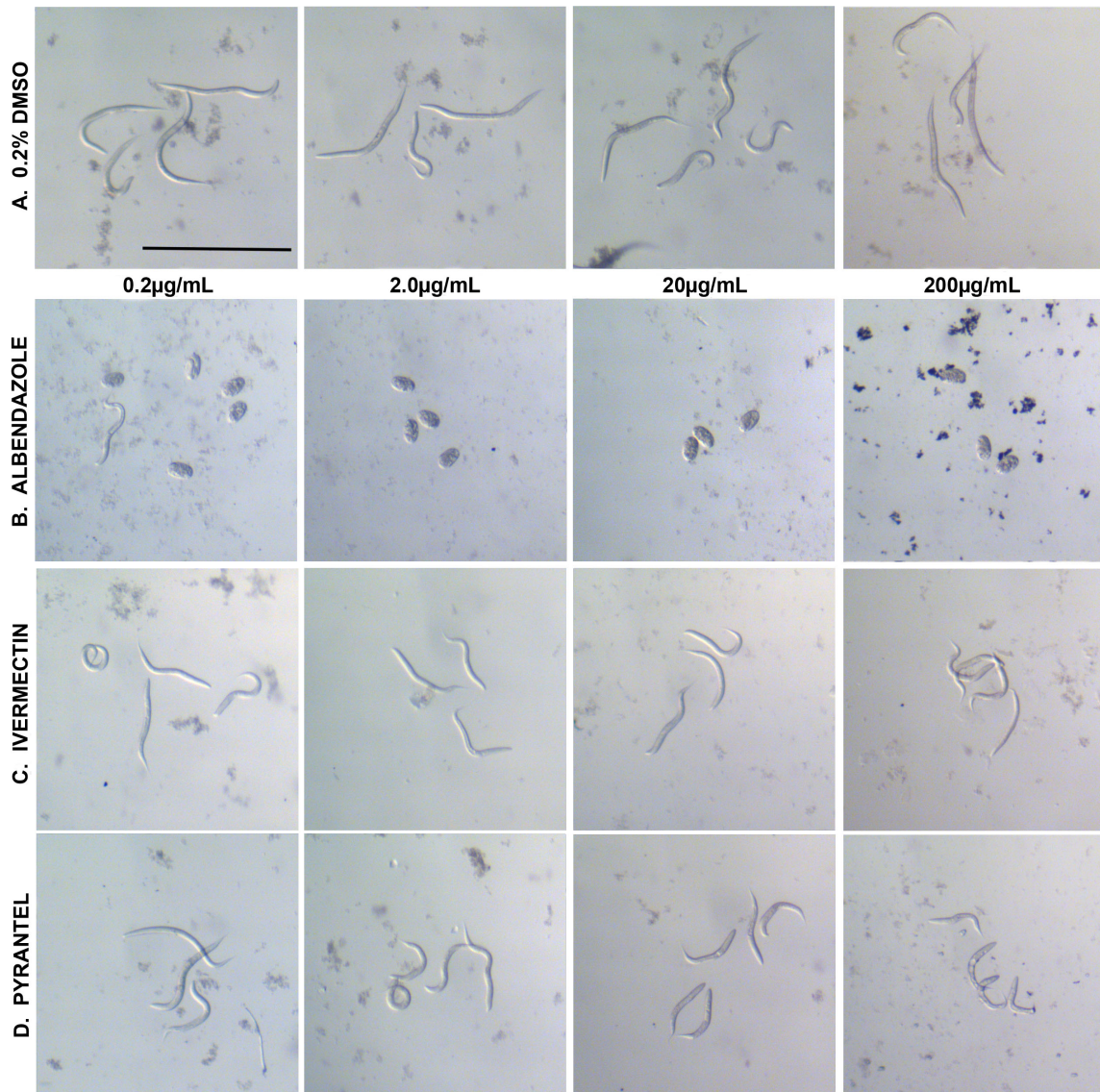


Figure 3: Dose-response of *H. bakeri* to a variety of known anthelmintics. *H. bakeri* eggs were cultured in the presence of B) albendazole, C) ivermectin, and D) pyrantel pamoate in a tenfold dose-response fashion: 0.2-200 µg/mL from left to right. A) Worms cultured in 0.2% DMSO in the absence of drug served as negative control. Scale bar in the top left panel is 0.5 mm. Note that images are not representative of hatch rates at each given dose – hatched or ill worms were preferentially photographed.

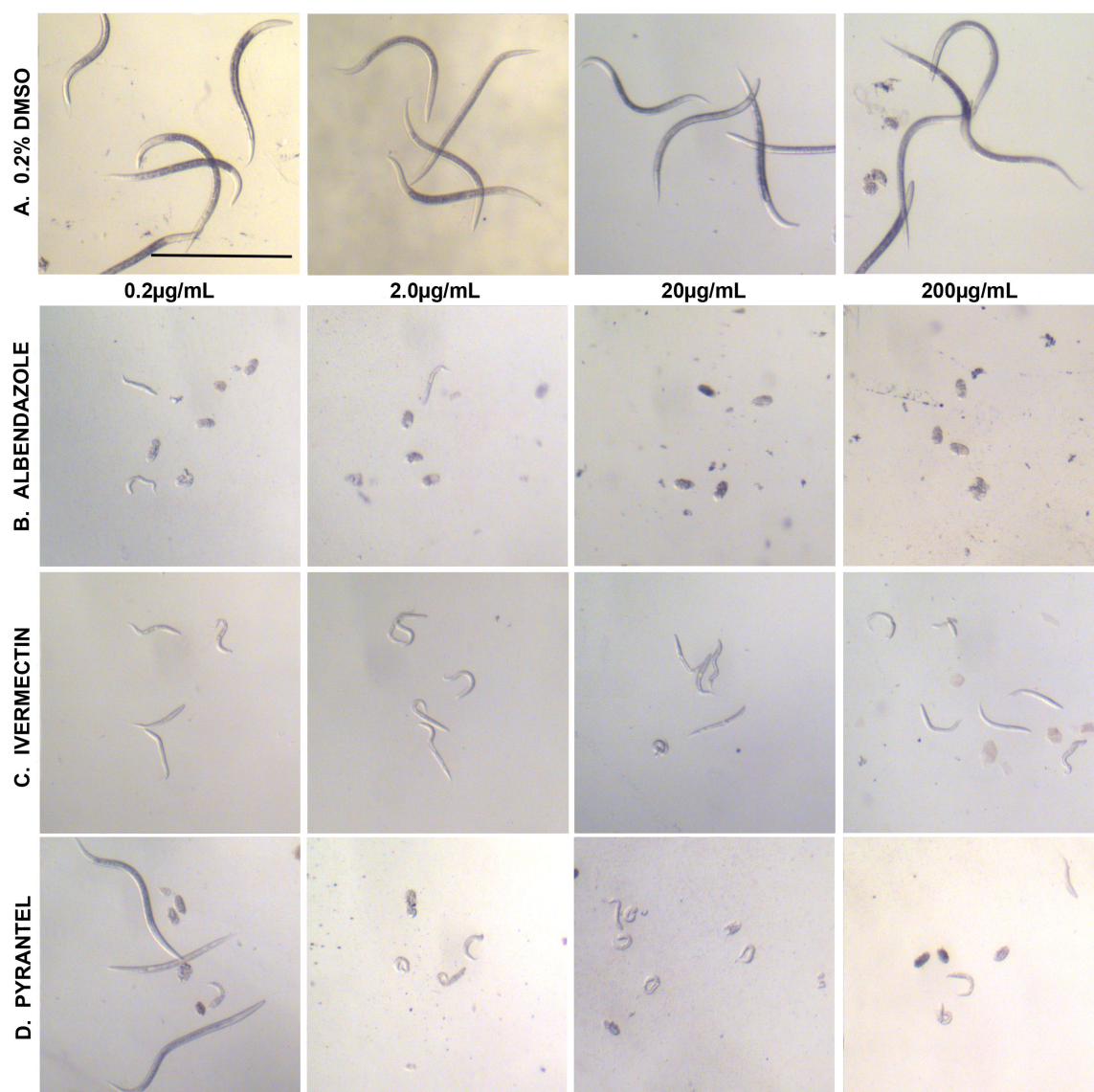


Figure 4: Dose-response of *A. ceylanicum* to a variety of known anthelmintics. *A. ceylanicum* eggs were cultured in the presence of B) albendazole, C) ivermectin, and D) pyrantel pamoate in a tenfold dose-response fashion: 0.2-200 µg/mL from left to right. A) Worms cultured in 0.2% DMSO in the absence of drug served as negative control. Scale bar in top left panel is 0.5mm. Note that images are not representative of hatch rate at each given dose – hatched or ill worms were preferentially photographed.

