UNIVERSITY OF CALIFORNIA, SAN DIEGO

Methods of Drug Screening Against Soil-Transmitted Helminthes

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

in

Biology

by

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Committee in charge

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Professor Sharon L. Reed
Professor Elina Zuniga

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

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Chair

University of California, San Diego

2014

I dedicate this thesis to my father Chris and mother Jessie, who gave me endless love and support throughout my whole life and provided me with opportunities I never could have imagined. Thank you Dad for all the wise advice when I felt lose and thank you Mom for giving me rest when I felt too tired to go on. I am truly lucky to have such supportive and loving parents who shaped me into a person that pursues the betterment of our world.

To my sister Tina, who looks up to me and drives me to be a better person and role model. Thank you for the encouragement and making me laugh in totally random and crappy situations.

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ABSTRACT OF THE THESIS

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by

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Master of Science in Biology

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Professor Raffi V. Aroian, Chair

Intestinal roundworm parasites infect two billion people in the world. In much of the developing world, children have intestinal worms that make them sick and anemic.

Furthermore, physical and cognitive stunting as a result of worm infections directly impacts the children's ability to attend school, keeping them in a cycle of poverty. Studies show that deworming kids is a cost effective way to keep kids in school, yet there is only one drug with adequate efficacy to be used for mass drug administration. As a result drug resistance is a huge problem and discovery of new anthelmintics is of utmost importance. In regards to this problem we ask, can we develop a high-throughput system for screening drug compounds that kill or intoxicate these parasites? To answer this question I optimize a high-throughput drug screening method using 384-well plates to screen against *A. ceylanicum*, a zoonotic hookworm. In addition I optimized a medium-throughput drug screening system using 96-well plates to screen on two parasites: *A. ceylanicum* and *Trichuris muris* (whipworm) and use it to test an FDA-approved library consisting of drugs that are approved for human use. Finally, experiments were done on different adult stage nematodes with two goals in mind: First to verify the validity of potential drug hits found by this screening system, and secondly to test potential drug candidates on a range of different nematodes. Discovery of a drug that is effective on multiple parasites would be a huge advantage in the fight to cure worm infections.

CHAPTER 1

INTRODUCTION

1.1 Soil-Transmitted Helminthes

Soil-transmitted helminths (STHs) refer to a group of intestinal nematode parasites that infect humans and cause diseases that are together known as soil-transmitted helminthiasis. As estimated by the CDC in the year 2013, approximately 807-1,121 million people are infected with *Ascaris*, 604-795 million with whipworm, and 576-740 million with hookworm. These infections are most prevalent in developing countries and are considered neglected tropical diseases (NTDs).

Soil-transmitted helminthes live in the intestine and can cause chronic infections that last up to 10-20 years. The eggs of these parasites are passed in the feces of infected individuals and become infective as they mature in the soil. *Ascaris* and whipworm infections occur when eggs are ingested. On the other hand, hookworm larvae in the soil can penetrate the skin and thus infections can be transmitted simply by walking barefoot on soil. Those living with intestinal parasites are subject to a variety of symptoms that cause a range of health problems. Heavy infections can cause blood and protein loss, resulting in anemia and gastrointestinal complications. Pregnant women are more

vulnerable to birth complications or maternal death, while in young children STH infections often lead to both cognitive and physical growth retardation (Wang et al, 2013). Furthermore, STH infections play a role in proliferating other serious diseases by dampening the immune response. For example, in one study done in Tanzania it was found that malaria parasites were significantly more prevalent in children and pregnant women infected with hookworm than those who were hookworm free (Kinung'hi et al, 2014). Helminth infections also accentuate the morbidity of HIV/AIDS and impair vaccine efficacy (Lustigman et al, 2012).

Given the symptoms that are caused by STH infections, it is generally accepted that these parasites contribute to keeping these people in a cycle of poverty. Studies have shown that deworming kids in Kenya decreased school absenteeism by 25% and is not only effective, but cost-effective with a single pill costing a few pennies (Miguel & Kremer, 2004). Furthermore, children who had been dewormed earned more money than other children years later, demonstrating a lasting impact on the lives of those living with infectious worms. Without mass drug administration to deworm these populations, STH infections remain vicious and persistent. Many populations living in developing countries remain trapped in a repeated cycle of infection throughout their lives, resulting in general decreased productivity and socioeconomic development.

1.2 Current Anthelmintics

Currently, there exist a few major classes of anthelmintics which include the benzimidazoles, nicotinic acetylcholine receptor agonists, and macrocyclic lactones. However, only the benzimidazoles (albendazole, mebendazole) and the nicotinic

acetylcholine receptor agonists (pyrantel, levamisole) are currently approved by the World Health Organization to treat STH infections. Furthermore, there is a tendency to rely heavily on only one drug, albendazole, as it is the most efficacious single-dose treatment against hookworms, does not require knowledge of the patient's weight, and is cost effective. The reliance on albendazole inevitably breeds resistance which has been emerging in parasites of both livestock and humans (Stepek et al, 2006). Thus, there is an urgent need for the discovery of new drugs. To make situations worse, currently there are *zero* anthelmintics that are effective in a single dose therapy against whipworms (Keiser et al, 2008). Whipworm infections account for a third of STH infections worldwide (WHO, 2014), yet a drug with satisfactory efficacy against whipworm has yet to be found. It is also important to note that polyparasitism is common in many populations, and concurrent infections significantly increase the risk of anemia. One study found that 69.8% of children examined in Tanzania were infected with one or more parasites (Kinung'hi et al, 2014). A drug that is effective against multiple parasites would provide a significant advantage in deworming these populations.

In the present study we seek to develop a method of drug screening which is capable of screening large compound libraries quickly and at low cost. From previous studies done in the Aroian Lab, it was found that the hookworm *A. ceylanicum* is a particularly sensitive and useful model for anthelmintic studies (Hu et al, 2013). Thus we chose it as the parasite of choice for initial drug screens for broad-spectrum human soil-transmitted helminth therapies. Additionally, development of a method for drug screening against whipworm *specifically* would be a step toward finally discovering a drug that is

effective on whipworm. For our purposes, using larval stages in the initial stage of drug screening provides several advantages. Firstly, eggs and larvae can be obtained in large numbers for high content screening without having to sacrifice animal hosts for each experiment. Secondly, it allows us to look at a broader and more metabolically active range of the parasitic life cycle. For example, it enables us to detect drugs that inhibit hatch rate as well as cause developmental arrest. By screening on hookworm and whipworm directly, we can identify the potential drug compounds that have the most relevance to treat human STH infections.

CHAPTER 2

A. CEYLANICUM DRUG SCREENING

Previous studies done by a fellow master's student in the Aroian Lab, Arash Safavi, and myself showed that the hookworm *A. ceylanicum* is a good parasite to screen drug libraries against because it is a zoonotic human hookworm and because of its responsiveness to current anthelmintics (*i.e.*, validated using current drugs). *A. ceylanicum* has significant genetic similarity to the primary human hookworm, *A. duodenale*, and is useful in the laboratory because an infection can be maintained in hamsters. Thus, a large part of my project was to optimize and improve on current drug screening methods against *A. ceylanicum*. To briefly describe the life cycle of *A. ceylanicum*: the adult hookworm resides in the host's small intestine and it is there that it reproduces and lays eggs which are passed in the feces into the environment. Eggs subsequently hatch in the soil where larvae develop from the L1 stage to the L3 infectious stage and are able to infect another host through ingestion or penetration of the skin. In the laboratory, *A. ceylanicum* eggs are isolated from hamster feces and cultured to obtain the free-living larval stages.

2.1 Screening Drug Libraries on *A. ceylanicum*

The Aroian lab received two drug libraries: one from Bill Gerwick which will be referred to as the "SIO purified compound library", and a library consisting of current FDA-approved drugs which will be referred to as the "NIH clinical library". These two libraries were screened for potential drug candidates on *A. ceylanicum* using a medium-throughput method developed with Arash Safavi. *A. ceylanicum* eggs were isolated from feces collected from infected hamsters by using a series of flotation gradient centrifugation. Six to twenty grams of hamster feces are soaked in 30 mL of 13% NaCl and mechanically broken up using a spatula and vortexing, then filtered through a strainer. This mixture is centrifuged at 3.5 rpm for 5 minutes at a time to allow the eggs to float to the top. The supernatant is then poured into a 50 mL conical tube and filled with water, which is then centrifuged again at 3.5 rpm for 5 minutes to allow the eggs to sink to the bottom. The supernatant is aspirated and the pellet is resuspended in 5 mL of 17% sucrose, then centrifuged again at 3.5 rpm for 5 minutes. The eggs will float to the top of the sucrose and this supernatant is collected in a new tube. This mixture is then washed at a concentration of 10% bleach for one minute in order to get rid of any contaminants from the feces. Finally a series of washes using sterile ddH_2O is done to wash the bleach from the eggs. The eggs are washed with 15 mL of sterile ddH_2O, centrifuged at 2.2 rpm for 2 minutes to collect the eggs at the bottom, and the dirty water is aspirated. The wash is repeated 5 times before finally resuspending the eggs in hookworm culture medium (HCM). Hookworm culture medium consists of RPMI-1640 culture medium with 25 mM HEPES (pH 7.2), 50% fetal bovine serum (FBS), and antibiotics: 100 IU/mL of penicillin,

100 mg/mL of streptomycin, and 1 ug/mL amphotericin B. The RPMI-1640 is a rich culture medium that is used as a major component of both hookworm and whipworm culture medium (which will be referred to throughout this thesis), while the HEPES is used to maintain the medium's pH at an optimal 7.2 for culturing nematodes. Fifteen to thirty hookworm eggs are added into each well of a 96-well U-bottom plate (Falcon cat#35-3077) along with 5 uL of *E. coli* bacteria (laboratory strain OP50 grown overnight in LB, OD_{600nm} = 3.0) resuspended in RPMI+HEPES as a food source and HCM to a total volume of 100 uL per well. 0.5 uL of each compound from the drug library was added to each well for a final concentration of 0.025 mM in 0.25% dimethyl sulfoxide (DMSO). The plate is then incubated at 25^0 C for 7 days before being scored. In the negative control condition of 0.25% DMSO eggs will hatch and larvae will develop healthily into infectious stage L3i worms within 7 days of incubation. Scoring for potential drug hits is based on the hatch rate, worm size, coloration, motility, and shape of worms.

The SIO purified compound library was graciously received from Bill Gerwick's lab at the Scripps Institution of Oceanography at UCSD. Eighty compounds were screened from this library and resulted in two "hits" being found as potential drug candidates against *A. ceylanicum* larvae (Figure 1 and Table 1). One of these compounds, dolastatin, was particularly interesting as it has been tested in clinical trials as an anti-cancer drug. Follow-up experiments were done with this drug compound, which will be discussed in a later chapter of this thesis.

The NIH clinical library of 727 compounds was purchased from Evotec as a set of ten 96-well plates with 50 µL of each compound at 10 mM concentration in 100% DMSO stored at -20^{0}C. Before screening, each plate of drug compounds (kept sealed and unopened) is placed in a dessicator and thawed on ice. The NIH clinical library was screened in the same 96-well plate format on *A. ceylanicum* as described above. Each drug compound was tested in duplicate wells during the initial screen and positive hits that were identified are repeated twice thereafter. Thirty-two compounds were found as potential drug candidates, which are listed in Table 1 (see also Figure 1). Among the positivie hits were albendazole and mebendazole (screened blindly in the sense I did not know which wells they are in during the screening process), which are known anthelmintics, thus validating the drug screen. Furthermore, *all* compounds that are identified as hits in the screen display one of two results: zero hatch rate, or significant developmental arrest that result in very small worms compared to negative controls. These are two very clear indicators of an effective drug compound that are not subjective, given the obvious difference compared to negative controls and drug compounds which are ineffective.

Table 1: Summary of NIH Clinical Library hits on *A. ceylanicum* larvae

Compound	Function	Plate and well #
Doxycycline (Tautomer) $C_{22}H_{24}N_2O_8$	A synthetic TETRACYCLINE derivative with similar antimicrobial activity. Animal studies suggest that it may cause less tooth staining than other tetracyclines. It is used in some areas for the treatment of chloroquine-resistant falciparum malaria	3078 - C2
Albendazole	A benzimidazole broad-spectrum anthelmintic structurally related to MEBENDAZOLE that is effective against many diseases.	3078 - A3
Mitoxantrone	An anthracenedione-derived antineoplastic agent	3078 - B4
Mebendazole	A benzimidazole that acts by interfering with CARBOHYDRATE METABOLISM and inhibiting polymerization of MICROTUBULES.	3078 - A6
Flecainide	A potent anti-arrhythmia agent, effective in a wide range of ventricular and atrial ARRHYTHMIAS and TACHYCARDIAS	3078 - C6
Rimantadine	An RNA synthesis inhibitor that is used as an antiviral agent in the prophylaxis and treatment of influenza	3078 - D10
Minocycline Hydrochloride $C_{23}H_{28}ClN_3O_7$	treatment of acne, arthritis...?	2900 - F3
Amitriptyline	Tricyclic antidepressant with anticholinergic and sedative properties. It appears to prevent the re-uptake of norepinephrine and serotonin at nerve terminals, thus potentiating the action of these neurotransmitters. Amitriptyline also appears to antagonize cholinergic and alpha-1 adrenergic responses to bioactive amines	2900 - C8
Nortriptyline	A metabolite of AMITRIPTYLINE that is also used as an antidepressive agent. Nortriptyline is used in major depression, dysthymia, and atypical depressions	2900 - D11
Fluoxetine	The first highly specific serotonin uptake inhibitor. It is used as an antidepressant and often has a more acceptable side-effects profile than traditional antidepressants	3167 - E5
Sertraline	A selective serotonin uptake inhibitor that is used in the treatment of depression	3167 - H6

Table 1: continued

Compound	Function	Plate and well #
Amoxapine	The N-demethylated derivative of the antipsychotic agent LOXAPINE that works by blocking the reuptake of norepinephrine, serotonin, or both. It also blocks dopamine receptors.	2989 - D4
Chlorpromazine	The prototypical phenothiazine antipsychotic drug. Like the other drugs in this class chlorpromazine's antipsychotic actions are thought to be due to long-term adaptation by the brain to blocking DOPAMINE RECEPTORS. Chlorpromazine has several other actions and therapeutic uses, including as an antiemetic and in the treatment of intractable hiccup	2989 - B5
Nicotine	Nicotine is highly toxic alkaloid. It is the prototypical agonist at nicotinic cholinergic receptors where it dramatically stimulates neurons and ultimately blocks synaptic transmission. Nicotine is also important medically because of its presence in tobacco smoke	2989 - C8
Thiabendazole	2-Substituted benzimidazole first introduced in 1962. It is active against a variety of nematodes and is the drug of choice for STRONGYLOIDIASIS. It has CENTRAL NERVOUS SYSTEM side effects and hepatototoxic potential.	2989 - C10
Thioridazine	A phenothiazine antipsychotic used in the management of PHYCOSES, including SCHIZOPHRENIA	2989 - D10
Demeclocycline $C_{21}H_{21}ClN_2O_8$	A TETRACYCLINE analog having a 7-chloro and a 6-methyl. Because it is excreted more slowly than TETRACYCLINE, it maintains effective blood levels for longer periods of time.	2989 - E11
duloxetine	a drug which primarily targets major depressive disorder (MDD), generalized anxiety disorder (GAD), pain related to diabetic peripheral neuropathy and in some countries stress urinary incontinence. Duloxetine is a selective SNRI (selective serotonin-norepinephrine reuptake inhibitor). Duloxetine is a systemic drug therapy which affects the body as a whole. Known also under the code name LY248686, it is a potent dual reuptake inhibitor of serotonin (5-hydroxytryptamine, 5-HT) and norepinephrine (NE), possessing comparable affinities in binding to NE- and 5-HT transporter sites. It is a less potent inhibitor of dopamine reuptake	2658 - E3
vesamicol	Neuromuscular Depolarizing Agents - Drugs that interrupt transmission at the skeletal neuromuscular junction by causing sustained depolarization of the motor end plate. These agents are primarily used as adjuvants in surgical anesthesia to cause skeletal muscle relaxation	2658 - B5
Prochlorperazine	A phenothiazine antipsychotic used principally in the treatment of NAUSEA; VOMITING; and VERTIGO	2658 - A6
bifemelane	antidepressive agent	2658 - A7

Table 1: continued

Compound	Function	Plate and well #
indatraline	a non-selective monoamine transporter inhibitor that has been shown to block the reuptake of dopamine, norepinephrine, and serotonin with effects similar to those of cocaine	2658 - E7
cinanserin	Serotonin Antagonists - Drugs that bind to but do not activate serotonin receptors, thereby blocking the actions of serotonin or SEROTONIN RECEPTOR AGONISTS.	2658 - C7
Maprotiline	A bridged-ring tetracyclic antidepressant that is both mechanistically and functionally similar to the tricyclic antidepressants, including side effects associated with its use	2658 - H8
SMR000059010	a clinically well-established anti-mitotic chemotherapy medication (that is, it interferes with cell division)	2692 - A6
flubendazole	Antinematodal Agents - Substances used in the treatment or control of nematode infestations. They are used also in veterinary practice.	2692 - A9
tegaserod	a 5-HT4 agonist manufactured by Novartis and used for the management of irritable bowel syndrome and constipation	2760 - E5
CGS 12066B	Serotonin Receptor Agonists - Endogenous compounds and drugs that bind to and activate SEROTONIN RECEPTORS. Many serotonin receptor agonists are used as ANTIDEPRESSANTS; ANXIOLYTICS; and in the treatment of MIGRAINE DISORDERS	2827 - H5
Sertraline	A selective serotonin uptake inhibitor that is used in the treatment of depression	2726 - F6
triptolide	Antineoplastic Agents, Alkylating; Antispermatogenic Agents; Immunosuppressive Agents	2726 - G7
duloxetine hydrochloride	selective serotonin and norepinephrine reuptake inhibitor (SSNRI)	2794 - D6
rimcazole	Anticonvulsants - Drugs used to prevent SEIZURES or reduce their severity; Antipsychotic Agents; Neuroprotective Agents - Drugs intended to prevent damage to the brain or spinal cord from ischemia, stroke, convulsions, or trauma. Some must be administered before the event, but others may be effective for some time after. They act by a variety of mechanisms, but often directly or indirectly minimize the damage produced by endogenous excitatory amino acids	2794 - H7
dolastatin	anti-microtubule agent, anti-cancer therapy	SIO library 2-C10
kabiramide C	antifungal macrolide	SIO library 2-C8

