UNIVERSITY OF CALIFORNIA SAN DIEGO

Microtubule-modulating agents as potential treatments for neglected tropical diseases

A thesis submitted in satisfaction of the requirements for the degree Master of Science

in

Chemistry

by

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The Thesis of Lawrence Jon Liu is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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LIST OF ABBREVIATIONS

HAT…… Human African Trypanosomiasis

SAR…… Structure Activity Relationship

RT…….. Room Temperature

BZ……... Benzimidazole

ABZ…… Albendazole

MBZ…… Mebendazole

MT…….. Microtubule

TPD……. Triazolopyrimidine

PPD……. Phenylpyrimidine

IMD...……...Imidazole

BBB……. Blood Brain Barrier

PP……. Pyrantel Pamoate

NGM…… Nematode growth medium

MTD……. Maximum tolerated dose

DMSO…….. Dimethyl sulfoxide

MOA……..…….. Mechanism of action

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ABSTRACT OF THE THESIS

Microtubule-modulating agents as potential treatments for neglected tropical diseases

by

Lawrence Jon Liu

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Professor Conor R. Caffrey, Chair
Professor Michael Sailor, Co-Chair

Protozoan and helminth parasites of humans are common in areas of extreme poverty. Treatment of these infections relies on often antiquated drugs that are toxic and/or for which resistance has developed. New drugs are needed. Microtubule (MT)-modulating agents are established treatments for cancer and, more recently, as experimental treatments for neurodegenerative indications such as Alzheimer's disease. Further, tubulin is a known molecular

target in both parasitic protozoa and helminths. Herein, I first developed and optimized a robust automated assay to measure the motility of C. *elegans,* the nematode which is often used as a convenient parasite surrogate for the screening of anthelmintics. This assay was augmented by a visual assay to measure egg hatching. Next, a collection of 38 MT-modulating agents was then screened at 50 μM in both assays and a number of 'hits' were discovered. Further optimization of the egg hatching assay and additional screening of MT-modulating agents will be pursued. Lastly, in relation to Human African Trypanosomiasis (HAT), one MT-modulating agent was tested in a mouse model of *Trypanosoma brucei* infection for anti-parasite efficacy. After intraperitoneal administration at 5, 7.5 and 10 mg/kg to infected mice, the compound decreased parasitemia in the blood to below detectable levels and significantly extended the survival of mice relative to vehicle-treated infected controls. The data encourage the further pursuit of MT-modulating agents for the therapy of HAT.

Introduction

Infectious diseases of poverty, caused by eukaryotic parasites, remain a major impediment to the economic and social advancement of the communities affected [1]. These diseases cause high mortality and/or morbidity and are common in lower income regions where access to clean water and sanitation is precarious [2]. In addition to their medical importance, parasites place an enormous burden on agricultural productivity with economic losses of the order of billions of dollars worldwide each year [3, 4]. Parasites are broadly separated into protozoa (single-cell organisms) and metazoa (multicellular organisms such as helminths (worms) and arthropods) [5]. This thesis will focus on an example of each, namely *Trypanosoma brucei*, the causative agent of Human African Trypanosomiasis (HAT; Sleeping sickness) and *Caenorhabditis elegans,* which although not a parasite, is used as a model for studying parasitic nematodes, especially in relation to screening for new anthelmintics [6].

1.1 Human African Trypanosomiasis (Sleeping sickness)

This vector-borne disease is caused by *T. brucei* which comprises three main sub-species, *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense*, all of which are transmitted by the bite of the tsetse fly vector [7]. *T. b. gambiense* and *T. b. rhodesiense* infect approximately 10,000 people each year primarily in West Africa and East Africa, respectively, whereas *T. b. brucei* infects only certain mammals, including mice, and is, therefore, used as a model trypanosome in the laboratory [7-9]. Although the number of infections has decreased over time, the World Health Organization states that the true infection rate is difficult to define given that the disease is mainly found in rural and isolated parts of Africa [10]. After being bitten by an infected tsetse fly, the flagellated trypanosome initially invades the blood and lymphatic systems (Stage 1 infection) and, eventually, the brain and central nervous system (CNS; Stage 2 infection). Clinical symptoms include drastic

changes in behavior, *e.g.*, paranoia and sleep/wake cycles that progress to coma and death [11]. The life-cycle of the parasite continues when the tsetse fly takes a blood meal from an infected human or animal (Fig. 1).

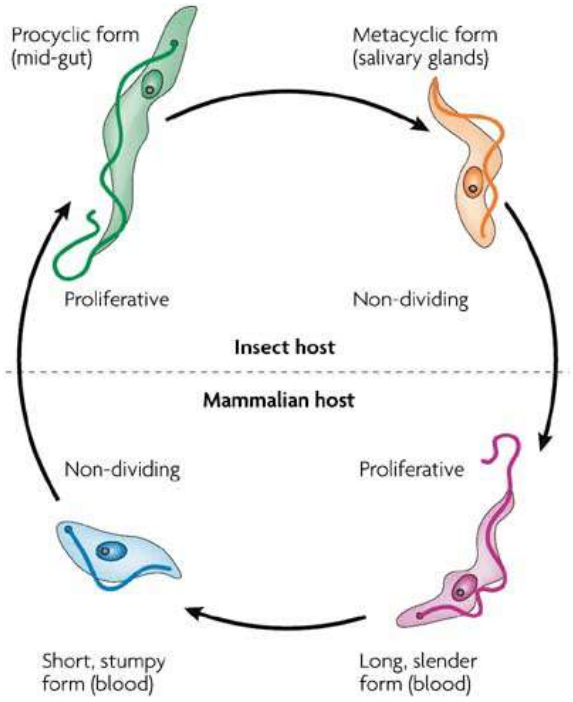
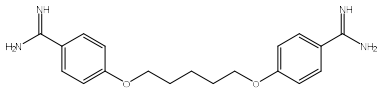
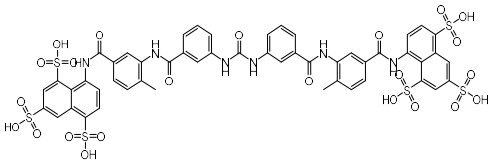
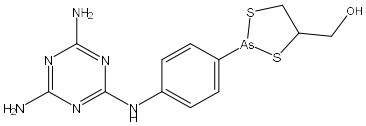
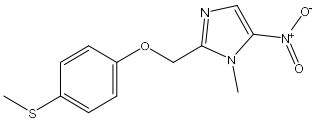


Figure 1. The life cycle of *T. brucei*. The four major developmental stages of *T. brucei* are indicated and each is adapted to survival in the insect or mammalian host. The parasite's length ranges from 14 to 33 μm in length [12, 13].

There is no vaccine for HAT and therapy relies heavily on a handful of antiquated and toxic drugs that can only be delivered systemically and under expert medical supervision, which, in rural sub-Saharan Africa, is difficult to find [11] (Table 1).

Table 1. The four principal drugs and their dosing regimens used to treat Stage 1 and 2 HAT.

Name	Structure	Dose and regimen	Route
Pentamidine		**Stage 1:** 4 mg/kg daily for 7-10 days	im or iv
Suramin		**Stage 1:** 20 mg/kg daily on days 1, 3, 7, 14, 21	iv
Melarsoprol		**Stage 2:** 3.6 mg/kg daily every three days for three weeks	iv
Fexinidazole		**Stage 2:** 20 mg/kg daily for up to 10 days	po

List of approved drug treatments for HAT [14-18]. im = intramuscular; iv = intravascular; po = oral.

In addition, side effects such as vomiting, hypertension, hypoglycemia and nephrotoxicity have been recorded, thus, negatively impacting patient compliance and proper treatment [14]. Finally, there is resistance to many of these drugs which further complicates an already challenging medical situation [19, 20]. In 2019, fexinidazole entered the market as an oral tablet taken once a day for 10 days (Table 1) [17]. As encouraging as this development is, it behooves the scientific community to continue the search for new drugs given the ever-present threat of drug resistance.

1.2 Parasitic helminths

Helminths infect more than 1.5 billion people with at least 300 species that use humans as a host [20, 21]. Infections are most concentrated in tropical and sub-tropical areas such as sub-Saharan Africa and South-East Asia [21] (Fig. 2A). Helminths are large multicellular organisms [22] and transmission can be fecal-oral, or involve invertebrate vectors or direct penetration of the skin [21-23]. Though helminths do not replicate like protozoa they are prodigiously fecund with the ability to produce hundreds to thousands of eggs/day depending on species [23-24, 25]. For example, the human hookworm, *Necator americanus*, lays 10,000 eggs in a day which, when released in human feces, results in significant environmental contamination [26, 27]. Once established in the mammalian host, helminth infections can persist for years, decades or even a lifetime [26].

Drugs that kill or expel helminths from hosts are called anthelmintics [28, 29]. The medical and veterinary communities rely on about 10 commonly prescribed drugs to treat all helminth infections [28]. For human infestations, two of the first line drugs are the benzimidazoles (BZs): albendazole (ABZ; Fig. 2B) and mebendazole (MBZ; Fig. 2C) [28]. BZs causes parasite death by inhibiting proper tubulin function and preventing glucose uptake [30, 31]. Loss of tubulin function results in paralysis and leads to the expulsion of gastro-intestinal worms or the killing of systemic nematodes [32].