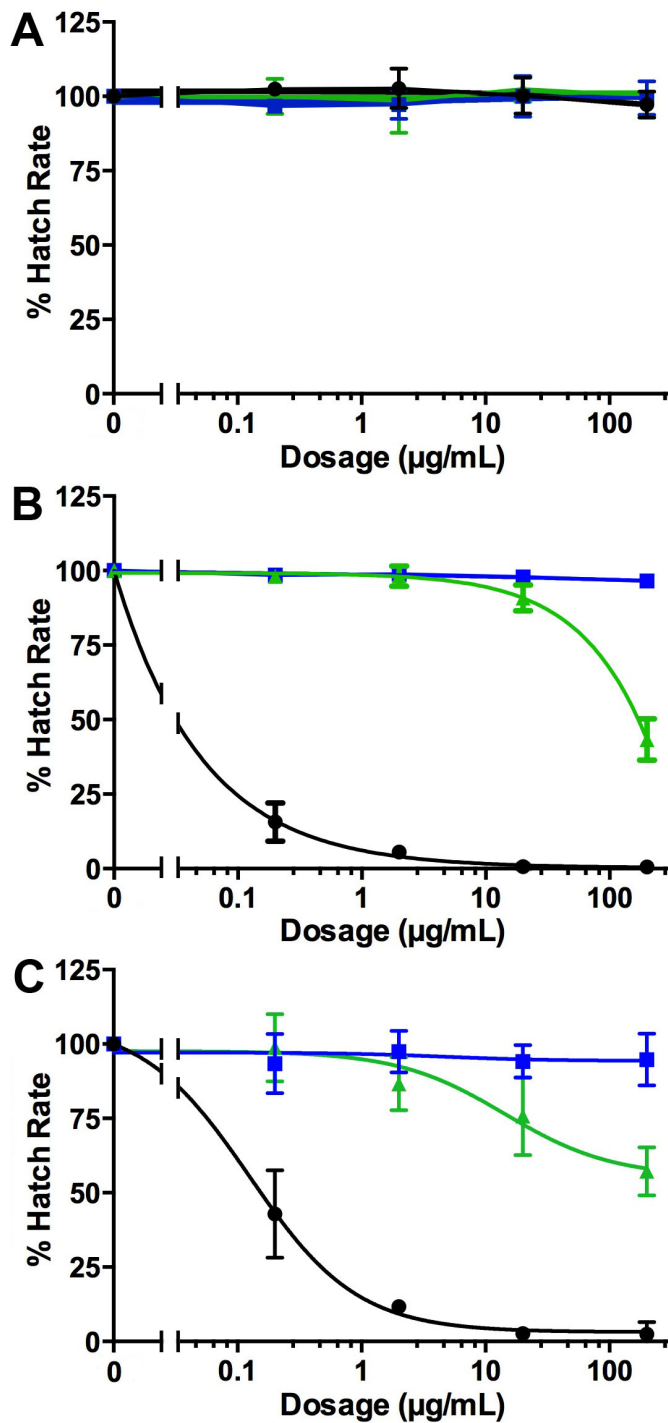


Figure 5: Dose-response hatch rates of three nematodes in response to three drugs. The hatch rates of *C. elegans* (A), *H. bakeri* (B), and *A. ceylanicum* (C) are displayed when assayed against albendazole (black), ivermectin (blue), and pyrantel pamoate (green). Values are calculated as a percent normalized to control (no drug, 0.2% DMSO).

2.4 Development of a Preliminary Whipworm Assay

Whipworm infections account for up to a third of STH infections, and of the three groups of parasites, they are the most lacking in terms of a satisfactory single-dose curative chemotherapy (Bethony et al., 2006). Consequently, any anthelmintic discovery effort would arguably have the greatest impact if it could contribute to the discovery of a compound with high-efficacy against whipworms. The primary organism implicated in human whipworm infections is *Trichuris trichiura* (Keiser & Utzinger, 2010). The phylogenetic relatedness of this organism to the mouse whipworm *Trichuris muris* has encouraged the use of *T. muris* as an infection model for human whipworms (Klementowicz, Travis, & Grencis, 2012). The whipworm life cycle is unique from the other STHs in that it does not have any free-living larval stages. Rather, parasitic eggs are consumed and hatch within the small intestine of the host organism. Notably, this makes it difficult to culture eggs into larvae within an *in vitro* screening system.

The recent revelation that hatching of whipworm eggs can be induced by various cues which collectively serve to imitate *in vivo* conditions has facilitated the opportunity to create a viable *in vitro* larval assay (Hayes et al., 2010). Building on the work of Hayes and colleagues, we tested hatching of whipworm eggs under variable conditions in 96-well U-bottom plates as was previously done with *H. bakeri* and *A. ceylanicum*. Parameters tested included incubation of isolated eggs in the presence of particulate or homogenized intestinal and colon extracts, cultured in HCM with variable ratios of RPMI to FBS, in the presence of various individual or combinations of gram-negative (OP50, JM101, HT115, DH5 α , XL10-GOLD) and gram-positive (*S. aureus*, *S. aureus* α -mutant, *B. cereus*, *B. subtilis*) bacteria, and a range of DMSO concentrations (0-2%). To further

mimic *in vivo* conditions, we additionally tested the effects of hypoxic environments on hatch-rate.

The whipworm eggs were not isolated from feces. Rather, infected mice were scarified after 22 or more days post-infection and their large intestine was harvested. Intestines were sliced longitudinally to expose the inner membrane and placed into 37°C pre-warmed HBSS. These were placed in a 37°C incubator (5% CO₂) for 1-2 hours, which allowed the adult worms to dislodge from the intestines. Worms were individually picked into pre-warmed culture media with up to 10 males and 10 females per well in a 6-well plate (5mL total culture per well, RPMI+HEPES, 100U pen/strep, 1µg/mL amphotericin B). After 24, 48, and 72 hours, worms were picked into new wells and media from old wells containing eggs was combined and centrifuged at 600 x g for 2 minutes. Pelleted eggs were then rewashed 5 times with sterile drinking water. The concentration of eggs was diluted to less than 4,000 eggs/mL and transferred to a p100 plate and wrapped in foil. The eggs were allowed to embryonate for 5-6 weeks and then stored for up to 6 months at 4°C.

Optimizing a uniform set of parameters for a whipworm developmental assay proved more difficult than previously optimized parasite assays. Batches of eggs harvested from different strains of mice seemingly responded differently to each of the assay parameters. For example, *T. muris* eggs isolated from AKR mice displayed a high hatch-rate and reasonable motility with 5µL OP50 in 100µL total culture (OD_{600nm} = 3). On the other hand, eggs from STAT-6^{-/-} mice had the best combined hatch-rate and motility outcome when cultured with a 5µL cocktail of *S. aureus*, OP50, and *B. cereus* (var. vietnami) in a 3:1:1 ratio respectively. Consistently, eggs from infected AKR mice

had a higher hatch-rate than those from STAT-6^{-/-} mice with their respective culture conditions (98.7% and 82.4%, respectively; average from three trials, no statistical analysis available). Furthermore, eggs harvested from different groups of mice of the same strain (AKR for example) showed consistent hatch-rates within a group when repeatedly tested, but there was variation between different groups. At this time, it is unclear what precise factors contribute the differences in viability of eggs isolated from different strains of mice. A common predictor of hatching was visible approximately 3-4 hours post-incubation. Eggs that would go on to eventually hatch would aggregate at the center of each well, seemingly attached at their opercula. Those that did not hatch remained scattered in the wells. This is consistent with the finding of Hayes and colleagues that bacterial fimbriae attach at the poles and induce egg hatching.

The following optimal parameters were selected: 20-30 eggs per well (isolated from worms harbored in AKR mice), cultured in a total of 100μL HCM (section 2.3), with 5μL OP50, and 0.2% DMSO. Assays were incubated at 37°C and oxygen was depleted (BD GasPak cat# 260001). The assays were examined after 24 hours of incubation and larvae in control wells displayed non-stimulated motility for up to 3 minutes after removal from the incubator. Incubation at 37°C for 30 minutes restored motility, and larvae were viable for up to 5 days. An existing *T. muris* assay with similar, but not identical parameters was recently published (Klementowicz et al., 2012). The group that developed this assay additionally controlled for pH and experimented with the presence of host stomach enzymes to stimulate hatching, but did not find these to have an effect. Other notable differences in their assay include the absence of FBS from culture

media and no control of oxygen saturation in the culture. This group reports a hatch-rate of approximately 70%, while our assay attains approximately 98% hatching.