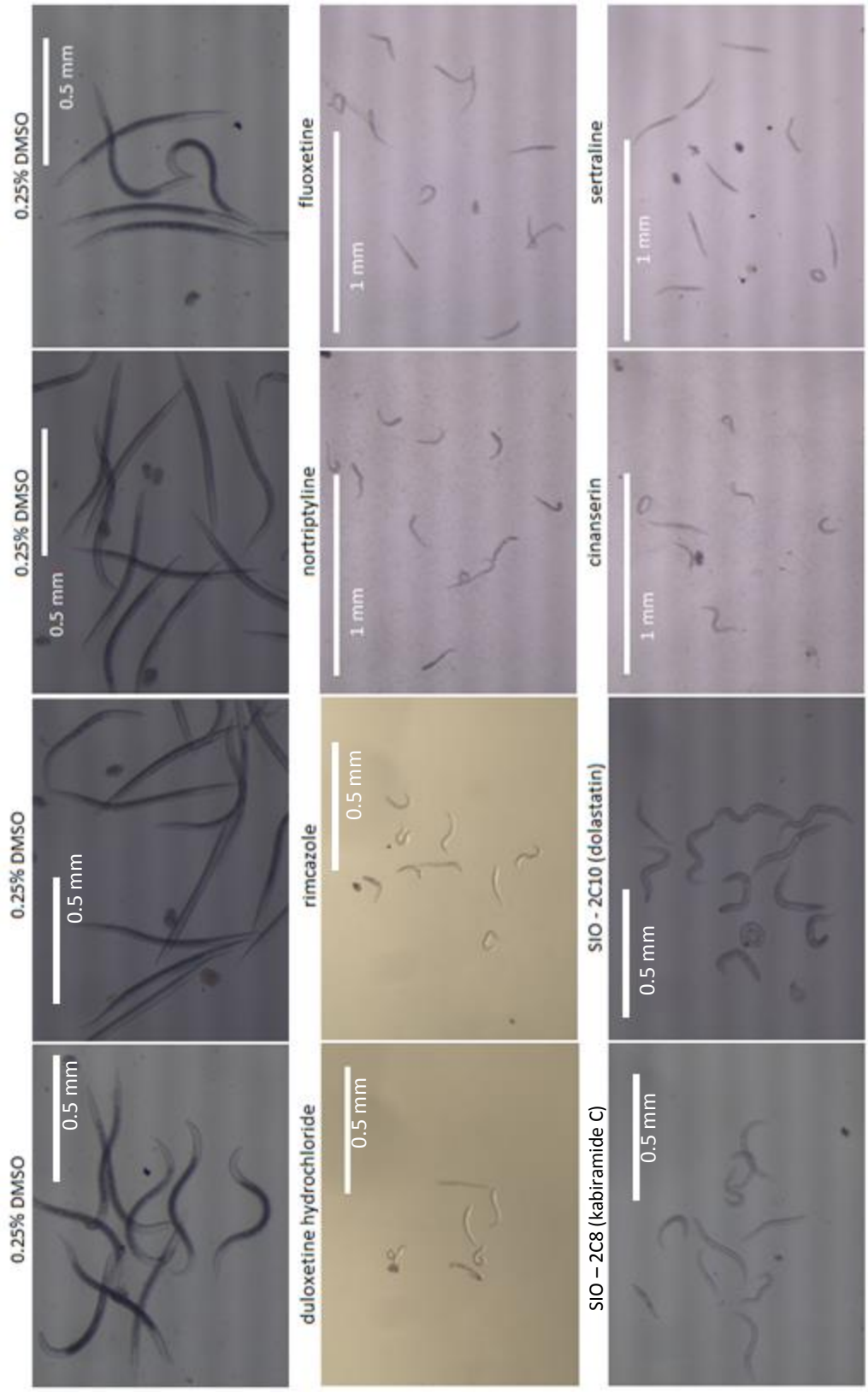
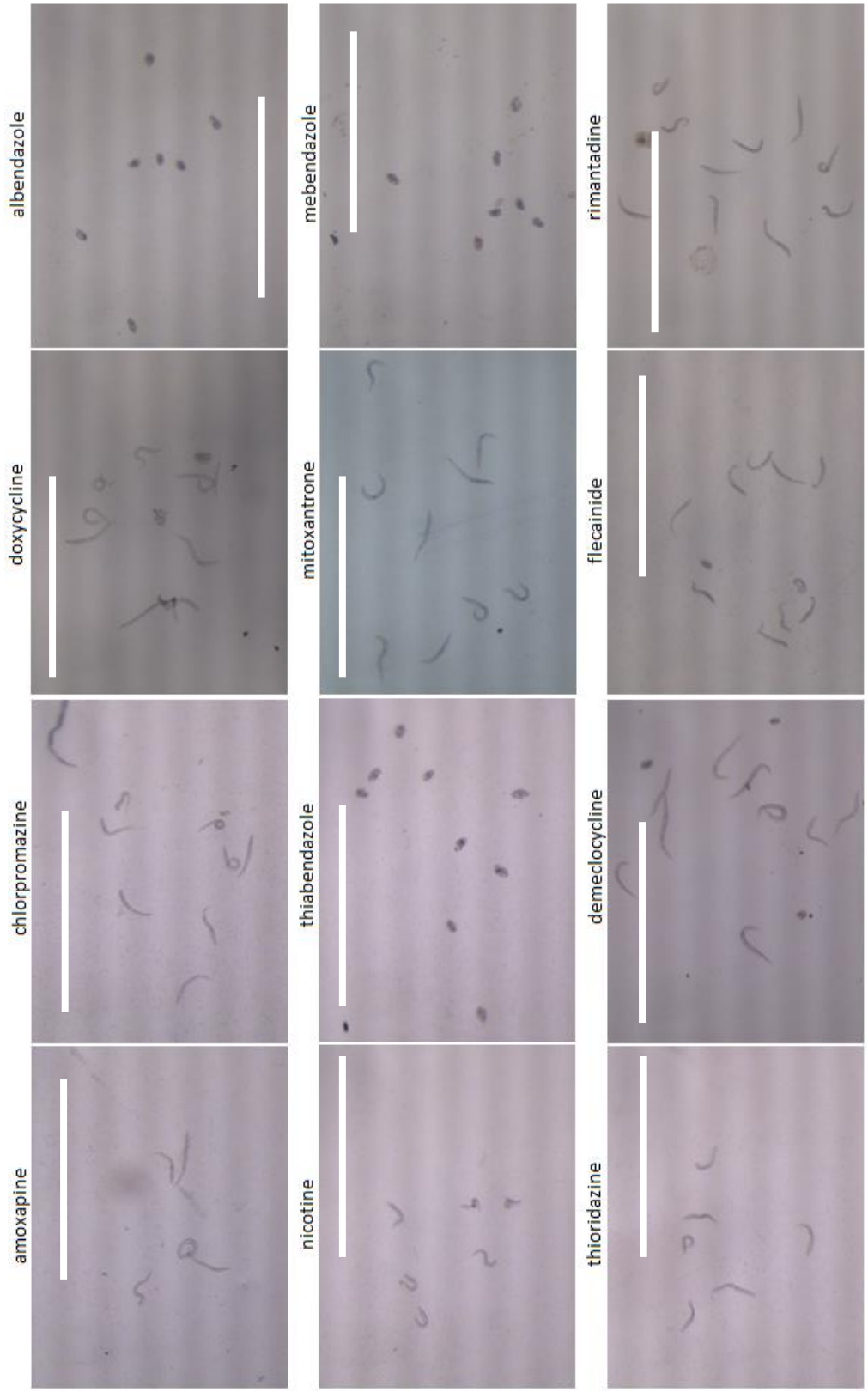


Figure 1: Examples of hits from NIH Clinical Library on *A. ceylanicum* larvae



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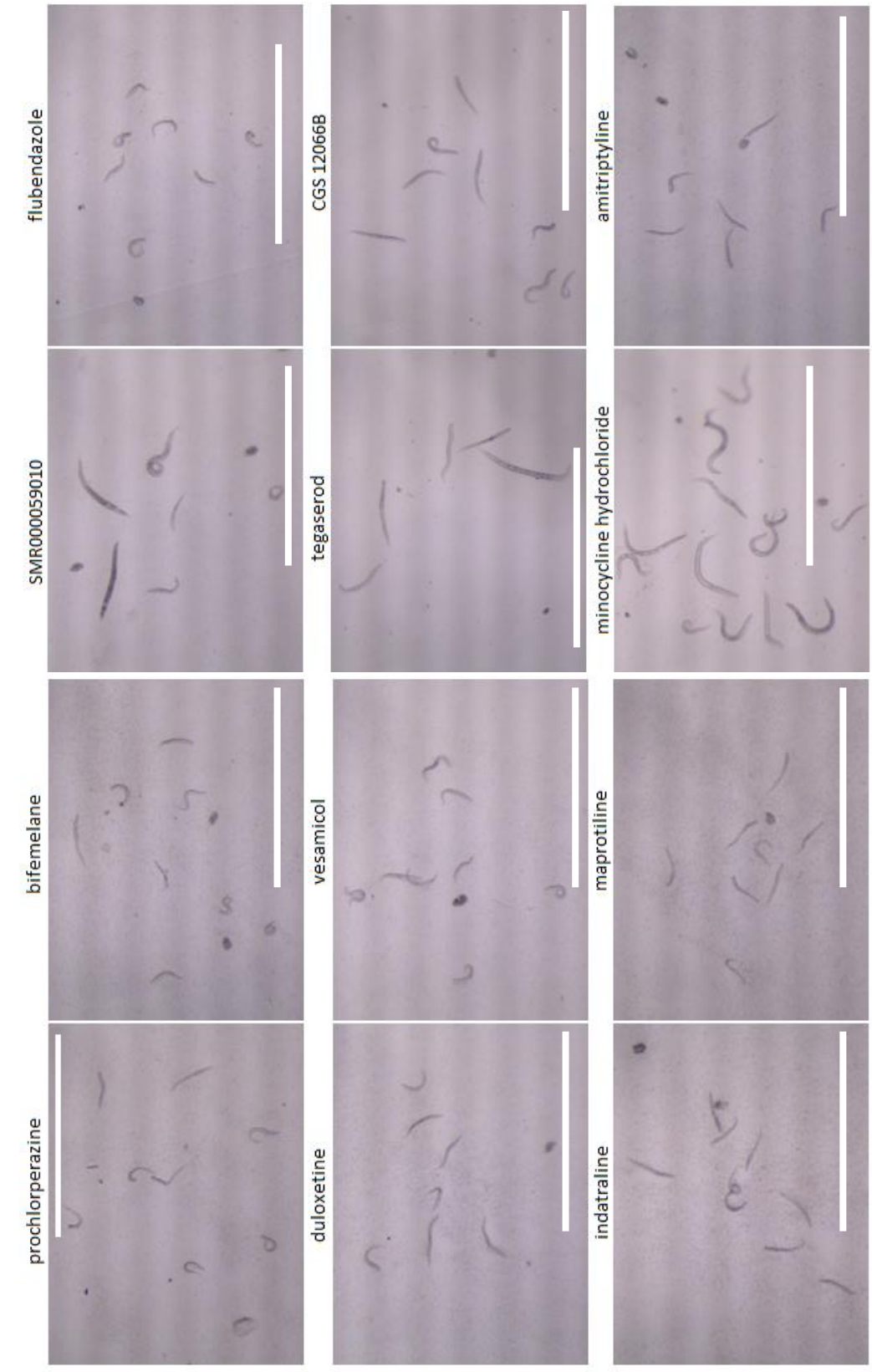

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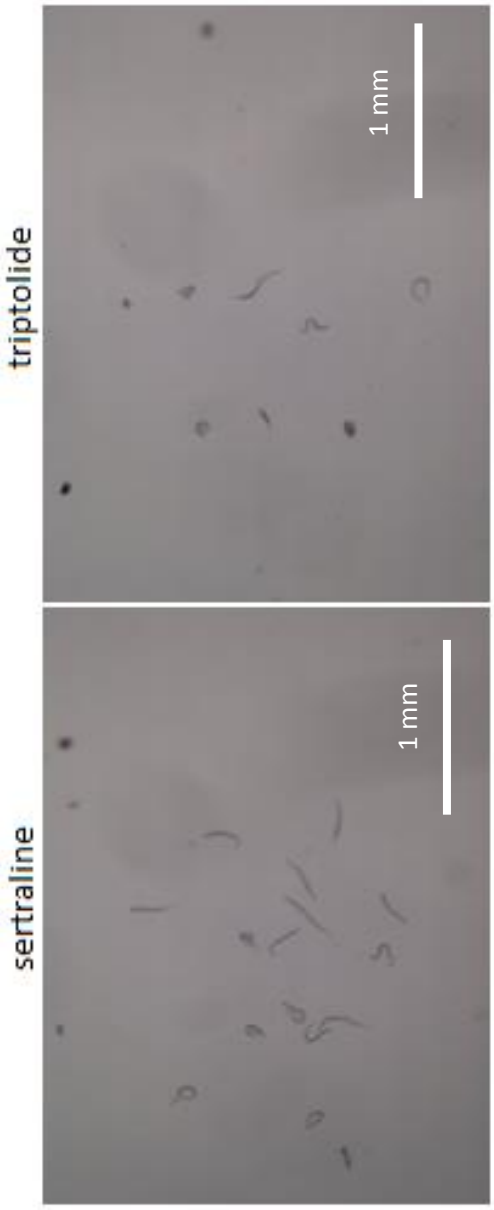


Figure 1: continued

2.2 Optimization of Larval Developmental Assays

The *A. ceylanicum* larval developmental assay measures a drug dose response based on a nematode's size. There is variation in size among individual animals as they develop in each assay that could influence the interpretation of results. Minimizing this variation by creating more uniformity among the worms during development would help to control this factor and optimize this assay. Hookworm eggs are isolated as previously described from hamster feces using flotation gradient centrifugation. When *A. ceylanicum* eggs are isolated from feces they are not all at the same stage of embryonation, thus some eggs hatch earlier while others hatch later, causing the larvae to develop at different times. It was previously thought that allowing the eggs a period of two days to hatch without the presence of any food source, and then adding the bacterial food source after two days would synchronize the larval development because all hatched larvae would be starved (arrested at the L1 stage) and subsequently exposed to food at the same exact time to proceed in growth. However, we observed that some larvae remained stunted and did not develop to the L3 infectious stage after this procedure. The cause for this may be that larvae which hatched earlier than others were starved for too long and became too sick to develop fully. In order to alleviate this, 0.5 µL of OP50 (OD_{600nm} = 3.0) is added along with hookworm culture medium to the *A. ceylanicum* eggs (approximately 10-15 per well) in 100 µL total volume at the beginning of assay set up in the 96-well U-bottom plate. This allows for larvae that hatch the earliest to have a minimal amount of food to survive. After allowing 48 hours for all larvae to hatch, an additional 4.5 µL of OP50 is added to each well, bringing the food availability to an optimal level for healthy

development. This procedure results in a uniform development to the L3 infectious stage of *all* larvae in the negative control wells (Figure 3A). Subsequently the worms are given a "recovery period" of one hour before drugs are added to each well. For this experiment, 10 mg of albendazole and tribendimidine were serially diluted in 100% dimethyl sulfoxide (DMSO) and resuspended in RPMI+HEPES culture medium (a rich medium that is used as a major component in hookworm culture medium). The final concentration for albendazole doses are as follows: 0.003125, 0.00625, 0.0125, 0.025, 0.05, 0.1, and 0.2 µg/mL. The final concentration for tribendimidine doses are as follows: 0.04375, 0.085, 0.175, 0.35, 0.7, 1.4, and 2.8 µg/mL. The negative control is 0.2% DMSO and we also include a "non-feeding control" consisting of worms that do not have any bacterial food source for the duration of the experiment. The assay is incubated at 25^0 C and scored on day 7. Scoring is done using a computer program called LabVIEW developed by the company National Instruments. LabVIEW is a software program that is able to calculate the size of a worm based on area by analyzing an image put under a microscope. In order to capture the cleanest image, the well contents are transferred from the 96-well plate to a spot plate. The spot plate is placed on ice until the worms stop thrashing and an eyelash pick is used to gather all worms to the center of the spot plate. The spot plate is then put under the microscope where the LabVIEW program takes a still image and highlights all objects it identifies in the spot plate. Within the program we can then manually eliminate any objects that are not worms and calculate the area of each individual object that is a worm. An average area of worm size in units of μm^2 is calculated for each condition, and this data is graphed as a dose response curve (Figure 2). This data can also be analyzed in terms of percent growth. The percent growth of worms in each drug condition is

normalized to the maximum potential growth that is possible of healthy worms in the negative control. The maximum potential growth of a worm is calculated by this equation: the size of negative control worms minus the size of non-feeding control worms. Thus we calculate the percent growth by the equation: (Area of negative control worms – Area of drug treatment worms) divided by (Area of negative control worms – Area of non-feeding control worms). All worms in the negative control wells of each experiment grow uniformly to approximately the same size, although this size may vary slightly between different groups of animals (between different experimental repeats). A general trend we see from albendazole is a decrease in worm size at doses 0.0125 µg/mL and higher (Figure 3B). For tribendimidine a mixture of both large worms and tiny worms are seen at dose 0.7 µg/mL and lower, and there is a significant decrease in the size of worms at higher doses (Figure 3C). Typically wells exposed to tribendimidine at the lower doses exhibit a mixture of large and small worms (Figure 3C), thus a much larger standard deviation is seen in the size of worms at these doses, but this can be attributed to the drug effect and not a natural variance of individual animals. By optimizing the uniformity of larval growth, we can normalize the percent growth to the negative control worms and say with more confidence that a worm that shows arrested development is a result of the drug's effect. Furthermore, using the LabVIEW program to calculate area (size) of worms provides another method of *quantifying* data when testing drugs against worms that provides objectivity.

