A rotifer-derived paralytic compound prevents transmission of schistosomiasis to a mammalian host

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Abstract

Schistosomiasis is a neglected tropical disease afflicting 200 million people worldwide, caused by parasitic flatworms of the genus *Schistosoma*. For decades, the treatment of schistosome infection has relied on a single drug, praziquantel. However, limitations in mass drug administration programs and the emergence of schistosomiasis in non-tropical areas indicate the need for new strategies to prevent infection. It has been known for several decades that rotifers colonizing the schistosome's snail intermediate host produce a water-soluble factor that paralyzes cercariae, the life-cycle stage infecting humans. In spite of its potential for preventing infection, the nature of this factor and its underlying mechanism of action have remained obscure. In this work, we report the purification and chemical characterization of Schistosome Paralysis Factor (SPF), a novel tetracyclic alkaloid produced by the rotifer *Rotaria rotatoria*. We show that this compound paralyzes schistosome cercariae and prevents infection. Structural search reveals analogous compounds (Ht-13 analogs) that phenocopy SPF. Based on their chemical structure, we hypothesize that these compounds work through bioamine G protein-coupled receptors (GPCRs). To determine if biogenic amines, particularly neurotransmitters, play a role in regulating cercaria motility, we screen a library of neurotransmitter-related compounds and find that increasing dopamine signaling in cercaria leads to paralysis. Utilizing a luciferase-based cell assay, we identify a schistosome dopamine receptor that is activated by these cercaria-paralytic compounds, suggesting the mode of action of SPF involves dopamine signaling. Furthermore, we show that Ht-13 analogs also activate various human serotonin and dopamine receptors, suggesting their potential for drug development against neurological diseases.

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Chapter 1: Introduction

1.1 Introduction to schistosomiasis

Schistosomiasis is a devastating yet neglected tropical disease caused by parasitic flatworms of the genus *Schistosoma*. The three main species infecting humans are *S. haematobium, S. japonicum,* and *S. mansoni* (WHO, 2017). *S. haematobium* is considered the most common species occurring in sub-Saharan Africa. *S. japonicum* is mainly found in China and Philippines (Colley et al., 2014), while *S. mansoni* is endemic to sub-Saharan Africa, South America and the Caribbean (Kurup and Hunjan, 2010). It is estimated that 200 million out of the 250 million people currently infected worldwide with this disease live in Africa (McManus et al., 2018; Steinmann et al., 2006; WHO, 2017). Moreover, conservative estimates place the death toll in Africa at approximately 200,000 deaths per year (McManus et al., 2018). In addition to the vast population that must cope with this disease, there are a further 779 million individuals worldwide that are at risk for infection (McManus et al., 2018; Steinmann et al., 2006).

Life cycle of schistosomes

Schistosomes have a complex life cycle that consists of two parasitic and two free-living stages. During the parasitic phases of the life cycle, schistosomes colonize an intermediate snail host and then move on to a definitive mammalian host (e.g., humans). On the other hand, the free-living stages occur in freshwater environments, serving as a bridge between the two parasitic stages (Basch, 1991; McManus et al., 2018).

The eggs of mature adult schistosomes are released into freshwater environments such as lakes, streams, rivers, etc. through the feces of their mammalian host. In response to temperature and

light, eggs will hatch upon contact with the water into their first free-living stage, the miracidium. These highly ciliated larvae must actively swim to find their intermediate host, the freshwater snail, as they are nonfeeding and only infective for approximately 10 hours post hatching. Each schistosome species exhibits a preference for a particular genus of snail host. Most common host snail genera are *Biomphalaria* (*S. mansoni*)*, Oncomelania* (*S. japonicum*)*,* and *Bulinus* (*S. haematobium*) (Basch, 1991; McManus et al., 2018).

Once miracidia enter the snail by penetrating its soft tissue, they shed their ciliated plates and develop into mother sporocysts. Driven by stem cells, mother sporocysts undergo multiple rounds of asexual reproduction to produce numerous daughter sporocysts (Wang et al., 2013). These daughters migrate to the liver and gonads of the snail where they are able to further grow. After 2-4 weeks, cercariae start to develop inside these daughter sporocysts (Basch, 1991; McManus et al., 2018).

About 30 days after the initial snail infection, cercariae are released from the snails in response to sunlight and enter their second free-living stage. Similar to miracidia the cercariae are nonfeeding and short-lived; thus, they must find a human host within a few hours (Whitfield et al., 2003). Unlike the ciliated miracidia, cercariae use a forked muscular tail to propel themselves in water. Once an unsuspecting host enters a river or stream, cercariae are given a window of opportunity to penetrate the skin of their human host. This penetration may be attracted by fatty acids present on the host; furthermore, proteases are secreted from acetabular glands to help the cercarial heads penetrate the host skin (McKerrow and Salter, 2002). While entering the skin, the cercariae transform into schistosomula by casting off their tail and outer membrane and form a

new intravascular, double lipid bilayer tegument (Skelly and Shoemaker, 2000). The passage through the skin takes a few minutes. Once inside the skin, schistosomula will move on to locate a venule.

The schistosomula enter the venous blood vessels and are subsequently transported to the lungs via the right side of the heart in a process that takes four to seven days (Miller and Wilson, 1980). By elongating themselves, schistosomula pass through the small capillaries of the lung and arrive at the left side of the heart. Flowing with the blood via pulmonary veins, they enter arterial circulation (Nation et al., 2020). This circulation leads them to a checkpoint in the hepatic portal system from which they further migrate to the mesenteric veins of the liver (*S. japonicum* or *S. mansoni*) (Wilson, 2009). *S. haematobium*, however, lives in the pelvis venous plexus. Feeding on host blood, schistosomula develop into sexually mature males or females, which are determined by their chromosomes (Ribeiro-Paes and Rodrigues, 1997).

As schistosomes sexually mature (approximately 4-5 weeks), the females and males become morphologically distinguishable from each other. Females appear more slender than the males. Both sexes also have two suckers; an oral sucker for feeding and a ventral sucker for attachment. Furthermore, the males develop a gynecophoral canal on their ventral side where the female resides. In this paired position, females continuously lay eggs; each female produces over 300 eggs per day (Loverde and Chen, 1991). These eggs are released into the blood stream. As they pass through the intestinal walls, some of the eggs are excreted from the body in feces to continue the life cycle. However, the rest of the eggs are passively transported to the liver (*S.*

japonicum or *S. mansoni*) or bladder (*S. haematobium*) via the bloodstream causing intestinal or urinary schistosomiasis (McManus et al., 2018).

Pathology

While acute schistosomiasis is considered to be a toxemic and allergic reaction to the migrating schistosomula, the pathology of chronic schistosomiasis is not directly caused by parasites but by their eggs (Jaureguiberry et al., 2010). Due to an immune response towards the eggs that are trapped in liver (*S. japonicum* or *S. mansoni*) and bladder (*S. haematobium*), granulomas form in these organs. The granulomas serve to isolate the egg from the host and they eventually resolve themselves once the eggs die. However, a fibrotic plaque is left behind, which is the basis for most of the symptoms that arise due to schistosome infections. As more and more eggs get lodged in the liver and bladder, the amount of fibrotic plaques will also increase. In chronic infections, such damage is usually irreversible, eventually leading to organ failure and even death if left untreated. Furthermore, there is emerging evidence showing that schistosome eggs can act as a primary carcinogen causing bladder and colorectal cancers (Badawi et al., 1995; Mostafa et al., 1999; OE et al., 2010).

Current Treatment

Currently, there are limited treatment options for individuals infected with schistosomes. The disease is treated and controlled through the administration of an anti-schistosomal drug, praziquantel (PZQ), to patients diagnosed with schistosomiasis and to travelers returning from endemic areas (Inobaya et al., 2014; McManus et al., 2018). For over 30 years, PZQ has served as a curative therapy option that can be used repeatedly until the infection is eliminated. Even

though the exact mechanism of its action has yet to be determined, PZQ has shown its efficacy against adult worms. Furthermore, the use of PZQ has shown only minor, temporary side-effects with no long-term consequences (Erko et al., 2012; Kabatereine et al., 2003; Midzi et al., 2008; Reta and Erko, 2013).

Shortcomings of the treatment

PZQ has proven to be an effective means of treating schistosomiasis. Through massive drug administration (MDA) programs, the annual global burden of 4.5 million disability-adjusted life years (DALYs) in 2002 was reduced to 1.9 million DALYs in 2016 (GBD 2016 DALYs and HALE Collaborators, 2017; King et al., 2005; WHO, 2002). However, there are some growing concerns regarding this drug among *Schistosoma* researchers. For example, PZQ cannot prevent reinfection and has no effect on immature schistosomes (Caffrey, 2007; Dejon-Agobe et al., 2019). In addition, it is believed that resistance to PZQ may develop in the near future. Certain species have already exhibited a decrease in susceptibility to the drug in the field (Crellen et al., 2016; Gryseels et al., 2006; Ross et al., 2002). Moreover, PZQ resistance has been induced under laboratory conditions (Ismail et al., 1994). Thus, it may only be a matter of time until a similar resistance is observed in the wild.

PZQ mode of action

PZQ is a tetracyclic tetrahydroisoquinoline derivative administered as a racemic mixture. The activity of the eutomer (R)-PZQ has been established in both *in vitro* and *in vivo* experiments (Mehlhorn et al., 1981; Staudt et al., 1992). PZQ elicits a rapid uptake of $^{45}Ca^{2+}$ in adult male schistosomes and leads to muscular contraction and tegument disruption (Pax et al., 1978). Due

to muscle contraction, the paralyzed schistosomes are flushed into the liver (hepatic shift). Furthermore, the tegument disruption facilitates antigen exposure and promotes immunological clearance. Although the underlying mechanism of PZQ is not well understood, removal of Ca^{2+} from the media blocks both responses suggesting PZQ might act through Ca^{2+}-dependent processes (Wolde Mussie et al., 1982). Schistosome voltage-gated calcium channel subunit $Ca_V\beta$s has been previously implicated in the action of PZQ. While mammalian calcium channel $Ca_V2.3$ $\alpha1$ subunit expressed alone in *Xenopus* oocytes does not respond to 100 nM PZQ, co-expression with schistosome $Ca_V\beta$s results in a significant response (Kohn et al., 2001). More recent work demonstrates that PZQ activates a schistosome transient receptor potential (TRP) channel *Sm.*$TRPM_{PZQ}$, revealed by electrophysiological analysis and Ca^{2+} imaging in HEK293 cells. The EC_{50} for the eutomer (R)-PZQ is 68±7nM, suggesting its high potency (Park et al., 2019).

On the other hand, (R)-PZQ is also a partial agonist of the human serotonin receptor HTR2B, which plays a role in regulating vascular tone (Chan et al., 2017). Despite its deleterious effect on schistosomes, (R)-PZQ treatment leads to vasoconstriction of mesenteric vessels where schistosomes reside. Increasing blood flow due to vasoconstriction may help flush PZQ-paralyzed parasites towards the liver where they get eliminated. Such cross species polypharmacology could be a useful strategy for novel anthelmintics development.

Vaccine development

In parallel to combating the disease head on, for the past few decades researchers have also investigated a preventative approach towards dealing with schistosomiasis. These studies led to

the development of multiple vaccines for this disease (Molehin, 2020). One vaccine candidate recombinant 28-kDa glutathione S-transferase of Sh (rSh28GST) reached phase 3 trials on human test subjects; however, the results left more to be desired due to low efficacy (Riveau et al., 2018). The test subjects were cleared of any underlying schistosome infections using PZQ prior to the administration of either the placebo or the vaccine. Recurrence of urinary schistosomiasis was observed in 84.8% of the vaccinated group and in 89.6% of the control group (Riveau et al., 2018). Although the vaccine was immunogenic and well tolerated by the infected subjects, the lack of efficacy of the vaccine to prevent a recurrence of schistosomiasis has proven to be a hurdle that remains to be overcome.