A dose-response was conducted with these optimal parameters similar to the other nematode assays with the addition of levamisole and oxantel. Oxantel is an N-type nAChR-agonist and a derivative of pyrantel that has been shown to have reasonable efficacy against whipworm infections in the past (Martin, Clark, Trailovic, & Robertson, 2004). After 24 hours of incubation, *T. muris* eggs in negative control wells hatched and were visibly motile (fig. 6a). Albendazole (fig. 6b) and ivermectin (fig. 6c) had no visibly significant effects on hatching or motility of the worms at any dose. No image is available for worms treated with albendazole at 200µg/mL due to diminished visibility from drug precipitation and inability to manipulate worms due to size. Pyrantel, levamisole and oxantel (in order of increasing activity) had a significant impact on the hatch-rate of whipworm eggs. Due to limited resources, these values are an average of only two trials. This is a preliminary assay with the potential to be a useful screening tool with more work.

2.5 Maintenance of Parasitic Life Cycles

Three-week-old male golden Syrian hamsters of the HsdHan:AURA outbred strain and five-week- old female Swiss Webster white mice were purchased from Harlan Laboratories (USA). All animals were kept in makrolon cages under environmentally controlled conditions and had free access to water and rodent food. After a 3-day acclimation period, hamsters were infected with 150-180 infectious stage (L3i) *A. ceylanicum* and mice were infected with 200 *H. bakeri* L3i by oral gavage. The *A. ceylanicum*, and *H. bakeri* life cycles have been maintained at UCSD since June 2009,

January 2009, respectively. The UCSD Institutional Animal Care and Use Committee (IACUC) approved this research, and the protocol numbers are S09067 and S08140, respectively. The maintenance and care of experimental animals complied with the University of California's Animal Care Program's guidelines for the humane use of laboratory animals.

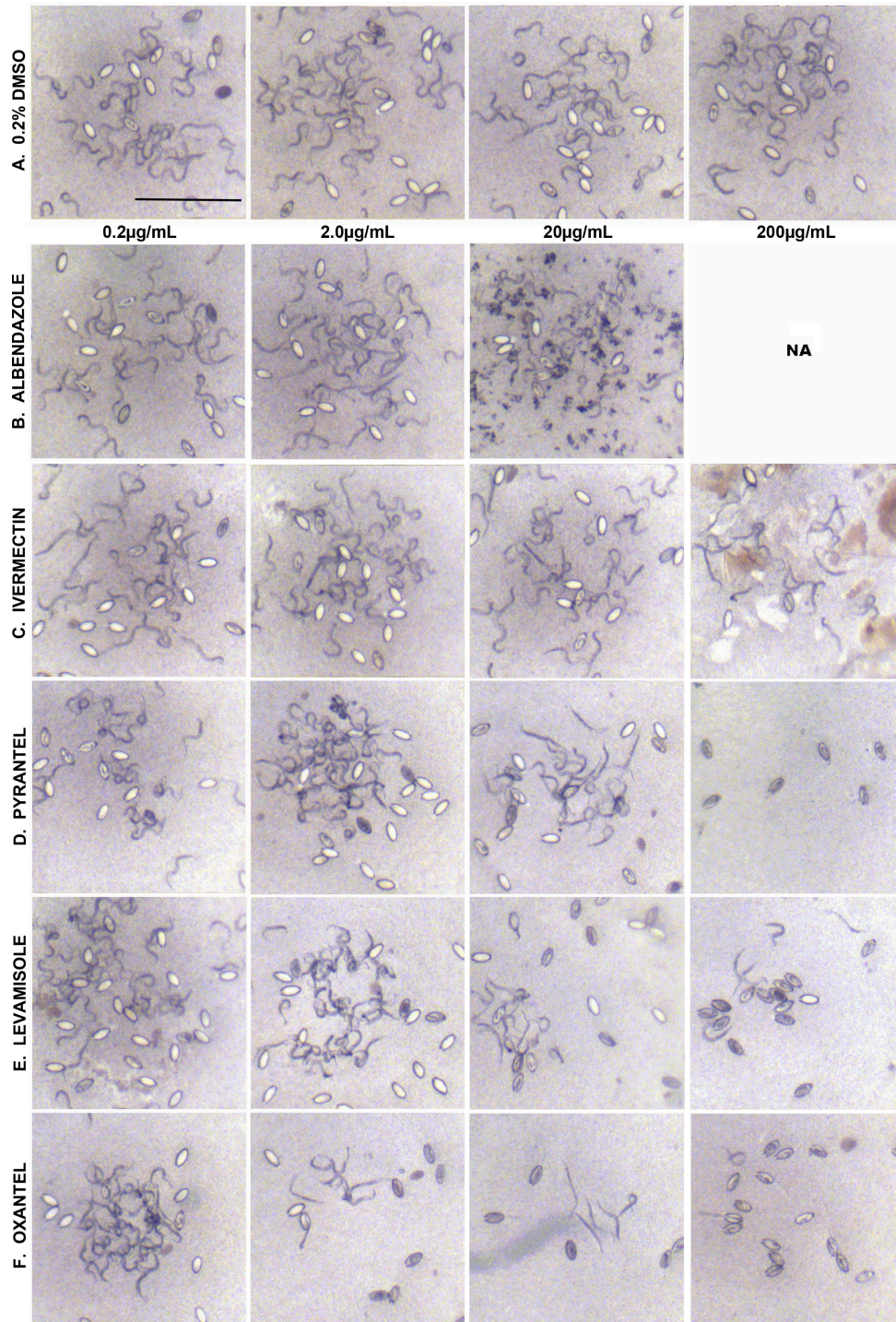


Figure 6: Dose-response of *T. muris* to a variety of known anthelmintics. *T. muris* eggs were cultured in the presence of B) albendazole, C) ivermectin, D) pyrantel, E) levamisole, and F) oxantel in a tenfold dose-response fashion: 0.2-200µg/mL from left to right. A) Worms cultured in 0.2% DMSO in the absence of drug served as negative control. Scale bar in the top left panel is 0.5mm. Photographed in wells, not spot plates.

CHAPTER 3

DEVELOPMENTAL ASSAY FOR LIBRARY SCREENING

After validation with the dosage response experiments, it was clear that the assay had matured into a basic platform with the capacity to screen compounds for anthelmintic activity against the nematodes *C. elegans*, *H. bakeri*, and *A. ceylanicum*. A general outline for a potential approach to anthelmintic screening against larval parasites is provided in figure 8. A primary screen may be conducted against larval stages, and a handful of uncovered hits can be prioritized due to apparent potency, toxicity against mammalian cells, and characteristic pharmaceutical chemistries of interest. These would then be tested in hit-to-lead follow-up studies *in vivo* to better rank potential candidates for drug development.

With a potential biodiversity of over 1,000,000 marine nematode species, we hypothesized that some marine bacterial organisms may have evolved nematode-specific toxins as a means to avoid predation (Heywood, Watson, & United Nations Environment Programme., 1995). As such, a small library of 640 marine Actinomycete compound mixtures was initially selected for screening purposes. Screening this library against the

three nematodes independently would provide some idea of the general rate of hits that overlap with all three worms or are unique to one nematode.

3.1 Screening Nematodes Against a Marine Compound Library

The marine compound library stock solutions consisted of extract mixtures (metabolites) from marine Actinomycetes in each well concentrated at 25mg/ml in 100% DMSO. From each stock, 0.5 μ L was pipetted into the corresponding assay well of a 96-well plate containing 200 μ L culture media, OP50, and nematode eggs. From this plate, 100 μ L per well was transferred to a parallel 96-well plate. The final mixture and DMSO concentration in all working conditions were 62.5 μ g/mL and 0.25%, respectively. Negative controls were no OP50 (starved), no DMSO, and 0.25% DMSO. The positive control was 50 μ g/mL pyrantel pamoate in 0.25% DMSO. Each control had four replicates per plate. Library compounds were only considered positive hits if they hit in both parallel plates and were confirmed by a single repetition in two additional parallel plates. A well was characterized as a hit when it caused inhibition of hatching, larval developmental arrest, or death (assumed from no motility and cuticle degradation). The assays were scored using a binocular dissecting microscope (objective 10/20/40x, eyepiece 2x).