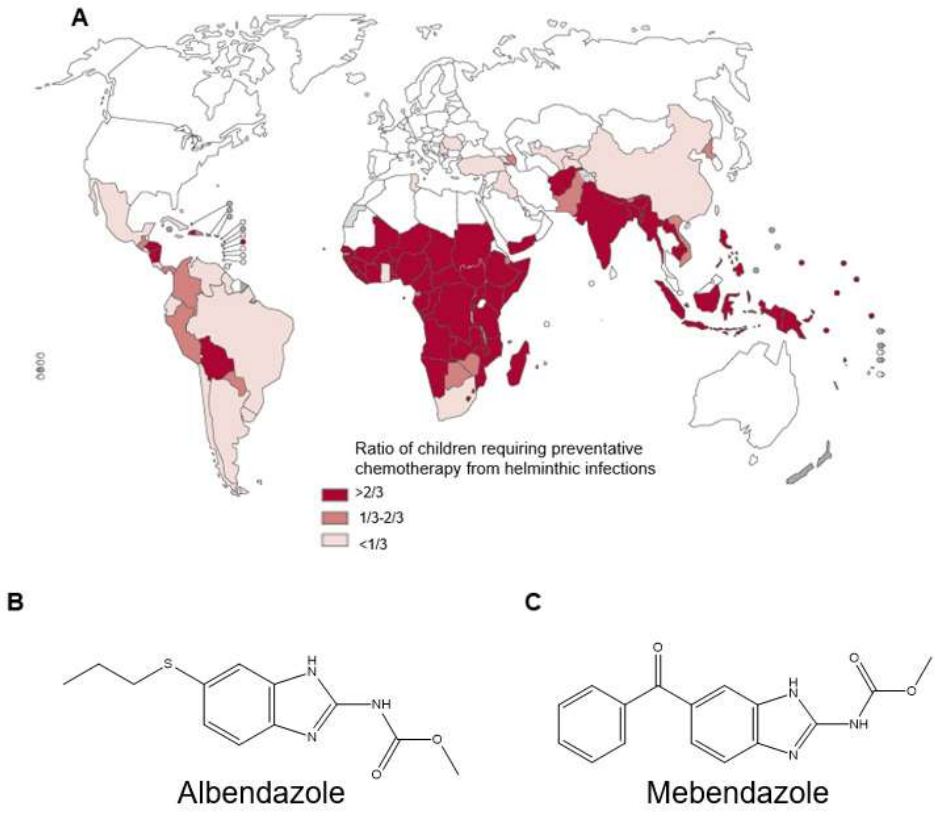


Figure 2. Global distribution of helminth parasites and the commonly prescribed benzimidazole anthelmintics. A. Global distribution of helminthiases [33]. The ratio describes the children requiring chemotherapy relative to the given population. **B.** and **C.** Structures of ABZ and MBZ, respectively.

1.3 Drug resistance in helminths

Reports of resistance to the BZs in helminths infecting humans are anecdotal but, nonetheless, are a constant source of worry [34, 35]. By contrast, resistance to the BZs is widespread in livestock, particularly sheep in Europe, Australia, South Africa and South America [36, 37]. For instance, in the UK, the total losses for 2010 in the sheep sector were £84 million [38].

1.4 *C. elegans* as a model nematode

Unlike the situation with a self-replicative organism like *T. brucei*, obtaining and culturing parasitic helminths in the lab is difficult, time-consuming and expensive. Helminths often require the use of (often sub-optimal) animal hosts, dedicated housing and specialized expertise. As a convenient alternative, *C. elegans* can be cultured in the lab using simple reagents and equipment [39]. The adult worm is also fecund, laying 4-10 eggs/h and being able to store up to 15 eggs in the uterus at any given time [40]. Another benefit of *C. elegans* is its quick life cycle, being able to grow from egg to an egg-laying hermaphrodite in 48 h [40,41]. The worm is also amenable to a variety of genetic manipulations that have greatly advanced our understanding of molecular and cellular biology in general [42-44]. Finally, the worm is a proven drug discovery/screening tool; both industrial and academic labs have employed *C. elegans* in the identification of anthelmintics [44, 45] and the determination of modes of action [46, 47].

1.5 Tubulin as an anti-parasitic drug target

As noted, drugs for treatment of parasitic diseases are limited by choice, variable efficacy (including as a consequence of resistance) and toxicity. One molecular target that has been well-researched in relation to both the treatment of HAT and helminthiases is tubulin [47-49]. Tubulin is part of the cytoskeleton for all eukaryotic cells and the dynamics associated with microtubule (MT) -construction and -deconstruction (Fig. 3) are highly coordinated to maintain cell-shape and -division, and facilitate the intracellular transport of proteins and vesicles [50, 51].

Tubulin is composed of two subunits, alpha and beta tubulin, which polymerize into long hollow MTs (24 nm in diameter) that provide structural support to the cell and are key to cell motility, intracellular transport and mitosis [50,51]. Because MTs are dynamic structures that are

spatially- and temporally-regulated [52, 53], interfering with MT-dynamics can lead to the arrest of the cell-cycle and death [54].

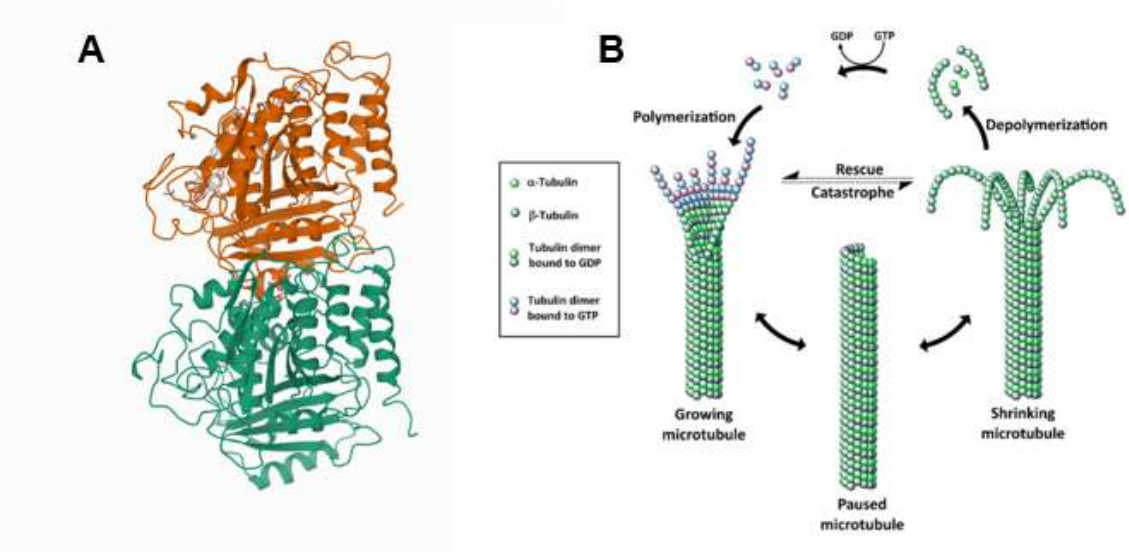


Figure 3. General scheme of the alpha-beta tubulin polymer and MT dynamics. A. Structure of the alpha-beta tubulin monomer (the orange molecule is the beta unit). **B.** MT dynamics showing both the stabilization and destabilization mechanisms [55]. The addition of GTP-tubulin dimers lengthens the MT structure (stabilization), whereas a "catastrophe event" rapidly switches MT growth into a shortening (destabilization) state [52, 56].

Indeed, tubulin and MTs are well-defined targets in cancer therapy [57-60]. MT-modulating agents work by two distinct mechanisms of actions (MOA) wherein binding to either the alpha or beta tubulin monomer can induce a cascade of changes that either stabilize of destabilize the whole MT polymer [61, 62]. Binding sites have been named after studies with specific natural products, namely laulimalide, taxane/epothilone, vinca alkaloid and colchicine [63, 64]. Binding to the first two sites promotes MT stability whereas binding to the last two sites induces MT destabilization [63, 64]. Mechanistically, cancer therapeutics modify normal MT-dynamics by primarily targeting one of the four binding sites listed above [63]. MT-stabilization or -destabilization depends on the individual drug but, ultimately, the ensuing antimitotic effect prevents the replication of cancerous cells [64].

In trypanosomes, subpellicular (sub-surface), flagellar, cytoplasmic and mitotic MTs are key to locomotion and mitosis, and in the maintenance of cell-shape [65, 66]. Thus, molecules that interfere with the polymerization state of tubulin and/or MT-dynamics are trypanocidal [48, 67-69]. In helminths, it is known that tubulin is the target of the anthelmintics ABZ and MBZ [28, 30, 32]. Mechanistically, BZs target cytoplasmic MTs (beta-tubulin) which, in turn, impairs and depletes several sources of glucose and cholinesterase [70]. Also, inhibition of beta-tubulin has a secondary effect wherein egg cells inside adult worms cannot divide, thus, BZs are ovicidal [71].

1.6 MT-modulating agents as potential anti-parasitic drugs

Although MT-modulating agents are fundamental in the chemotherapy of various cancers, well characterized MT-modulating agents, such as paclitaxel and vinblastine, cannot be used for diseases of the CNS due to their poor penetrance of the blood-brain barrier (BBB) [72, 73]. However, research over the last decade with neurodegenerative diseases, such as Alzheimer's disease, has shown that heterocyclic small molecules such as triazolopyrimidine (TPD) MT-modulating agents *can* cross the BBB *and* stabilize neuronal MTs [74-76]. This is viewed as an attractive chemotherapeutic strategy for neurodegenerative diseases in the knowledge that enhancing MT-stabilization can compensate for the progressive loss of the endogenous MT-stabilizing *tau* protein, which is associated with cognitive decline [77, 78]. TPDs, and the related phenylpyrimidines (PPDs), compete with vinblastine for binding to tubulin although it's not clear whether this is due to binding to the vinblastine site itself and/or via a possible allosteric site [79].

Given that HAT is a disease of the brain and CNS, and in the knowledge that tubulin is essential for trypanosome function, my supervisor's research group is leveraging the opportunity to test PPDs and TPDs as potential anti-trypanosomal agents. Indeed, recent studies with *T. brucei*

in vitro have already shown that some of these small molecules are as potent as the current Stage 1 HAT drug, pentamidine, with 50% effective concentration (EC_{50}) values in the low nanomolar range [80].

1.7 Goals of this thesis

From a collection of 38 MT-modulating agents available in the lab, the first aim of this thesis was to understand whether one or more of these possess anthelmintic activity against *C. elegans*. This aim was enabled by small molecule screening assays that I developed, as described below. Secondly, based on the established *in vitro* potency of TPDs *vs. T. brucei* [80], I tested one brain penetrant TPD analogue in a mouse model of *T. brucei* infection to understand whether the *in vitro* potency could be translated into *in vivo* efficacy.