Preventative strategies

Currently, PZQ is able to help with the treatment of schistosomiasis, however, the growing concerns of schistosomal resistance to this drug must not be overlooked. Furthermore, PZQ is merely a treatment for the disease. Any symptoms or complications that arise from contracting schistosomiasis, prior to PZQ administration, may prove to be life-long or fatal. Thus, the future of mitigating this disease should also be aimed at disease prevention. Although recent hurdles have halted phase 3 vaccine trials for schistosomiasis, there are still other avenues to explore to prevent this devastating disease from occurring.

The most logical path to preventing schistosomiasis lies in merely avoiding contact with potentially infective bodies of water. However, in the rural regions of the world where the disease is endemic, this option is far from ideal. Individuals in these areas rely on limited sources of water for their everyday lives, thus, restricting them from this daily necessity is not an option.

Since the infective cercaria are shed from snails, another option would be to eliminate this intermediate host. By introducing mass quantities of molluscicide into the water sources in endemic areas, the cercariae-producing source would be annihilated (de Carvalho Augusto and Mello Silva, 2018). However, before this option can be introduced into practice, rigorous and extensive research needs to be conducted on the effects of molluscicide upon the surrounding ecosystems and to human health.

Instead of treating the source of cercariae, it may be more straightforward to hinder the ability of cercariae to infect their hosts. Motility is a good target as they have to actively swim towards their host while adjusting direction constantly. Moreover, after interaction with host is initiated, penetration does not occur immediately. Instead, cercariae have been observed exploring short distances on the skin before attempting to penetrate it (McKerrow and Salter, 2002). Therefore, interruption of cercarial motility will most likely lead to a prevention of successful infection.

Cercaria biology

Since disrupting the ability of cercariae to infect the host is a potential step in preventing schistosomiasis, it is important to understand cercarial biology and development. Since cercariae are positively phototropic, they tend to congregate towards the surface of the water once they emerge from the infected snail (McKerrow and Salter, 2002). This promotes human infection as the shallow waters are where human contact is maximized. The body of the cercaria consists of a head and tail region. The size of the cercariae can vary due to its capacity to extend and contract, but it is roughly 300-500 um long (Dorsey et al., 2002). Once contact with human skin is

established, cercariae respond to chemical signals as an indication for epidermal invasion (Salafsky et al., 1984). However, skin invasion must occur within a few hours after the cercaria leaves the intermediate snail host because cercariae do not feed and rely solely on their glycogen stores as their energy source (McKerrow and Salter, 2002).

The head of the cercaria consists of an oral sucker and mouth. This region also possesses strong musculature that is believed to aid in migration throughout the skin. A ventral sucker (otherwise known as the acetabulum) is located dorsally (Stirewalt, 1974). Anterior and posterior to the acetabulum, the unicellular pre- and post- acetabular glands are found, with their cytoplasmic processes extending towards the oral sucker (Stirewalt, 1974).

The tail of the cercaria is highly specialized and is the key to the animal's motility. This organ consists of several myocytes and neurons that aid in motility. The tail musculature consists of an inner longitudinal, a subtegumental, and three outer circular muscle layers (Collins et al., 2011). The tail must also satisfy the enormous energy demands of the cercariae and it is able to accomplish this with the large numbers of mitochondria and glycogen stores lining this organ (Dorsey et al., 2002).

Rotifers and cercariae

Both quality and quantity of cercarial production by snails have been very important for any cercaria study. However, variability has been observed routinely and across different labs (Lewis et al., 1986). In 1981, one source of such variability was found to be associated with the presence of rotifers, small zooplankton, that live as commensals on snail shells (Stirewalt and Lewis,

1981). In their study, Stirewalt and Lewis reported that not only the physical presence of rotifers, but also rotifer-conditioned water reduced cercarial motility and infectivity. This suggested that rotifers are releasing water-soluble molecules that negatively affect cercariae. Such a naturally occurring molecule could be a good candidate as an anti-cercarial/anti-schistosomal drug. Revealing the underlying mechanism would also provide more insights into developing preventative strategies. However, neither the identity of the molecule nor its the mechanism of action had been successfully characterized for almost 40 years.

Inspired by this observation, my thesis project aimed to uncover the identity of this anti-cercarial molecule and its underlying mechanism of action. In Chapter 2, I present methods to purify the Schistosome Paralysis Factor (SPF) and characterize its chemical structure. I show that this compound paralyzes schistosome cercariae and prevents infection, and does so more effectively than analogous compounds (Ht-13 analogs). Based on the evidence that SPF contains a serotonin backbone, I hypothesize that SPF might act through neurotransmitter GPCR. To test this hypothesis, as presented in Chapter 3, I screen a library of neurotransmitter-related compounds and find that increasing dopamine signaling in cercaria leads to paralysis. Utilizing a luciferase-based cell assay, I identify a schistosome dopamine receptor that is activated by these cercaria-paralytic compounds. Altogether, this evidence suggests that SPF might act through dopamine signaling. The role of serotonin signaling is yet to be excluded until all other schistosome dopamine/serotonin GPCRs are tested, as discussed in Chapter 4. In addition, I also propose long-term goals, including SPF synthesis and drug tests on other schistosome life stages. Besides the work presented here, the observation of SPF-induced cercarial paralysis also evokes many interdisciplinary questions which have yet to be addressed. Future studies to answer these

questions might contribute to multiple fields such as pharmacology, chemical ecology, cell biology and neurobiology.

Chapter 2: A rotifer-derived paralytic compound prevents transmission of schistosomiasis to a mammalian host

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2.1 Abstract

Schistosomes are parasitic flatworms that infect over 200 million people, causing the neglected tropical disease, schistosomiasis. A single drug, praziquantel, is used to treat schistosome infection. Limitations in mass drug administration programs and the emergence of schistosomiasis in non-tropical areas indicate the need for new strategies to prevent infection. It has been known for several decades that rotifers colonizing the schistosome's snail intermediate host produce a water-soluble factor that paralyzes cercariae, the life-cycle stage infecting humans. In spite of its potential for preventing infection, the nature of this factor has remained obscure. Here, we report the purification and chemical characterization of Schistosome Paralysis Factor (SPF), a novel tetracyclic alkaloid produced by the rotifer *Rotaria rotatoria*. We show that this compound paralyzes schistosome cercariae and prevents infection, and does so more effectively than analogous compounds. This molecule provides new directions for understanding cercariae motility and new strategies for preventing schistosome infection.

2.2 Introduction

Schistosomiasis – caused by parasitic flatworms of the genus *Schistosoma* – is a major neglected tropical disease, affecting over 200 million people, with over 700 million people at risk of infection (Gryseels et al., 2006; Steinmann et al., 2006; WHO, 2017). Praziquantel is currently the only drug used for treating schistosomiasis. Concerns about the emergence of drug resistance (Doenhoff et al., 2008; Wang et al., 2012) as well as limitations observed in mass drug administration programs (Assare et al., 2016; Crellen et al., 2016; Inobaya et al., 2014; Ross et al., 2015) highlight the need to devise new strategies for preventing infection by these parasites. This need is amplified by the recent identification of people infected with human/livestock hybrid schistosomes and the geographical expansion of schistosomiasis to temperate regions (Kincaid-Smith et al., 2017; Leger and Webster, 2017; Webster et al., 2013).

Schistosomes have a complex life cycle that alternates between an intermediate host (snail) and a definitive host (mammal) via two free-living, water-borne forms called miracidia and cercariae, respectively (Basch, 1991) (Fig 2.1A). For decades, inconsistency in cercarial production by snails and infectivity of mammalian hosts has been observed in most schistosome laboratories (Lewis et al., 1986). Intriguingly, Stirewalt and Lewis reported that rotifer colonization on shells of the snail intermediate host (*Biomphalaria glabrata*) significantly reduced cercariae output, motility, and infectivity (Stirewalt and Lewis, 1981). Furthermore, they observed that cercarial motility was affected not only by the presence of rotifers, but also by rotifer-conditioned water, indicating that rotifers released water-soluble molecules with paralytic activity. Almost 40 years have passed since this important finding, yet this factor's identity has remained a mystery.

2.3 Results and Discussion

Purification of the rotifer-derived compound

Encouraged by this anti-cercarial effect and its potential to prevent schistosome infection, we sought to purify this paralyzing agent. We isolated individual rotifers from snail shells and found two species, *Rotaria rotatoria* (Fig 2.1B) and *Philodina acuticornis* (Fig 2.1C), as previously reported (Stirewalt and Lewis, 1981). To identify which rotifer was responsible for the paralytic effect, we grew clonal isolates of each species, producing rotifer-conditioned artificial pond water (APW). Adding *Rotaria*-conditioned APW to freshly collected cercariae resulted in gradual paralysis within five minutes (Fig 2.1D). Most cercariae stopped swimming and sank to the bottom of the dish. Tapping the dish could stimulate their movement, but their response was limited to writhing on the dish bottom or short-distance swimming before becoming paralyzed again. In contrast, *P. acuticornis*-conditioned water had no effect (Fig 2.1E).

To purify the paralyzing agent, we performed molecular weight cut-off filtration (MWCO) of rotifer-conditioned water and found that the activity was present in the <650 Da fraction. The <650 Da filtrate was fractionated by reversed-phase high-performance liquid chromatography (RP-HPLC) (Fig 2.2A) and each fraction was tested on cercariae. Paralysis was only observed following treatment with a peak eluting at 25-27 min (Fig 2.2B). As expected, this peak was detected only in *R. rotatoria*- but not *P. acuticornis*-conditioned water (Fig 2.2B). A second round of HPLC on this peak revealed one peak (eluting at 24-26 min) with paralytic activity (Fig 2.2C). A predominant signal of 273.16 Da (M+H) in this peak was revealed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (Fig 2.2D). Consistent with the paralysis assay, this signal (m/z 273.16) was detected exclusively in the fraction eluting at 24-26

min but not in the fractions before or after (Fig 2.2E). These results suggested that the component with m/z 273.16 was the paralyzing agent, which we named "Schistosome Paralysis Factor" (SPF). We then determined the monoisotopic mass for protonated SPF using high-resolution quadrupole time-of-flight (Q-TOF) MS, 273.1595 Da (Fig 2.2F), suggesting $C_{16}H_{20}N_2O_2$ as the best-fitting formula for SPF.

SPF is a novel tetracyclic alkaloid

To elucidate its structure, we purified ~0.1 mg SPF from 25 L *R. rotatoria*-conditioned water. Nuclear magnetic resonance (NMR) spectroscopy revealed a novel tetracyclic structure. Briefly, ^{1}H spectra showed the presence of 19 protons in the compound (S2.1 Fig), which agrees with the best-fitting formula and Hydrogen/Deuterium exchange mass spectrometry (MS) analysis (S2.2 Fig). Heteronuclear single quantum coherence spectroscopy **(**HSQC) revealed three methyl, two methylene, six methine groups, and five quaternary carbons (S2.3 Fig). Total correlation spectrometry (TOCSY) showed that aliphatic protons, except two methyl groups, are from one spin system (S2.4 Fig). The connectivity of the neighboring groups was derived from correlation spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) spectra (S2.5 Fig and S2.6 Fig). Overall, the aliphatic region is composed of a dimethylpyrrolidine structure which is linked to an indole via a CH_2 group and an oxygen. Nuclear Overhauser effect spectroscopy (NOESY) suggested (*R, S, S*) or (*S, R, R*) configurations on the chiral centers (S2.7 Fig). Altogether, combined NMR analysis led to two possible structures (Fig 2.2G, 2.2H and S2.1 Table).

SPF and its analogs paralyze cercariae in a dose-dependent manner

To test its dose dependency, we examined the paralytic effect of serially diluted SPF on cercariae by quantifying their movement over time. In the absence of SPF, over 82% of cercariae were free-swimming over three minutes (Fig 2.3A). In 2.5 nM SPF, the percentage of free-swimming cercariae dropped to 67% three minutes after drug treatment. As the concentration of SPF increased, so did the rate of paralysis, and more cercariae were paralyzed at the end of treatment. We observed maximum effects in 250 nM and 2.5 μM SPF, with the majority of cercariae paralyzed within 30 s.