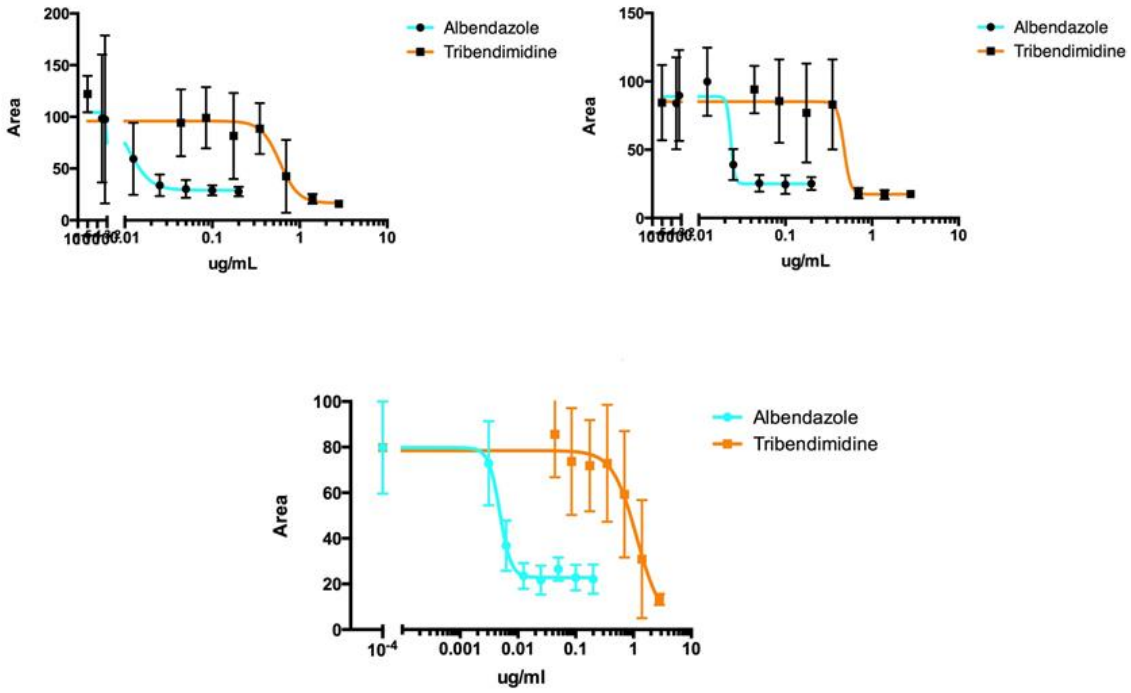


Figure 2: Dose-response based on worm size (area) in response to albendazole and tribendimidine. Area is calculated in units of μm^2. Error bars represent standard deviation. Each graph is one independent replicate.

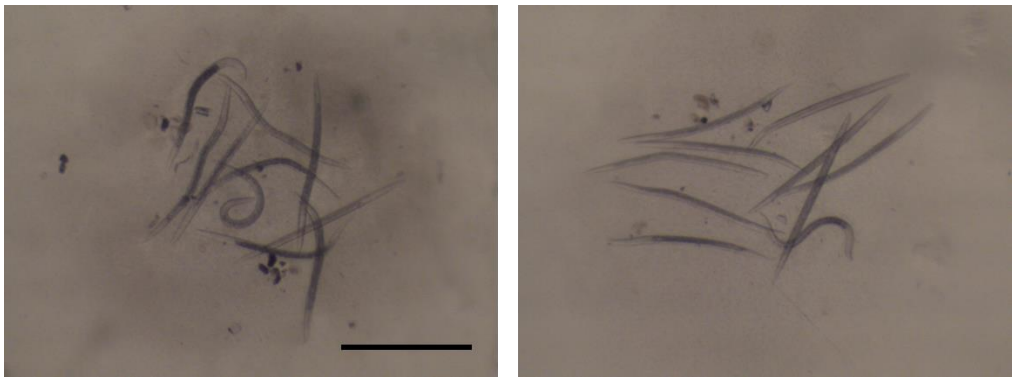


Figure 3A: Negative control (no drug, 0.2% DMSO) of *A. ceylanicum* larvae for larval development assays showing how all larvae develop uniformly in size. Scale bar represents 0.5 mm.

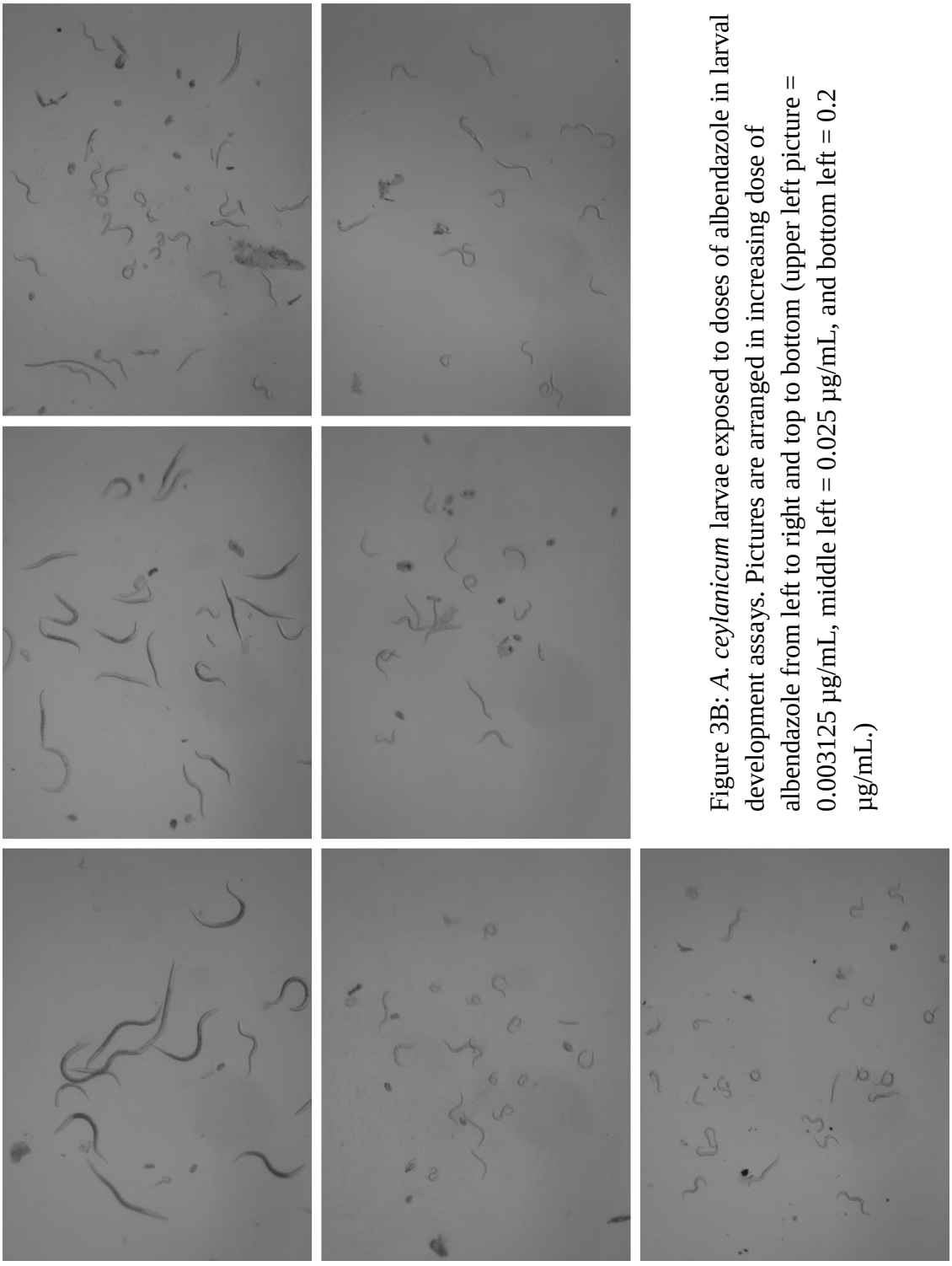


Figure 3B: *A. ceylanicum* larvae exposed to doses of albendazole in larval development assays. Pictures are arranged in increasing dose of albendazole from left to right and top to bottom (upper left picture = 0.003125 µg/mL, middle left = 0.025 µg/mL, and bottom left = 0.2 µg/mL.)

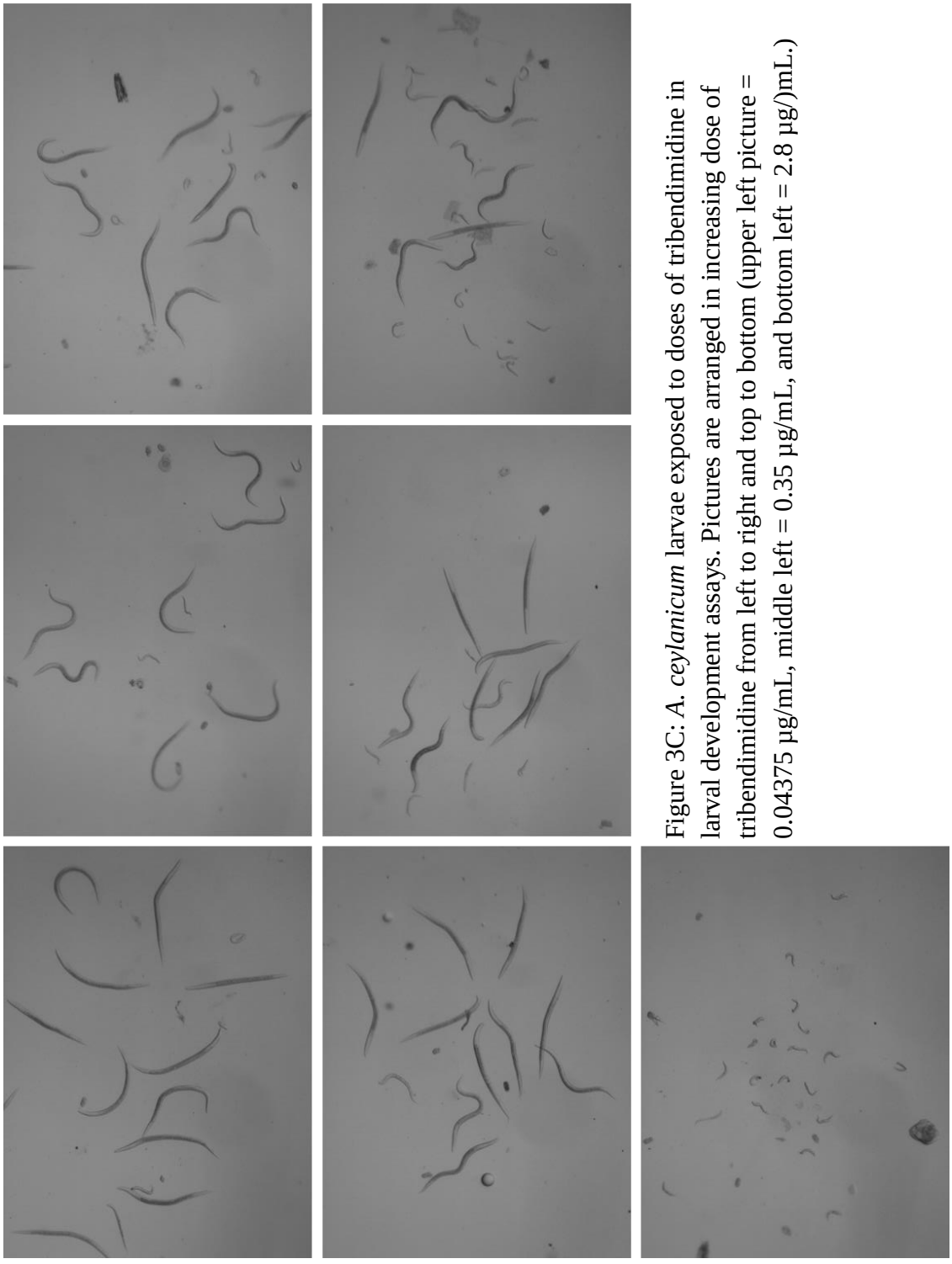


Figure 3C: *A. ceylanicum* larvae exposed to doses of tribendimidine in larval development assays. Pictures are arranged in increasing dose of tribendimidine from left to right and top to bottom (upper left picture = 0.04375 µg/mL, middle left = 0.35 µg/mL, and bottom left = 2.8 µg/)mL.)

2.3 Developing a 384-well Drug Screen Assay

A high-throughput drug screen method is needed in order to screen large drug libraries quickly and more cost effectively. Using a 384-well plate format would not only allow more drugs to be screened at a time, but also reduce the amount of drug that needs to be added per well due to a smaller well size. *A. ceylanicum* eggs were isolated again from hamster feces using the same protocol described for the 96-well plate format. Multiple parameters were tested and optimized to allow for *A. ceylanicum* larvae to be able to develop healthily into the L3i stage within the wells. It was found that 3 uL of OP50 (OD_{600nm} = 3.0), approximately 10 eggs per well, and a total well volume of 50 ul made up of HCM allowed *A. ceylanicum* eggs to hatch and develop into healthy L3i within the wells of the 384-well flat-bottom plate (Nunc cat# 242757). In these conditions the larvae are able to develop healthily in 0.2% DMSO, thus allowing us to use this condition as a negative control in drug screening. A drug dose response was tested on this assay using three known anthelmintics of different drug classes: albendazole, ivermectin, and pyrantel. Albendazole is in the class of benzimidazoles that work by inhibiting microtubule polymerization by binding to β-tubulin (Nayak et al, 2011). This inhibition of the assembly of microtubules causes inhibition of glucose uptake in the worms and thus kills them. Ivermectin binds and activates glutamate-gated chloride channels, causing disruption of the nervous system and muscle function of the worms (Moreno et al, 2010). Pyrantel is a nicotinic acetylcholine receptor agonist that works as a depolarizing neuromuscular blocking agent that eventually paralyzes the worms, causing death. The effects of these drugs on hookworm in a 96-well format are shown (Figures 4A, 5A, and

6A), thus they can be used to verify if the effects can be repeated in a 384-well format. Albendazole, pyrantel pamoate, and ivermectin were tested at a final concentration of 0.2, 2, 20, and 200 µg/mL dissolved in 0.2% DMSO. Albendazole, ivermectin, pyrantel pamoate, and DMSO were purchased from Sigma-Aldrich (cat# A4673-10G, P6210-5G, I8898-250MG, D8418-250ML respectively). For each drug, 10 mg were initially dissolved in 100% DMSO and serially diluted, finally raising the volumes with RPMI+HEPES culture medium to achieve the desired final concentration. The assay was incubated at 25^0C for 7 days before being scored for hatch rate, worm size, coloration, motility, and shape of worms. The drug effects in the 384-well flat-bottom plate were the same as those seen in previous experiments done in 96-well plates, thus verifying the ability to use the 384-well format in drug screens (Figures 4, 5, and 6). The albendazole was extremely effective, killing all larvae at a dose of 0.2 µg/mL and preventing all hatching at each of the higher doses (Figure 4B). Figure 4B does not show the result of albendazole at the 200 µg/mL dose due to drug precipitation which obscures the ability to view unhatched eggs. The ivermectin stunted larval growth at the L1 stage and inhibited motility in every dose (Figures 5B and 5C). The pyrantel pamoate had a moderate effect at the 0.2 µg/mL dose, stunting the growth of only some larvae while many were still able to develop to a larger size. At all higher doses of pyrantel pamoate worms had extreme developmental arrest and were killed, causing the curled shrunken shape of the larvae (Figures 6B, 6C, and 6D). The pictures taken in the 384-well flat-bottom plate depict the same results as worms in a 96-well U-bottom plate, however, it can be quite difficult to see the worms because sick larvae tend to be extremely small and spread around the edges of the well. The same occurrence was observed with larvae in 96-well

flat-bottom plates, which is the reason U-bottom plates are used. Thus, different types of 384-well plates were also tested. However, the only 384-well plates with a curved bottom surface that our lab could obtain were not transparent in color, making the results still difficult to view. 384-well V-bottom plates (cat# 95040000) proved to be especially convenient as larvae were able to develop healthily to the L3 infectious stage in negative controls and also reproduce drug-dose responses in the presence of albendazole, ivermectin, and pyrantel. Figures 5B and 6D are shown as they depict larvae exposed to ivermectin and pyrantel, and were easiest to be seen. Unfortunately, albendazole typically inhibits hatch rate at all doses (with zero hatching above 0.2 µg/mL), and unhatched eggs are extremely hard to detect without a transparent plate bottom.

Although a 384-well format would reduce the cost by allowing significantly more compounds to be screened at once and reducing the amount of drug needed in the screen, a faster method of scoring would be a huge advantage as well. In this regard, the Operetta system was tested to be a more automated method of scoring worms. The Winzeler Lab at UCSD graciously helped with this project by lending us the use of the PerkinElmer Operetta High Content Imaging System which allows for automated image acquisition. A Greiner 384-well plate (Cat# 789071-G) is used with the same parameters as above (50 µL total volume consisting of HCM, OP50, and about 10 eggs per well in 0.2% DMSO). After placing the plate in the Operetta machine, it automatically acquires images of the well contents (Figure 7). The program also has some capability of measuring length and size of an object of interest, and allows one to input a threshold on the length such that anything below a given length will not be counted, or only objects below a certain length

will be counted. As shown in Figure 1, all compounds that are considered hits (but allow hatching of eggs) have a striking effect of arresting larval development such that all sick worms have a length that is significantly smaller than negative control worms. This is a phenotype that occurs in 100% of the hits that do not inhibit hatch rate, whereas worms exposed to compounds which have no effect will develop to a healthy L3 stage length within 5 days. Hits that inhibit hatch rate show zero hatching which means that eggs will be the only objects detected by the Operetta. Thus, it would be convenient to use the Operetta's capability of measuring length and setting a threshold to automate scoring.