MATERIALS AND METHODS

2.1. Maintenance of *C. elegans*

C. elegans wild-type N2 was kindly donated by Dr. Emily Troemel of the Department of Biology, UCSD. Growth and maintenance of *C. elegans* was according to a procedure outlined in WormBook, a peer-reviewed collection of open-access chapters covering protocols and research from various *C. elegans* labs around the world. Briefly, *C. elegans* was grown in 100 x 10 mm petri dishes on nematode growth medium (NGM) agar that contained a thin layer of dried *E. coli* (OP50 strain – courtesy of the Troemel lab) as a food source. Twice a week, adult worms were transferred with a thin plastic rod to fresh agar plates to prevent the initiation of the dauer diapause, a developmentally-arrested stage that is triggered by a lack of food in addition to a variety of other adverse environmental stimuli [81]. Worm-growth and egg-laying were checked daily using a Zeiss Stemi 305 Stereo Microscope. Development of *C. elegans* from egg to egg-laying adult takes approximately 48 h [40, 41].

2.2 Maintenance of *E. coli* OP50

E. coli was maintained using a protocol derived from WormBook. *E. coli* was streaked across a LB Broth (Lennox) agar plate every month and left to grow at 4 º C. A liquid suspension was used for seeding NGM plates. When more liquid suspension was needed, a colony was scraped from the agar plate and mixed with 100 ml LB Broth (Lennox) to grow overnight at 37 º C. *E. coli* solutions and plates can be stored at a 4 º C for several months.

2.3 Synchronizing the growth of *C. elegans*

2.3.1 Bleaching adult *C. elegans*

Once a NGM agar plate is covered by ~800-1,500 adult worms, which are identifiable by eggs in the reproductive tract, a modified bleaching protocol is employed to isolate eggs (Fig. 4) [82]. Specifically, worms on agar plates were washed with M9 medium, transferred to a 15 ml falcon tube and centrifuged at 250 x *g* for 3 min to remove debris and excess OP50. This procedure was repeated three times until the supernatant had become clear of *E. coli*. After the final wash, the supernatant was removed without disturbing the worms and 3 ml bleach solution (3 parts sterile H_2O:2 parts 5% hypochlorite:1 part 5 M NaOH) were added and vortexed (typically 5 min) until all the worms had become stiff and motionless. Fresh M9 buffer was then added to a final volume of 11 ml to stop the reaction and the mixture was then centrifuged at 250 x *g* for 1 min. Bleaching was repeated again and stopped when all worms had disintegrated. The remaining eggs were washed three times with S Medium and left to rotate at 20 rpm for 24 h at room temperature (r.t.) to allow the eggs to develop to L1 larvae (Fig. 4). Typically, 6,000 - 7,000 eggs are harvested using this bleaching procedure.

2.3.2 Culturing synchronized *C. elegans*

L1 larvae were centrifuged for 3 min at 250 x *g* to remove excess S medium. Using a plastic Pasteur pipette, approximately 1,000 L1 were placed onto 15 cm agar plates that had been seeded with *E. coli* OP50. Larvae were allowed to grow to the L4 stage for 48 h at 20 ^{0}C (Fig. 4).

2.4 *C. elegans* phenotypic screening assays

2.4.1 Measuring motility via small molecule incubation

Synchronized L4 worms were manually removed and washed three times in M9 buffer with centrifugation for 3 min at 250 x *g*. The supernatant was removed and the worms were washed twice in S medium. A final wash involved pipetting the worms up and down over a 100 µm mesh to mechanically remove any *E. coli* that had become embedded on the surface of the animals. This rigorous washing ensured that the worms were sufficiently clean to record motility and facilitate viewing under a microscope.

Small molecule PPDs and TPD (kindly supplied by Dr. Carlo Ballatore, SSPPS) were dissolved as 20 mM stocks in DMSO and stored at –20 C (Supp. Table 1). Screens were performed in 96w plates whereby 1 µl compound was dispensed onto the bottom of the well such that the final compound concentration was 50 µM. Then, 60 worms in 100 µl S medium were added and the plate was mixed for 20 sec using a microplate mixer (Scientific Genie; SI-0400A). The plate was then inserted into the WMicroTracker plate reader (PhylumTech, Argentina). Worm motility was measured every 20 mins for 24 h at r.t. (Fig. 4). The WMicroTracker system detects the movement of organisms via the interference of infrared light beams (two beams/well; wavelength 880 nm, 100 micrometers in diameter, power <1mW / mm2; patents # US12515723, EP208640881) [83,84].

2.4.2 Measuring egg hatching

Apart from measuring the direct effects of small molecules on worm motility as a read-out for viability, measuring a decrease in nematode egg hatching would be important from the standpoint of reducing environmental contamination by the parasite and, consequently, the risk of

acquiring infection [85, 86]. After the 24 h period of incubation with small molecules, egg hatching was evaluated by first vigorously mixing the contents of each well and then placing 10 µl culture medium onto a slide and counting under a microscope. The number of larvae in each well was estimated by multiplying the number on the slide by 10.

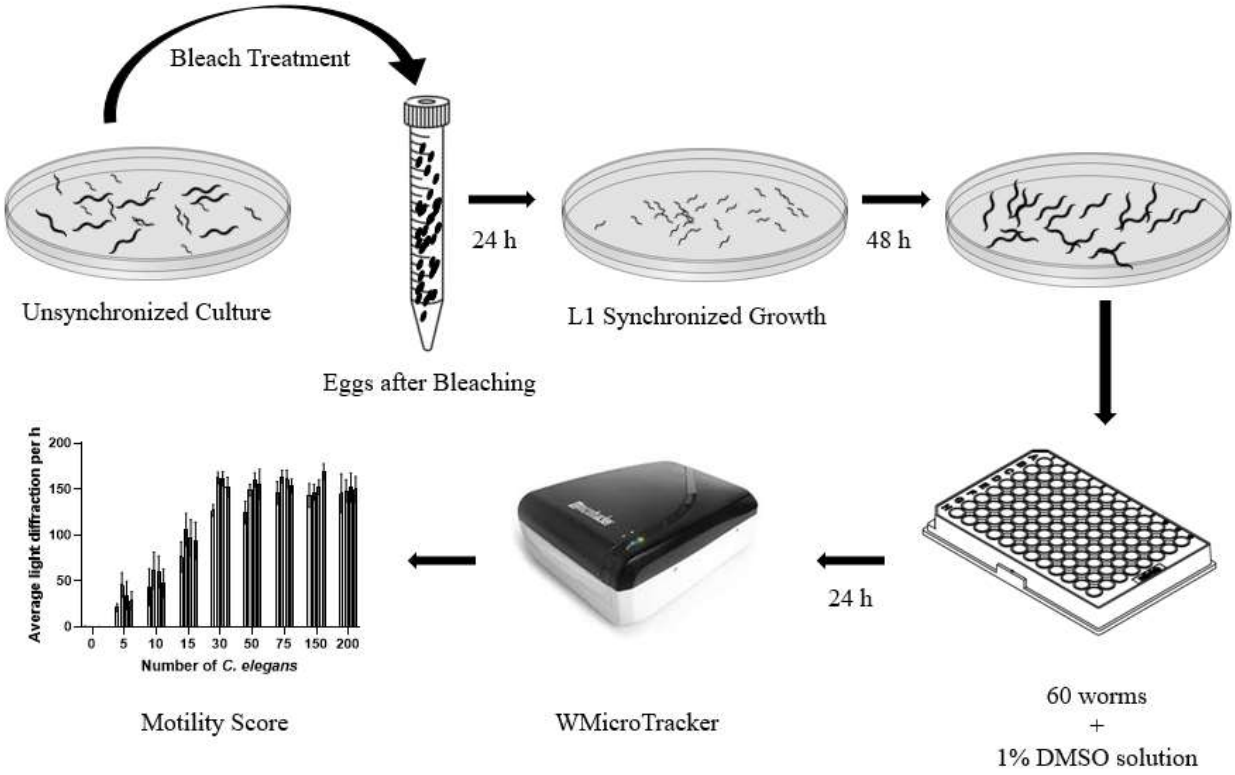


Figure 4. Scheme showing the preparation of *C. elegans* and measurement of motility. Upon completion of the assay after 24 h, any L1 larvae hatched were counted manually.

2.5 Maintenance of *T. brucei in vitro*

T. b. brucei Lister 427 was aseptically and continuously maintained in 5 ml HMI-9 medium (modified according to Hirumi) [87] in T25 flasks. Cell density was counted by transferring 10 µl culture into a hemocytometer. The hemocytometer displays parasites in a 4x4 grid and using a 10x magnification the animals can be counted (Supp. Fig. 1). The number counted is scaled up to

calculate the cell density in the original culture using the formula: number of parasites in hemocytometer x 10000 = number of parasites/ml in culture. Generally, cell density was never allowed to exceed 1 x 10^7 parasites/ml to avoid rapid death of the parasite. Cultures were maintained by splitting 10-500 µl of culture into 5 ml fresh HMI-9 medium every 2 to 3 days.

2.6 Efficacy studies in a mouse model of *T. brucei* infection

On the day of infecting mice, parasite density was calculated from *in vitro* culture and diluted so that 100,000 parasites were injected per mouse in 100 µl HMI-9. Groups of five, 6-week-old female mice received a single intra-peritoneal (ip) dose of parasites using a 27-gauge needle attached to a 1 ml syringe. After 48 h, to allow the parasite to establish infection, mice were treated with compound **1** (**Fig. 5**) that had been dissolved in vehicle (9% DMSO, 91% corn oil) at differing concentrations. Negative controls comprised infected mice that had been treated with vehicle alone and positive controls were those that had been treated with 4 mg/kg pentamidine dissolved in the same vehicle.

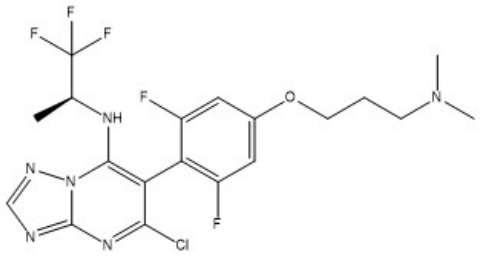


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Figure 5. Structure of compound 1 tested against *T. brucei* within mice.