Two natural compounds from *Streptomyces sp.*, ht-13-A and ht-13-B, are structurally related to SPF; they were isolated based on their affinities for human serotonin receptors (Kamigauchi and Yasui, 2000). All three alkaloids share a novel oxepineindole framework fused with a pyrrolidine ring (Fig 2.3A-C; note the serotonin backbone highlighted in red in Fig 2.3A). Although synthesis of SPF has not been achieved, total syntheses of ht-13-A and ht-13-B have been reported (Tao et al., 2016; Zhang et al., 2015; Zhang et al., 2016). To test whether this shared tetracyclic scaffold is responsible for the paralytic effect, we analyzed structure-activity relationships by using ht-13-A, ht-13-B, three ht-13-A derivatives (18), and one epimer in cercarial paralysis assays. Importantly, ht-13-A, although not as potent as SPF, also had a paralytic effect on cercariae (Fig 2.3B and). In contrast, ht-13-B did not paralyze cercariae, suggesting that the extra methyl group disrupts interaction with the target (Fig 2.3C). Of the three ht-13-A analogs, only ht-13-A-pr effectively paralyzed cercariae; it was more potent than ht-13-A, indicating that the nature of the side chain is important for proper target interaction (Fig 2.3D-E). In contrast to ht-13-A and Ht-13-A-pr, the epimer was unable to paralyze cercariae;

these results support the (*R, S*) configuration of SPF at C12, 13 as the biologically active form (Fig 2.2G).

SPF prevents mammalian infection

Since motility of the cercarial tail is essential for swimming and provides force for skin penetration (Haas, 1992, 2003; Whitfield et al., 2003), we examined whether SPF prevented infection. We treated ~200 cercariae with different concentrations of SPF for 10 mins, and then tested their infectivity by exposing them to mouse tails for 30 min (N=6 for each condition). Six-weeks post infection, we euthanized the mice, counted schistosomes recovered after hepatic portal vein perfusion, and examined liver pathology. From controls, we recovered 83 adult worms on average (Fig 2.4B and Appendix 1), consistent with typical recoveries of ~40% (Lewis, 2001). Livers from these mice appeared dark and contained extensive granulomas (Fig 2.4A). In contrast, we did not recover any adult worms from mice after treatment with 250 nM or 2.5 μM SPF (Fig 2.4B and Appendix 1) and no granulomas were observed (Fig 2.4A). Histological examination confirmed that these livers were free of schistosome eggs (Fig 2.4E and Appendix 1), suggesting complete inhibition of infection. These data are consistent with the full paralysis observed after treatment with 250 nM or 2.5 μM SPF (Fig 2.3A). Although 25 nM SPF paralyzed most cercariae *in vitro*, the effects on mouse infection were not as severe (Fig 2.4A). Mechanical and/or chemical stimuli from mouse tails may overcome SPF-induced paralytic effects at low SPF concentrations. Notably, neither Ht-13-A nor Ht-13-A-pr blocked infection as completely as 250 nM SPF, even at 25 μM (Fig 2.4A, 2.4C, 2.4D, 2.4F, 2.4G and Appendix 1). Under more realistic infection conditions, in which mouse tails were lifted 1-2 cm from the bottom of the test tube containing cercariae, so they had to swim actively towards the tail to

infect the mouse, Ht-13-A and Ht-13-A-pr were still not as effective as SPF, which completely blocked infection (S2.8 Fig and Appendix 1).

2.4 Conclusion

This work has identified a novel tetracyclic alkaloid, produced by the rotifer *R. rotatoria,* that paralyzes the infective larvae of schistosomes. Although its mechanism of action remains unknown, its chemical structure provides important clues. SPF contains a serotonin backbone, suggesting that SPF might antagonize serotonin signaling, perhaps via G protein-coupled receptors (GPCRs) or serotonin-gated channels. Consistent with this idea, the structurally related compounds, ht-13-A and ht-13-B, bind several human serotonin receptors (Kamigauchi and Yasui, 2000). In schistosomes, serotonin has been implicated in neuromuscular functions in multiple life-cycle stages (Boyle and Yoshino, 2005; Boyle et al., 2000; Chan et al., 2016; Ribeiro et al., 2012); knocking down a serotonergic GPCR (Sm5HTR) in schistosomulae and adult worms led to decreased movement (Patocka et al., 2014). Interestingly, praziquantel partially activates the human serotonin receptor, HT2BR, suggesting that it may also target schistosome serotonergic GPCRs (Chan et al., 2017).

The chemical ecology underlying *R. rotatoria*'s production of SPF is also unclear. Whether SPF is used naturally to combat other aquatic creatures (e.g., to prevent other rotifers from colonizing areas where *R. rotaria* live) and, thus, the effect on schistosome cercariae is indirect, or whether SPF benefits the rotifer's commensal host will require further study. Because compounds with structural similarities to SPF are produced by *Streptomyces sp*., it will be important to examine the possibility that SPF is not directly produced by the rotifer, but rather by constituent(s) of its own microbiome. However, given that horizontal gene transfer is well documented in rotifers (Flot et al., 2013; Gladyshev et al., 2008), it is also possible that *R. rotatoria* has acquired the synthetic machinery to produce SPF on its own. Future work will help reveal the source of SPF and its biosynthetic pathway.

In the past few decades, the discovery and development of natural products have helped combat parasitic diseases (Shen, 2015). Based on its ability to block infection, SPF holds great promise as an anti-schistosomal agent. Identifying the biologically active chemical scaffolds and understanding SPF's mode of action are expected to provide important clues for preventing schistosomiasis.

2.5 Materials and Methods

Artificial pond water

Four stock solutions were prepared to make artificial pond water (Collins et al., 2011): 1) 0.25 g/L $FeCl_3 \cdot 6H_2O$, 2) 12.9 g/L $CaCl_2 \cdot 2H_2O$, 3) 10g/L $MgSO_4 \cdot 7H_2O$, and 4) 34 g/L KH_2PO_4 1.5 g/L $(NH_4)_2SO_4$, pH 7.2. For 1L artificial pond water, we added 0.5 mL of $FeCl_3$ solution, 2.5 mL $CaCl_2$ solution, 2.5 mL $MgSO_4$ solution and 1.25 mL phosphate buffer.

Obtaining *S. mansoni* cercariae

Infected *B. glabrata* snails provided by Biomedical Research Institute (BRI, Rockville, MD) were maintained in artificial pond water and fed Layer Crumbles (chicken feed) (Rural King, Mattoon, IL). To obtain *S. mansoni* cercariae, *B. glabrata* snails were exposed to light at 26°C for 1-2 hrs. Artificial pond water containing cercariae was passed through a 100 μm cell strainer (Falcon) to remove snail food and feces. Cercariae were then collected using custom-made 20 μm cell strainers.

Rotifer culture

Since both rotifer species reproduce parthenogenetically, we clonally expanded each species into one-liter cultures from a single rotifer. Individual rotifers (*R. rotatoria* and *P. acuticornis*) were initially isolated from the shell of *B. glabrata* and cultured in artificial pond water in 24-well plates. Each individual colony was expanded into ever-larger culture volumes and ultimately maintained in two-liter flasks. Both species were fed Roti-rich liquid invertebrate food (Florida Aqua Farms Inc). Rotifer-conditioned water was collected every month by filtering out the

rotifers using a 20 μm cell strainer. Filtered rotifers were then passaged to fresh artificial pond water to propagate the cultures.

Crude rotifer-conditioned water preparation

One liter rotifer media was lyophilized, reconstituted with 50 mL dH_2O and filtered through 10,000 and 650 (MWCO) Pall Minimate TFF Capsules with Omega membrane (Ann Arbor, MI). Filtrate (<650 Da) was freeze dried. For RP-HPLC, 300 mg of the dried material was dissolved in dH_2O and run on a RP-HPLC – Merck Chromolith semi-prep RP-18e column (Darmstadt, Germany) at 5 ml/min using a gradient of 100% A (water) to 60% B (acetonitrile) in 60 min. 10 mL fractions were collected and assayed for biological activity. Fractions containing biological activity were saved for further study.

Further purification of rotifer media

The bioactive fractions were pooled, freeze dried with SpeedVac (Savant, MA), reconstituted with 500 μL dH_2O and injected into a 4.6mm diameter × 25cm Symmetry column (Waters, MA). Breeze2 analytical LC system (Waters, MA) was employed for separation at 0.5 mL/min with the following solvents and gradients: Solvent A, 0.1% formic acid (FA); solvent B, methanol with 0.1% FA; 0—10 min 0—10% B, 10—30 min 10—35% B, 30—33 min 35—80% B, 33—37 min 80—80% B, 37—40 min 80—0% B. Eluents were collected manually based on peak elution. All fractions were lyophilized, reconstituted with water and analyzed with MALDI-MS. Fractions containing biological activity were saved for future use.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis

For each collected fraction, 1 μL of sample solution was spotted on ground steel MALDI target and mixed with 1 μL of alpha-cyano-4-hydroxy-cinnamic acid (CHCA, Sigma-Aldrich, MO) solution (10 mg/mL CHCA in 50% acetonitrile solution with 0.005% trifluoroacetic acid). Mass calibration, spectra acquisition and analysis were performed under conditions as previously described (Tillmaand et al., 2015).

High-resolution quadrupole time-of-flight mass spectrometry (Q-TOF MS) analysis

1 μL of the bioactive fraction was separated on a Magic 0.1 × 150mm column (Michrom, CA) and analyzed with maXis 4G mass spectrometer (Bruker, MA) using previously established methods for metabolite study (Aerts et al., 2014). The separation was performed at 300 nl/min by use of solvent A (95% water, 5% acetonitrile with 0.1% FA) and solvent B (5% water, 95% acetonitrile with 0.1% FA) with the following gradient conditions: 0—5 min 4% B, 5—50 min 4—50% B, 50—52 min 50—90% B, 52—60 min 90% B, 60—70 min 90—4% B, 70—90 min 4% B.

Hydrogen/deuterium (H/D) exchange analysis

Acidified deuterated methanol (CD_3OD, methanol—d4, Sigma—Aldrich, MO) was made by adding 1 μL of deuterated FA into 1 mL of CD_3OD. 2 μL of the bioactive fractions were added into 18 μL of acidified methanol above. 15 μL of the mixture were analyzed by direct infusion into a modified 11 Tesla FTMS (Thermo Scientific, MA) using a NanoMate robot (Advion, NY) (Lee et al., 2010). Full spectra were acquired with resolution set at 100k.

Nuclear magnetic resonance (NMR) analysis

Purified bioactive materials were dissolved in 250 μL of CD_3OD and transferred into a 5 mm Shigemi NMR tube with a glass magnetic plug with susceptibility matched to CD_3OD on the bottom. All NMR data were collected at 40°C on an Agilent VNMRS 750 MHz spectrometer equipped with a 5 mm Varian indirect detection probe with z gradient capability. Collected NMR data included 1H spectrum, gradient selected correlation spectroscopy (gCOSY), total correlation spectroscopy (TOCSY), nuclear Overhauser enhancement spectroscopy (NOESY) with a mixing time of 500 ms, heteronuclear single quantum coherence spectroscopy (^1H—^{13}C HSQC) and heteronuclear multiple-bond correlation spectroscopy (^1H—^{13}C HMBC). The NMR spectra were analyzed using Mnova NMR software (Mestrelab Research, Spain).

Determination of SPF concentration

The proton quantification experiments were performed at 23ºC on an Agilent 750 MHz VNMRS NMR spectrometer equipped with a 5mm triple-resonance ($^1H/^{13}C/^{15}N$) indirect-detection probe with XYZ PFG gradient capability. The probe was calibrated using the qEstimate tool in the Agilent VnmrJ4.2 software with a known standard. The proton spectrum of the sample was collected with a 90-degree pulse angle of 8.5 ms, 16 scans and 10.4 s delay between scans. The Agilent VnmrJ4.2 software was used to determine the concentration of the sample based on the integration values of proton peaks. A total of 5 well-resolved proton peaks (7.12ppm (1H), ~6.89ppm (2H), 4.41 (1H), 3.83 (3H), and ~3.58 (2H)) was used, and the concentration of the sample was 1.55 ± 0.07 mM. All concentrations used in the cercarial paralysis assay were calculated based on this value.