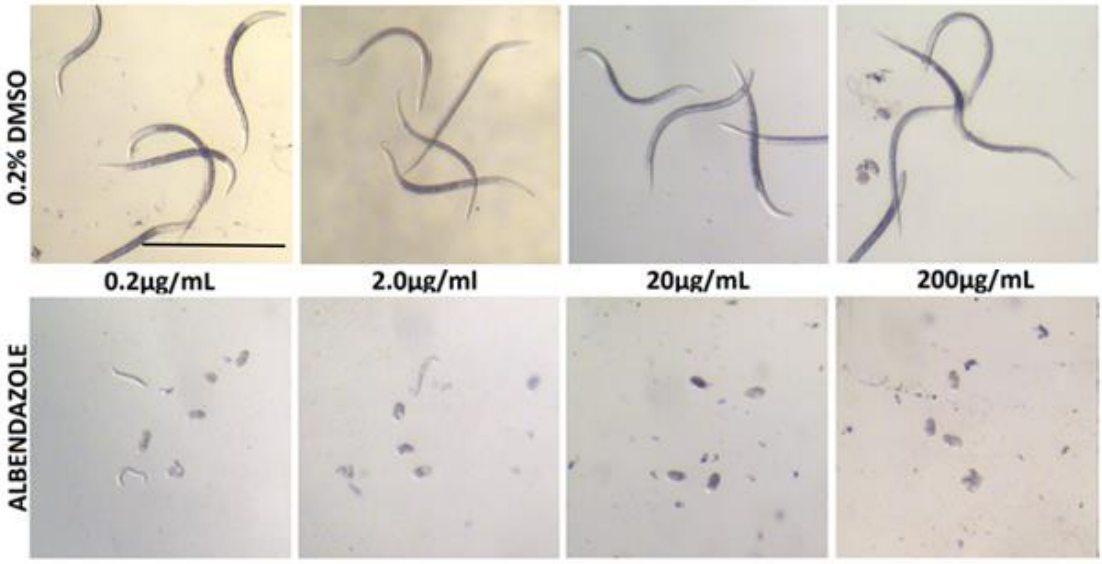


Figure 4A: Dose response of *A. ceylanicum* larvae to albendazole in 96-well U-bottom plate, as published by Arash Safavi. Worms cultured in 0.2% DMSO (no drug) served as a negative control. Scale bar represents 0.5 mm.

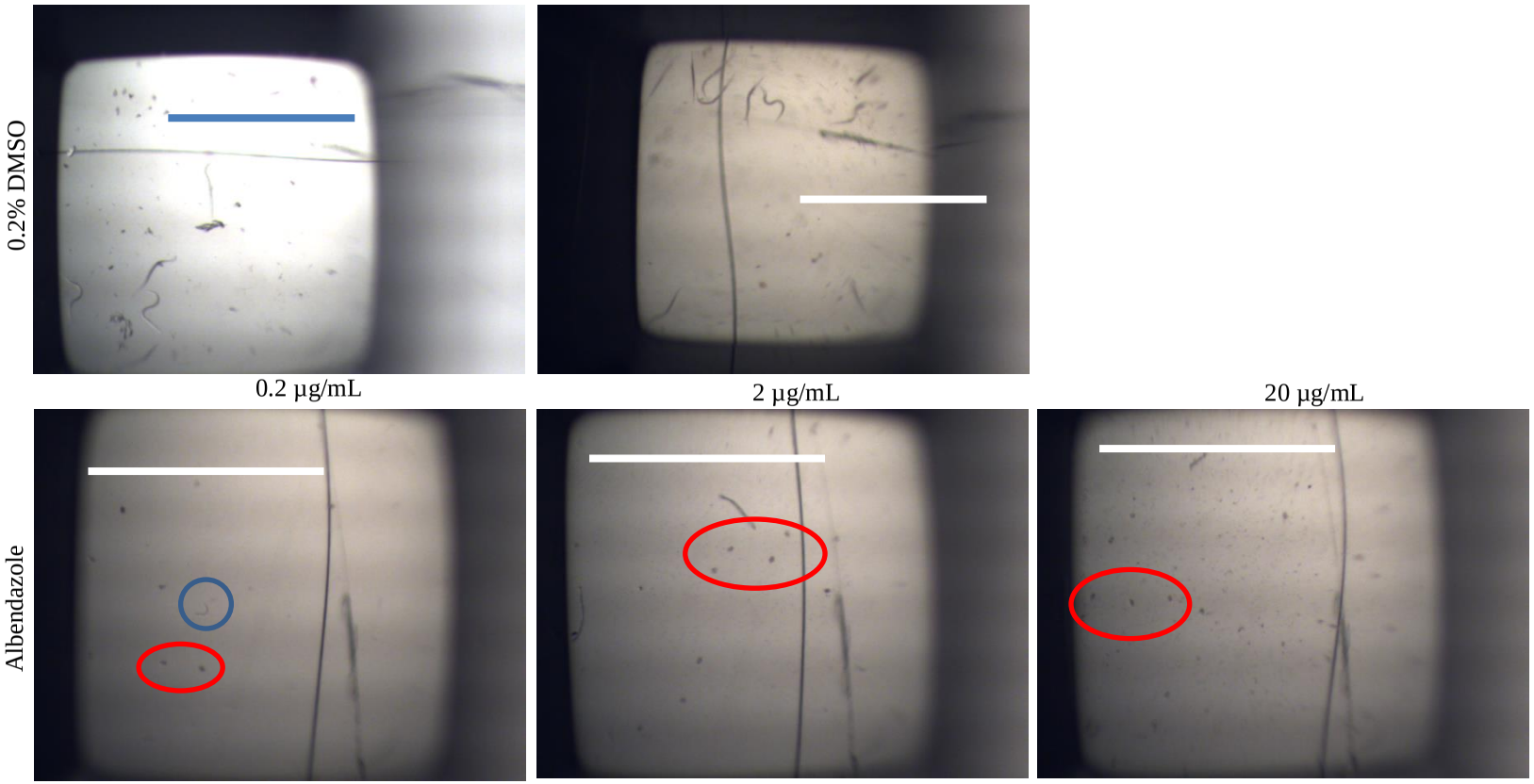


Figure 4B: Dose response of *A. ceylanicum* larvae to albendazole in 384-well flat-bottom plate. Worms cultured in 0.2% DMSO served as a negative control. Blue circles highlight hatched larvae, red circles highlight unhatched eggs. The results of the 200 µg/mL dose are not depicted due to drug precipitation that obscures vision of unhatched eggs. Scale bar represents 1 mm.

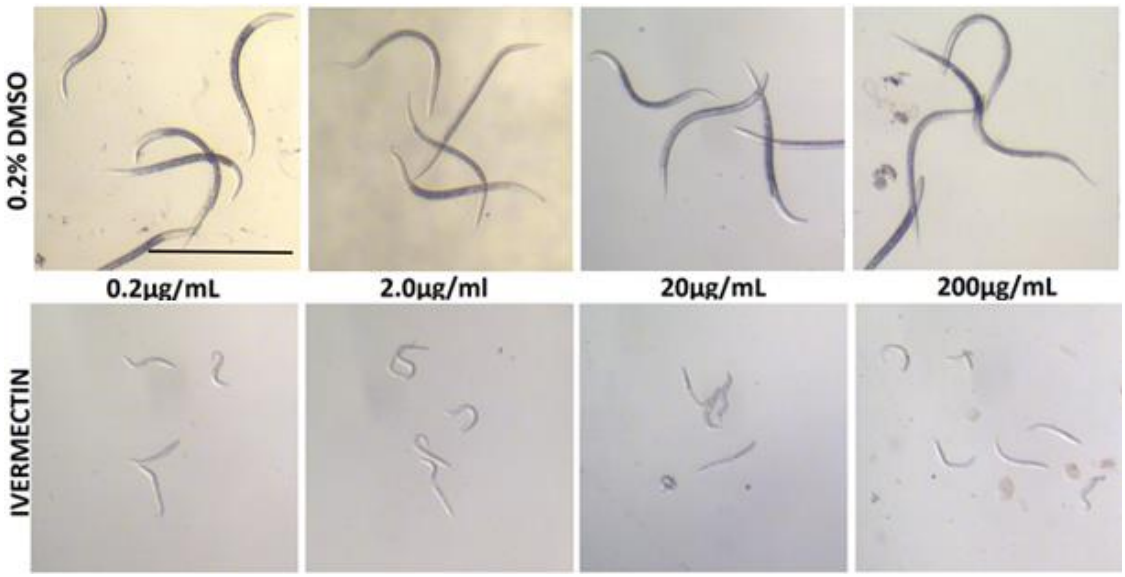
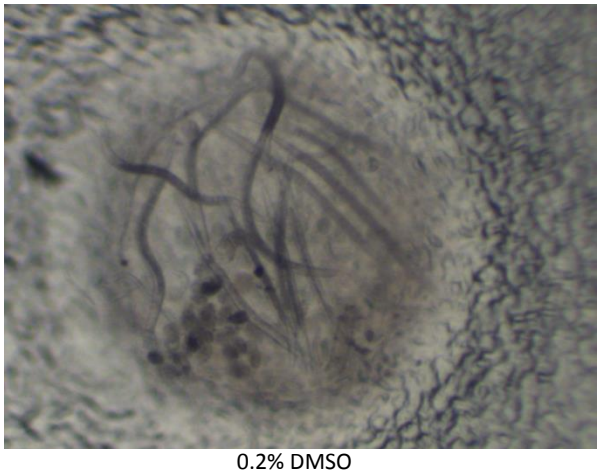
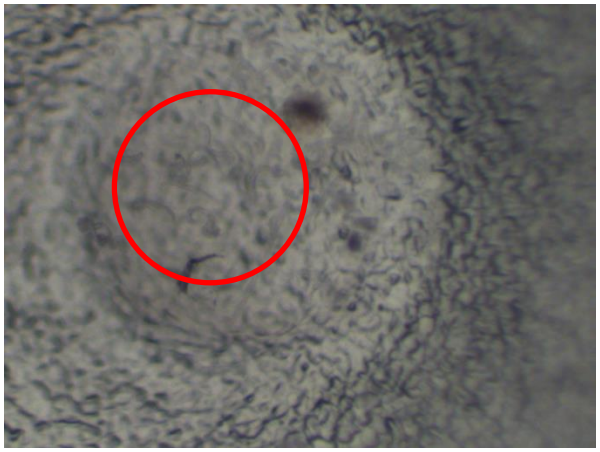


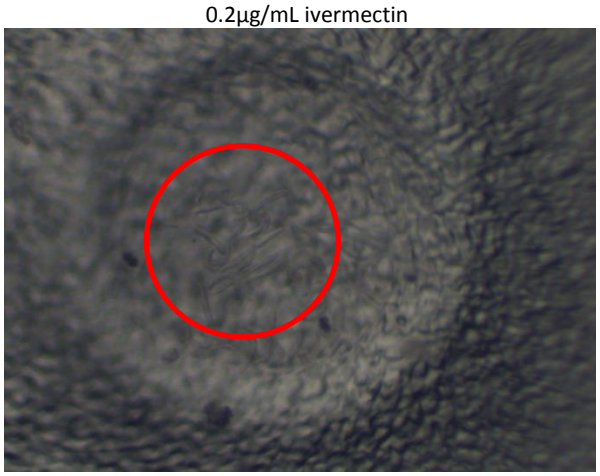
Figure 5A: Dose response of *A. ceylanicum* larvae to ivermectin in 96-well U-bottom plate, as published by Arash Safavi. Worms cultured in 0.2% DMSO (no drug) served as a negative control. Scale bar represents 0.5 mm.



0.2% DMSO



0.2µg/mL ivermectin



20µg/mL ivermectin

Figure 5B: Dose response of *A. ceylanicum* larvae to ivermectin in 384-well V-bottom plate. Worms cultured in 0.2% DMSO served as a negative control. Red circles highlight sick larvae. Pictures were selected for doses with worms that were most visible (given the nature of the plate color).

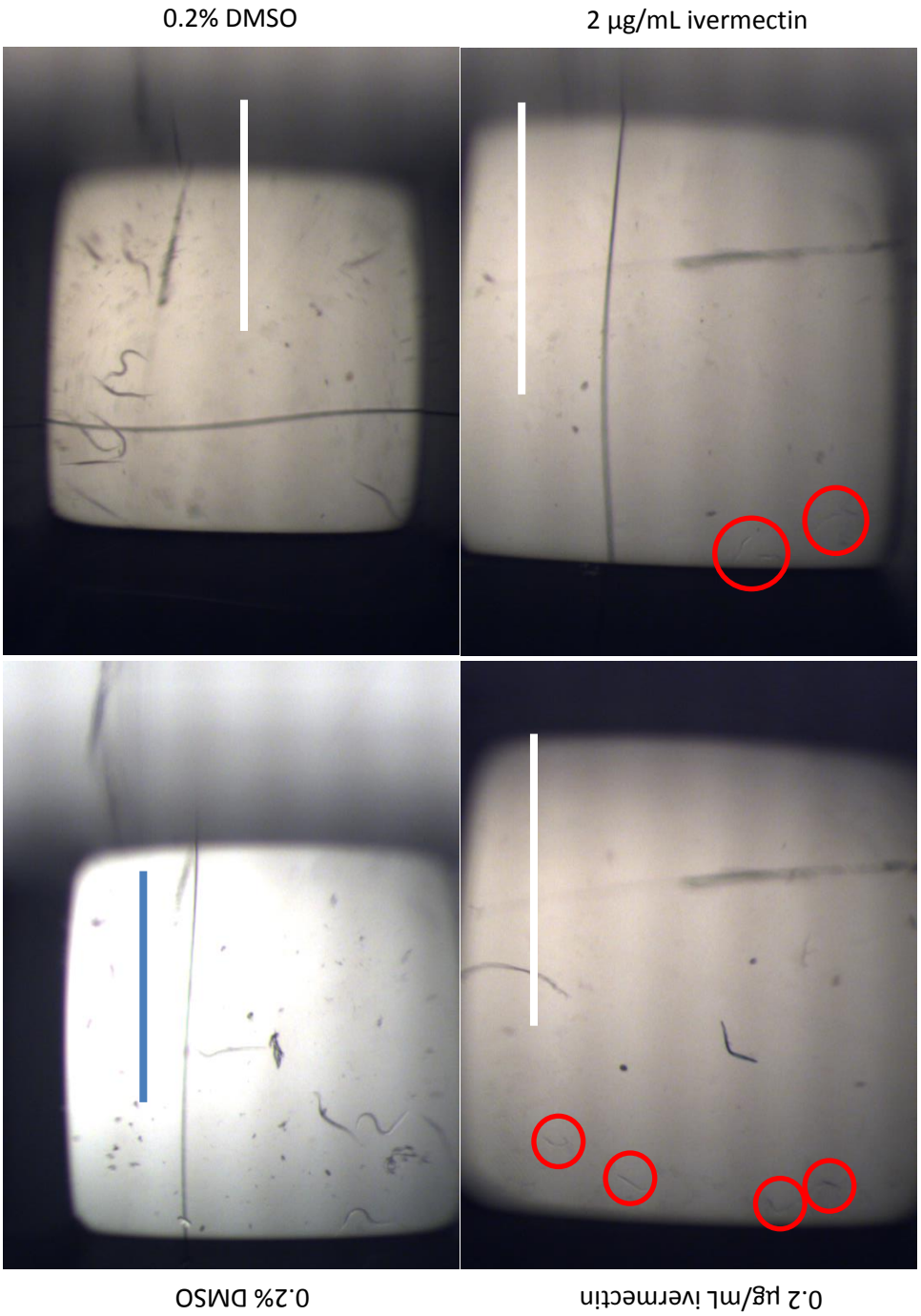


Figure 5C: Dose response of *A. ceylanicum* larvae to ivermectin in 384-well flat-bottom plate. Worms cultured in 0.2% DMSO served as a negative control. Red circles highlight sick larvae. Scale bar represents 1 mm.

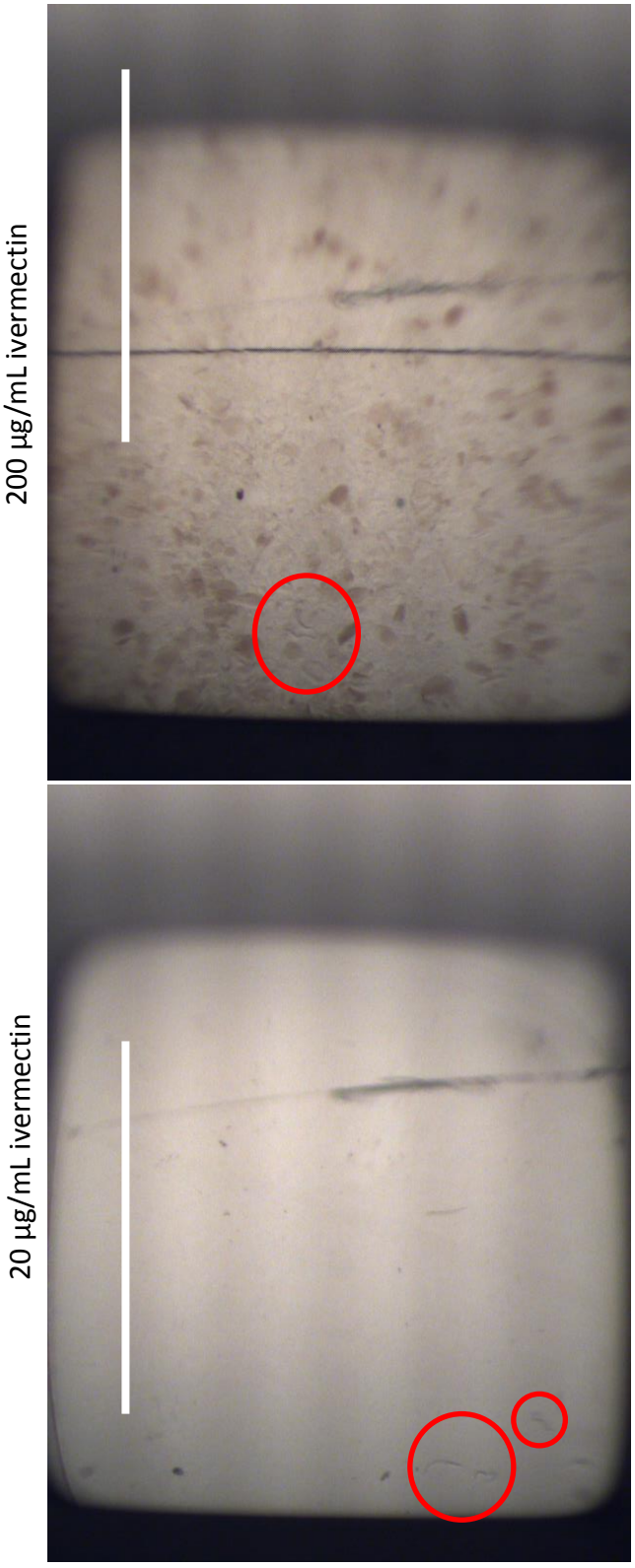


Figure 5C: continued

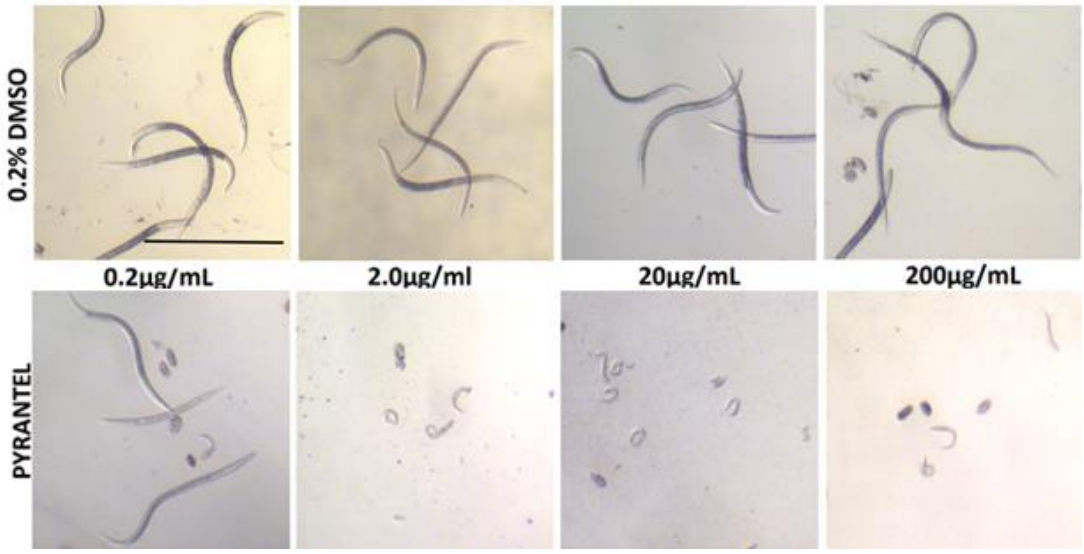


Figure 6A: Dose response of *A. ceylanicum* larvae to pyrantel pamoate in 96-well U-bottom plate, as published by Arash Safavi. Worms cultured in 0.2% DMSO (no drug) served as a negative control. Scale bar represents 0.5 mm.