Mice were monitored daily and those demonstrating abnormal behavior (*e.g.*, social isolation or decreased mobility) or appearance (*e.g.*, unkempt hair) as a consequence of acute drug

toxicity and/or infection were humanely euthanized by carbon dioxide and cervical dislocation. Blood parasitemia was checked every 24 to 48 h by withdrawing blood via a tail tip amputation. Specifically, 5 µl blood was withdrawn via a P20 pipette and diluted immediately with 5 µl heparin solution (0.5 mg/ml in sterile water). Samples were diluted a further 100-fold in PBS at 37°C and the parasites counted in 10 µl using a hemocytometer. The number of parasites per ml within each mouse was calculated by multiplying the number of parasites in the hemocytometer by 2,000,000. The distinct shape and movement of *T. brucei* compared to red blood cells facilitates the measurement of parasitemia.

All experiments with mice were performed under a protocol that was approved by the Institutional Animal Care and Use Committee (IACUC) at UCSD. Approval from UCSD-IACUC is granted under two policies: The Animal Welfare Act and Regulations (AWAR) Act and the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals.

The research described referring to C. elegans will be prepared as a manuscript for publication with authors in the following order: Liu, Lawrence; Monti, Ludovica; Alle, Thibault; Oukoloff, Killian; Lucero, Bobby; Yang, Wenqian, Ballatore, Carlo, and Caffrey, Conor. The research described referring T. brucei to will be prepared as a manuscript for publication with authors in the following order: Monti, Ludovica; Liu, Lawrence; Bobby Lucero; Yang, Wenqian; Ballatore, Carlo; and Caffrey, Conor.

RESULTS

3.1 *C. elegans* phenotypic screen for motility – assay performance

We tested two plate formats in the WMicrotracker to understand which might offer better assay performance (sensitivity and consistency); black round-bottom well plates (Corning No: 07-000-143) which concentrate the worms into one central field of view and black half-area well plates (Corning No: CLS3994) which also concentrate the worms into a tighter field of view in a volume that is 1/3 that of a regular 96 well (100 *vs*. 300 µl).

The data in Fig. 6 illustrate three principal findings. First, the half-area well plate offered better sensitivity as a function of worm number/well. For example, at 15 worms/well, the number of light beam disruptions caused by worm motility in the half-area well plate was 80-100 per h for all four time points measured (Fig. 6A). In contrast, in the round-bottom plate with the same number of worms/well, the detection of worm movement was at best 25 light beam disruptions/h (Fig. 6B). Second, sensitivity was independent of the time point in the half-area well plate which was not the case with the round-bottom geometry. Finally, the variability in movement measured, as demonstrated by the SD values, was 2-40% of the mean in the half-area well format but was 10-91% of the mean in the round-bottomed format. Overall, the assay performance of the half-area well plate geometry was superior with the added value of requiring only 1/3 of the reagent volume compared to a regular 96-well plate. Based on the data presented in Fig. 6A, 60 worms/well were used for further assays in the half-area well plate format.

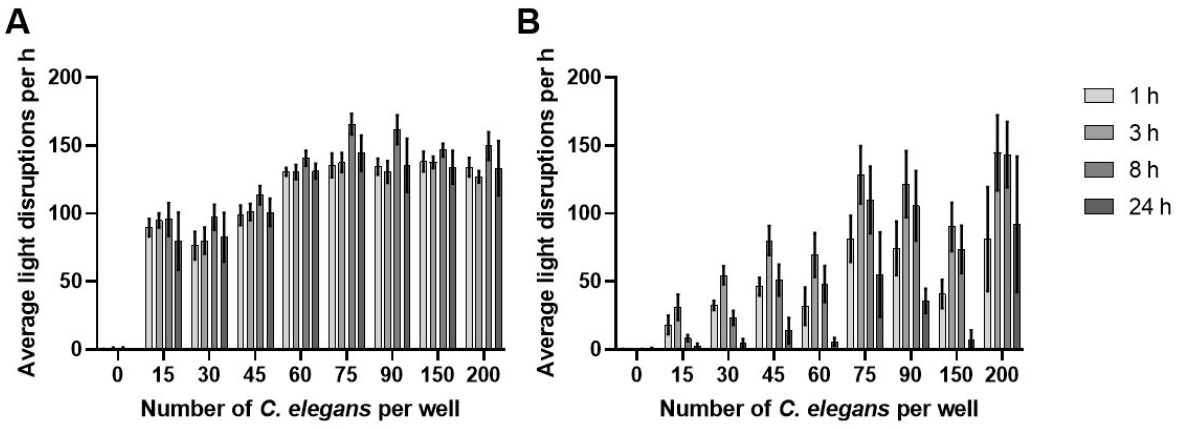


Figure 6. Effect of assay plate format on assay performance. A. Half-area well plate. **B.** Round-bottom well plate. Data are derived from three biological experiments each performed in duplicate (n=6).

3.2 Identifying small molecule control compounds that interfere with C. *elegans* motility and egg hatching

Positive control small molecules were first sought that interfere with motility and/or egg hatching. The data in Fig. 7 demonstrate the differential effects of two known anthelmintics on motility and egg hatching. Thus, pyrantel, a methyl-substituted carboxamidine in its pamoate salt form (Fig. 7A), generated a concentration-dependent decrease in motility with an EC_{50} value of 1.42 µM ± 0.8 (Fig. 7B). By contrast, ABZ had no effect on motility up to 50 µM but elicited a concentration-dependent decrease in egg hatching with an EC_{50} of 2.7 µM ± 1.6 (Fig. 7C): PP had no measurable effect except at the greatest concentration of 50 µM.

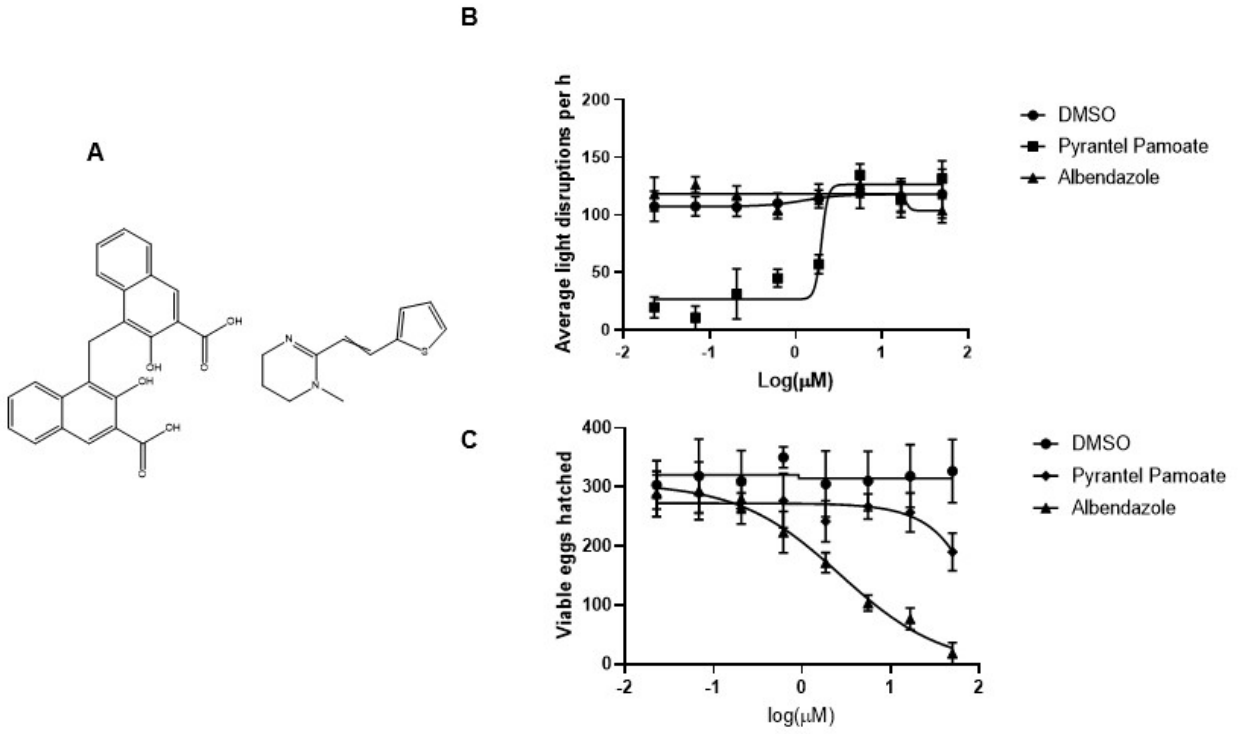


Figure 7. Effects of established anthelmintics on the motility of and egg hatching by *C. elegans.* A. Structure of pyrantel pamoate (PP). **B.** Worm motility as a function of drug concentration. **C.** Egg hatching as a function of drug concentration. Data are derived from three biological experiments each performed in duplicate (n=6). The range of concentrations tested was 34 nM - 50 μM and the final concentration of DMSO was 1%.

3.3 Effect of tubulin-modulators on adult *C. elegans* motility and egg hatching

Once the phenotypic assays for motility and egg hatching were established, a collection of 38 MT-modulating agents comprising PPDs, TPDs and imidazoles (IMDs; all kindly supplied by Dr. Carlo Ballatore, SSPPS) was evaluated at 50 μM. In the presence of the TPDs, **2** and **3,** and the PPD, **32** (Fig. 8A), light beam disruption counts/h were 115 ± 23, 101 ± 24 and 121 ± 19, respectively (Fig. 8B). Compared to the counts for the DMSO control (135 ± 5), each compound decreased motility by 21, 31 and 17%, respectively. As expected, the drug control, PP, decreased motility by 87.6%, whereas ABZ only decreased motility by approximately 11%. Data for the complete set of 38 compounds are presented in Supp. Fig. 2.