Cercarial paralysis assay

To capture the whole field while avoiding excess reflected light in a well, we used the lid of 96-well plate (Costar). 40 μL of artificial pond water containing ~50 cercariae were added to each shallow well on the lid. 10 μL of SPF (dissolved in APW) was then added to reach the final concentration indicated. Using a high-speed camera (Olympus i-SPEED TR), attached to a stereomicroscope (Leica MZ125), we recorded cercariae movement at 20-60 fps at 1.25X magnification just prior to addition of test compounds until 3-4 min after treatment started. Raw movies were converted to .avi files using i-SPEED Viewer and compressed into JPEG format using ImageJ (addition of compound is considered time 0). We then counted the numbers of free swimming/paralyzed cercariae every 5 s for 1 min and every 30 s thereafter for 3 min. The number of dead cercariae (those that never swim before and after SPF treatment) were subtracted from data. Experiments were performed in biological triplicate.

Mouse infectivity assay

Swiss Webster mice (female) were purchased from Taconic Biosciences and bred by RARC SPF Mouse Breeding Core (University of Wisconsin-Madison, Madison, WI). Mouse infections were performed by exposing mouse tails to *S. mansoni* cercariae according to standard protocol from BRI (Lewis, 2001) with slight modifications. Briefly, we secured mice in rodent restrainers (Thomas Scientific, Cat #551-BSRR) and put them vertically on top of a rack with grids. We pipetted 100 μL of each drug at proper concentration into a skinny glass tube (Fisher Scientific, Cat #14-958A) inside a 12 X 75 mm holding glass tube (VWR, Cat # 47729-570). 300 μL of APW containing ~200 cercariae were pipetted into each skinny tube and incubated for 10 mins before we inserted the mouse tail. Mouse tails were wiped with APW-moistened Kimwipes,

inserted into the skinny tube, and exposed to cercariae for 30 mins. The mouse tail was touching the bottom of the test tube unless otherwise specified. We euthanized and perfused these mice six week-post infection according to standard protocols (Lewis, 2001). For each drug, we initially used three mice for controls (APW only) and three mice for each concentration tested except for 25 nM Ht-13-A and Ht-13-A-pr. We then repeated the experiments again with three mice for each condition. In addition to that, we included six mice for 25 nM Ht-13-A and Ht-13-A-pr. Adult worms were recovered by hepatic portal vein perfusion, and males and females were unpaired by a brief incubation in 2.5% Tricaine (Sigma) to facilitate counting. We counted total numbers of adult worms under a stereomicroscope (Leica MZ75). Livers from infected mice were fixed in 4% formaldehyde in PBS overnight. Largest liver lobes (left lobe) were submitted to University of Wisconsin-Madison Histology Core Facility for sectioning and Hematoxylin and Eosin staining. Each left lobe was evenly cut into 4-6 pieces and paraffin embedded on a large cassette. One slide (4-6 liver sections) for each liver was used for histological examination, which provided a representative view throughout the whole liver lobe. We took a tiled image of the whole slide using a Zeiss Axio Zoom microscope and used ImageJ to determine the area of each section. Total numbers of eggs in each section were counted and normalized to the area. In adherence to the Animal Welfare Act and the Public Health Service Policy on Humane Care and Use of Laboratory Animals, all experiments with and care of mice were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-Madison (protocol approval number M005569).

Statistical analysis

GraphPad Prism (Version 7) was used for all statistical analyses. One-way ANOVA test followed by Dunnett's multiple comparison test was used. Mean ± S.D. is shown in all figures.

2.6 Acknowledgments

B. glabrata snails were provided by the NIAID Schistosomiasis Resource Center of the Biomedical Research Institute (Rockville, MD) through NIH-NIAID Contract HHSN272201700014I for distribution through BEI Resources. We thank: Melanie Issigonis, Umair Khan, Jayhun Lee, and Tania Rozario for helpful discussions and comments on the manuscript; Tracy Chong, Jayhun Lee, and Janmesh Patel for help maintaining the schistosome life cycle; Melanie Issigonis for solving the pond water crisis; Björn Söderberg and Yanxing Jia for providing Ht-13-A, -B, and derivatives; Lingyang Zhu for expert assistance with NMR; Brian Imai for assistance with SPF purification; as well as Peg Stirewalt, James Leef, Tom Nerad, and Paul Mazzocchi for their early efforts to help solve this puzzle.

2.7 Figures

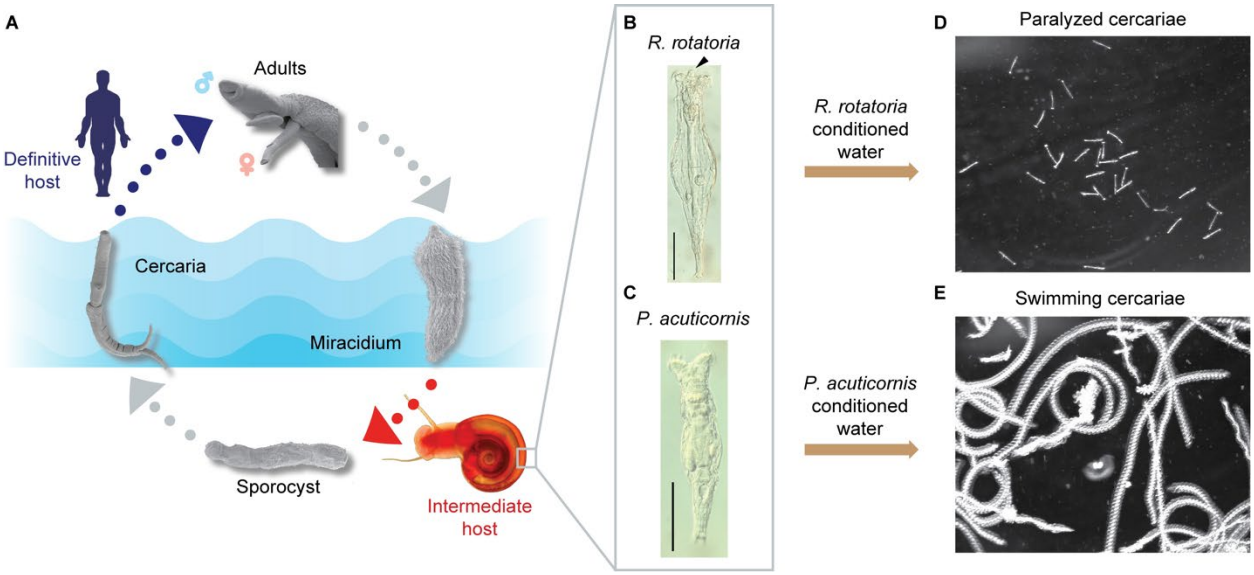


Fig 2.1. *R. rotatoria*-conditioned water paralyzes *S. mansoni* cercariae.

(A) Life cycle of *S. mansoni*. Adult parasites, residing in the mammalian host vasculature, lay eggs (not shown). Upon exposure to fresh water, eggs release miracidia, which infect the appropriate snail host. Inside the snail the parasite reproduces asexually, ultimately producing large numbers of free-swimming infective larvae (cercariae) that can penetrate mammalian skin to continue the life cycle (adapted from (Collins and Newmark, 2013)). (B and C), Nomarski differential interference contrast microscopy images of *R. rotatoria* and *P. acuticornis* (arrowhead indicates the rostrum in *R. rotatoria,* which is lacking in *P. acuticornis*). Scale bars: 100 μm. (D and E) Maximum intensity projection (5 s, 150 frames) of cercariae motility after treatment with *R. rotatoria*- or *P. acuticornis*- conditioned water.

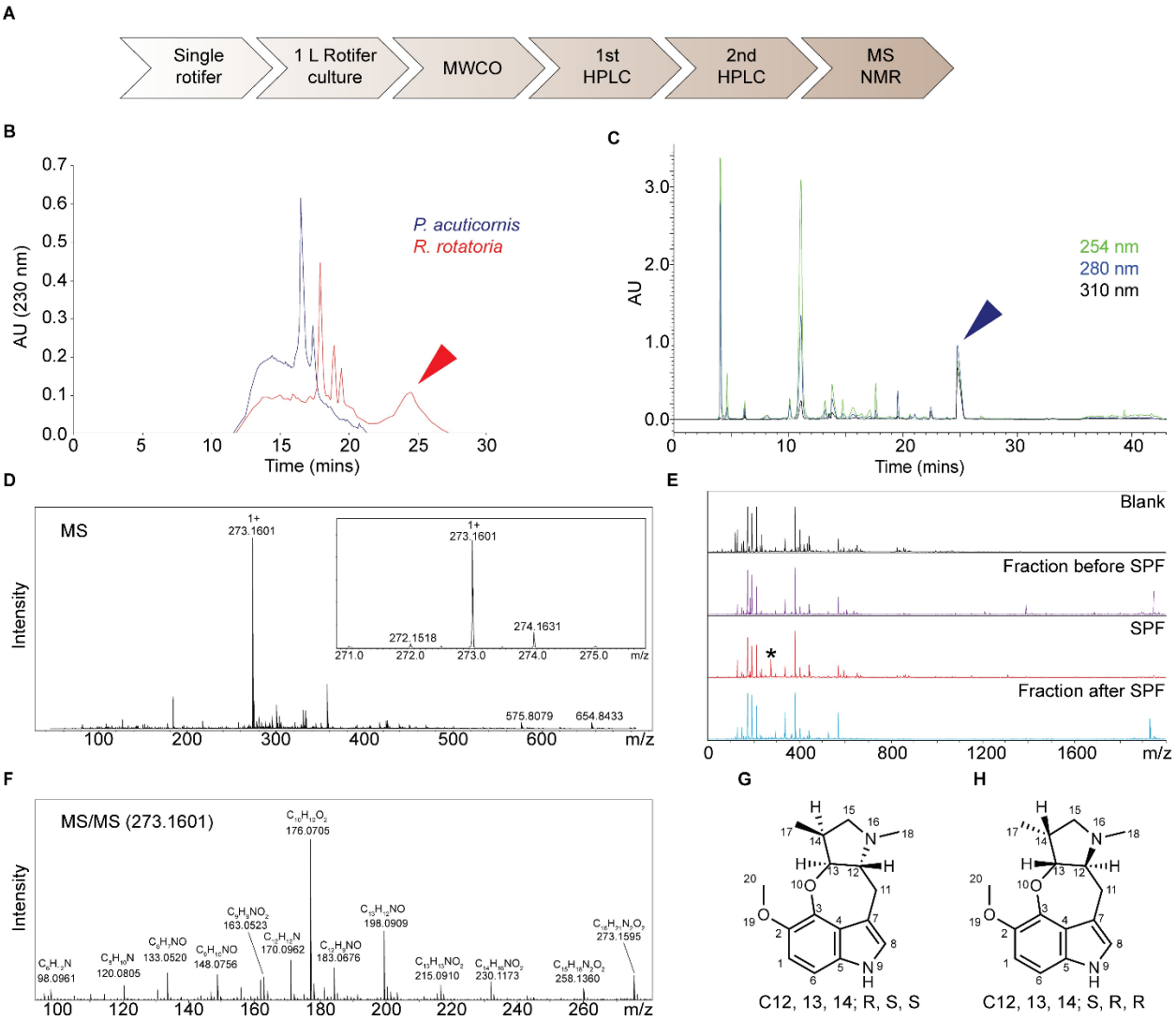


Fig 2.2. SPF is a novel tetracyclic alkaloid.