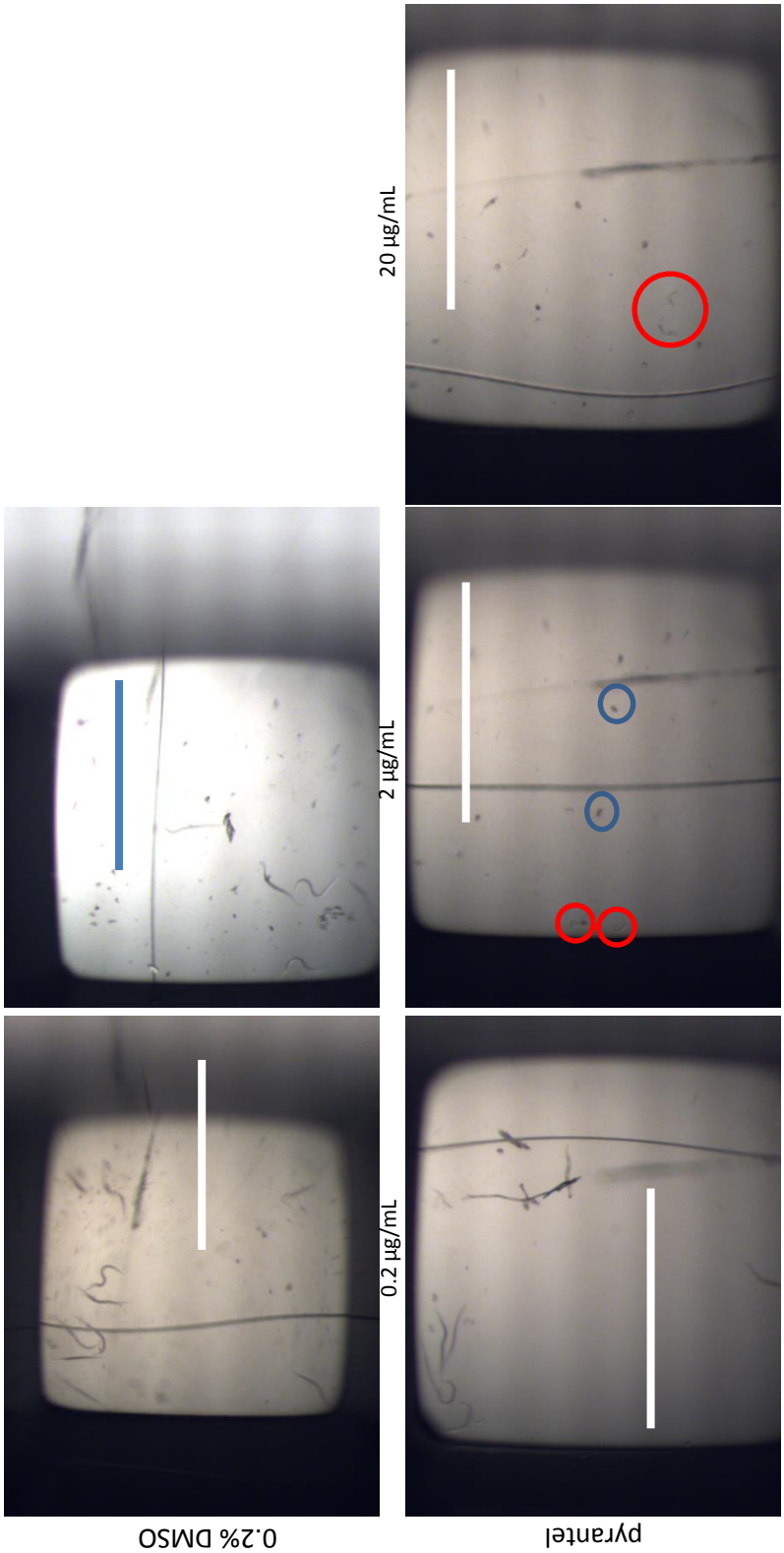


Figure 6B: Dose response of *A. ceylanicum* larvae to pyrantel pamoate in 384-well flat-bottom plate. Worms cultured in 0.2% DMSO served as a negative control. Red circles highlight hatched larvae, blue circles highlight unhatched eggs. Scale bar represents 1 mm.

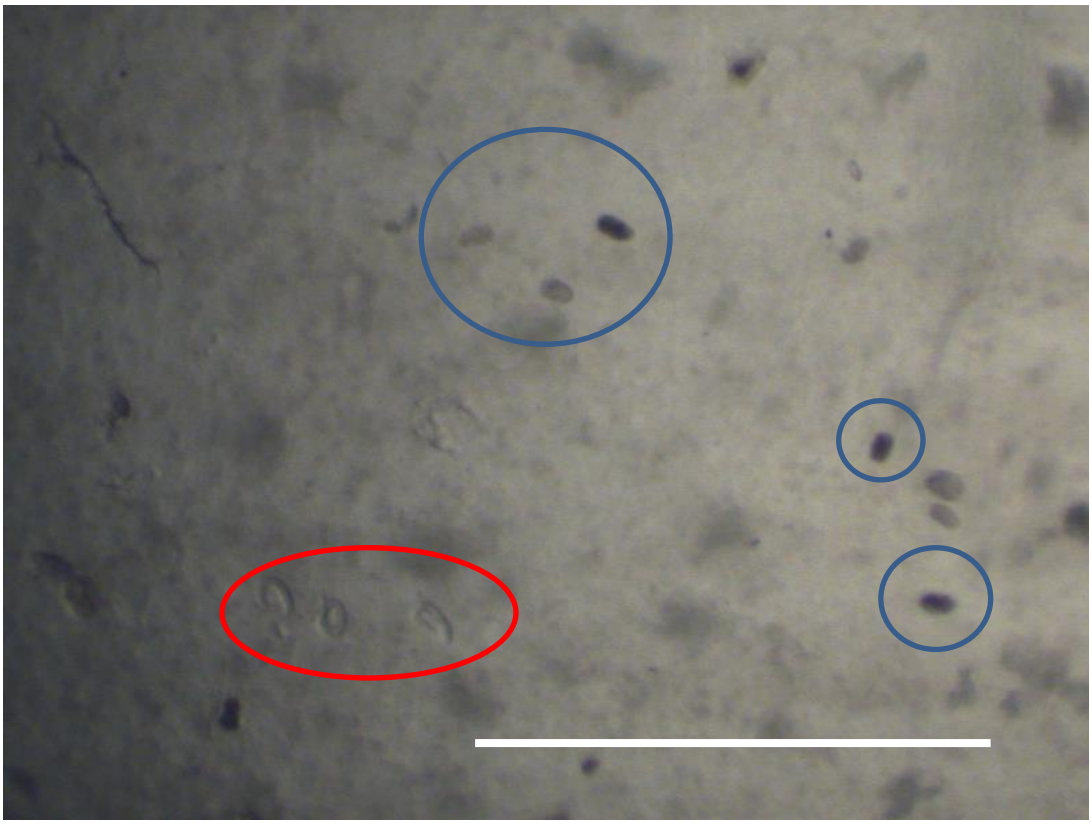


Figure 6C: Magnified picture of *A. ceylanicum* exposed to 200 µg/mL pyrantel pamoate within one well of a 384-well plate. Blue circles highlight unhatched eggs, red circles highlight hatched larvae. Scale bar represents 0.5 mm.

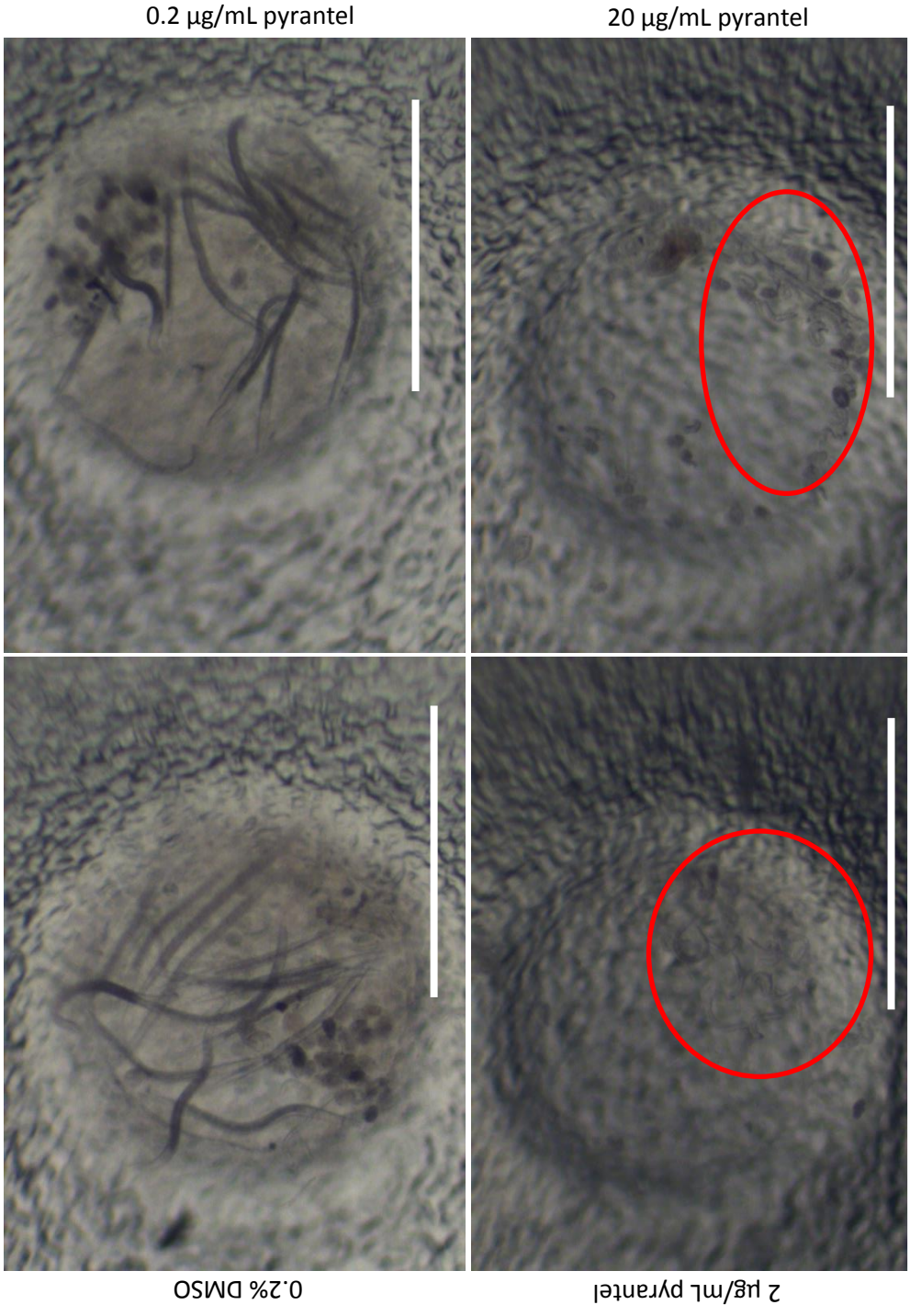


Figure 6D: Dose response of *A. ceylanicum* larvae to pyrantel pamoate in 384-well V-bottom plate. Worms cultured in 0.2% DMSO served as a negative control. Red circles highlight sick larvae. Pictures were selected for doses with worms that were most visible (given the nature of the plate color). Scale bar represents 1mm.

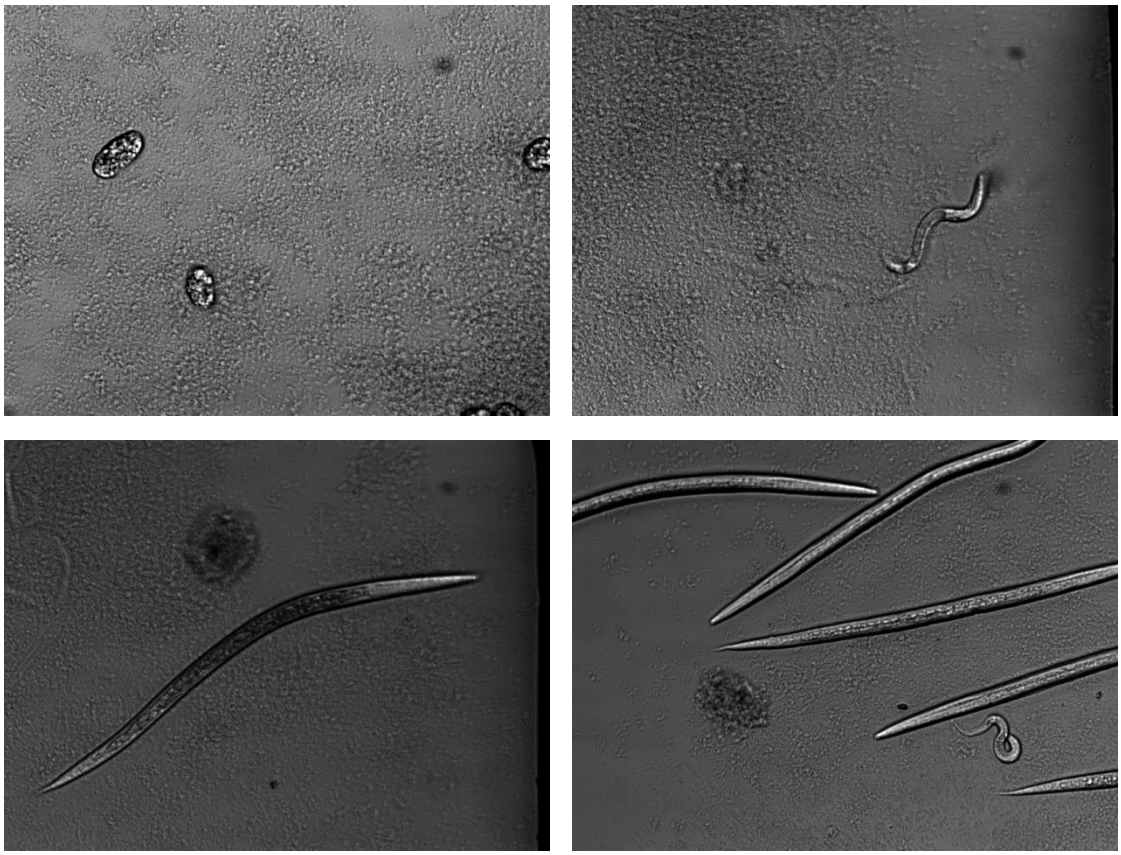


Figure 7: Sample pictures generated from the Operetta for a 384-well flat-bottom plate. Upper left: unhatched *A. ceylanicum* eggs. Upper right: *A. ceylanicum* larva in arrested development. Lower left: Healthy *A. ceylanicum* infectious L3 stage larva. Lower right: Multiple *A. ceylanicum* larvae within one well.

CHAPTER 3

Trichuris Muris DRUG SCREENING

Up to 795 million people in the world are infected with *Trichuris trichiura* (human whipworm), accounting for about a third of soil-transmitted helminth infections (WHO, 2014). Yet we are lacking an effective single-dose cure for trichuriasis (whipworm disease). *Trichuris muris* is a whipworm parasite that infects mice and is closely related to the human whipworm parasite *Trichuris trichiura*. The whipworm has a unique life cycle compared to other major human intestinal parasitic nematodes. It has no free-living stage, but instead, unembryonated eggs are passed in feces and embryonated eggs are ingested. Within the host the eggs hatch in the small intestine and migrate to the cecum, where the larvae reside and form tunnels composed of the host's own enterocyte cells (Huttemann et al, 2007).

There is an urgent need to discover a new drug that can cure trichuriasis. Additionally, if a drug is discovered that is effective against *Ascaris,* whipworms, and hookworms, it would be an amazing discovery as whipworm, hookworm, and *Ascaris* account for an overwhelming burden of parasitic infections worldwide. The phylogenetic tree for the phylum Nematoda shows that *T. muris* diverged much earlier and thus may not have evolved homologous receptors compared to many other nematodes including

hookworm, *C. elegans*, and *H. bakeri* (Blaxter et al, 1998). Therefore, if a drug is effective on both *T. muris* and *A. ceylanicum*, then it may indicate a drug that is effective on a wide range of nematodes. Hence, the objective is to develop a drug screen on *T. muris* complimentary to that of the *A. ceylanicum* drug screen and compare drug hits between the two parasites.