The screen identified a total of nine compounds with potential nematicidal activity. Of these, 3 hit on all of the nematodes, 2 hit on both *A. ceylanicum* and *C. elegans*, 2 were unique to *A. ceylanicum*, 1 was unique to *C. elegans*, and 1 was unique to *H. bakeri* (summarized by the Venn diagram in figure 7). The compounds of interest selected from this screen would ideally be killing worms because of some specific anti-nematode properties and not just because they are generally toxic. The Fenical Lab

previously conducted toxicity screens of a collection of these metabolites against a human carcinoma cell line, HCT-116. Of the compounds that hit on *A. ceylanicum*, those that displayed toxicity against HCT-116 or had no toxicity data available were disregarded, leaving 138-F3 and 142-E6 as the only two fractions of interest. The former was detected in screens by all three nematodes, while the latter was detected only with the hookworm screen. This data summarized in table 1.

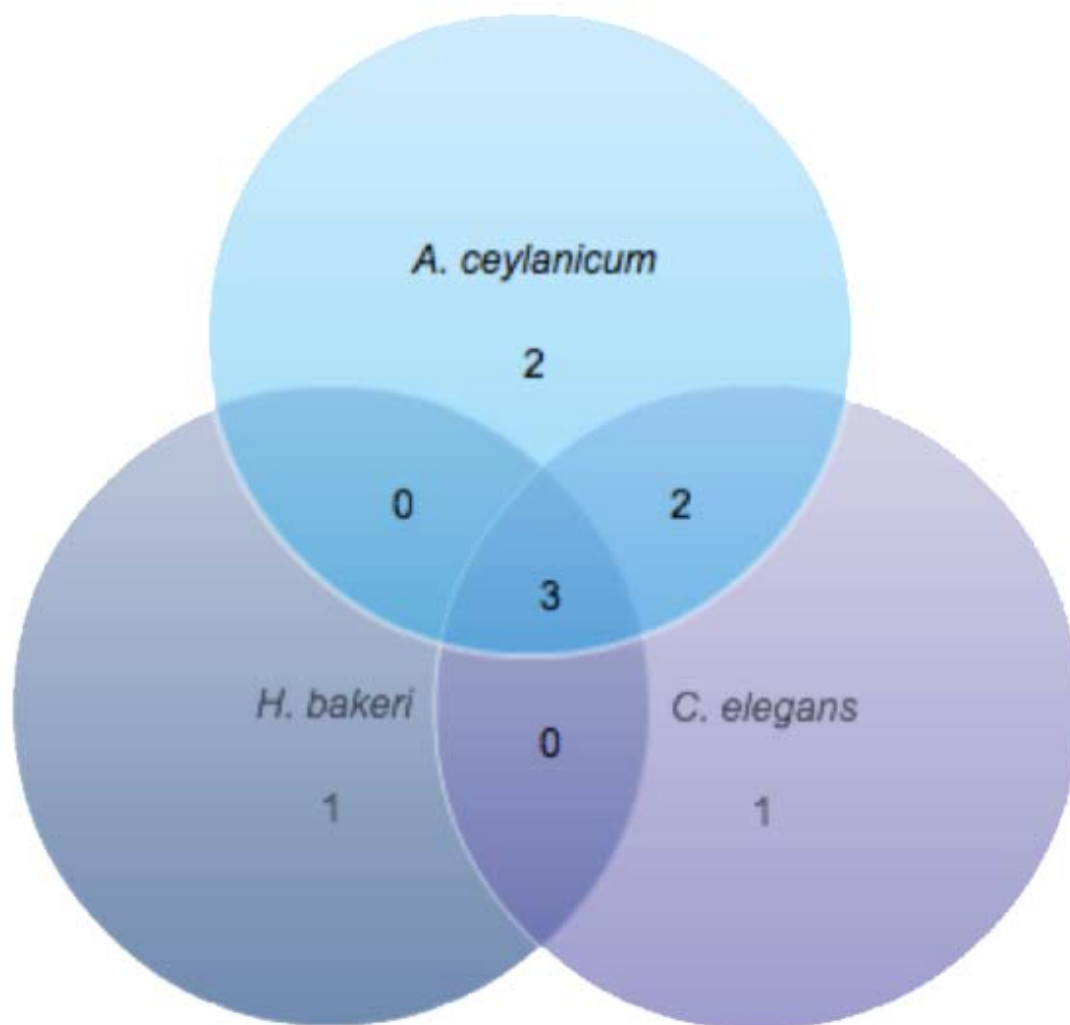


Figure 7: A visual demonstration of marine library hits overlapping in developmental screens of three nematodes. Numbers in individual circles represent number of fractions that hit only on the individual respective nematode. Numbers in the overlapping space of two or more circles indicate fractions that hit on two or more respective nematodes.

Table 1: A summery of active fractions found. Ac, Hb, and Ce are abbreviations for *A. ceylanicum*, *H. bakeri*, and *C. elegans*, respectively. And “X” in a given box indicates that the fraction had activity against the respective nematode and was considered a hit. Toxicity information for the fractions indicated Inhibitory Concentration (50%) values against HCT-116. NSA means no significant activity was detectable.

MCL Hit	Ac	Hb	Ce	Toxicity
136-G1	X	-	-	IC50=6.478
136-H6	-	X	-	No Data
137-F2	X	-	X	No Data
138-C4	X	X	X	No Data
138-F3	X	X	X	NSA
139-F6	-	-	X	NSA
140-B6	X	X	X	IC50=0.076
140-F3	X	-	X	IC50=0.076
142-E6	X	-	-	NSA

3.2 Screening Nematodes Against LOPAC

Upon inspection, data from the marine metabolite screen proved insufficient to make general assumptions about the *C. elegans* and *H. bakeri* assays in terms of predictive capacity for identifying compounds that would hit using the *A. ceylanicum* assay. Sigma's Library of 1280 Pharmaceutically Active Compounds (or LOPAC-1280) was chosen to provide more insight through a second round of screening. The LOPAC library stocks contained 10mM individual compounds per well dissolved in 100% DMSO. A primary screen of the LOPAC-1280 was conducted against *A. ceylanicum* using the same protocol used to screen the marine metabolites, with final drug concentrations diluted to 25 μ M in 0.25% DMSO in each well. This initial screen uncovered 53 compounds with potential anthelmintic activity against hookworms. Amongst these, the only two anthelmintics in the library (levamisole and ivermectin) were blindly detected as hits, serving as further positive affirmation of the assay.

The collection of 53 compounds was consequently screened using the *C. elegans* and *H. bakeri* assays. *A. ceylanicum* assays were set-up parallel to each experiment to insure that compounds retained efficacy after each freeze-thaw cycle. Those that lost efficacy against *A. ceylanicum* through the course of the experiment were discounted from the study, bringing the final count to 50 compounds. Of these 20 compounds (40%) were detected by the *C. elegans* assay and 11 compounds (or 22%) were detected by the *H. bakeri* system with both positive and negative hits confirmed by repetition (fig. 9). The compounds fell into four categories: those that also hit on *C. elegans* (fig. 10B), those that also hit on *H. bakeri* (fig. 10C), those that hit on all three nematodes (fig. 10A),

and perhaps most importantly those that hit only on *A. ceylanicum* (fig. 10D). The complete list of compound and their activity against the nematodes is provided in table 2.

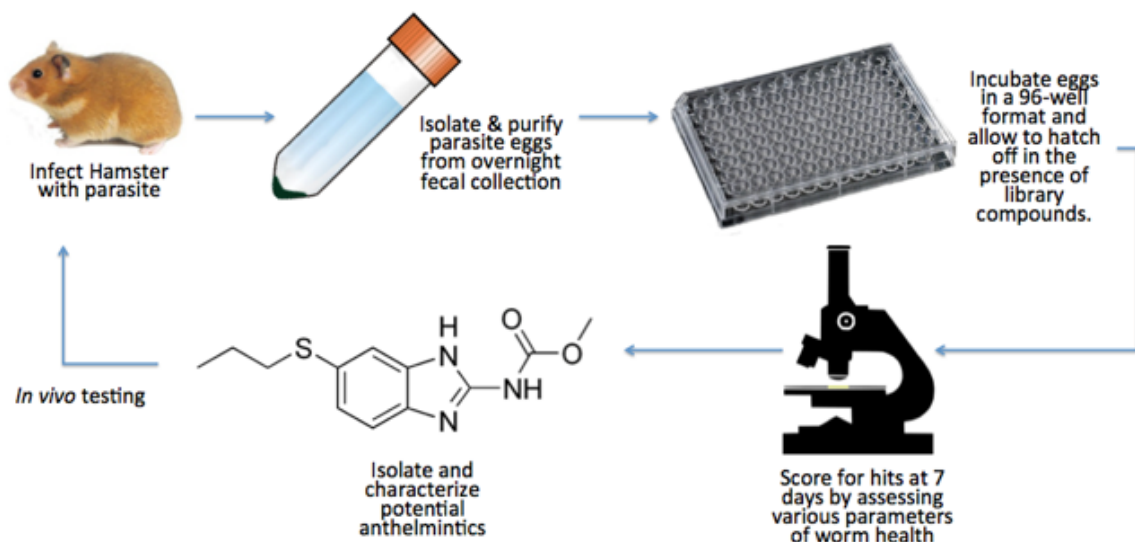


Figure 8: Proposed model for compound screening against larval parasites. Infected hamsters yield eggs (1) which can then be isolated and purified (2). These can be cultured in the presence of various compounds (3) and screened for viability (4). Compounds with apparent activity against the nematodes (5) can be ranked and a handful of promising candidates can be tested *in vivo* (6).