Regarding egg hatching, six compounds that were restricted to the TPD chemotype (Fig. 8A) and decreased hatching by at least 30% relative to the DMSO control were identified (Fig. 8C). Compounds **2**, **3**, **4**, **21**, **23** and **30** decreased egg hatching by 49, 69, 44, 40, 31 and 49%, respectively. As expected, ABZ decreased egg hatching by 80%, whereas PP only decreased hatching by approximately 12%. Data for the complete set of 38 compounds are presented in Supp. Fig. 3.

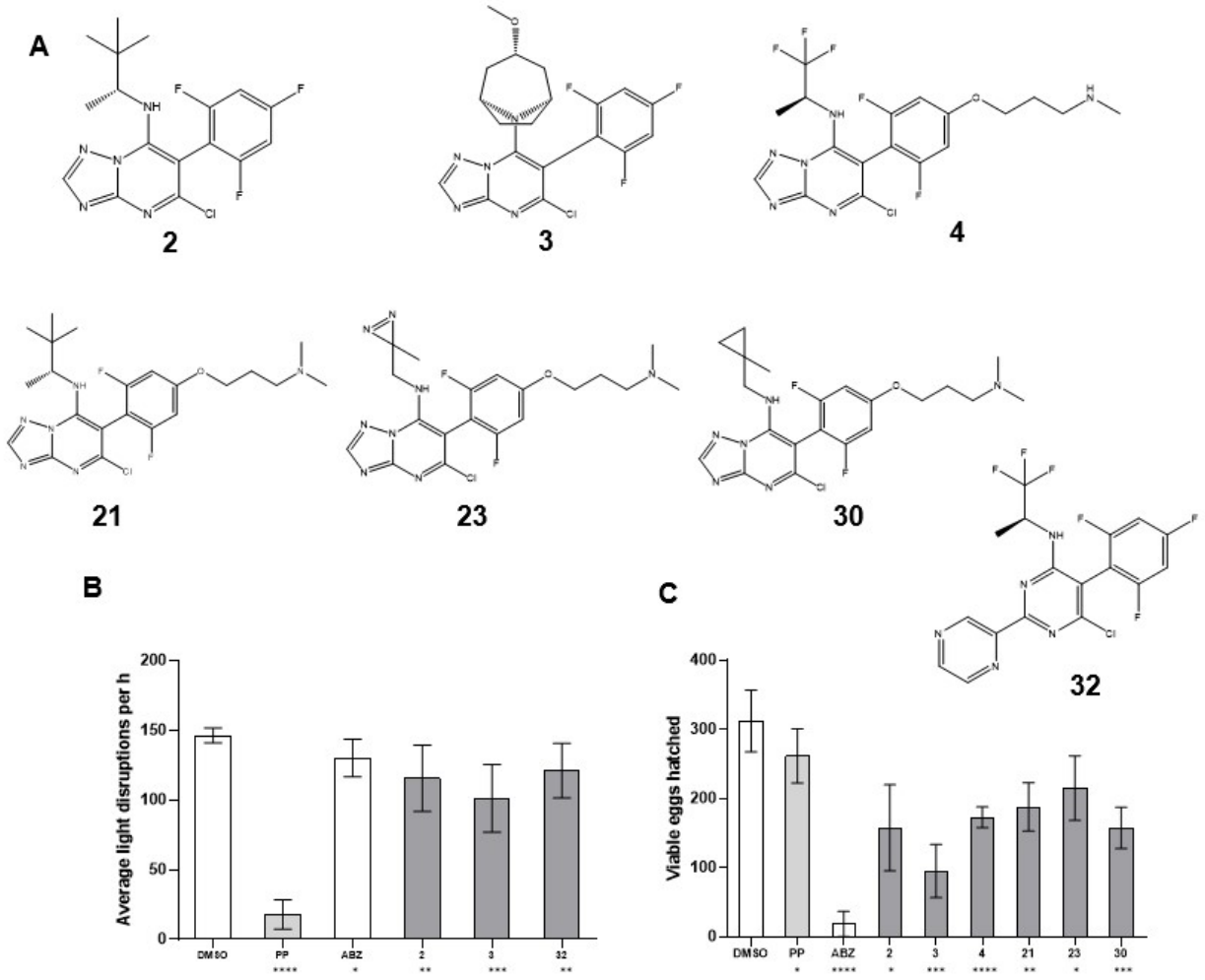


Figure 8: A screen of 38 MT-modulating agents identifies TPD and PPD compounds that decrease *C. elegans* motility and/or egg hatching. A. Structures of **2**, **3**, **4**, **21**, **23**, **30** and **32**. **B.** and **C**. Data for motility and egg hatching, respectively. Data are derived from two biological experiments each performed in duplicate (n=4). *P < 0.05, ** P < 0.005, *** P < 0.001, **** P < 0.0001 by the Student's paired t-test. 1. Representative data are shown: full data are shown in Supp. Figs. 2 and 3.

3.4 Efficacy of a MT-modulating agent *vs. T. b. brucei in vivo*

3.4.1. Compound 1 decreases parasitemia in mice infected with *T. b. brucei*

As shown in Fig. 9, two days after infection with 10^5 *T. b. brucei* Lister 427 parasites, parasitemia ranged from 2.5 – 3.5x10^7 parasites/ml. A single ip dose of 5, 7.5 or 10 mg/kg of compound **1** (Fig. 5), or 4 mg/kg of pentamidine, was then administered. The next day (Day 3 post-infection), a dose-dependent decrease in parasitemia was measurable. Thus, parasitemia was decreased to below the detectable threshold of 2x10^5 parasites/ml in all five of the mice treated with 10 mg/kg compound **1** and in four of the five mice treated with 7.5 mg/kg. At 5 mg/kg, compound **1** was less effective but still recorded a 9-fold decrease in parasitemia. As expected, parasitemia was decreased to below the detectable threshold in all of the mice treated with 4 mg/kg pentamidine. By contrast, mice treated with vehicle only recorded a 29-fold increase in parasitemia on Day 3.

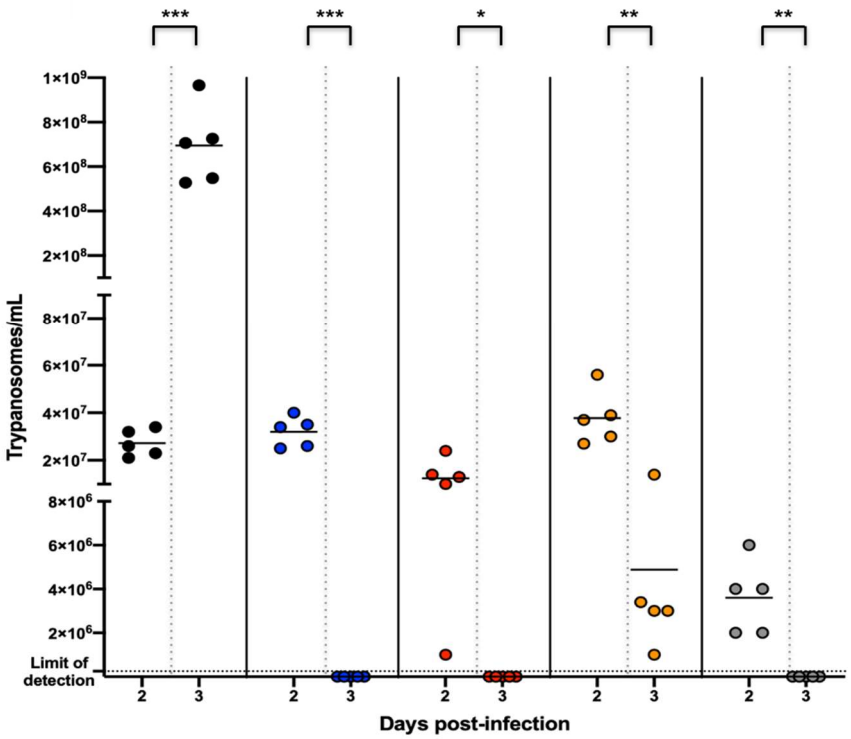


Figure 9. Compound 1 decreases parasitemia in a mouse model of *T. b. brucei* infection. Parasitemia was measured on Day 3 post-infection 24 h after administration of vehicle alone (black), compound **1** at 10 (blue), 7.5 (red) or 5 mg/kg (yellow), or pentamidine at 4 mg/kg (grey). Groups of 5 mice were used per treatment. *p <0.05, **p <0.01 and ***p <0.001 by the Student's paired *t*-test.

3.4.2. Compound 1 increases the survival of mice infected with *T. b. brucei*

Having established that **1** is effective in decreasing parasitemia, we next asked whether the infected mice treated with **1** survive longer than those mice receiving vehicle. All of the mice treated with 4 mg/kg pentamidine were free of blood parasitemia and survived until the end of the experiment on Day 16 post-infection, whereas mice treated with vehicle all died on Day 4 post-infection (Fig. 10). By contrast, three of five mice treated with **1** at 5 mg/kg survived until Day 7 post-infection, with two mice either dying or being euthanized on Day 6 post-infection. All of the mice treated with either 7.5 mg/kg or 10 mg/kg survived to Day 9 post-infection at which point

parasites were found in the blood (recrudescence) and the decision was taken to administer a second dose of 7 and 5 mg/kg, respectively. This decision was based on the knowledge that parasitemia, once detected, will quickly overwhelm the mice within 24 - 48 h. Twenty-four hours after administration of the second dose, two and three mice, had died in the 7.5 and 10 mg/kg groups, respectively, but the remaining mice survived until Days 15 and 16 post-infection, respectively. At these time points, parasitemia was once again detected and the mice were euthanized (Fig. 10). Overall, compound **1** extended the survival of mice infected with *T. b. brucei*, however, it did not provide cure as was apparently the case for pentamidine. Also, there is some concern for compound toxicity due to a portion of the mice dying 24 h after dosing. However, the possibility of direct compound toxicity is confounded by the presence of an active parasitic infection and will need to be specifically investigated by maximum tolerated dose (MTD) experiments in non-infected mice.