(A) Flowchart for SPF purification. (B) 1st HPLC plots of *R. rotatoria*- and *P. acuticornis*-conditioned water. All fractions were tested for bioactivity; the red arrowhead indicates the only active peak. (C) 2nd HPLC plot of the bioactive fraction (red arrowhead in B). All peaks were tested for bioactivity; the blue arrowhead indicates the only peak containing activity. (D) MS showing the dominant signal of m/z 273.1601 from the peak (blue arrowhead). (E) MS plots showing this signal (asterisk, m/z 273.1601) was only detected in the fraction eluting at 24-26 min. (F) Tandem MS acquired from high-resolution Q-TOF analysis. (G and H) NOESY

resolved the relative stereochemistry of three chiral centers and narrowed it down to two possible configurations.

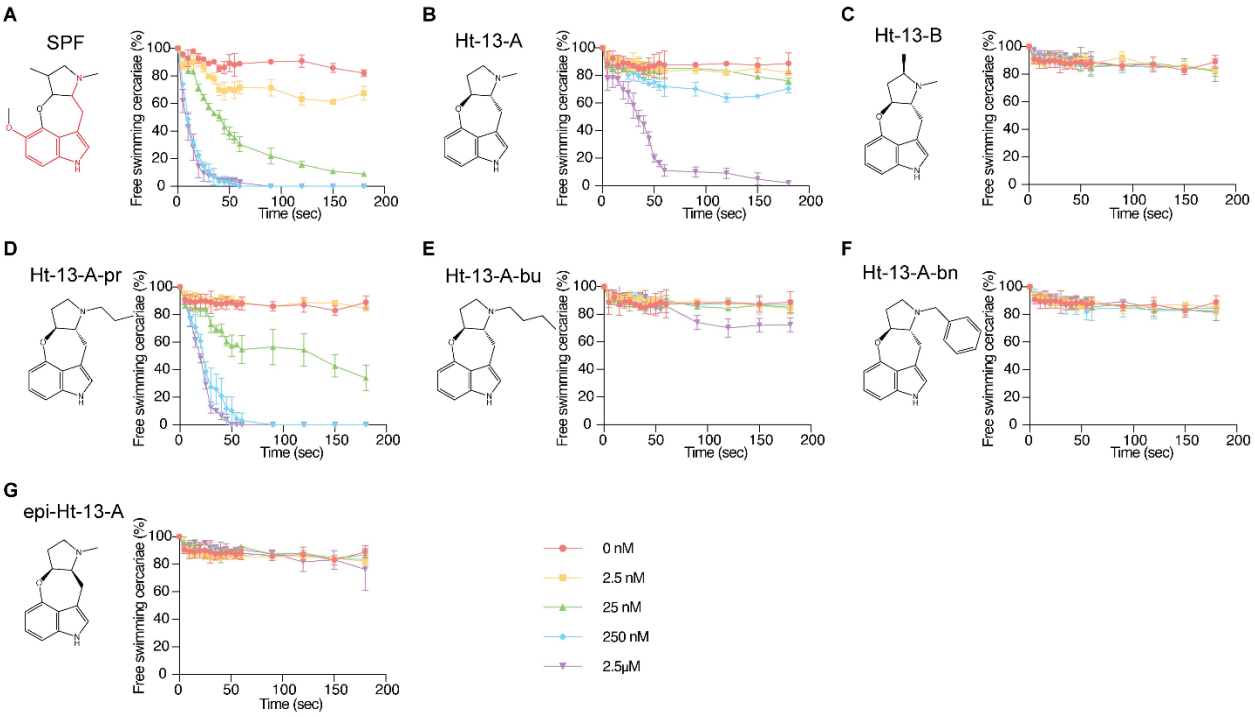


Fig 2.3. Structure-activity relationships of SPF and related compounds as measured by cercarial motility assays.

(A-G), Percentage of cercariae (~50) continuing to swim over three minutes after addition of each compound at specified final concentrations. Triplicates were performed. Data are mean ± S.D. Serotonin structure in SPF is outlined in red.

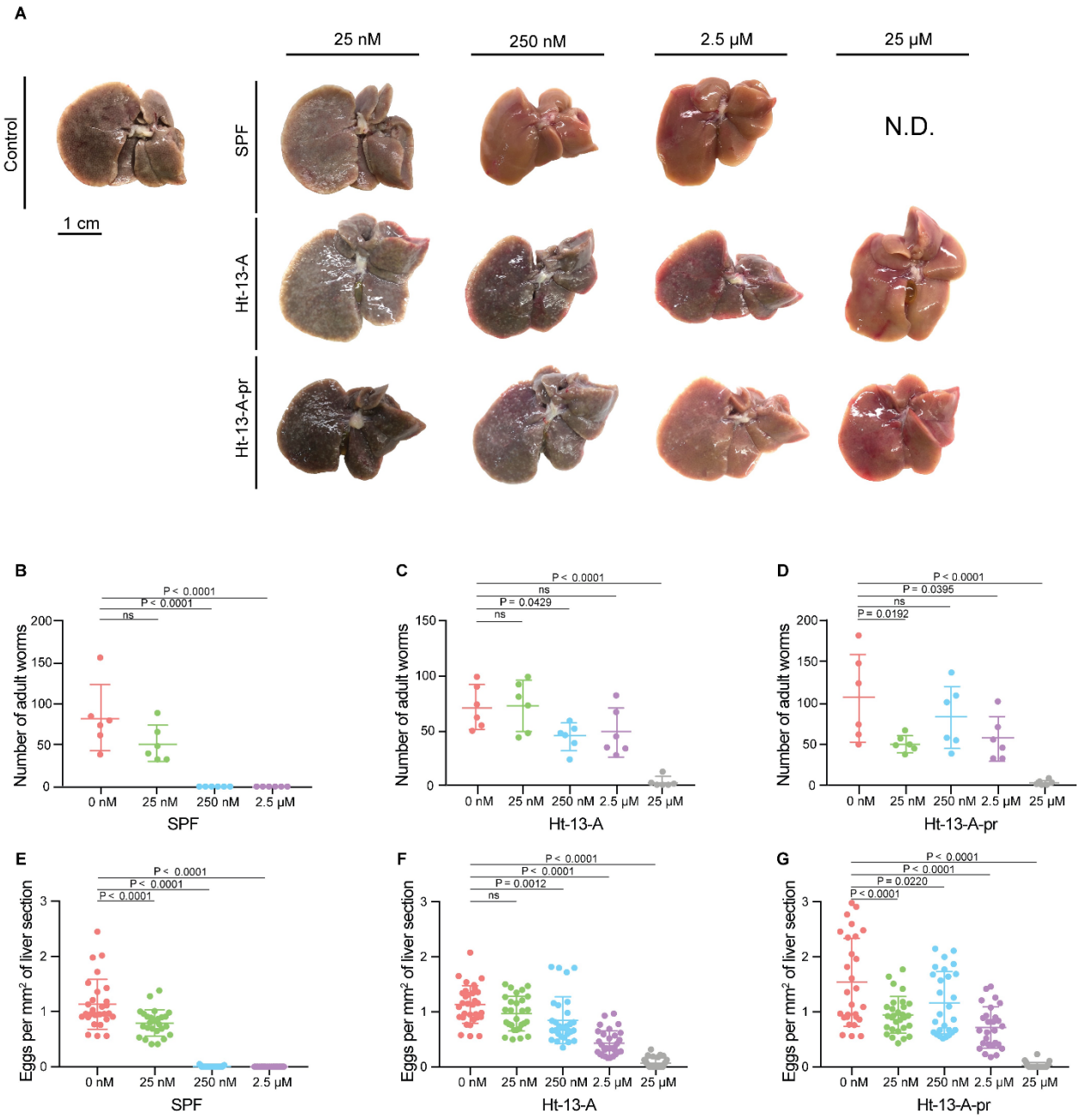
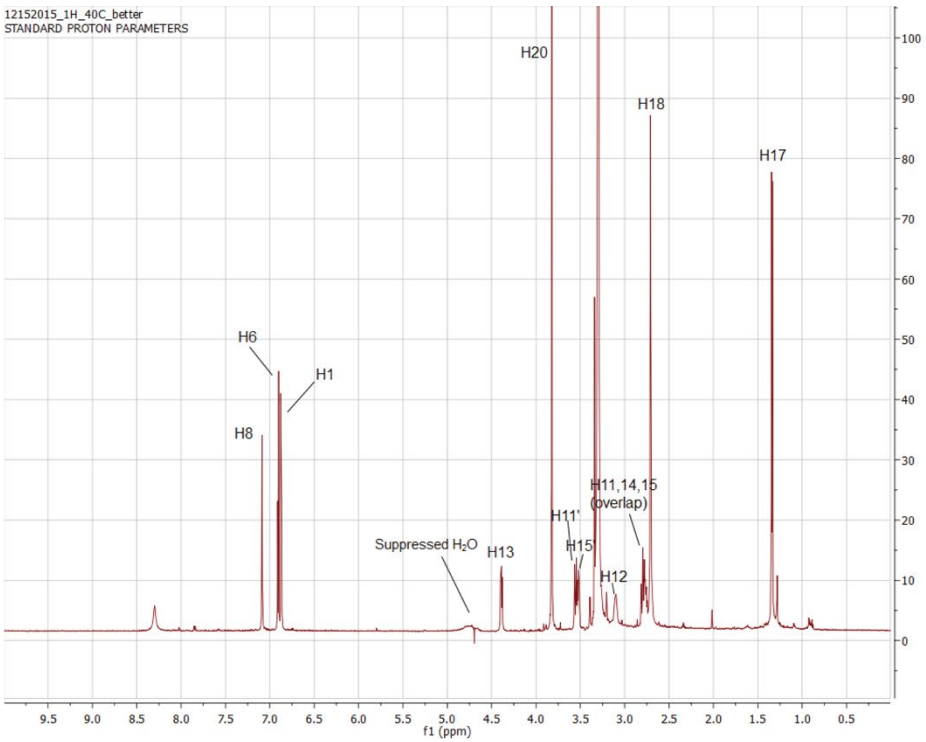


Fig 2.4. Treating cercariae with SPF, Ht-13-A or Ht-13-A-pr blocks schistosome infection and alleviates pathology.

(A) Representative livers (post perfusion) from mice (N=6) exposed to drug-treated cercariae. Livers from mice treated with control and lower drug concentrations were darker in color and contained more granulomas (white spots). With higher drug concentrations, livers had normal

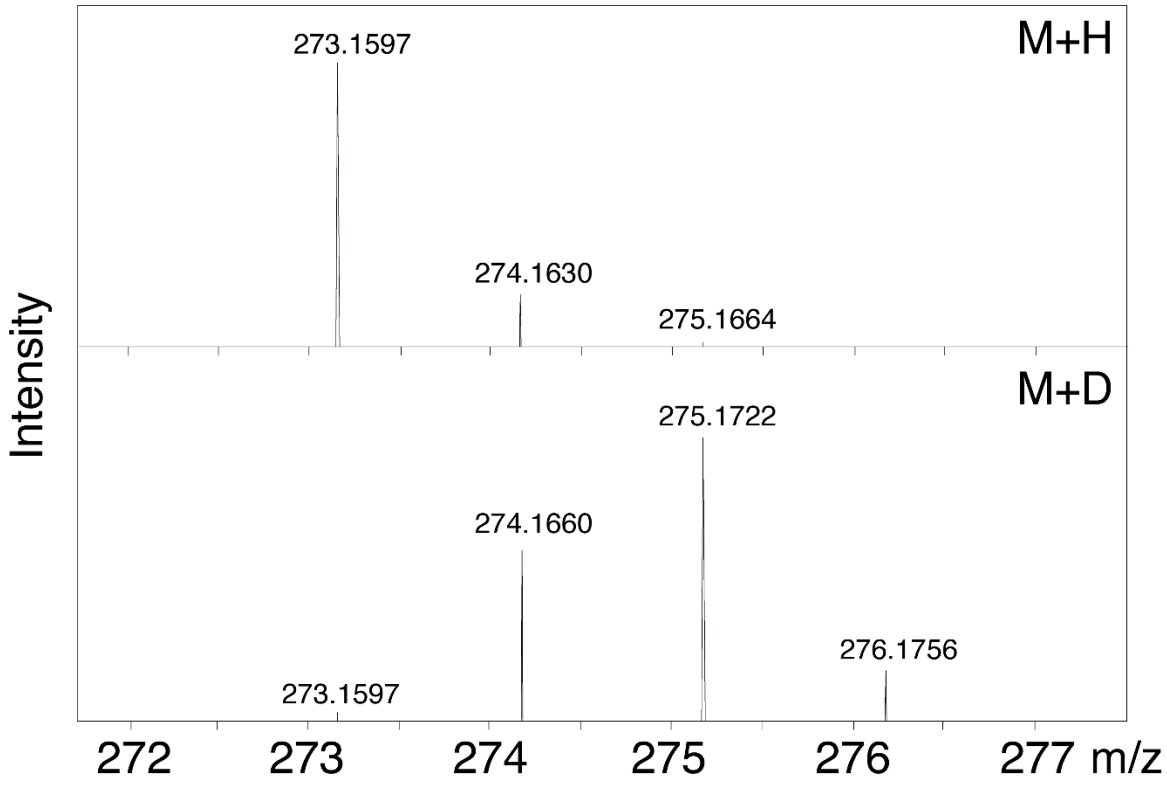
morphologies with few or no granulomas. 25 μM SPF treatment was not determined (N.D.) due to limited amounts of purified SPF. (B-D) Numbers of adult worms recovered from exposed mice (two experiments for each drug, 6 mice total for each condition). (E-G) Numbers of schistosome eggs per area (/mm^2) from liver sections (4-6 sections per mouse). Data (B-G) are mean ± S.D. Statistics: One-way ANOVA, post Dunnett's test.