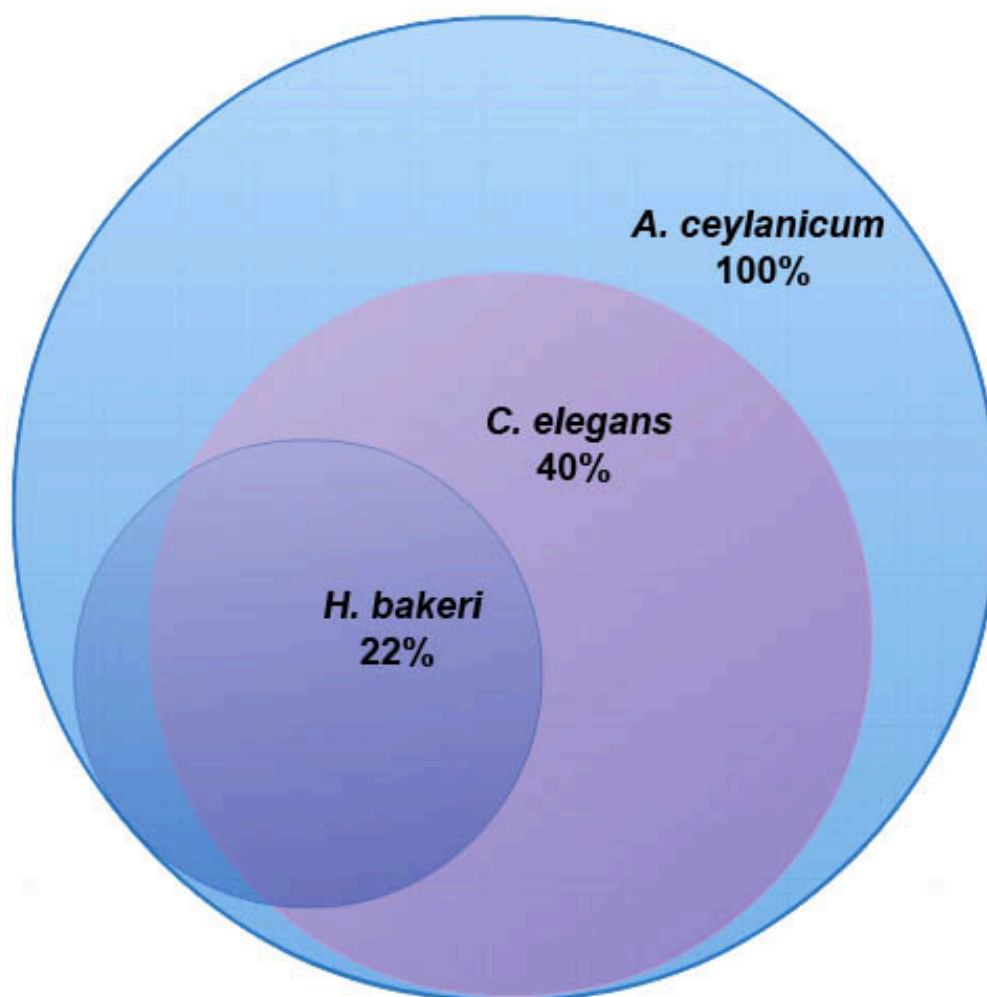


Figure 9: Percent overlap of LOPAC-1280 hookworm hits on *C. elegans* and *H. bakeri*. A primary screen was conducted against *A. ceylanicum* (by definition 100% of hits, largest circle) and these were assayed in secondary screens with *C. elegans* (middle circle) and *H. bakeri* (smallest circle, 22%).

Table 2: Summary of LOPAC hits. Hits are marked with “X.”

Well	Compound (original hits)	<i>A. ceylanicum</i>	<i>C. elegans</i>	<i>H. bakeri</i>
2-B6	Paroxetine hydrochloride	X	X	-
2-D3	Amitriptyline hydrochloride	X	-	-
2-D7	Amoxapine	X	-	-
2-E8	Alaproclate hydrochloride	X	-	-
2-G5	Lercanidipine hydrochloride hemihydrate	-	-	-
2-H2	ABT-418 hydrochloride	X	-	-
3-A5	Brefeldin	X	-	-
3-B7	CP-100356 monohydrochloride	X	-	-
3-D3:	Benazoline	X	-	-
5-B3:	CGS-12066A maleate	X	X	X
5-B9	Dequalinium chloride hydrate	X	X	-
5-E8	Diphenyleneiodonium chloride	X	X	-
5-H7	SANT-1	X	-	-
6F11	Emetine dihydrochloride hydrate	X	-	-
6H6:	AC-93253 iodide	X	-	X
6-H7	2,3-Dichloro- α -methylbenzylamine	X	-	-
7-A5	Eticlopride hydrochloride	X	-	-
7-C10	GW2974	X	-	-
7-H9	Fluoxetine hydrochloride	X	-	-
8-C4	Haloperidol	-	-	-
8-E9	Hexahydro-sila-difenidol hydrochloride	X	-	-
9-B10	VER-3323	X	X	-
9-B8	U73343	X	-	-
9-E11	Levamisole hydrochloride	X	X	X
9-E5	Indatraline HCl	X	X	X
9-F4	Ivermectin	X	X	X
9-F5	Iofetamine HCl	X	-	-
10-B4	Nocodazole	X	X	-
10-D3:	L-703,606 oxalate salt hydrate	X	X	X
10-F10	Mitoxantrone	X	X	X
11-B3	Maprotiline hydrochloride	X	-	-
11-B5	Nemadipine-A	X	X	-
11-G11	Sertraline hydrochloride	X	X	X
11-G3	Methoctramine tetrahydrochloride	X	-	-
11-G9	Nortriptyline hydrochloride	X	-	-
12-E6	Pentamidine isethionate	X	X	X
13-A11	Quinacrine dihydrochloride	X	-	-
13-B5	Protriptyline hydrochloride	X	-	-
13-C4	PD173952	X	X	-
13-C5	Pergolide methanesulfonate	X	-	-
13-H3	Phorbol 12-myristate 13-acetate	X	X	-
13-H5	Prochlorperazine dimaleate	X	X	-
14-B11	PD-166285	X	X	-
14-B7	SKF 86466	X	-	-
14-D2	Ruthenium red	X	-	-
14-F2	Rutaecarpine	X	-	-
14-H3	Rotenone	X	X	X
15-G11	Aprindine hydrochloride	X	-	-
15-H5	Tetramisole hydrochloride	X	X	X
16-A9	Vincristine sulfate	X	-	-
16-C9	Vesamicol hydrochloride	-	-	-
16-H5	CP-31398	X	-	-
16-H7	U-101958	X	-	X