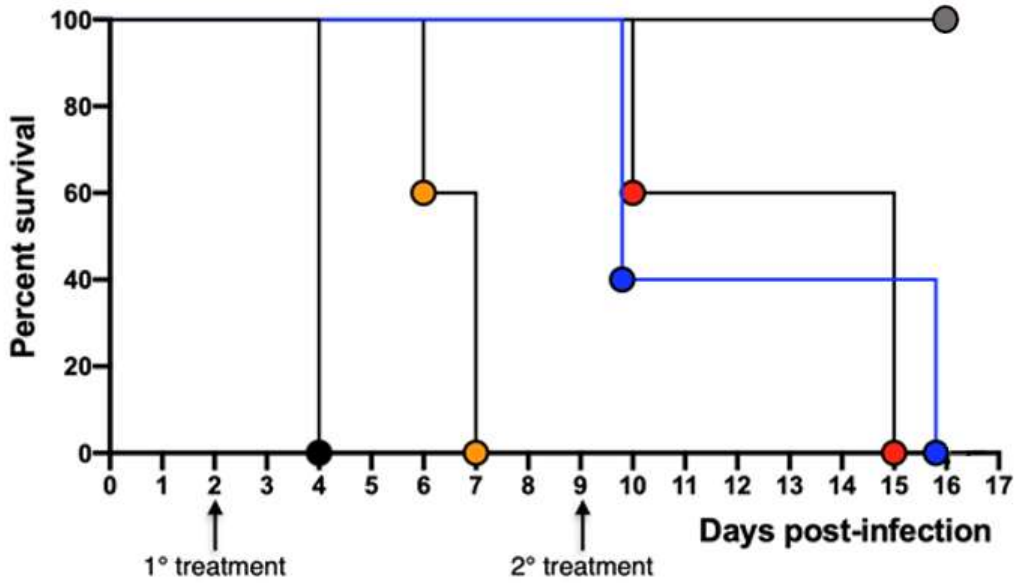


Figure 10. Compound 1 increases the survival of mice infected with *T. b. brucei*. Survival curves for groups of mice (n=5) after treatment with vehicle (black), pentamidine at 4 mg/kg (grey), or 5, 7.5 or 10 mg/kg **1** (yellow, red and blue, respectively). On the recrudescence of parasitemia on Day 9 post-infection, a second treatment of 5 and 7 mg/kg of **1** was given to the 10 and 7.5 mg/kg groups, respectively.

The research described referring to C. elegans will be prepared as a manuscript for publication with authors in the following order: Liu, Lawrence; Monti, Ludovica; Alle, Thibault; Oukoloff, Killian; Lucero, Bobby; Yang, Wenqian, Ballatore, Carlo, and Caffrey, Conor. The research described referring T. brucei to will be prepared as a manuscript for publication with authors in the following order: Monti, Ludovica; Liu, Lawrence; Bobby Lucero; Yang, Wenqian; Ballatore, Carlo; and Caffrey, Conor.

Discussion

Drug discovery for parasitic diseases of humans is hampered for a number of reasons. First, the market is small relative to big-earner medical indications such as cancer or pain management [88] and for which the underlying financial incentive is to intervene in *chronic*, potentially, life-long, conditions rather than the *short course* therapies typically used for parasitic diseases [89]. This has only been exasperated in the attritional 'mergers and acquisitions' environment of the pharmaceutical industry over the last 30 years in which companies have increasingly focused on 'big earner' indications with many other companies having long ago abandoned the anti-parasitic area [88]. Lastly, many parasitic diseases are now only the concern of poorer countries where a drug consumer/payer base is weak or absent, thus further discouraging R&D for anti-parasitics [88, 90]. Today, anti-parasitic R&D often involves public-private partnerships (PPPs) that include private and public donors, and the know-how of the pharmaceutical industry in a shared-risk business model to bring drugs to market [91]. A case in point is the successful development of fexinidazole (Table 1) for the treatment of HAT [92] by the Drugs for Neglected Diseases *initiative* (DND*i*). DND*i* is also involved in repurposing drugs from animal health into the medical arena, *e.g.*, moxidectin for treatment of human filariasis [93]. It is against this background that the anti-parasitics discovery research reported in this thesis for both helminth and protozoan parasites is especially relevant.

C. elegans is a well-established model organism for human diseases [94-96] and has been used for anthelmintic screening for the past 40 years [44, 97]. Its ease of manipulation, low cost of maintenance and well-understood responses to common anthelmintic agents makes *C. elegans* an attractive "surrogate" parasite [44]. Herein, I developed assays for screening anthelmintics that was able to record two important biological parameters of *C. elegans* - motility and egg hatching.

For motility, subjective scoring/counting methods have been traditionally used [98] being replaced by automated and much more expensive instruments that are often too costly for most labs [99]. As an alternative, I employed the WMicroTracker (investment of $10,000) to capitalize on the machine's ability to continuously record worm motility for as long as needed. Also, the assay format was optimized in terms of plate geometry (1/2 area well *vs.* round bottom plates) and number of worms per well.

Because the WMicroTracker motility assay is non-invasive, I was then able to manually count egg hatching as a second readout for anthelmintic activity. Counting is labor intensive, however, and I am currently investigating the option of using a single-metric readout for chitinase activity. Chitinase is released as a consequence of nematode egg hatching and should be a simple alternative to manual counting as already demonstrated for *C. elegans* by Geary and colleagues [100]. For each of the motility and egg hatching assays, the differential inhibitory activities of PP and ABZ were demonstrated and these are consistent with previous data [101].

Tubulin is well-established as drug target for anti-parasitics, including against nematodes [102] and *T. brucei* [80, 103]. Because of a collaboration between my supervisor, Dr. Caffrey and Dr. Carlo Ballatore at the SSPPS, I was able to test 38 of Dr. Ballatore's TPD, PPD and IMD MT-modulating agent that had been originally developed as part of a program for treatment of Alzheimer's disease due to their ability to stabilize MTs in the brain [79]. In relation to *T. brucei,* the collection was especially attractive as a number of the compounds also cross the BBB [80].

Against *C. elegans,* two TPDs and one PPD, out of the collection of 38 compounds screened at 50 μM had a slight effect on motility but were much less active than the positive drug control, PP. Interestingly, six compounds from the TPD chemotype impacted egg hatching. In general, out of the 38 compounds screened, **3** gave the best results for decreasing both motility and

egg hatching. Screening activities will continue with a larger collection of TB-modulators which might reveal an underlying structure-activity relationship.

In addition to the progress reported here regarding the development and implementation of *C. elegans* screening assays, additional research focused on the efficacy testing of a TPD, compound **1**, in a murine model of *T. brucei* infection. This compound was selected based on its potent activity against *T. brucei in vitro* (L. Monti; unpubl. data), and for its ability to penetrate the BBB *and* stabilize neuronal MTs [80]. The ability to penetrate the BBB is a prerequisite of any new treatment for HAT [104].

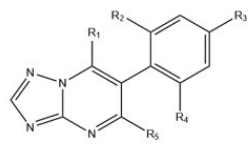
The data from these *in vivo* efficacy tests against *T. brucei* show that compound **1** decreased blood parasitemia and extended the survival of infected mice well beyond that of mice that had been treated with vehicle alone. Although **1** is not curative, the efficacy demonstrated supports the investigation of an expanded set of related MT-modulating agents as a possible treatment of HAT. Part of this process will require additional medicinal chemistry to improve potency and selectivity while decreasing toxicity, which is often a limiting factor for MT-binders [105]. Furthermore, a murine infection model that encompasses both Stage 1 and 2 *T. brucei* infection will need to be incorporated into the development program, *e.g*., the *T. b. brucei*-GVR35 strain which expresses a red-shifted luciferase and can be used to quantify parasitemia in the blood and brains of infected mice [106]. Our current *T. b. brucei* Lister 427 strain cannot enter the brain but is useful due to the quick time-to-result whereby untreated mice die within four days of infection (the GVR35 strain requires 28 days from infection to accrual of data) [107].

Conclusion

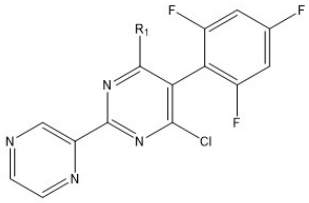
This thesis describes the development and implementation of assays for *C. elegans* to (i) measure motility using WMicroTracker and (ii) egg hatching using manual counting as part of a program to screen for anthelmintics. Using the optimized assays, a screen of 38 MT-modulating agents, comprising TPDs, PPDs and IMDs, and which were originally developed for Alzheimer's disease, identified a number of compounds with statistically significant anthelmintic activity. The thesis also describes how a related TPD, compound **1**, was identified as a potent antitrypanosomal agent in a murine model of *T. brucei* infection. Although not curative, the efficacy data for **1** provide proof-of-concept that MT-modulating agents are effective antitrypanosomal agents *in vivo* and encourage a program to improve potency and selectivity, and decrease toxicity.