3.1 Development of *T. muris* Drug Screen Assay

T. muris eggs were obtained from culturing adult *T. muris* worms *in vitro*. Infected mice were sacrificed 22 days post infection and their large intestines harvested. Intestines were opened to expose the adult *T. muris* inside and incubated at 37^0C (5% CO_2) in Hank's Balanced Salt Solution (HBSS) for an hour. This allows time for the worms to release from the intestine membrane. Subsequently a mixture of male and female worms are picked into a 6-well plate containing 5 mL total culture medium per well (RPMI+HEPES, 100U pen/strep, 1ug/mL amphotericin B). RPMI (Roswell Park Memorial Institute 1640) medium is a rich medium that comprises the major component of whipworm culture medium, while HEPES is used to maintain the optimal pH. Worms are moved into new wells at 24, 48, and 72 hours. The medium from the old wells now contain eggs laid by the adult worms and is centrifuged at 600 x g (2.2 rpm) for 2 minutes in order to pellet them and wash them with sterile drinking water for a total of 5 times. Eggs are then placed in a p100 petri dish, wrapped in foil, and left at room temperature for 5-6 weeks to allow the eggs to embryonate. Embryonated eggs are collected in a conical tube and stored at 4^0C for experimental use.

Next, the embryonated eggs were used to develop optimal parameters for healthy hatching and development of larvae *in vitro*. Experiments done by Hayes et al concluded that within the host, *T. muris* eggs encounter a set of "cues" that must be present in order for eggs to hatch, most notably temperature and bacterial cues (Hayes et al., 2010). Thus, if we can mimic these cues *in vitro* and cause *T. muris* eggs to hatch and develop into healthy larvae, they can be used in a drug screen. 96-well U-bottom plates were chosen as the screening format, as was done previously with *A. ceylanicum*. Several parameters were tested including: a variety of bacterial strains, temperature, oxygen levels, culture mediums, and presence of mouse colon extracts. The optimal temperature turned out to be 37^0C, which matches the environment of worms in their natural host, and is also noted by Hayes et al. A variety of bacterial strains were tested for optimal hatching and motility of hatched larvae. Finally, a 1:1 ratio combination of *Staphylococcus aureus* and *Lactobacillus reuteri* was chosen; *S. aureus* induced almost 60% hatch rate reported by Hayes et al, while *L. reuteri* is a bacterium that naturally inhabits the intestines of *T. muris*' natural host animal. The *S. aureus* is grown overnight in LB medium at 37°C with shaking. The *L. reuteri* is grown overnight in a culture tube with 7 mL of MRS medium (a bacterial growth medium suitable for *lactobacillus*) at 30°C without shaking (because it grows under anaerobic conditions). Oxygen levels seemed to be a relevant parameter in order to more closely replicate the natural environment of *T. muris* in the host, as the cecum of mice is an anaerobic environment. Furthermore, studies by Huttemann et al on the cells of *T. muris* intestines revealed that they contain glycogen and electron light granules, but no mitochondria, suggesting that *T. muris* intestinal cells have an anaerobic energy metabolism (Huttemann et al., 2007). Therefore, the assay was kept in an airtight

Ziploc with a Gaspak (BD GasPak cat#260001) which converts oxygen to CO_2 for the duration of the incubation. The total well volume is 200 µL (double the volume for *A. ceylanicum*) so that the increased depth of the liquid column will also allow less oxygen to reach the worms during scoring (since the plate is removed from the ziploc and gaspak while scoring under the microscope). Thus the final parameters for the assay in 96-well U-bottom plates is: 190 µL of hookworm culture medium (HCM), 3 µL of *S. aureus* (OD_{600nm} = 3.0) resuspended in RPMI+HEPES, 3 µL of *L. reuteri* (OD_{600nm} = 3.0) resuspended in RPMI+HEPES, 3 µL eggs (approximately 30 eggs), and 0.5 µL of drug compound. This results in a final drug concentration of 0.025 mM in 0.25% DMSO, which is the same for the *A. ceylanicum* larval drug screen. The negative control is 0.25% DMSO and positive control is 20 µg/mL of oxantel, which is a dose low enough to allow larvae to hatch and high enough to make the worms sick and immotile. The assay was wrapped in a wet paper towel to keep humidity and sealed in a Ziploc with a Gaspak before being incubated at 37^0C (5% CO_2) for up to 48 hours. In these conditions negative control wells exhibit decent hatch rate and good motility of *T. muris* larvae (which can be viewed in video format on computers at the Aroian Lab at the University of California, San Diego).

From previous experiments done, it was seen that there is variation in hatch rate between different batches of eggs, possibly related to the strain of mice that the adult *T. muris* was harvested from. For example, eggs obtained in the Aroian lab from AKR mice showed up to 98% hatch rate while eggs from STAT-$6^{-/-}$ mice showed about an 82% hatch rate. Eggs received from Joe Urban and harvested from STAT-6 mice showed a

hatch rate of 33%. Even eggs harvested from different groups of the same strain of mice would sometimes show variability in hatch rate. It is unclear what causes this variability and thus could pose a problem for an efficient drug screen. Additionally, there is variation in how well the eggs become embryonated within the 5-6 weeks after harvesting the unembryonated eggs from adult *T. muris*. A well-embryonated egg that is expected to hatch will have a lighter semi-clear color and collect at the center of the well where the bacteria aggregate, whereas an egg that is not well-embryonated will be dark in color and scattered throughout the well. Another pitfall of developing one standard protocol for this drug screen seemed to be the variation in hatching time. For example, 24 hours of incubation was sufficient for eggs obtained in the Aroian Lab from STAT-$6^{-/-}$ mice to hatch and be scored. On the other hand, it was discovered that eggs obtained from Joe Urban needed to be incubated for 48 hours for the majority of the viable eggs to hatch. Collectively, this seemingly random variability between egg batches would be inadequate for a streamlined and efficient drug screen method. However, if eggs can be consistently obtained from a single source that consistently yields a "good" hatch rate (such as above 70%), drug screening is achievable.

In order to have a basis for scoring sick worms, the drug oxantel was used as a positive control, as it has previously been shown to be effective against whipworm (Keiser et al, 2013). An oxantel drug dose response was done on *T. muris* eggs and larvae. 10 mg of oxantel was serially diluted in 100% DMSO and brought to a final concentration of 0.2, 2, 20, and 200 µg/mL in 0.2% DMSO by bringing the volume up using RPMI + HEPES. When the drug at these doses is exposed to *T. muris* eggs, there is

zero hatching at 200µg/mL and a very low hatch rate (7-10%) at 20 µg/mL (Figure 8). At 2 µg/mL the hatch rate is a bit higher, with hatched larvae being largely immotile. At 0.2 µg/mL there seemed to be only a small effect on *T. muris* hatching and motility of larvae, with an average of 25.6% hatch rate, compared to negative control that had a hatch rate of 43.4%. These drug doses were also tested on *T. muris* larvae that were first hatched off in a 96-well plate in order to view the drug effect on larvae itself (disregarding hatch rate) (Figure 9). Eggs were incubated in a 96-well plate with HCM and *S. aureus* + *L. reuteri* for about 20 hours until the larvae hatched out, and drugs were subsequently added at a dose of 0.2, 2, 20, and 200 µg/mL in 0.2% DMSO. The negative control (0.2% DMSO) larvae display a curvaceous shape and are motile. At the lowest dose of oxantel there was no significant difference seen compared to negative controls. At 2 µg/mL some worms resemble negative control with a curvaceous shape and motility, while other worms are straight and rod-like with no motility. At the 20 and 200 µg/mL doses all worms are straight and rod-like instead of having the healthy curvaceous shape. In addition, the larvae's body texture appeared to be degenerating in the sense that the body looks as if it is disintegrating (Figure 9).

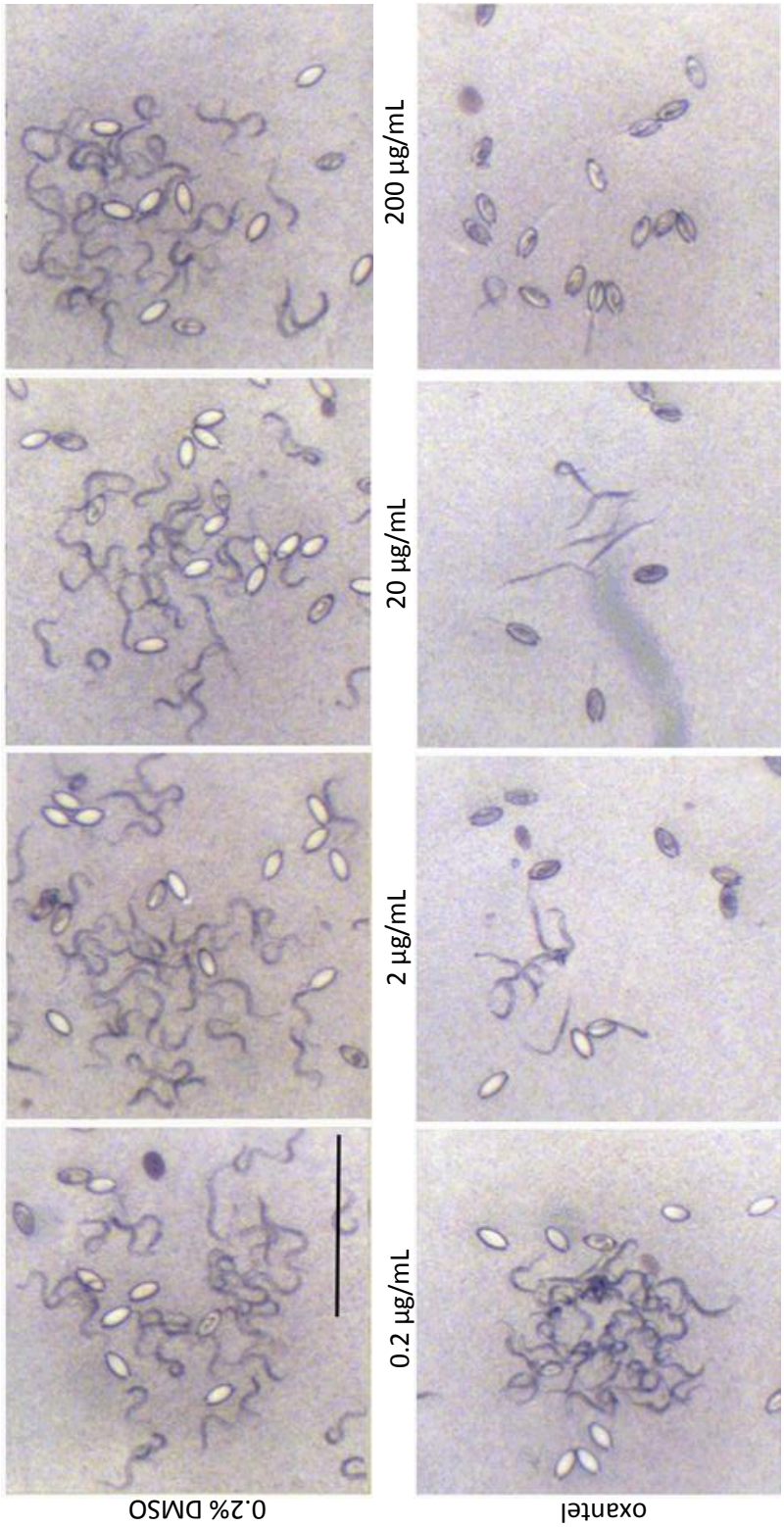


Figure 8: Dose response of *T. muris* to oxantel. *T. muris* eggs were cultured in the presence of oxantel in a tenfold dose-response fashion. Scale bar represents 0.5 mm.

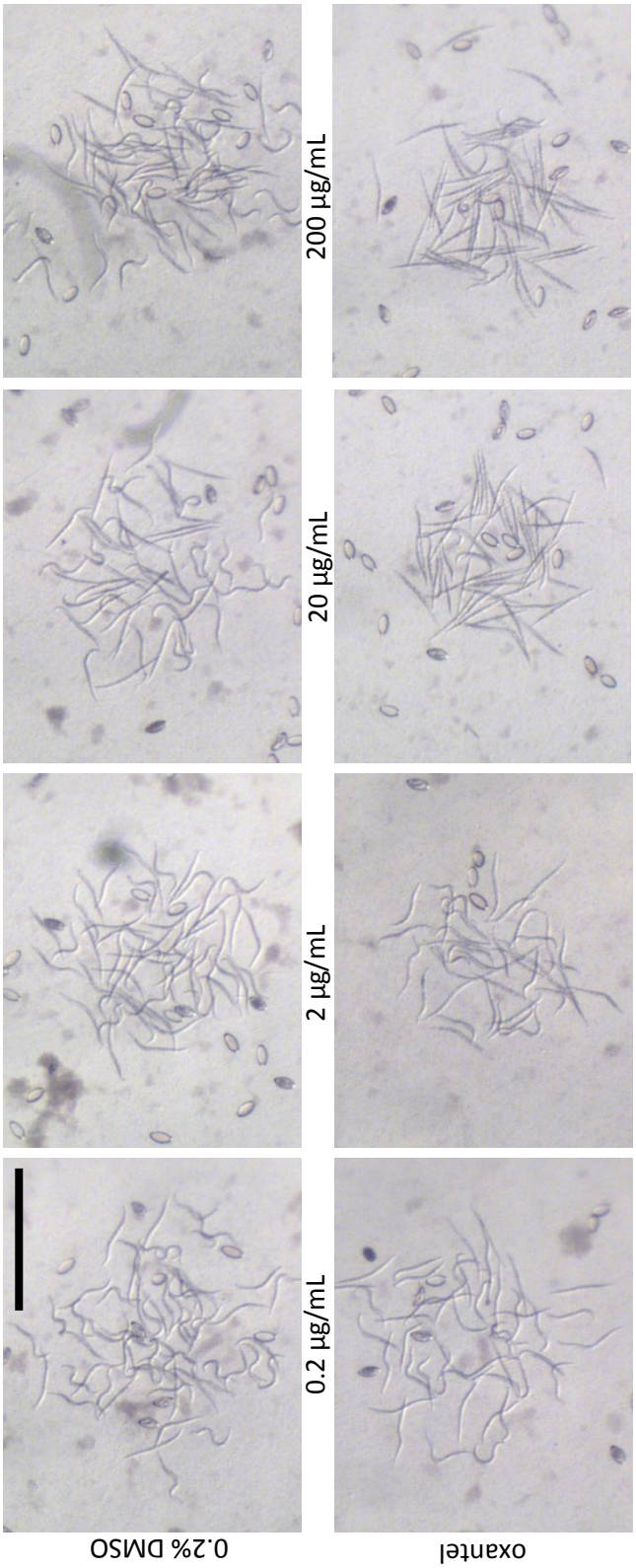


Figure 9: Dose response of *T. muris* larvae to oxantel. *T. muris* eggs were hatched off in the presence of bacteria only for 20 hours, and doses of oxantel were added subsequently in a tenfold dose-response fashion. Scale bar represents 0.5 mm.

3.2 Screening NIH Clinical Library against *T. muris*

Three hundred and sixty of 727 compounds of the NIH Clinical Library were screened with the parameters described in the previous section (chapter 3.1), yielding a total of 6 potential hits (Table 2). The screen was done with a batch of eggs received graciously from Joe Urban. Typically for this particular batch of eggs, negative control wells yielded a 33% to 40% hatch rate and larvae had good motility. After the initial screen, wells that were considered a hit were repeated in duplicate wells. The scoring was done based on hatch rate and motility, which are the most obvious effects observed from an effective drug compound because *T. muris* larvae do not grow in size *in vitro*. A compound is considered a hit if the hatch rate is less than 50% that of the negative control and/or the larvae that hatched are immotile. Nalbuphine had a 6.67% hatch rate (an average of three repeats) with no motility in hatched larvae. Flecainide had a 1% hatch rate with no motility; it is also notable that 2 out of the 3 repeats yielded a hatch rate of zero. Probenecid had a hatch rate of 11.3% with half of the worms being motile and half showing only occasional movement. Pyridine-2-aldoxime methochloride yielded a 9% hatch rate with half of the worms being motile while the other half had slow motility that was unlike the motility of negative controls. Loperamide had a 6.67% hatch rate with all hatched larvae having slow and minor movement. Finally, timolol had a 4% hatch rate with half of the worms being motile and half being immotile but not dead (twitches once in a while). See Figure 10 for pictures of these hits. Of the six hits found on *T. muris* larvae (Table 2), one compound also was a hit on *A. ceylanicum* larvae (flecainide). The reason only half of the compounds were screened is that the batch of eggs being used was

depleted, and when a different batch of eggs were tested with the same protocol the resulting hatch rate and motility were significantly lower. It is presumed that perhaps the eggs had been stored for too long (approximately 2 years), their viability diminished over time.

Table 2: Summary of NIH Clinical Library hits on *T. muris* larvae.