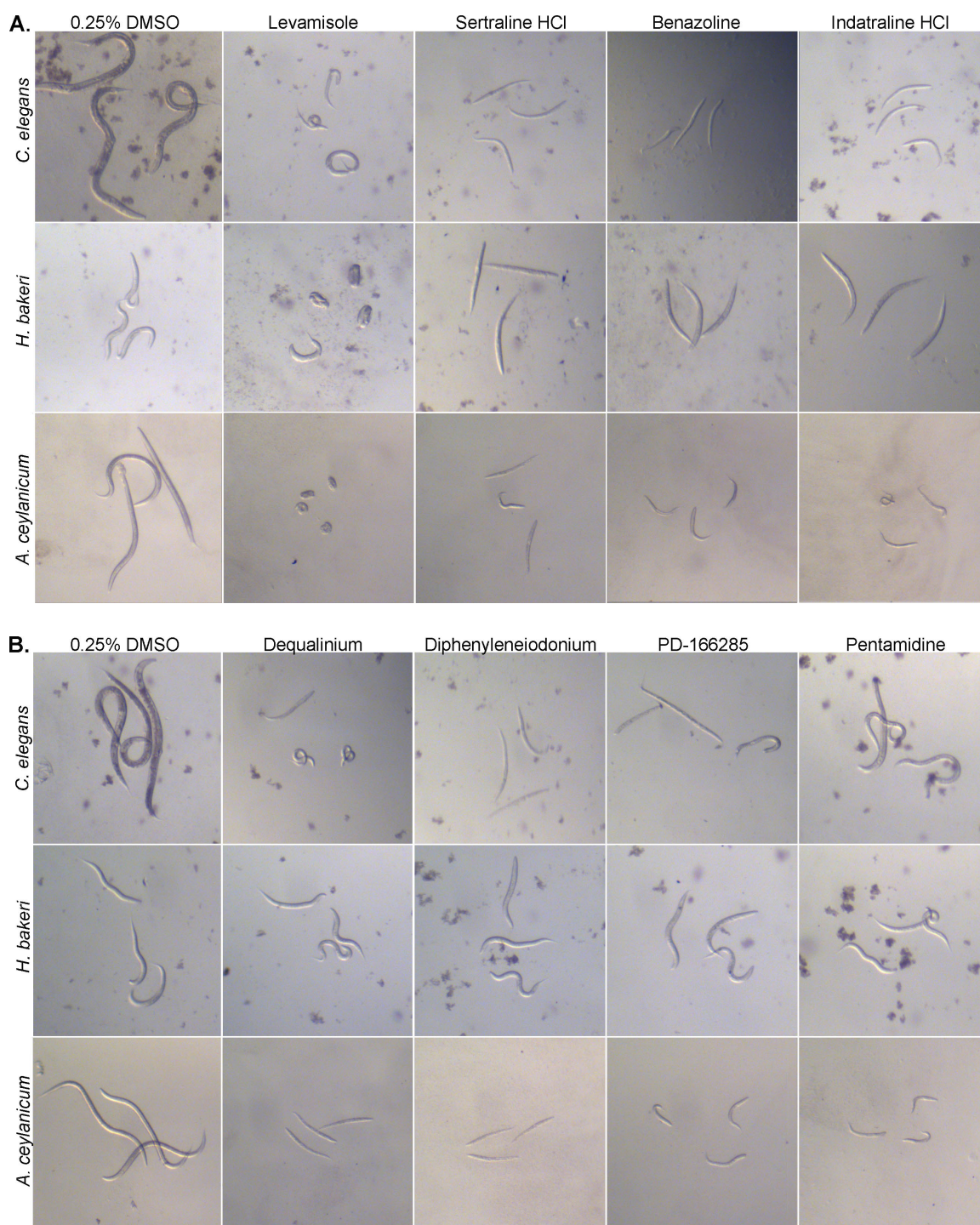


Figure 10: Examples of hits from LOPAC-1280. Pictures of compounds that (in addition to *A. ceylanicum*) hit on *C. elegans* and *H. bakeri* (A), *C. elegans* only (B), *H. bakeri* only (C), or only hit on *A. ceylanicum* (D).

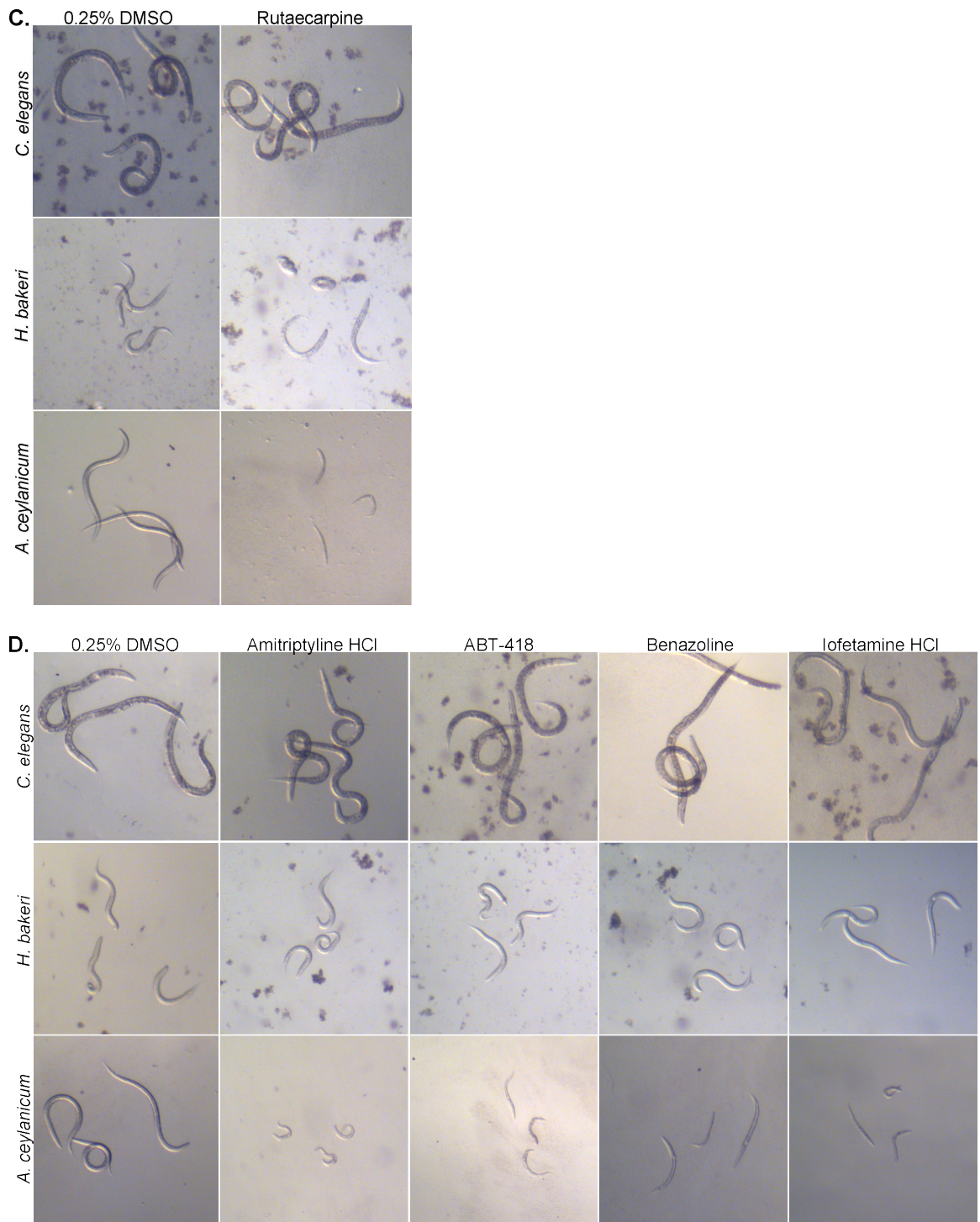


Figure 10 continued.

CHAPTER 4

DISCUSSION

I demonstrate here the degree of variability to which nematodes of different species will react to different compounds in multiple small library screens. By my estimation, when vivarium and host-animal costs are accounted for, the costs of nematode maintenance for *C. elegans*, *H. bakeri*, and *A. ceylanicum* are 1, 6, and 26 cents per compound screened. Clearly, the low cost of the former two nematodes would be a motivating factor in utilizing one of them for large-scale anthelmintic screens, not to mention bypassing BSL-2 biosafety concerns with the use of *C. elegans*. My screen of 640 fractions from a library of marine metabolites identified two significant compounds with activity against a human hookworm, only one of which was detected in alternative nematode assays. Another screen of a small library of pharmaceutically active compounds demonstrated that *C. elegans* replicated 40% of hookworm hits, and *H. bakeri* only 22% (or 60% and 78% rate of false-negatives, respectively).

Thus *C. elegans* presents a financially more feasible screening system, but at the cost of potentially missing a large number of active compounds. The next revolutionary anthelmintic with the potential to drastically improve the condition of people in the developing world may very well be overlooked if it is screened in *C. elegans* and falls

into that group of false-negatives. Using either the mouse parasite or soil-dwelling nematode alone in the LOPAC screen would have missed at least 3 major drug classes with multiple compounds that showed repeated anthelmintic activity against hookworm larvae, such as the tricyclic antidepressants (amitriptyline, amoxapine), norepinephrine reuptake inhibitors (maprotiline, protriptyline), and the muscarinic acetylcholine receptor antagonists (hexahydro-sila-difenidol, methoctramine). This makes it blaringly clear that using any system to screen for anthelmintics other than one that incorporates relevant parasites is likely a flawed approach.