Appendix

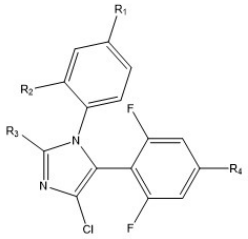
Supplementary Table 1. Structures of MT-modulating agents



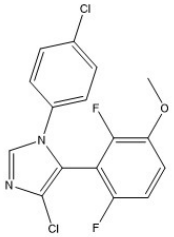
Triazolopyrimidine (TPD)



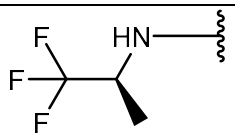
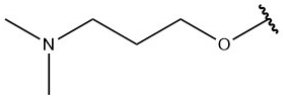
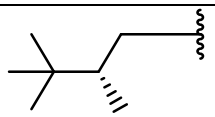
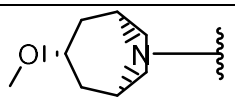
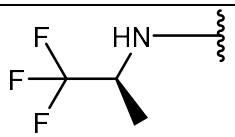
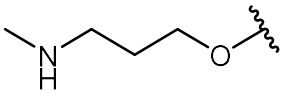
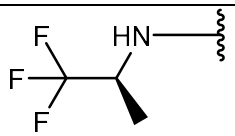
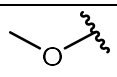
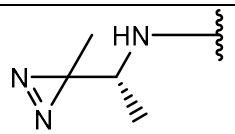
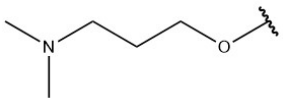
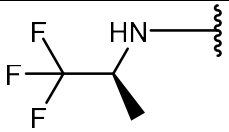
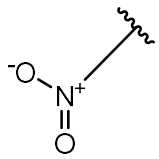
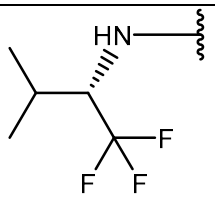
Phenylpyrimidine (PPD)



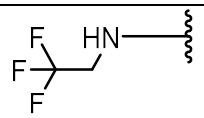
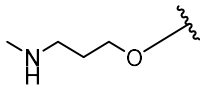
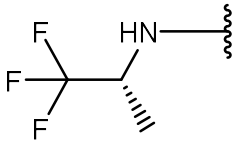
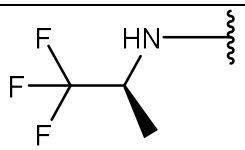
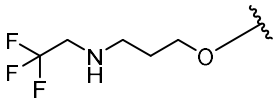
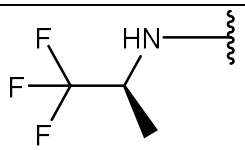
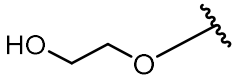
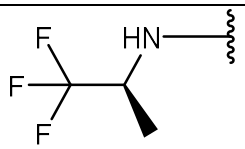
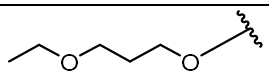
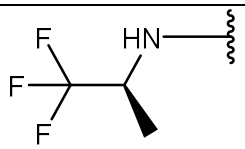
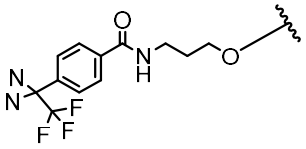
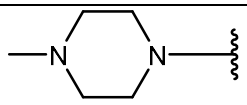
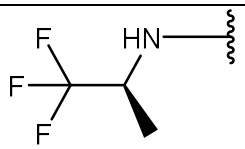
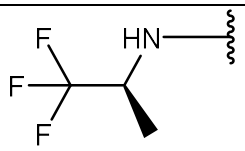
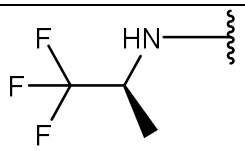
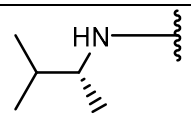
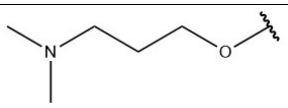
Imidazole (IMD)



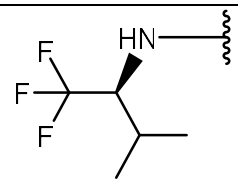
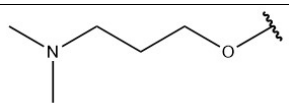
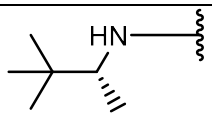
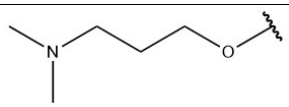
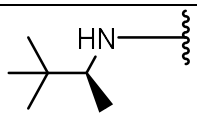
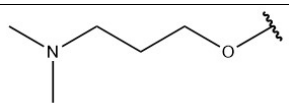
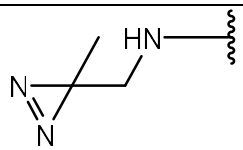
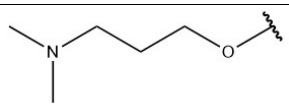
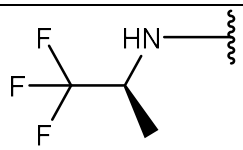
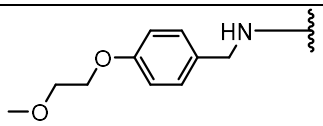
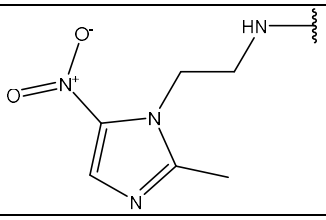
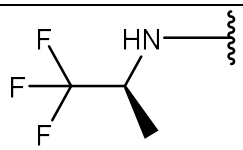
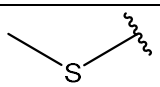
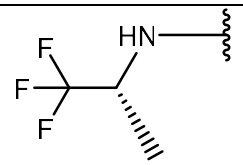
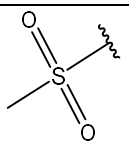
36

Molecule Class	Compound	R_1	R_2	R_3	R_4	R_5
TPD	**1**		-F		-F	-Cl
TPD	**2**		-F	-F	-F	-Cl
TPD	**3**		-F	-F	-F	-Cl
TPD	**4**		-F		-F	-Cl
TPD	**5**		-F		-F	-Cl
TPD	**6**		-F		-F	-Cl
TPD	**7**		-F		-F	-Cl
TPD	**8**		-F	-F	-F	-Cl

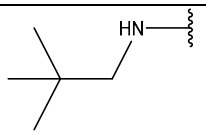
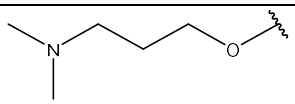
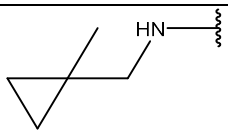
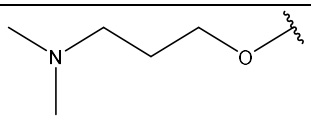
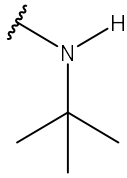
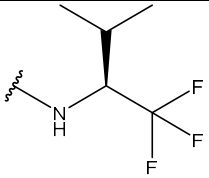
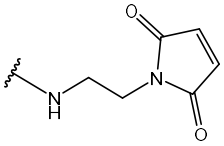
Supplementary Table 1. Structures of MT-modulating agents, Continued

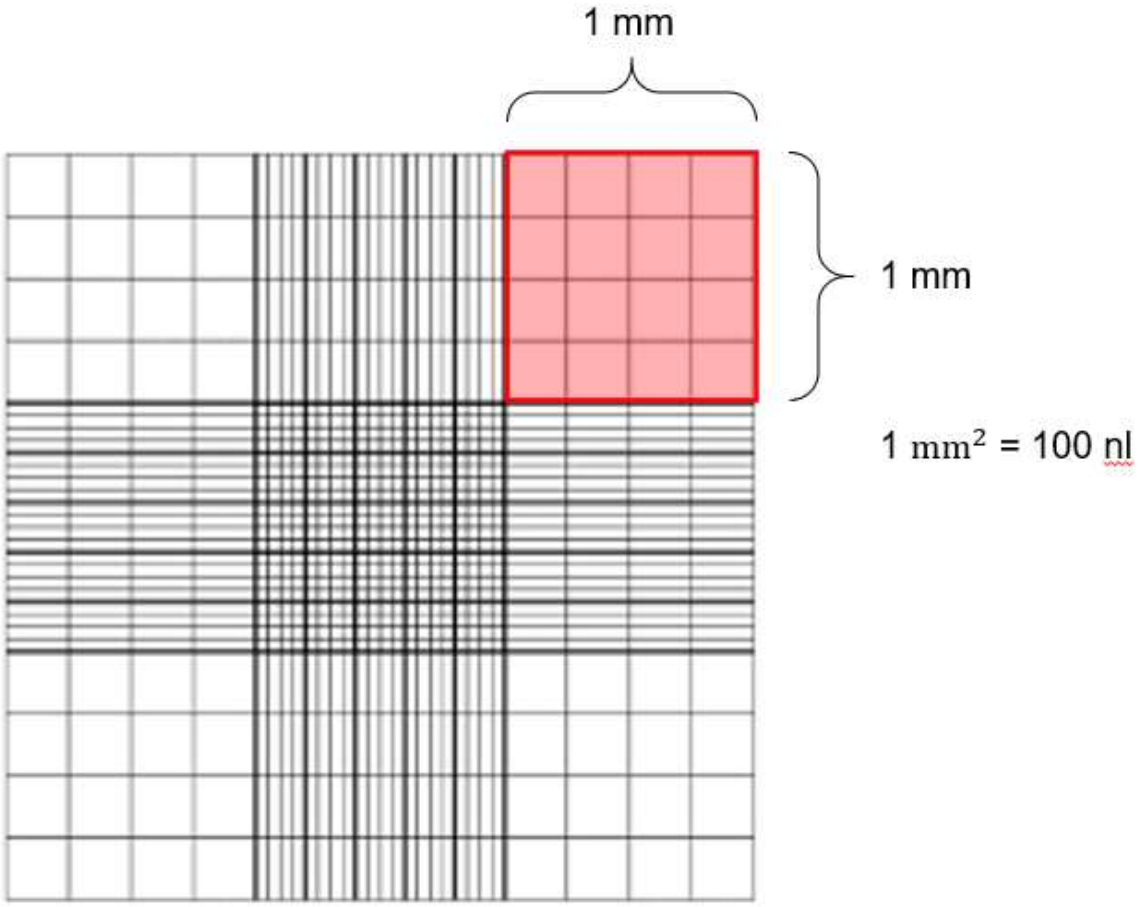
Molecule Class	Compound	R_1	R_2	R_3	R_4	R_5
TPD	**9**		-F		-F	-Cl
TPD	**10**		-F	-F	-F	-Cl
TPD	**11**		-F		-F	-Cl
TPD	**12**		-F		-F	-Cl
TPD	**13**		-F		-F	-Cl
TPD	**14**		-H		-F	-Cl
TPD	**15**		-F	-F	-F	-Cl
TPD	**16**		-H	-H	-H	-Cl
TPD	**17**		-F	-H	-H	-Cl
TPD	**18**		-H	-F	-H	-Cl
TPD	**19**		-F		-F	-Cl