Compound	Function	Plate and well #
Nalbuphine	semi-synthetic opioid agonist-antagonist analgesic of the phenanthrene series	2658 – A2
Flecainide acetate	Anti-Arrhythmia Agent, Voltage-Gated Sodium Channel Blockers	3078 – C6
Probenecid	Adjuvants, Pharmaceutic - Agents that aid or increase the action of the principle drug (DRUG SYNERGISM) or that affect the absorption, mechanism of action, metabolism, or excretion of the primary drug (PHARMACOKINETICS) in such a way as to enhance its effects	2989 – D2
Pyridine-2-aldoxime methochloride	Cholinesterase Reactivators - Drugs used to reverse the inactivation of cholinesterase caused by organophosphates or sulfonates	2989 – E2
Loperamide	Antidiarrheals	2989 – F7
Timolol	Adrenergic beta-Antagonists, Anti-Arrhythmia Agents	2989 – B10

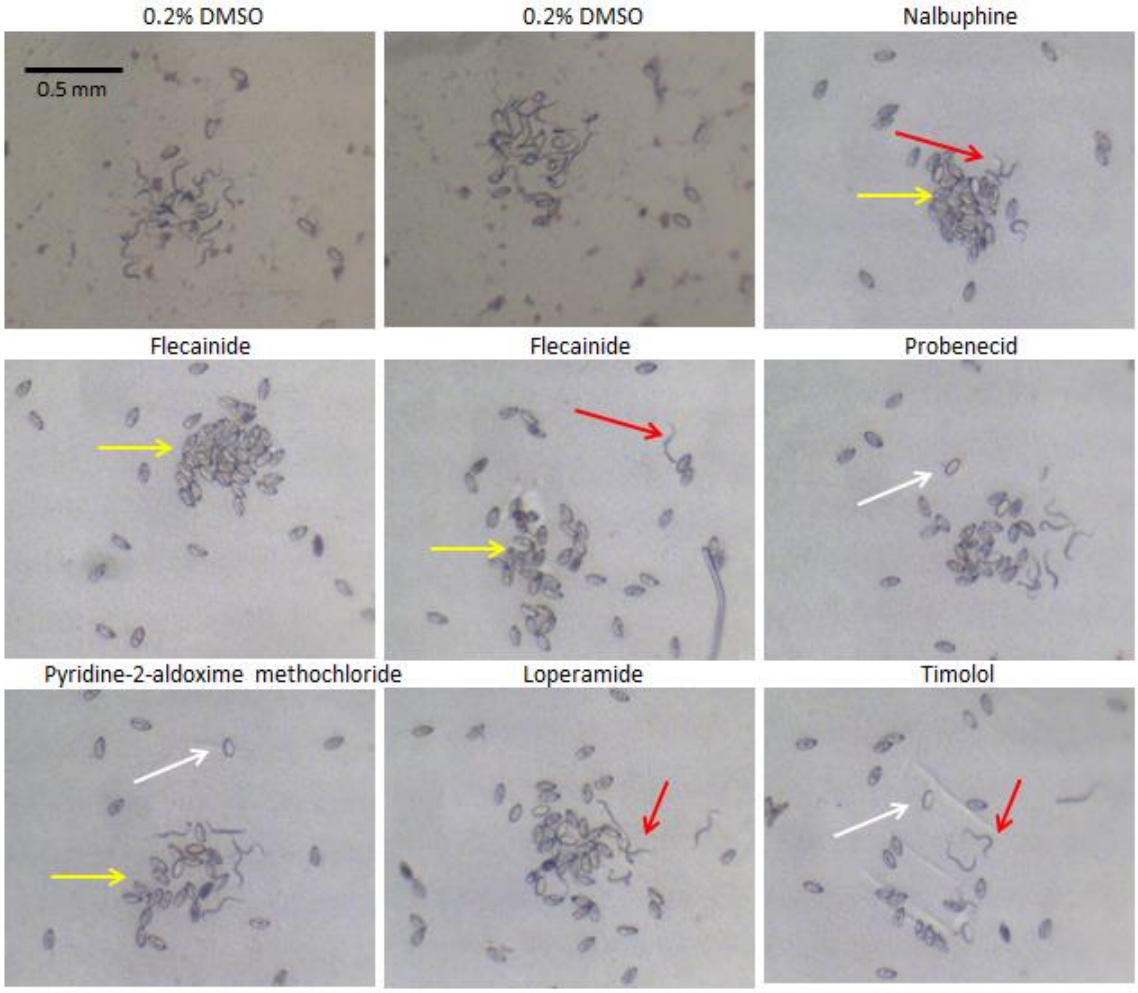


Figure 10: Examples of hits from NIH Clinical Library on *T. muris* larvae. Scale bar represents 0.5 mm. Red arrows indicate hatched larvae, white arrows indicate empty eggshells, yellow arrows indicate unhatched eggs.

3.3 Validation of *T. muris* larval drug screen

In order to validate the *T. muris* larval drug screen, the six hits that were found in Table 2 above were tested on adult *T. muris*, which is the relevant target for a drug to be used clinically. Adult parasites were isolated from a STAT6 mouse coinfected with *T. muris* (28 days post infection) and *H. bakeri* (9 days post infection). The mouse was sacrificed and the intestine was harvested. After opening the intestine using scissors, the intestine was placed in pre-warmed Hanks buffer for 45 minutes to allow the parasites to loosen. The small intestines were then placed in a wire mesh over a beaker of warm HBSS, allowing the released *H. bakeri* to fall to the bottom of the beaker and be collected for another experiment (to be discussed later). The adult whipworms were removed from the intestine by picking them individually, and placed into a petri dish with pre-warmed RPMI medium. The assay was set up in a 384-well flat bottom plate. 40 µL of *T. muris* culture medium (RPMI 1640 + 25 mM HEPES, pH=7.2, 100 U/mL Penicillin-Streptomycin, 1 ug/mL Amphotericin B) was added to each well. Next, 0.4 µL of drug compound from the NIH library plate was added to the wells. An additional 40 µL of *T. muris* culture medium was added to each well and 40 µL was subsequently transferred to their respective duplicate wells, giving a final concentration of 50 uM for each drug compound. Albendazole, oxantel, and pyrantel were serially diluted in DMSO and used as positive controls at a final concentration of 50 µM. One healthy *T. muris* adult was picked into each well. Each compound was tested in two wells (one as a replicate), for a total of two worms per compound. The assay was scored for motility every 24 hours for 4 days. Scoring was done on a 0 to 3 number system. A worm that is moving its whole

body continuously is scored as a 3 (healthy). A 2 is given if the worm is only moving part of its body or not moving as rigorously as the negative control, but still moves on its own without stimulation. A score of 1 is given if the worm is barely moving or only moves if stimulated by a worm pick. Finally a score of 0 means the worm has no motility even when stimulated (presumably dead). Out of the 6 potential candidates (2989 D2, 2989 E2, 2989 B10, 2989 F7, 2658 A2, 3078 C6), only one compound, flecainide (3078 – C6), showed an appreciable effect on the day 28 *T. muris* (Figure 11). The motility score of worms exposed to flecainide decreased to zero within 48 hours, proving to be almost as effective as the positive control anthelmintics oxantel and pyrantel. Flecainide has now been found to be effective against *A. ceylanicum* larvae, *T. muris* larvae, and adult *T. muris*.

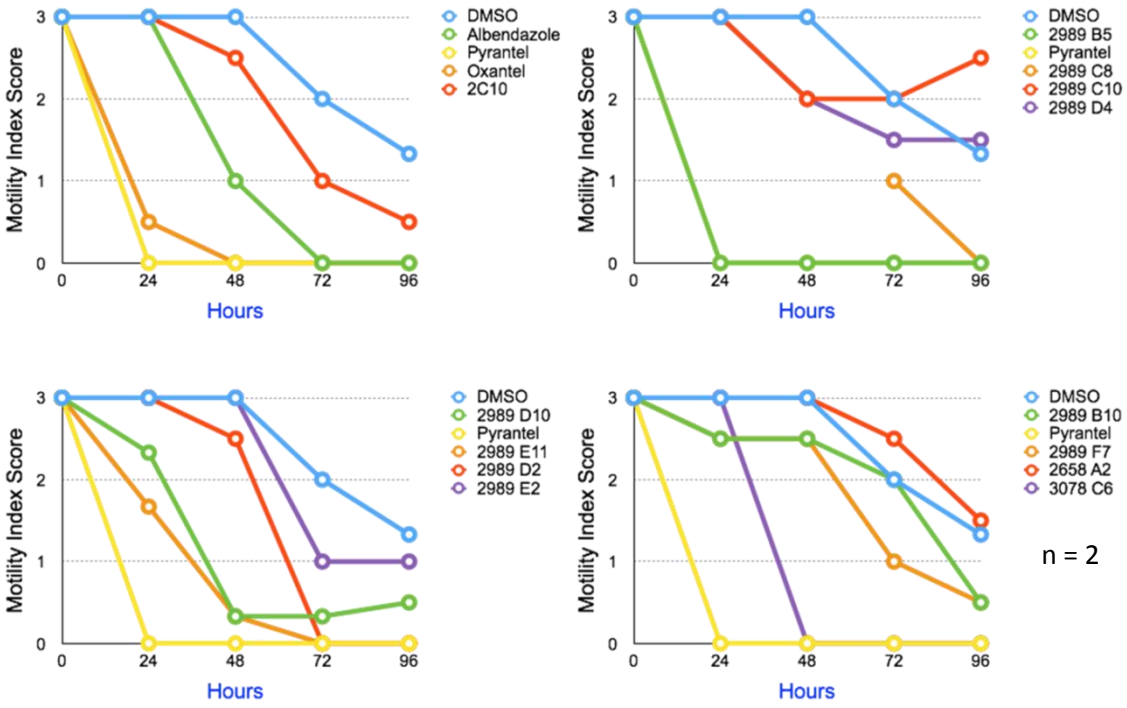


Figure 11: Graphs of motility index of adult *T. muris* worms exposed to different hits identified on *A. ceylanicum* larvae and *T. muris* larvae during initial screen of drug libraries. Worms were exposed to drugs at a concentration of 50 µM for 96 hours. "DMSO" represents negative control (no drug) condition.

Compounds identified to be effective on *A. ceylanicum* larvae: 2C10, 2989 B5, 2989 C8, 2989 C10, 2989 D4, 2989 D10, 2989 E11

Compounds identified to be effective on *T. muris* larvae:

- 2989 D2: probenecid
- 2989 E2: pyridine-2-aldoxime methochloride
- 2989 B10: timolol
- 2989 F7: loperamide
- 2658 A2: nalbuphine
- 3078 C6: flecainide acetate

CHAPTER 4

FOLLOW UP EXPERIMENTS ON POTENTIAL DRUG CANDIDATES

After an initial drug screen done on larvae, it is important to test the hits that are found on adult worms because that is the relevant target for a drug to cure infections *in vivo*. Sam Pan from the Aroian Lab takes the hits found on the *A. ceylanicum* larval drug screen and tests them on multiple adult nematodes using a variety of assays. I was fortunate enough to be given the opportunity of working with him to test different hits found from our initial drug screens, with focus on one particular drug compound of interest (dolastatin). Dolastatin was of particular interest for a couple of reasons. It has already been tested in clinical trials to be developed as an anti-cancer drug and completed phases I and II. This drug is a tubulin inhibitor, which works in the same class of drugs as albendazole (Gajula et al, 2013). However, it is believed that dolastatin does not work on the same targets as albendazole and therefore could potentially be a new drug that is effective against soil transmitted helminthes. Albendazole works by binding to the colchicine-sensitive site of tubulin, thus inhibiting its polymerization into microtubules. Dolastatin is a tubulin binding drug that is classified as a polymerization inhibitor which binds to the vinca domain. Vinca domain ligands interfere with the binding of vinblastine to tubulin and inhibit microtubule assembly. Thus these compounds prevent cell mitosis by binding to tubulin in the mitotic spindle and inhibiting polymerization into

microtubules (Islam et al, 2004). Working with Sam Pan, dolastatin was tested on adult *A. ceylanicum*, L4 stage *C. elegans*, adult *H. bakeri* (day 9), and adult *T. muris* (day 28).

4.1 Adult *A. ceylanicum*

Firstly, positive hits from an SIO clinical library received graciously from the Fenical Lab were tested on adult *A. ceylanicum*. The SIO library is a library of 640 marine Actinomycete compound mixtures at a stock concentration of 25 mg/mL in 100% DMSO that I initially screened on *A. ceylanicum* larvae with my partner, Arash Safavi, which is published in his thesis. Subsequently an additional plate of purified compounds was given to our lab which I screened and found two hits, including dolastatin. The combined hits from all SIO library plates were tested on adult *A. ceylanicum*. Adult parasites were isolated from day 17-18 infected hamsters. Hamsters were sacrificed and their intestines harvested. The intestines were opened and placed in pre-warmed Hanks buffer solution for 45 minutes to allow the parasites to release from the intestinal tissue. Hookworms were picked into a petri dish with pre-warmed RPMI + HEPES to be used in the experiment. A 24-well plate was used with a total volume of 500 µL per well. Each SIO drug compound was diluted and added to the well at a final concentration of 10 µg/mL, with hookworm culture medium making up the rest of the 500 µL volume per well. Albendazole and pyrantel were serially diluted and used as positive controls at a final concentration of 10 µg/mL. Six healthy adult *A. ceylanicum* were picked into each well. The assay was incubated at 37^0C (5% CO_2) and scored for motility every 24 hours for 5 days. Scoring was done on a 0-3 number system where a 3 equates to normal intensive movement, a 2 for continuous smooth movement but not as intense as a 3, 1 for

low motility after stimulation with a worm pick, and a 0 for no movement at all. The majority of the potential candidates tested had some effect of making the worms sick, although dolastatin ("2c10") was the only compound that showed lethality to adult *A. ceylanicum*. Furthermore, dolastatin proved to be even more effective than the positive control albendazole, having a lethal effect by 48 hours. Compounds 140B6 and 138C4 are also interesting candidates because they were previously found to be efficacious against *A. ceylanicum* larvae, *H. bakeri* larvae, and *C. elegans* in a drug screen (done by Arash Safavi), and now seem to have an effect on adult *A. ceylanicum* as well. See Figure 12 for these results.

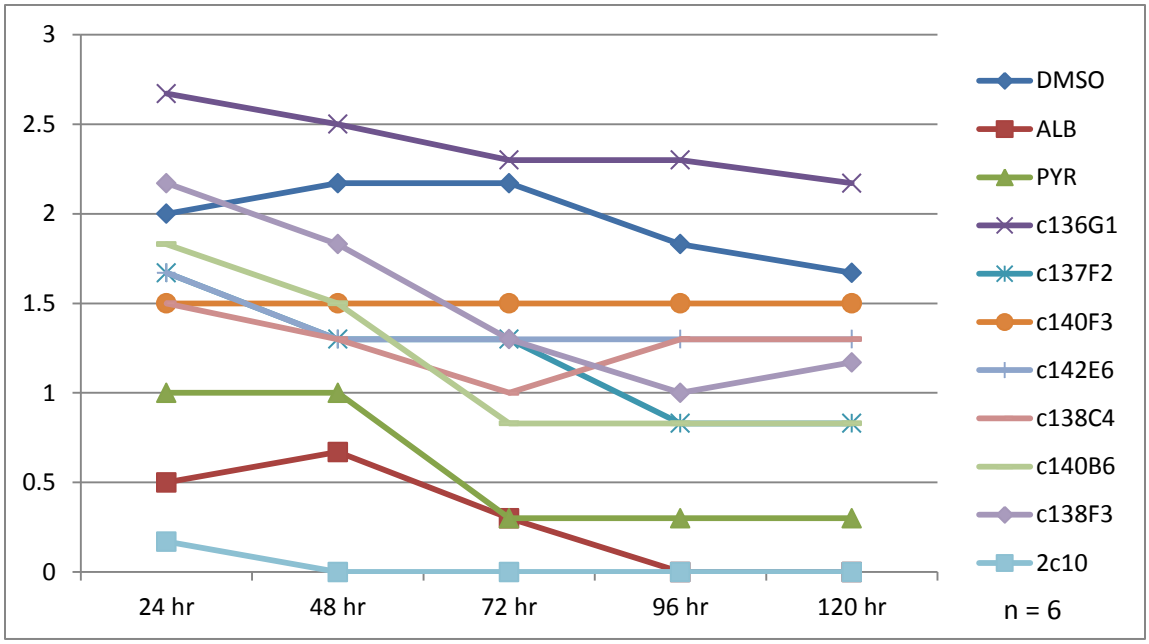


Figure 12: Graph of motility index of adult *A. ceylanicum* worms exposed to potential drug candidates over a period of 120 hours. The Y axis represents the average motility score of 6 worms. "DMSO" represents the negative control (no drug) condition, and drugs were tested at a final concentration of 10 µg/mL.

4.2 *C. elegans* Brood Assay

The *C. elegans* brood assay was done to test the effect of dolastatin on ben-1 (e1880) *C. elegans*. Ben-1 *C. elegans* is a strain with a tubulin mutation that shows partial resistance to albendazole. Both albendazole and dolastatin work by targeting tubulin, but they have different specific target sites. If this is true we should see a difference when comparing their effects on ben-1 *C. elegans*. In preparation for the experiment, ben-1 (e1880) or N2 (wild type) L4 *C. elegans* were picked from a maintenance plate onto a new plate with OP50 bacteria. The plates were incubated for 64 hours to allow L4 larvae to reproduce, and incubated for an additional 44 hours to allow progenies to develop into L4 larvae to be used for the experiment. The assay is done in a 48-well plate with each well containing: 140 µL of special S-medium (no $CaCl_2$), 40 µL of OP50 (OD_{600nm} = 3.0), and 20 µL of drug for a final concentration of 100 µg/mL. One ben-1 or N2 L4 stage *C. elegans* was picked into each well. The assay is incubated at 25^0C for 64 hours. In order to score for brood size the contents of each well are transferred to individual pre-warmed agar plates and allowed to air dry. The progenies are counted under the microscope (removing each worm from the plate by vacuum suction after it is counted).

The composite data shown in Figure 13 is a collection of data over 5 repeated experiments. Because ben-1 worms have partial resistance to albendazole, its brood size is not as high as the DMSO control but is higher than that of N2 worms (Figure 13). The dolastatin also has an overall greater effect on the brood size of both wild type and ben-1 worms compared to albendazole (Figure 13). There is a significant effect of dolastatin on both wild type and ben-1 mutant worms, but at this point we cannot make any

conclusions about whether the ben-1 mutation confers resistance for the worms. However, this is the type of experiment we could do to test the effects of dolastatin compared to albendazole.