Additionally, I have demonstrated a robust novel assay for *in vitro* drug screening against free-living stages of *A. ceylanicum*, a zoonotic human hookworm. This unique assay follows eggs hatched off in the presence of compounds through development to infectious L3 stage. Under the conditions of this assay, control worms always exhibit greater than 75% hatch-rate and development to L3i within 5 days of incubation. The assay is unique in that it looks at a broader, more metabolically active range of the parasitic life cycle, compared to existing egg-hatch or L3 assays. All of the free-living stages of the parasite occur in the same place, allowing for broader detection of sensitivity to drugs versus assays looking at a single developmental stage alone. In most cases where compounds are shown to have anthelmintic activity, they intoxicate the parasites shortly after hatching, during early larval development. Therefore, hits detected in this assay screen have demonstrated distinctive characteristics of early larval arrest and death – a hit or miss phenotype that essentially lacks subjectivity when compared to negative controls. The assay cannot necessarily deduce information about how these drugs will affect adult worms *in vivo*, but seemingly has the capacity to detect

anthelmintic activity of compounds on some level as demonstrated with the dosage response assays. Consequently, this assay can be used as a primary screening method to select potential candidates for hit-to-lead follow up studies in true infection models. The assay is currently a moderate-throughput approach to anthelmintic screening with the capacity to screen 1000 compounds per week, at most. When combined with an automated liquid dispensing system and high-content imaging pipeline, it may provide the foundation for a truly high-throughput anthelmintic discovery system.

Soil-transmitted helminths seem to have stayed true to their classification as a subclass of the so-called “neglected” tropical diseases. Since human worm infections are almost non-existent in the developed world, the primary profit for the manufacturers of anthelmintic drugs comes from treatment of livestock and domesticated pets. Although current anthelmintics are subpar in eradicating human intestinal worm infections, they are sufficient in suppressing parasitic infections in veterinary animals. Therefore, those with the power to drive anthelmintic discovery efforts have no motivation. Perhaps worse still, the developed world is almost entirely clueless as to the toll delivered by intestinal parasites to one in four people in other parts of the world – out of site, out of mind. Perhaps focused compound screens utilizing relevant parasites may one day change the status quo and guide us to the discovery of superior anthelmintics for humans. An effective approach to deworming populations of the developing world may be a significant step in helping these individuals to fight and overcome poverty. I hope that this assay is a step in that direction.

REFERENCES

- Alexander, P. E., & De, P. (2009). HIV-1 and intestinal helminth review update: updating a Cochrane Review and building the case for treatment and has the time come to test and treat? [Review]. *Parasite Immunol*, 31(6), 283-286. doi: 10.1111/j.1365-3024.2009.01100.x
- Bethony, J., Brooker, S., Albonico, M., Geiger, S. M., Loukas, A., Diemert, D., & Hotez, P. J. (2006). Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. [Review]. *Lancet*, 367(9521), 1521-1532. doi: 10.1016/S0140-6736(06)68653-4
- Bleakley, H. (2007). Disease and development: Evidence from hookworm eradication in the American South. *Quarterly Journal of Economics*, 122(1), 73-117.
- Borkow, G., & Bentwich, Z. (2006). HIV and helminth co-infection: is deworming necessary? [Review]. *Parasite Immunol*, 28(11), 605-612. doi: 10.1111/j.1365-3024.2006.00918.x
- Brooker, S., Hotez, P. J., & Bundy, D. A. (2008). Hookworm-related anaemia among pregnant women: a systematic review. [Research Support, Non-U.S. Gov't Review]. *PLoS Negl Trop Dis*, 2(9), e291. doi: 10.1371/journal.pntd.0000291
- Brown, L. A., Jones, A. K., Buckingham, S. D., Mee, C. J., & Sattelle, D. B. (2006). Contributions from *Caenorhabditis elegans* functional genetics to antiparasitic drug target identification and validation: nicotinic acetylcholine receptors, a case study. [Review]. *Int J Parasitol*, 36(6), 617-624. doi: 10.1016/j.ijpara.2006.01.016
- Burns, A. R., Wallace, I. M., Wildenhain, J., Tyers, M., Giaever, G., Bader, G. D., . . . Roy, P. J. (2010). A predictive model for drug bioaccumulation and bioactivity in *Caenorhabditis elegans*. [Research Support, Non-U.S. Gov't]. *Nat Chem Biol*, 6(7), 549-557. doi: 10.1038/nchembio.380
- de Hostos, E. L. and Nguyen, T. (2012) Anthelmintic Drugs: Tools and Shortcuts for the Long Road from Discovery to Product, in *Parasitic Helminths: Targets, Screens, Drugs and Vaccines* (ed C. R. Caffrey), Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany. doi: 10.1002/9783527652969.ch13
- Caffrey, C. (2012). *Parasitic helminths: Targets, screens, drugs and vaccines*. (Vol. 3). Wiley, John & Sons, Incorporated.

- Cappello, M., Bungiro, R. D., Harrison, L. M., Bischof, L. J., Griffiths, J. S., Barrows, B. D., & Aroian, R. V. (2006). A purified *Bacillus thuringiensis* crystal protein with therapeutic activity against the hookworm parasite *Ancylostoma ceylanicum*. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. *Proc Natl Acad Sci U S A*, 103(41), 15154-15159. doi: 10.1073/pnas.0607002103
- Conlan, J. V., Sripa, B., Attwood, S., & Newton, P. N. (2011). A review of parasitic zoonoses in a changing Southeast Asia. [Research Support, Non-U.S. Gov't Review]. *Vet Parasitol*, 182(1), 22-40. doi: 10.1016/j.vetpar.2011.07.013
- Datu, B. J., Gasser, R. B., Nagaraj, S. H., Ong, E. K., O'Donoghue, P., McInnes, R., . . . Loukas, A. (2008). Transcriptional changes in the hookworm, *Ancylostoma caninum*, during the transition from a free-living to a parasitic larva. [Research Support, Non-U.S. Gov't]. *PLoS Negl Trop Dis*, 2(1), e130. doi: 10.1371/journal.pntd.0000130
- Druilhe, P., Tall, A., & Sokhna, C. (2005). Worms can worsen malaria: towards a new means to roll back malaria? [Review]. *Trends Parasitol*, 21(8), 359-362. doi: 10.1016/j.pt.2005.06.011
- Elias, D., Mengistu, G., Akuffo, H., & Britton, S. (2006). Are intestinal helminths risk factors for developing active tuberculosis? [Research Support, Non-U.S. Gov't]. *Trop Med Int Health*, 11(4), 551-558. doi: 10.1111/j.1365-3156.2006.01578.x
- Fahmy, M. A. (1956). An investigation on the life cycle of *Nematospiroides dubius* (Nematoda: Heligmosomidae) with special reference to the free-living stages. *Z Parasitenkd*, 17(5), 394-399.
- Geary, T. G., & Thompson, D. P. (2001). *Caenorhabditis elegans*: how good a model for veterinary parasites? [Review]. *Vet Parasitol*, 101(3-4), 371-386.
- Geary, T. G., Thompson, D. P., & Klein, R. D. (1999). Mechanism-based screening: discovery of the next generation of anthelmintics depends upon more basic research. [Review]. *Int J Parasitol*, 29(1), 105-112; discussion 113-104.
- Greenwood, David. (2008). *Antimicrobial drugs : chronicle of a twentieth century medical triumph*. Oxford ; New York: Oxford University Press.
- Hall, A., Hewitt, G., Tuffrey, V., & de Silva, N. (2008a). A review and meta-analysis of the impact of intestinal worms on child growth and nutrition. [Meta-Analysis Research Support, Non-U.S. Gov't Review]. *Maternal and Child Nutrition*, 4 Suppl 1, 118-236. doi: 10.1111/j.1740-8709.2007.00127.x