Supplementary Table 1. Structures of MT-modulating agents, Continued

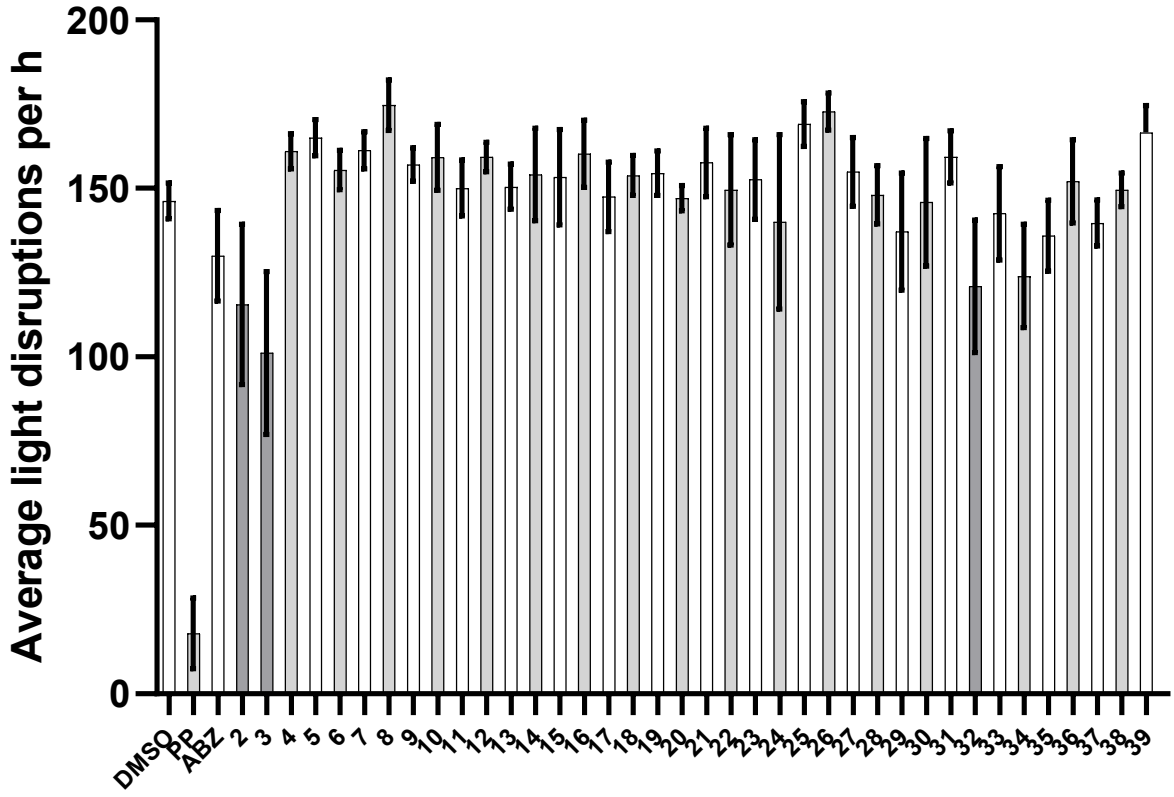
Molecule Class	Compound	R_1	R_2	R_3	R_4	R_5
TPD	**20**		-F		-F	-Cl
TPD	**21**		-F		-F	-Cl
TPD	**22**		-F		-F	-Cl
TPD	**23**		-F		-F	-Cl
TPD	**24**		-F	-F	-F	-H
TPD	**25**		-F	-F	-F	-Cl
TPD	**26**		-F	-F	-F	-Cl
TPD	**27**		-F		-F	-Cl
TPD	**28**		-F		-F	-Cl

Supplementary Table 1. Structures of MT-modulating agents, Continued

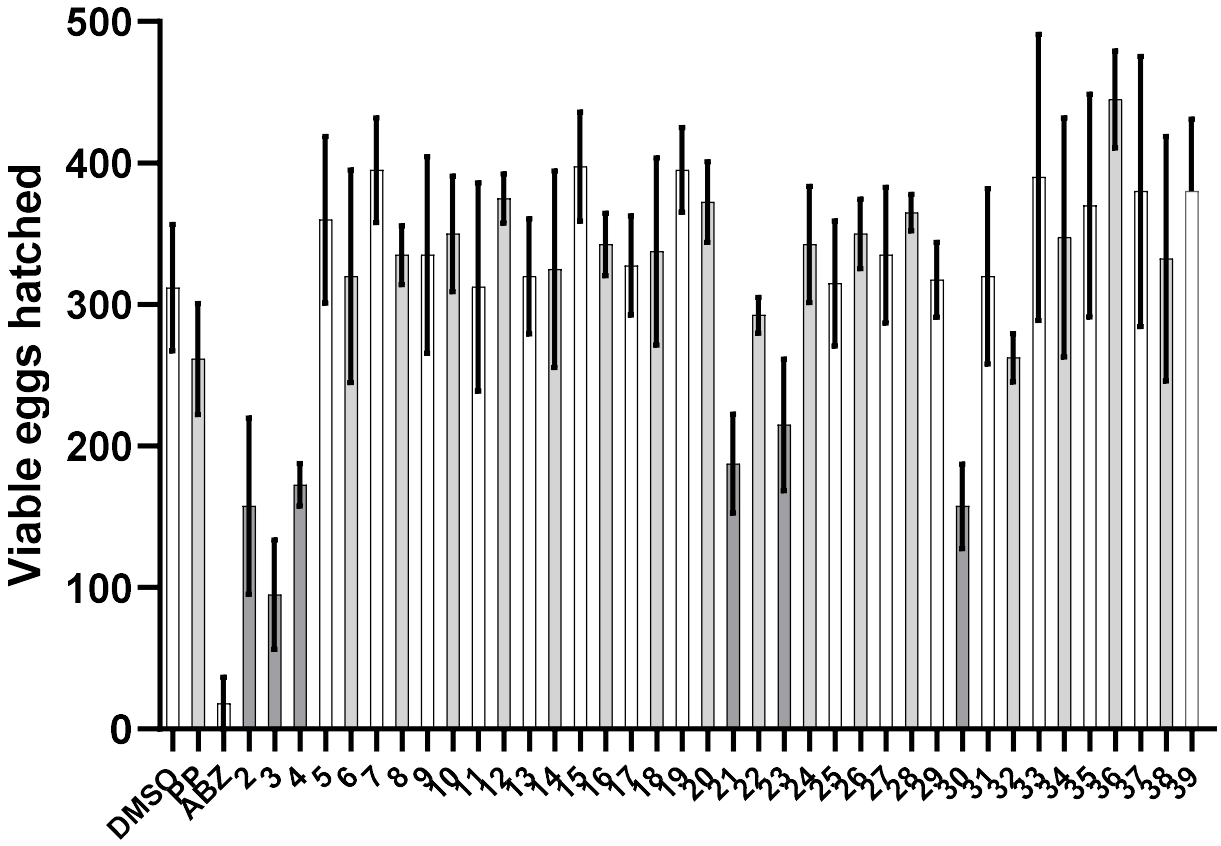
Molecule Class	Compound	R_1	R_2	R_3	R_4	R_5
TPD	**29**		-F		-F	-Cl
TPD	**30**		-F		-F	-Cl
PPD	**31**					
PPD	**32**					
PPD	**33**					
IMD	**34**	**-Cl**	**-H**	**-H**	**-F**	
IMD	**35**	**-Cl**	**-H**	**-CH_3**	**-OCH_3**	
IMD	**37**	**-Cl**	**-H**	**-H**	**-CH_3**	
IMD	**38**	**-OCH_3**	**-H**	**-H**	**-F**	
IMD	**39**	**-OCH_3**	**-H**	**-Cl**	**-F**	



Supplementary Figure 1. Dimensions of a hemocytometer



Supplementary Figure 2. Effects of 38 MT-modulating agents on the motility of *C. elegans*.



Supplementary Figure 3. Effects of 38 MT-modulating agents on egg hatching by *C. elegans*

Recipes

C. elegans

M9 Buffer:

- 3 g KH_2PO_4
- 6 g Na_2HPO_4
- 5 g NaCl
- 1 ml 1 M $MgSO_4$
- 1000 mL H_2O
- Sterilize by autoclave

S Medium:

- 5.85 g NaCl
- 1 g K_2 HPO_4
- 6 g KH_2PO_4
- 1 ml cholesterol (5 mg/ml in ethanol) – add after autoclave
- 1000 ml H_2O
- Sterilize by autoclave

Nematode Growth Medium (NGM) Agar Plates:

- NaCl
- Agar
- Peptone
- 975 ml H_2O

- Microwave until large clear bubbles form and seed onto Petri plates
- Add after cooling to 60 º C
    - 1 ml $CaCl_2$
    - 1 ml 5 mg/ml cholesterol in ethanol
    - 1 ml $MgSO_4$
    - 25 ml 1 M KPO_4

Bleach Solution:

- 3 parts Sterile H_2O
- 2 parts 5% hypochlorite
- 1 parts 5 M NaOH

Lennox Broth (LB) Media

- 10 g Bacto-tryptone
- 5 g Bacto-yeast
- 5 g NaCl
- 1000 ml H_2O
- pH to 7.0 using 1 M NaOH
- Sterilize by autoclave

Lennox Broth (LB) agar plate

- 10 g Bacto-tryptone
- 5 g Bacto-yeast
- 5 g NaCL

- 15 g agar
- 1000 ml H_2O, pH 7.5

T. brucei

Modified HMI-9 Medium

- 400 mL IMDM
- 50 mL HI-FBS
- 50 mL Serum Plus
- 5 mL 100 mM hypoxanthine (dissolve 4.0 g NaCl in 1 L of water and add 13.6 g hypoxanthine)
- 14 mg bathocuproine disulfonic acid
- 91 mg cysteine (add after bathocuproine)
- 55 mg (50 µL) pyruvic acid
- 5 mg uracil
- 5 mg cytosine
- 7 µL 2-mercaptoethanol
- Vacuum filter after all reagents are added

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