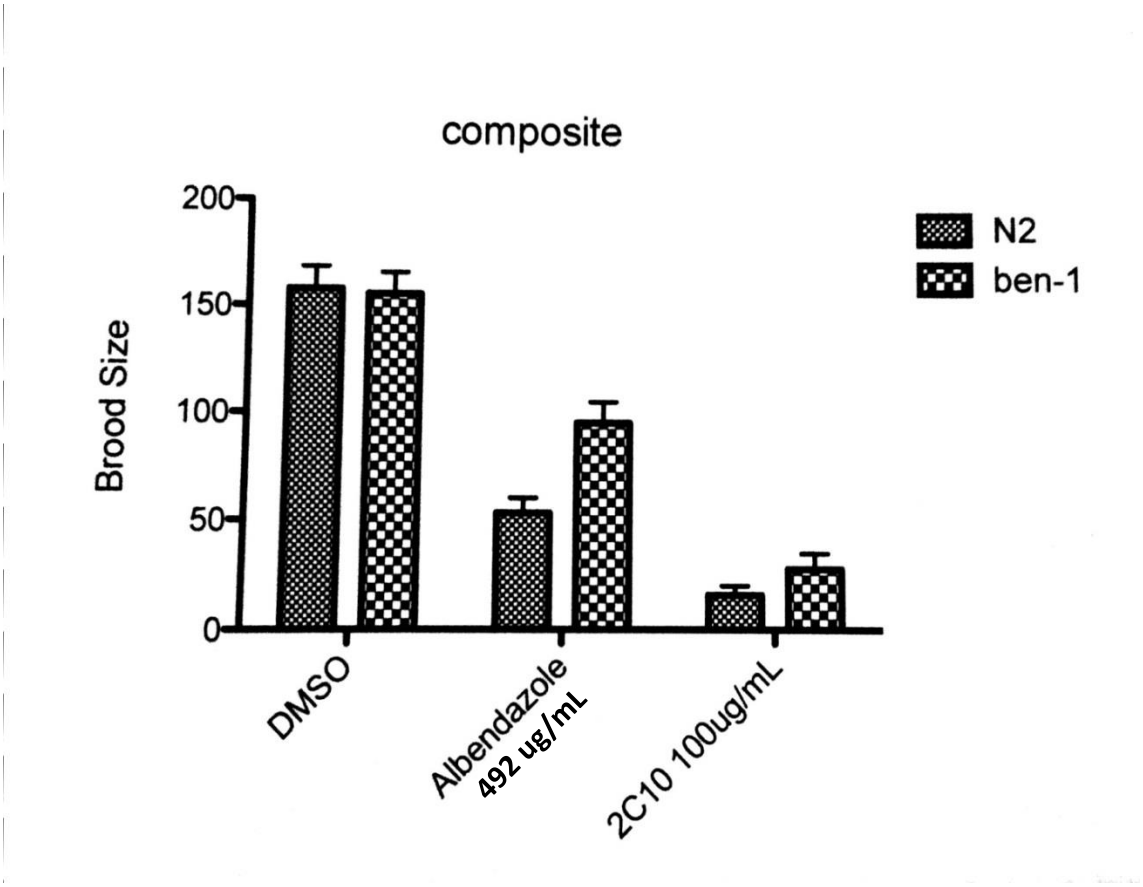


Figure 13: Brood size of N2 and ben-1 *C. elegans* exposed to dolastatin or albendazole. "DMSO" represents negative control (no drug) condition. Composite data represents averages obtained from 5 repeat experiments.

4.3 Adult *H. bakeri* Drug Screen

Heligmosomoides bakeri (*H. bakeri*) or *Heligmosomoides polygyrus* is a rodent intestinal parasite nematode. Adults have a unique appearance of being heavily coiled, thus it is given the name "polygyrus". *H. bakeri* has a high infection rate and is a good model for chronic intestinal nematode infections in mice. This parasite was used in the discovery of ivermectin, thus playing an important role in anthelmintic development. For this experiment, adult *H. bakeri* (9 days post infection) were obtained by sacrificing and harvesting the intestine of a STAT6 mouse co-infected with both *T. muris* and *H. bakeri*. As described previously and also here briefly, the intestine was opened and placed on a wire mesh over a warm beaker of HBSS in order to allow the parasites to release and collect at the bottom of the beaker to be used for the experiment. The assay was done in a 384-well flat bottom plate with 40 µL total volume of whipworm culture medium (RPMI 1640 + 25 mM HEPES, pH=7.2, 100 U/mL Penicillin-Streptomycin, 1 ug/mL Amphotericin B) and a final drug concentration of 50 µM. Oxantel and pyrantel were used as positive controls also at a concentration of 50 µM. Albendazole at a concentration of 50 µM was also tested, although albendazole typically has no effect on adult *H. bakeri*. Two to three healthy day 9 adult *H. bakeri* were picked into each well. The assay was incubated at 37^0C (5% CO_2) and scored every 24 hours for 4 days. Plate 2989 of the NIH clinical library was chosen to be screened, with particular interest in the hits that were previously found on *A. ceylanicum* larvae and *T. muris* larvae (Figure 14). Each drug compound was tested with duplicate wells in the same 384-well plate for this screen. Scoring for adult *H. bakeri* was again done on a 0-3 number system. A 3 is given for

tightly wound (coiled) worm that is usually quite motile (uncoils and recoils parts of its body constantly). A 2 is given for worms that are motile and have some parts of their body tightly coiled while other parts are loose (uncoiled). A worm with a score of 1 may move occasionally and if stimulated, but is generally uncoiled and loose, resembling a pile of loose ropes. A zero is given for worms that are uncoiled and immotile (presumably dead). A graph of the motility score is created by graphing the average scores of all worms for each compound on each 24 hour time mark (Figure 14).

The negative control worms remained healthy for 72 hours, and showed a drop in motility score between 72 to 96 hours. The positive control worms also showed typical results which are described here. Pyrantel is the most effective on the worms, causing them to be sick by 24 hours and killing them by 72 hours. Oxantel has a moderate effect on the worms but results in a more steady drop in health over the course of 96 hours. Albendazole is typically not effective on adult *H. bakeri*, although we see a steep drop in health between 72 and 96 hours similarly to the negative control. The results of the screen with regards to the whole plate 2989 in general showed no compounds with a significant effect on the worms save for one compound (chlorpromazine). With regards to the 6 hits found on *T. muris* larvae specifically (Table 2), none of them had a significant effect on adult *H. bakeri*. Finally, in regards to the 6 hits found on *A. ceylanicum* larvae (Table 1), only one compound (2989 – B5 = chlorpromazine) showed an interesting effect on the worms. Chlorpromazine (2989 – B5) caused a moderate and steady drop in health of the worms over the course of 96 hours similarly to the oxantel positive control. Although it is true that the biggest decrease in health occurred between 72 and 96 hours, which also

occurred in the negative control, this compound caught interest because it was also identified as a hit on *A. ceylanicum* larvae, and works against adult *A. ceylanicum* (experiment done by Sam Pan) and adult *T. muris* as well (to be discussed in the next section).

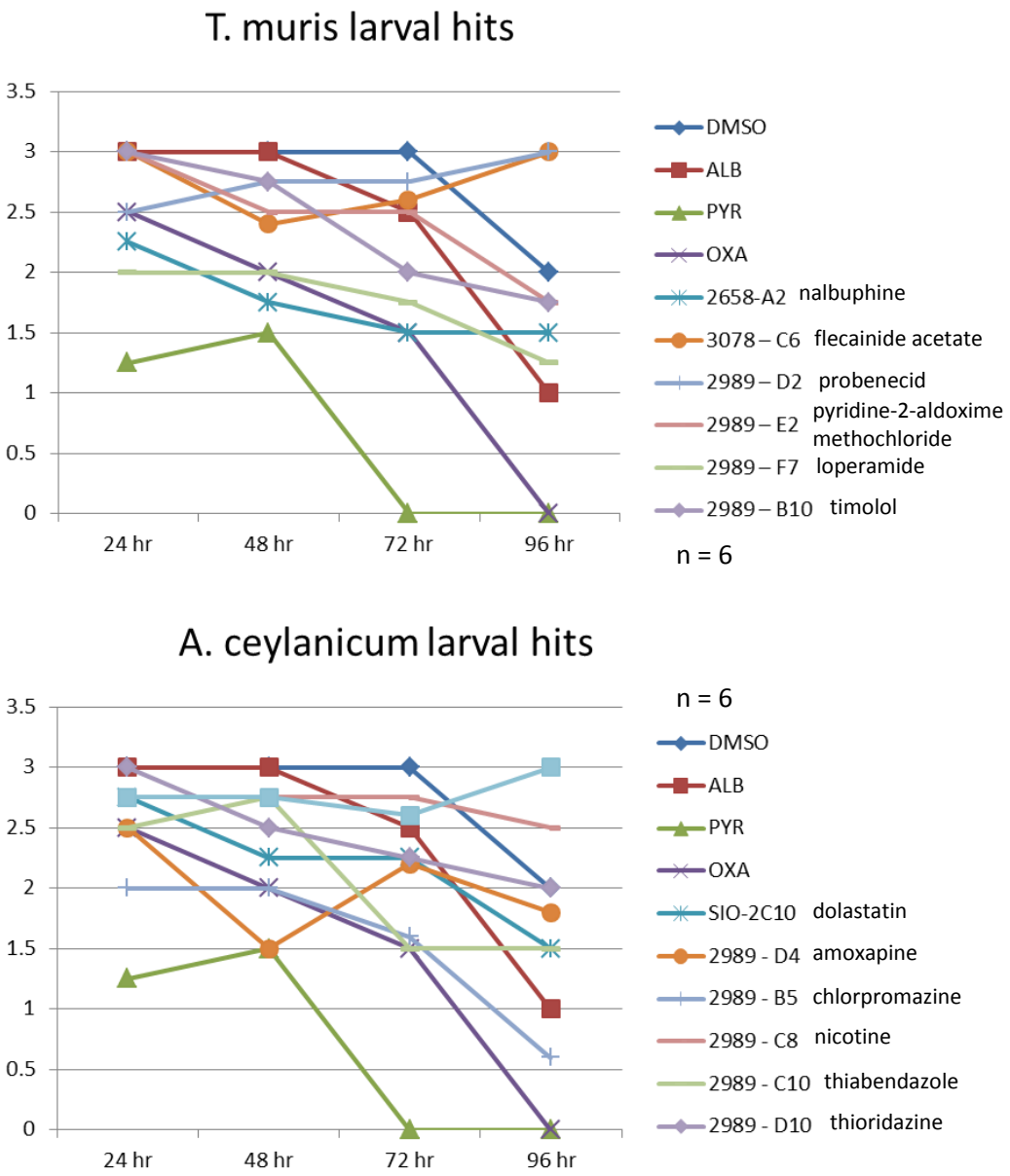


Figure 14: Graphs of motility index of adult *H. bakeri* worms exposed to hits identified from drug libraries over a period of 96 hours. Y- axis represents average motility score of 2 or 3 worms. The top graph displays data for hits identified on *T. muris* larvae during initial screen. The bottom graph displays data for hits identified on *A. ceylanicum* larvae during initial screen. "DMSO" represents the negative control (no drug) condition, and drugs were tested at a final concentration of 50 µM.

4.4 Adult *T. muris* Drug Screen

The experiment done on adult *T. muris* in order to validate the *T. muris* larval drug screen has been previously described (chapter 3.3). Simultaneously using the same protocol, the NIH clinical library hits found on larval *A. ceylanicum* were also tested on adult *T. muris* and is described in this section. Dolastatin, which was identified from the SIO clinical library to be effective on *A. ceylanicum* larvae is included. To summarize the experimental set up, adult parasites were isolated from a STAT6 mouse coinfected with *T. muris* (28 days post infection) and *H. bakeri* (9 days post infection). The assay was set up in a 384-well flat bottom plate with 40 µL of *T. muris* culture medium (RPMI 1640 + 25 mM HEPES, pH=7.2, 100 U/mL Penicillin-Streptomycin, 1 ug/mL Amphotericin B) per well. Drugs were tested at a final concentration of 50 uM for each drug compound. Albendazole, oxantel, and pyrantel were used as positive controls at a final concentration of 50 µM. One healthy *T. muris* adult was picked into each well and the assay was scored for motility every 24 hours for 4 days on a 0 to 3 number system. With regards to the six hits identified from the NIH Clinical Library to be effective on *A. ceylanicum* larvae, 4 compounds (2989 B5, 2989 C8, 2989 D10, 2989 E11), were also efficacious against the adult *T. muris* compared to the negative DMSO control (Figure 15). As briefly mentioned earlier, Chlorpromazine (2989 – B5) results in lethality of adult *T. muris* within 24 hours. The dolastatin (2c10) has only a moderate effect on adult *T. muris* at best (Figure 15).

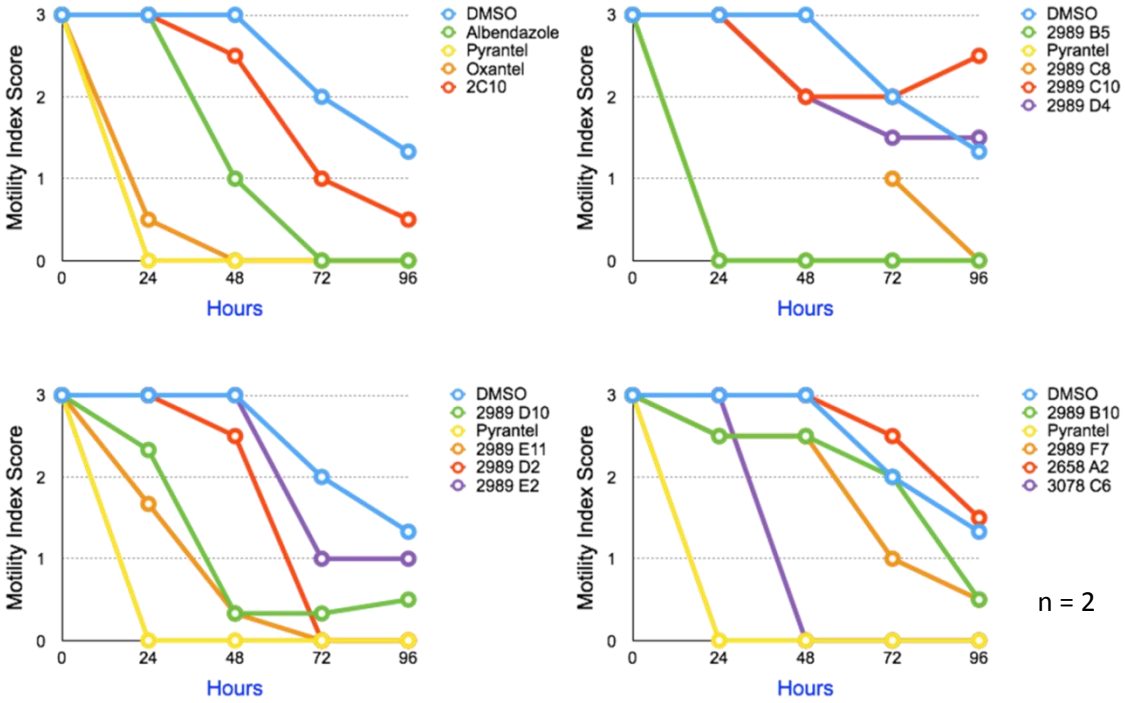


Figure 15: Graphs of motility index of adult *T. muris* worms exposed to different hits identified on *A. ceylanicum* larvae and *T. muris* larvae during initial screen of drug libraries. Worms were exposed to drugs at a concentration of 50 µM for 96 hours. "DMSO" represents negative control (no drug) condition.

Compounds identified to be effective on *A. ceylanicum* larvae:

- 2C10: dolastatin
- 2989 B5: chlorpromazine
- 2989 C8: nicotine
- 2989 C10: thiabendazole
- 2989 D4: amoxapine
- 2989 D10: thioridazine
- 2989 E11: demeclocycline

Compounds identified to be effective on *T. muris* larvae:

- 2989 D2: probenecid
- 2989 E2: pyridine-2-aldoxime methochloride
- 2989 B10: timolol
- 2989 F7: loperamide
- 2658 A2: nalbuphine
- 3078 C6: flecainide acetate

CHAPTER 5

DISCUSSION

I demonstrate here the ability to screen compounds from a drug library on both the zoonotic human hookworm *A. ceylanicum* and the whipworm *Trichuris muris*. The whipworm drug screen assay still has some pitfalls and at this point is inadequate for a reliable high-throughput screening system. One major pitfall is the variability in hatch rate between different batches of *T. muris* eggs which causes the system to be unreliable and inconsistent, yet the source of this variability is unknown. Scoring the *T. muris* larvae is also somewhat complicated and requires the eyes of trained individuals (which may be subjective) to assess the motility of larvae. Although the whipworm drug screen could be improved on, it was able to identify a drug compound that is effective on *A. ceylanicum* larvae, *T. muris* larvae, and adult *T. muris*, so the assay may still prove to be useful. Because the NIH clinical library is composed of drugs that are FDA approved and have a history of use in human clinical trials, they may be easier to develop into anthelmintics approved for treatment of STH infections in humans. Therefore, screening this library in particular against hookworm and whipworm parasites directly gives us valuable information on which drugs to pursue in the search for new anthelmintics.

Six drugs have been identified here that are effective against multiple nematodes of different stages in the parasitic life cycle. Flecainide (3078 – C6) was effective against

A. ceylanicum larvae, *T. muris* larvae, and adult *T. muris*, and also has some effect on adult *A. ceylanicum* within 72 hours. Dolastatin (SIO library 2C10) was lethal against adult *A. ceylanicum* and larval *A. ceylanicum*, but only moderately effective against adult *T. muris*. Chlorpromazine (2989 – B5) was lethal against *A. ceylanicum* larvae, adult *A. ceylanicum*, and adult *T. muris*, and causes a steady decline in health of adult *H. bakeri* over the course of 96 hours. Nicotine (2989 C8), thioridazine (2989 D10), and demeclocycline (2989 E11) which were lethal against larval *A. ceylanicum* were also efficacious against the adult *T. muris*. These drugs could be useful in treating people suffering from polyparasitism and should be further explored for *in vivo* effects.

I also demonstrate the ability to observe a drug dose response on *A. ceylanicum* in 384-well plates, and thus the ability to use this system for drug screening. *A. ceylanicum* is the most useful and relevant parasite to screen libraries on as it is a zoonotic human hookworm. Combining the use of an automated dispensing system such as a worm sorter and the automated imaging system Operetta, the 384-well format has great potential as a high-throughput drug screen assay. As observed from the NIH clinical library screen, compounds that are effective on *A. ceylanicum* show a distinct phenotype (zero hatch rate or severe developmental arrest) which can easily be detected objectively by a machine. The development of a high-throughput and low-cost drug screening method which can utilize relevant model parasites such as *A. ceylanicum* and *T. muris* is a step toward making the discovery of new anthelmintics more feasible.

Soil-transmitted helminth infections are considered neglected tropical diseases. They inflict tremendous disease burden throughout developing countries worldwide, but

there is little to no incentive for pharmaceutical companies to develop a cure since infections are nearly non-existent in the developed world. Because the NIH clinical library is composed of drugs whose safety profiles are already known, it is my hope that finding potential drug candidates from this library means there will finally be an interest to develop a new anthelmintic simply by using an already existing drug in a new way. Maybe one day STH infections will cease to be classified as a neglected tropical disease and mass drug administration will break the cycles of poverty that so many people are trapped in.

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