2.8 Supplementary materials



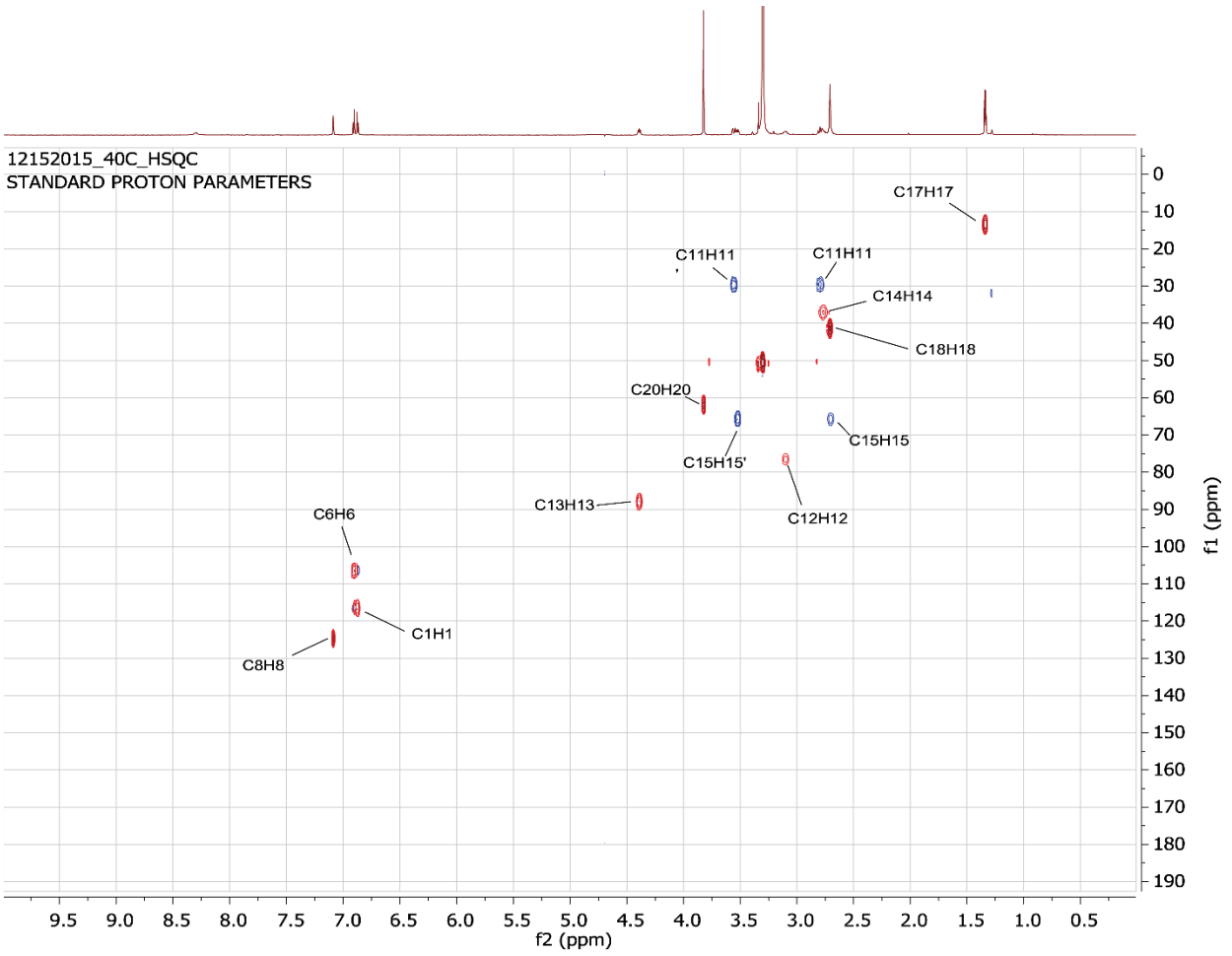
S2.1 Fig. ^{1}H Nuclear magnetic resonance (NMR) spectrum of SPF.

Peak areas of the non-overlapping peaks were integrated and protons (δH 1.34, 3.10, 3.52, 3.56, 3.81, 4.40, 6.86, 6.90 and 7.09) showed integer ratios, supporting the mass spectrometry results that their signals were from the same compound. After adding the integration of overlapping peaks (δH 2.70, 2.72, 2.77, 2.79), a total of 19 protons were discovered, consistent with the best-fitting formula from the mass spectrometry results: ($C_{16}H_{20}N_2O_2$).



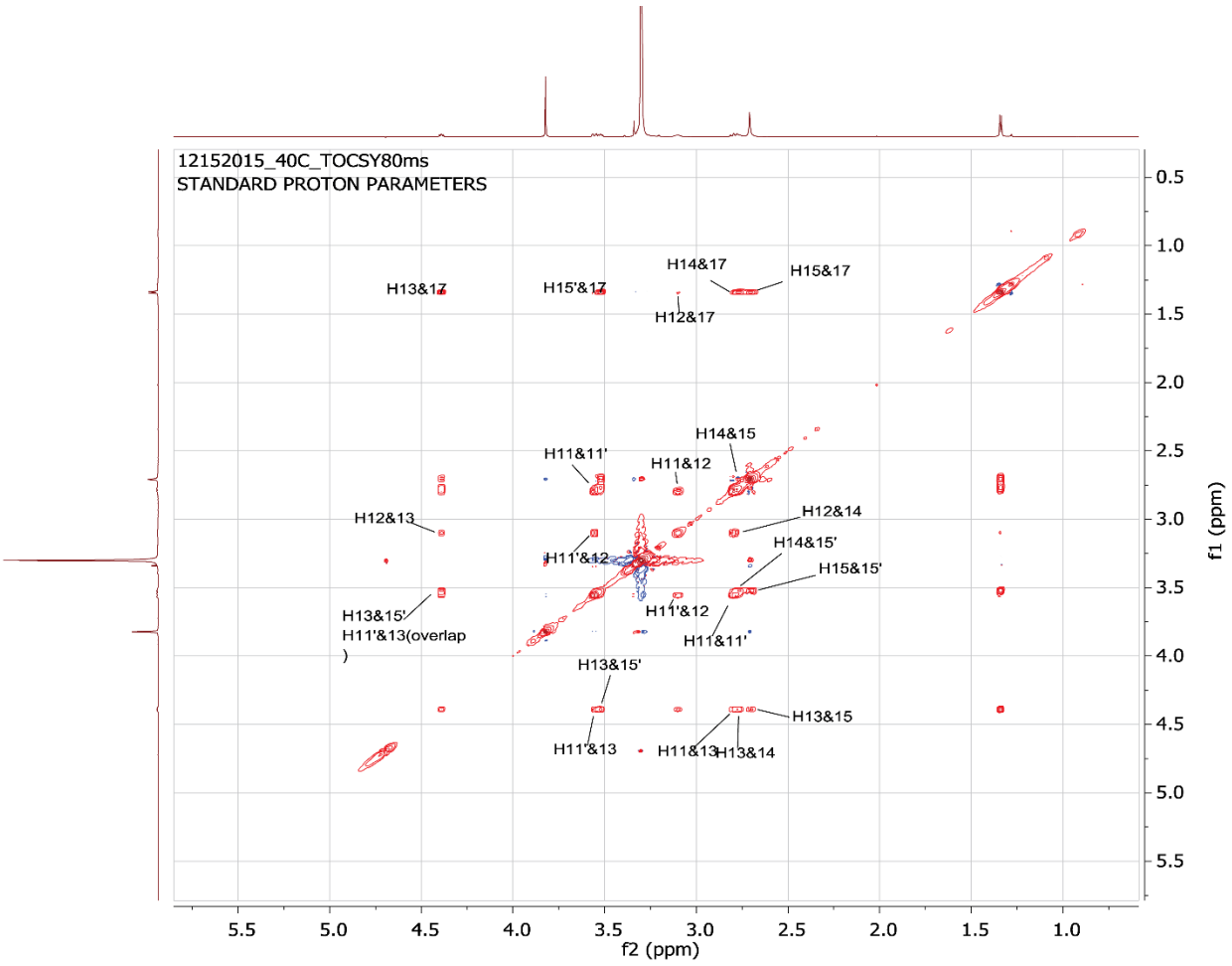
S2.2 Fig. Fourier-transform mass spectrometry (FTMS) determined the accurate *m/z* of the target molecule and revealed its isotopic pattern.

Before deuterium exchange (top panel), 273.1597 was the measured *m/z* of the target molecule. After deuterium exchange (bottom panel), m/z of the base peak increased to 275.1722 (deuterium singly charged target molecule with one proton replaced by deuterium), suggesting the presence of one exchangeable proton in SPF.