- Hall, A., Hewitt, G., Tuffrey, V., & de Silva, N. (2008b). A review and meta-analysis of the impact of intestinal worms on child growth and nutrition. *Maternal and Child Nutrition*, 4, 118-236.
- Hayes, K. S., Bancroft, A. J., Goldrick, M., Portsmouth, C., Roberts, I. S., & Grencis, R. K. (2010). Exploitation of the intestinal microflora by the parasitic nematode *Trichuris muris*. [Research Support, Non-U.S. Gov't]. *Science*, 328(5984), 1391-1394. doi: 10.1126/science.1187703
- Heywood, V. H., Watson, R. T., & United Nations Environment Programme. (1995). *Global biodiversity assessment*. Cambridge ; New York, NY, USA: Cambridge University Press.
- Hotez, Peter J., & American Society for Microbiology. (2013). *Forgotten people, forgotten diseases : the neglected tropical diseases and their impact on global health and development* (2nd ed.). Washington, DC: ASM Press.
- Johnson, S. S., E. M. Thomas, and T. G. Geary. (1999). *Intestinal Worm Infections*. London, U.K.: Academic Press.
- Keiser, J., & Utzinger, J. (2008). Efficacy of current drugs against soil-transmitted helminth infections: systematic review and meta-analysis. [Meta-Analysis Research Support, Non-U.S. Gov't Review]. *JAMA*, 299(16), 1937-1948. doi: 10.1001/jama.299.16.1937
- Keiser, J., & Utzinger, J. (2010). The drugs we have and the drugs we need against major helminth infections. [Research Support, Non-U.S. Gov't Review]. *Adv Parasitol*, 73, 197-230. doi: 10.1016/S0065-308X(10)73008-6
- Klementowicz, J. E., Travis, M. A., & Grencis, R. K. (2012). *Trichuris muris*: a model of gastrointestinal parasite infection. [Research Support, Non-U.S. Gov't Review]. *Semin Immunopathol*, 34(6), 815-828. doi: 10.1007/s00281-012-0348-2
- Kone, W. M., Vargas, M., & Keiser, J. (2012). Anthelmintic activity of medicinal plants used in Cte d'Ivoire for treating parasitic diseases. *Parasitol Res*, 110(6), 2351-2362. doi: Doi 10.1007/S00436-011-2771-Z
- Lewis, J. A., & Fleming, J. T. (1995). Basic culture methods. [Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S. Review]. *Methods Cell Biol*, 48, 3-29.
- Lubis, I. N., Pasaribu, S., & Lubis, C. P. (2012). Current status of the efficacy and effectiveness of albendazole and mebendazole for the treatment of *Ascaris lumbricoides* in North-Western Indonesia. *Asian Pac J Trop Med*, 5(8), 605-609. doi: 10.1016/S1995-7645(12)60125-4

- Martin, R. J., Clark, C. L., Trailovic, S. M., & Robertson, A. P. (2004). Oxantel is an N-type (methyridine and nicotine) agonist not an L-type (levamisole and pyrantel) agonist: classification of cholinergic anthelmintics in *Ascaris*. [Research Support, U.S. Gov't, P.H.S.]. *Int J Parasitol*, 34(9), 1083-1090. doi: 10.1016/j.ijpara.2004.04.014
- Mes, T. H., Eysker, M., & Ploeger, H. W. (2007). A simple, robust and semi-automated parasite egg isolation protocol. *Nat Protoc*, 2(3), 486-489. doi: 10.1038/nprot.2007.56
- Miguel, E., & Kremer, M. (2004). Worms: Identifying impacts on education and health in the presence of treatment externalities. *Econometrica*, 72(1), 159-217.
- Ngui, R., Ching, L. S., Kai, T. T., Roslan, M. A., & Lim, Y. A. (2012). Molecular identification of human hookworm infections in economically disadvantaged communities in Peninsular Malaysia. [Research Support, Non-U.S. Gov't]. *Am J Trop Med Hyg*, 86(5), 837-842. doi: 10.4269/ajtmh.2012.11-0446
- Ogawa, A., Streit, A., Antebi, A., & Sommer, R. J. (2009). A conserved endocrine mechanism controls the formation of dauer and infective larvae in nematodes. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. *Curr Biol*, 19(1), 67-71. doi: 10.1016/j.cub.2008.11.063
- Ray, D. K., & Bhopale, K. K. (1972). Complete development of *ancylostoma ceylanicum* (Looss, 1911) in golden hamsters, *mesocricetus auratus*. *Experientia*, 28(3), 359-361.
- Reiss, D., Harrison, L. M., Bungiro, R., & Cappello, M. (2007). Short report: an agar plate method for culturing hookworm larvae: analysis of growth kinetics and infectivity compared with standard coproculture techniques. [Research Support, N.I.H., Extramural]. *Am J Trop Med Hyg*, 77(6), 1087-1090.
- Sabatelli, L., Ghani, A. C., Rodrigues, L. C., Hotez, P. J., & Brooker, S. (2008). Modelling heterogeneity and the impact of chemotherapy and vaccination against human hookworm. [Comparative Study Research Support, Non-U.S. Gov't]. *J R Soc Interface*, 5(28), 1329-1341. doi: 10.1098/rsif.2007.1255
- Smout, M. J., Kotze, A. C., McCarthy, J. S., & Loukas, A. (2010). A novel high throughput assay for anthelmintic drug screening and resistance diagnosis by real-time monitoring of parasite motility. [Evaluation Studies Research Support, Non-U.S. Gov't]. *PLoS Negl Trop Dis*, 4(11), e885. doi: 10.1371/journal.pntd.0000885
- Soukhathammavong, P. A., Sayasone, S., Phongluxa, K., Xayaseng, V., Utzinger, J., Vounatsou, P., . . . Odermatt, P. (2012). Low efficacy of single-dose albendazole and mebendazole against hookworm and effect on concomitant helminth infection

- in Lao PDR. [Randomized Controlled Trial Research Support, Non-U.S. Gov't]. *PLoS Negl Trop Dis*, 6(1), e1417. doi: 10.1371/journal.pntd.0001417
- Stepek, G., Buttle, D. J., Duce, I. R., & Behnke, J. M. (2006). Human gastrointestinal nematode infections: are new control methods required? [Research Support, Non-U.S. Gov't Review]. *Int J Exp Pathol*, 87(5), 325-341. doi: 10.1111/j.1365-2613.2006.00495.x
- Stiernagle, T. (2006). Maintenance of *C. elegans*. [Review]. *WormBook*, 1-11. doi: 10.1895/wormbook.1.101.1
- Theodorides, V. J., Gyurik, R. J., Kingsbury, W. D., & Parish, R. C. (1976). Anthelmintic activity of albendazole against liver flukes, tapeworms, lung and gastrointestinal roundworms. *Experientia*, 32(6), 702-703.
- Tritten, L., Braissant, O., & Keiser, J. (2012). Comparison of novel and existing tools for studying drug sensitivity against the hookworm *Ancylostoma ceylanicum* in vitro. [Research Support, Non-U.S. Gov't]. *Parasitology*, 139(3), 348-357. doi: 10.1017/S0031182011001934
- Tritten, L., Nwosu, U., Vargas, M., & Keiser, J. (2012). In vitro and in vivo efficacy of tribendimidine and its metabolites alone and in combination against the hookworms *Heligmosomoides bakeri* and *Ancylostoma ceylanicum*. [Research Support, Non-U.S. Gov't]. *Acta Trop*, 122(1), 101-107. doi: 10.1016/j.actatropica.2011.12.008
- Tritten, L., Silbereisen, A., & Keiser, J. (2011). In vitro and in vivo efficacy of Monepantel (AAD 1566) against laboratory models of human intestinal nematode infections. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. *PLoS Negl Trop Dis*, 5(12), e1457. doi: 10.1371/journal.pntd.0001457
- von Samson-Himmelstjerna, G., Coles, G. C., Jackson, F., Bauer, C., Borgsteede, F., Cirak, V. Y., . . . Wirtherle, N. (2009). Standardization of the egg hatch test for the detection of benzimidazole resistance in parasitic nematodes. [Research Support, Non-U.S. Gov't]. *Parasitol Res*, 105(3), 825-834. doi: 10.1007/s00436-009-1466-1
- Wabo Pone, J., Fossi Tankoua, O., Yondo, J., Komtangi, M. C., Mbida, M., & Bilong Bilong, C. F. (2011). The In Vitro Effects of Aqueous and Ethanolic Extracts of the Leaves of *Ageratum conyzoides* (Asteraceae) on Three Life Cycle Stages of the Parasitic Nematode *Heligmosomoides bakeri* (Nematoda: Heligmosomatidae). *Vet Med Int*, 2011, 140293. doi: 10.4061/2011/140293
- Walson, J. L., Otieno, P. A., Mbuchi, M., Richardson, B. A., Lohman-Payne, B., Macharia, S. W., . . . John-Stewart, G. C. (2008). Albendazole treatment of HIV-1 and helminth co-infection: a randomized, double-blind, placebo-controlled trial.

[Randomized Controlled Trial Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. *AIDS*, 22(13), 1601-1609. doi: 10.1097/QAD.0b013e32830a502e

World Health Organization. "Soil-transmitted helminth infections." 1 June 2013. Online. Internet. Available: <http://www.who.int/mediacentre/factsheets/fs366/en/>

Wood, William Barry. (1988). *The Nematode Caenorhabditis elegans*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

Woods, D. J., & Williams, T. M. (2007). The challenges of developing novel antiparasitic drugs. [Review]. *Invert Neurosci*, 7(4), 245-250. doi: 10.1007/s10158-007-0055-1