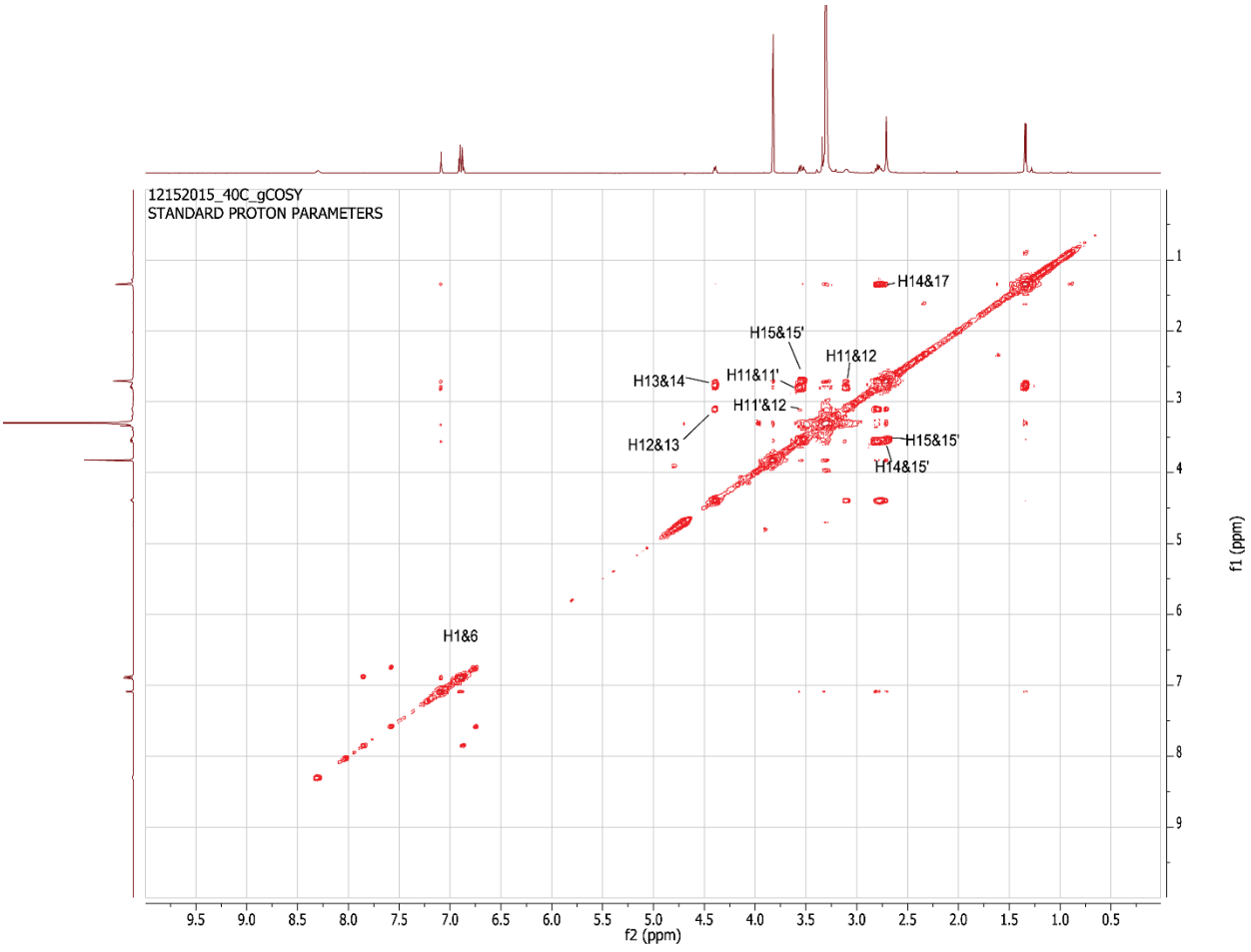
S2.3 Fig. ^{1}H—^{13}C Heteronuclear single quantum coherence spectroscopy (HSQC) NMR spectrum of SPF.

HSQC revealed the cross—correlation between directly bonded proton and carbon nuclei and determined the number of methyl, methylene and methine groups. 19 protons were attached to 11 carbons, including: three methyl groups (δC 14.0, δH 1.34; δC 41.7, δH 2.71; δC 62.2, δH 3.81); two methylene groups (δC 29.0, δH 2.79, 3.56; δC 65.8, δH 2.70, 3.52); and six methine groups (δC 116.8, δH 6.86; δC 106.6, δH 6.90; δC 124.9, δH 7.09; δC 76.7, δH 3.10; δC 88.2, δH 4.40; δC 37.2, δH 2.77). The other 5 carbons that did not show up in the HSQC spectrum are the quaternary carbons. Based on the carbon chemical shift, the two methyl groups (δC 41.7, δH 2.71 and δC 62.2, δH 3.81) are likely to be bound to nitrogen and oxygen, respectively.



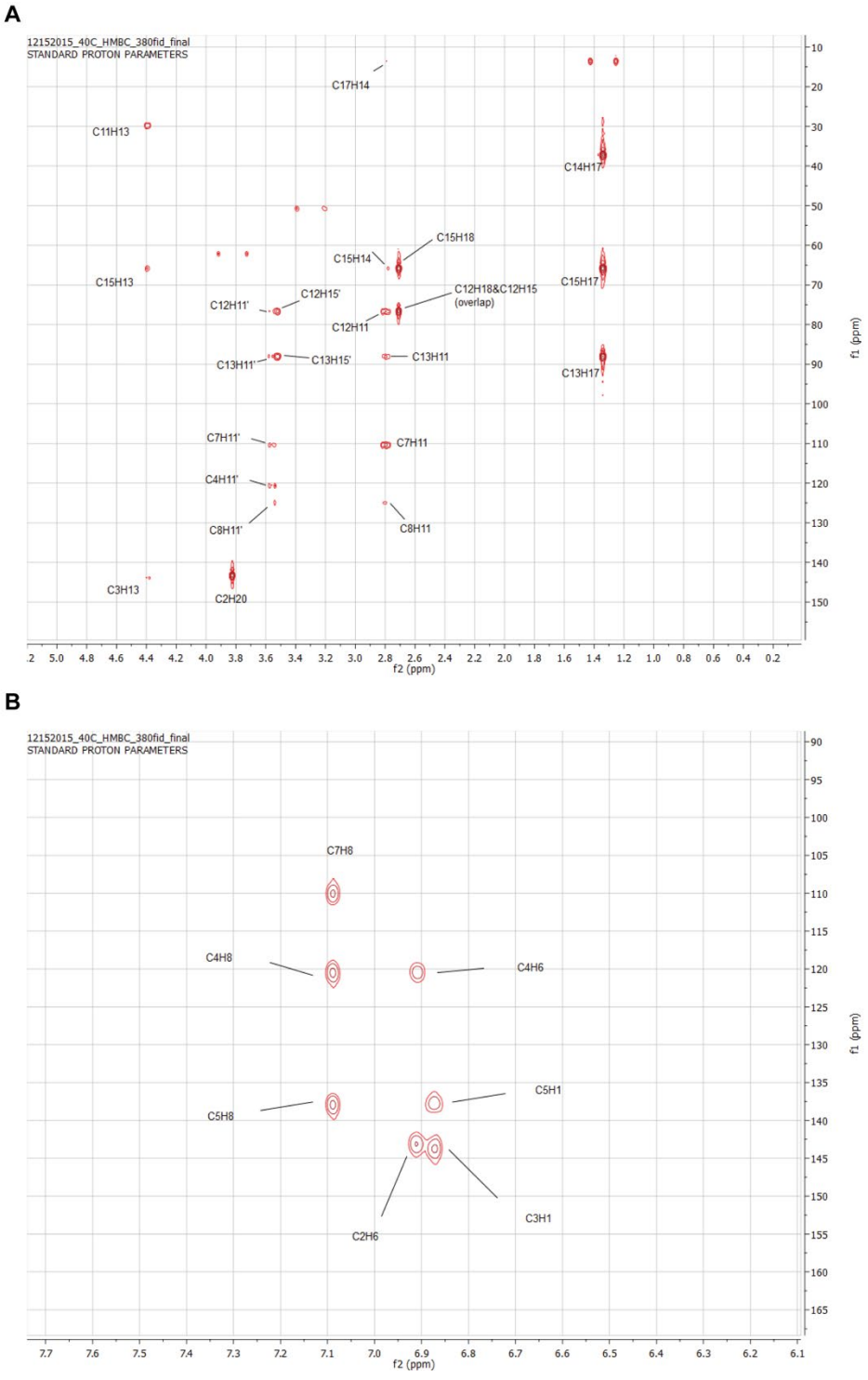
S2.4 Fig. Total correlation spectrometry (TOCSY) NMR spectrum of SPF.

TOCSY revealed that the aliphatic protons except the two methyl groups (δH 2.71 and 3.81) found binding to N and O in HSQC (S2.3 Fig) are from a single spin system. Cross-peaks were also observed among the aromatic proton δH 7.09 and the aliphatic protons (δH 3.56, 2.79 and 3.10), due to long-range couplings.



S2.5 Fig. Correlation spectroscopy (COSY) NMR spectrum of SPF.

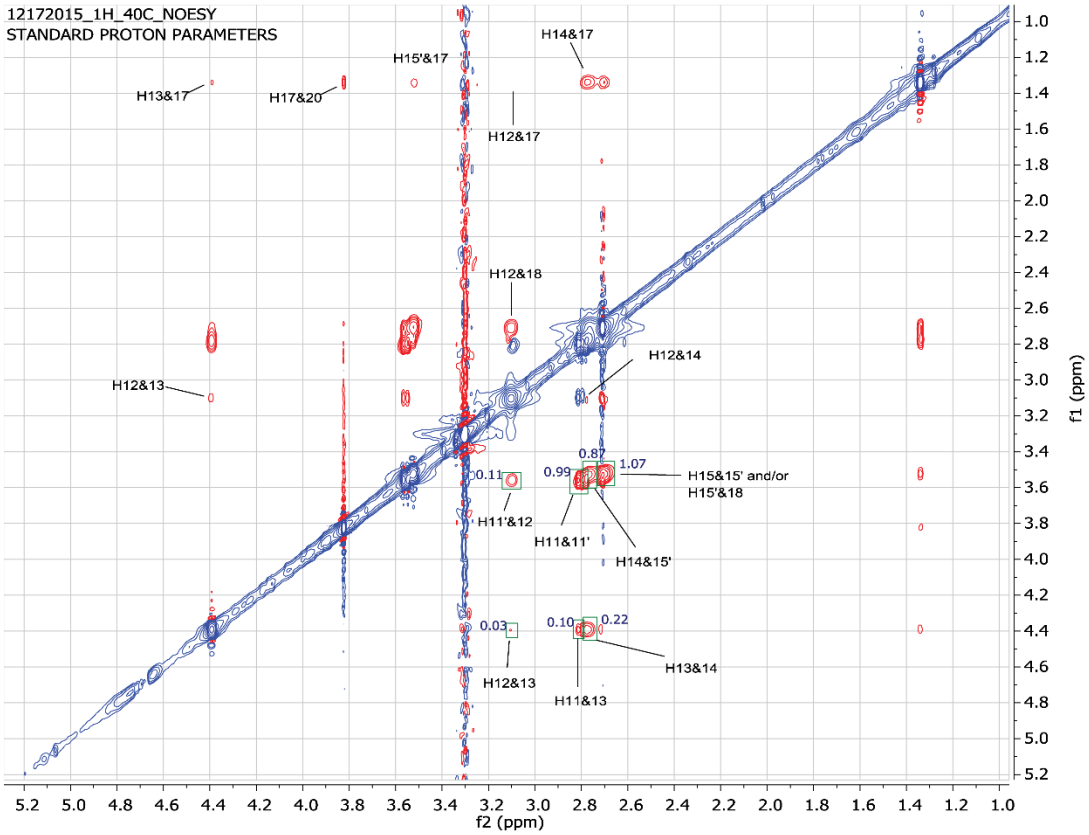
Based on HSQC, protons 11 and 11' (δH 2.79 and 3.56) are on the same carbon. Both have cross-peaks with proton 12 (δH 3.10) on COSY, which has an additional cross-peak with proton 13 (δH 4.40). This suggests CH_2 (C11, H11 and 11')-CH (C12, H12)-CH (C13, H13) connectivity. Similarly, proton 14 (δH 2.77) is connected to CH (C13, H13). Methyl group CH_3 (proton 17, δH 1.34) and CH_2 group (proton 15, 15', δH 2.70, 3.52) are directly connected to CH (proton 14).



S2.6 Fig. H—13C Heteronuclear multiple bond correlation (HMBC) NMR spectrum of SPF.

(A) Aliphatic region. Given the results from the COSY (S2.5 Fig) and the chemical shifts of C12 and C15 (δC 76.7 and 65.8), C12 and C15 are joined to a heteroatom. Since proton 18 (δH 2.71) has cross-peaks with both C12 and C15, it is a nitrogen atom that connects methyl group (δC 41.7, δH 2.71 on position 18), CH group (δC 76.7, δH 3.10) and CH_2 group (δC 65.8, δH 2.70 and 3.52). C13 has a chemical shift of 88.2 ppm, suggesting its connection to an oxygen. With HMBC, TOCSY, HSQC and COSY, the connectivity of the aliphatic portions are resolved. (B) Aromatic region. The connectivity-built aliphatic structure has the formula $C_7H_{13}NO$, which leaves C_9H_6NO after subtracting from the best-fitting formula. HSQC (S2.3 Fig) showed the existence of methoxyl group (δC 62.2, δH 3.81). Therefore, the aromatic region was composed of C_8H_3N. HMBC data showed that three aromatic protons were located in different rings, implying a fused aromatic ring structure with one nitrogen. A substituted indole was the most common structure utilized in organisms with the matching formula. In addition, HMBC showed that protons on the methoxyl group (δH 3.81) and the aromatic proton (δH 6.90) have cross-peaks with carbon (δC 143.1), suggesting they are meta to each other. The other proton (δH 6.86) was vicinal to proton (δH 6.90) because of their coupling seen in the COSY spectrum (S5). The aromatic singlet proton δH 7.09 showed cross-peaks with three aromatic carbons, two of those carbons (δC 120.6 and δC 138.1) had cross-peaks with protons (δH 6.86 and δH 6.90) respectively, consistent with an indole configuration. HMBC further confirmed C (δC 110.6) was linked to CH_2 (δH 2.79 and 3.56), and C (δC 143.7) was linked to the CH (δC 88.2, δH 4.40) across an oxygen atom.

A


12172015_1H_40C_NOESY
STANDARD PROTON PARAMETERS
H13&17
H17&20
H15'&17
H14&17
H12&17
H12&18
H12&13
H12&14
0.11
H11'&12
0.99
0.87
1.07
H15&15' and/or
H15'&18
H11&11'
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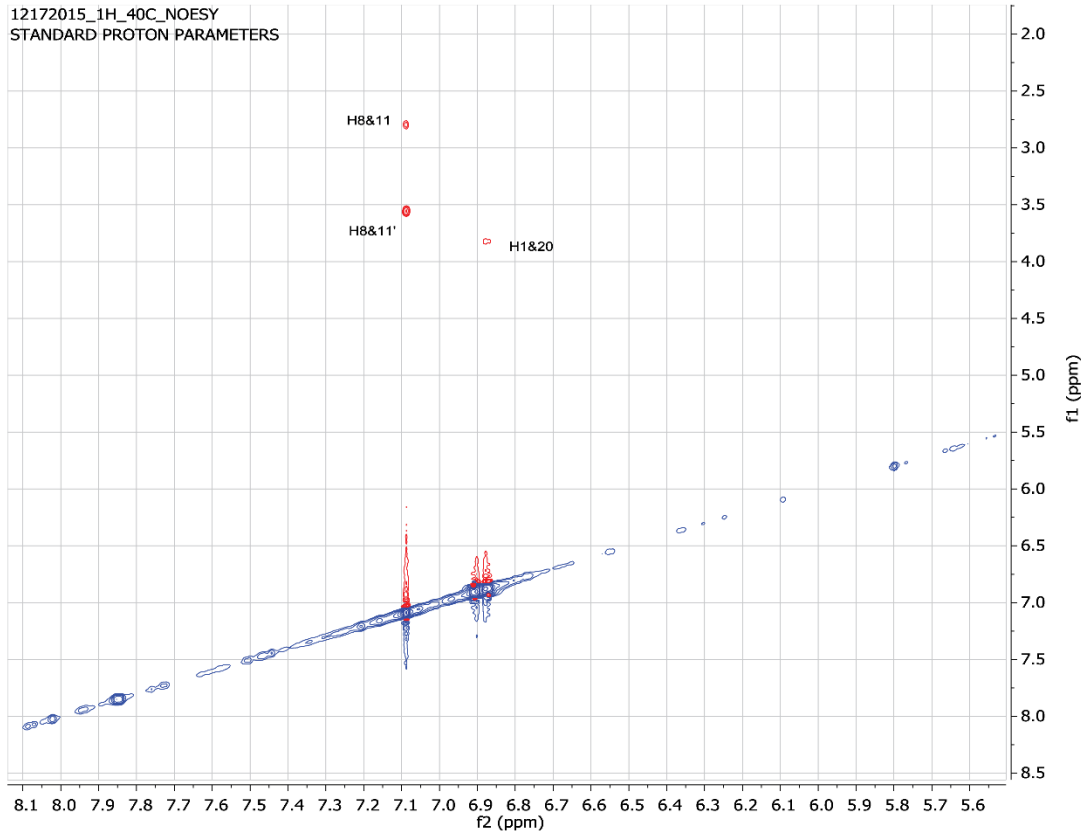
0.03
H12&13
0.10
0.22
H13&14
H11&13
f2 (ppm)
f1 (ppm)

B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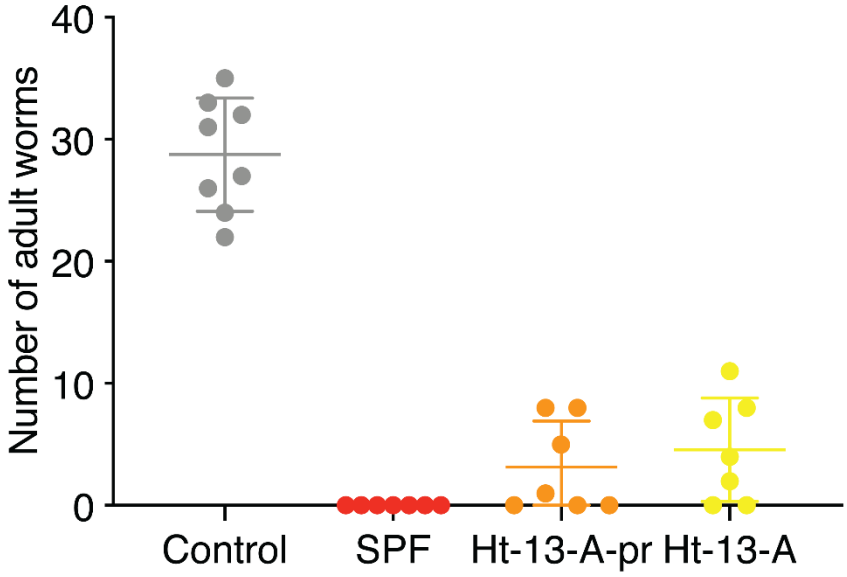
STANDARD PROTON PARAMETERS
H8&11
H8&11'
H1&20
f2 (ppm)
f1 (ppm)

S2.7 Fig. Nuclear Overhauser effect spectroscopy (NOESY) NMR spectrum of SPF. (A) Aliphatic region. The intensities of selected cross-peaks were integrated using Mnova software and shown in the spectrum. (B) Aromatic region. Results of the NOESY experiment support the final structures (Fig 2.2G and 2.2H) due to the presence of NOE signal between H (δH 3.81) and H (δH 1.34), which could only be observed between protons with short spatial distance. For protons on the three consecutive chiral centers, H (δH 4.40) had an intense cross-peak with H (δH 2.77), while a weak signal was observed between H (δH 4.40) and H (δH 3.10) and no signal was observed between H (δH 2.77) and H (δH 3.10). This suggests that H (δH 4.40) and H (δH 2.77) are close to each other and both are distant from H (δH 3.10), which corresponds to (*R, S, S*) or (*S, R, R*) configuration on C 12, 13, 14 (δC 76.7, 88.2 and 37.2). This was further supported by NOESY signals between H (δH 2.79, 3.56) and the three H on chiral centers. H (δH 4.40) had a cross-peak with H (δH 2.79) but no cross-peak with H (δH 3.56). However, the opposite was observed for H (δH 3.10) which had a cross-peak with H (δH 3.56) but no cross-peak with H (δH 2.79).



S2.8 Fig. Stringent mouse infection experiment.

Numbers of adult worms recovered from mice exposed to ~100 cercariae that were pre-treated with APW (N=8), 2.5 μM SPF (N=7), 2.5 μM Ht-13-A (N=7) or 2.5 μM Ht-13-A-pr (N=7). The mouse tail was lifted slightly during exposure so that its tip was 1-2 cm from the bottom of the test tube, avoiding direct contact with paralyzed cercariae. Data are mean ± S.D.

	^{13}C			^{1}H					
Position	δc (detected)	δc (predicted)	mult.	δ_H	Peak area	mult.	COSY	HMBC	NOESY
1	116.8	110.4	CH	6.86	1.12	**d** (*J*=8.6 Hz)	6	3, 5	18
2	143.1	148.2	C						
3	143.7	143.1	C						
4	120.6	120.3	C						
5	138.1	132.5	C						
6	106.6	103.7	CH	6.90	1.00	**d** (*J*=8.6 Hz)	1	2, 4	
7	110.6	110.8	C						
8	124.9	123.0	CH	7.09	1.00	s		4, 5, 7	11,11’
9			NH						
10			O						
11	29.0	32.5	CH_2	2.79		Overlap	11’, 12	7, 8,12,13	8,11’,13
11’	29.0	32.5	CH_2	3.56	1.04	**dd** (*J*=14.3 Hz, 3.8Hz)	11, 12	4,7,8,12,13	8,11,12
12	76.7	72.1	CH	3.10	1.16	br	11, 11’, 13		11’,13, 14,17, 18
13	88.2	84.5	CH	4.40	1.05	**dd** (*J*=9.4 Hz, 6.8Hz)	12, 14	3,11,15	11,12, 14,17
14	37.2	38.9	CH	2.77		Overlap	13, 15’, 17	15,17	12,13, 15’,17
15	65.8	62.6	CH_2	2.70		Overlap	15’	12	15’,17
15’	65.8	62.6	CH_2	3.52	1.08	**dd** (*J*=9.6 Hz, 6.8Hz)	14	12,13	14, 15,17, 18
16			N						
17	14.0	14.6	CH_3	1.34	3.01	**d** (*J*=6.8 Hz)	14	13, 14, 15	12,13, 14,15, 15’,20
18	41.7	43.7	CH_3	2.71		s		12, 15	12,15’
19			O						
20	62.2	56.1	CH_3	3.81	2.98	s		2	17

S2.1 Table. Summary of protons and carbons from ^{1}H, COSY, HSQC, HMBC and NOESY.

Chapter 3: Activating dopamine signaling inhibits cercaria motility

I designed and performed all experiments presented in this chapter. Janmesh D. Patel contributed to drug screen experiments (Appendix 2).

3.1 Abstract

Schistosomiasis is a neglected tropical disease afflicting hundreds of million people worldwide. Current treatment relies on one drug, praziquantel, which exhibits several shortcomings. Since a vaccine does not exist, there is an urgent need for alternative strategies for disease prevention. Previous studies identified a naturally occurring alkaloid produced by *Rotaria rotatoria*, Schistosome Paralysis Factor (SPF), that can paralyze the infective stage of schistosome (cercaria)(Gao et al., 2019). Structurally related compounds (Ht-13 analogs) also exhibited similar paralytic effects. Since cercarial motility is the key to infection, cercariae treated with SPF and related compounds failed to infect host mice. Such compounds hold great potential to prevent infection. However, the mechanism underlying SPF-mediated paralysis is unknown. Based on their chemical structures, we hypothesized that these compounds work through bioamine G protein-coupled receptors (GPCRs). In this study, we screened a library of neurotransmitter-related compounds and found that increasing dopamine signaling in cercaria led to paralysis. Utilizing a luciferase-based cell assay, we identified a schistosome dopamine receptor that is activated by these cercaria-paralytic compounds. Furthermore, Ht-13 analogs also activated various human serotonin and dopamine receptors, suggesting their potential for drug development against neurological diseases.

3.2 Introduction

Schistosomiasis is among the most socioeconomically devastating helminth infections, yet it remains a neglected tropical disease. Currently, over 200 million people worldwide are infected with *Schistosoma*, the causal agent of schistosomiasis (WHO, 2017). Hundreds of millions of people in endemic areas are at risk of acquiring this disease. However, there is no efficient way for prevention due to the lack of a vaccine. Current treatment relies on one drug, praziquantel (PZQ), which was approved about 40 years ago (Wilson, 2020). Although PZQ efficiently kills adult parasites, it fails to eliminate juveniles, and application of PZQ does not prevent reinfection (Caffrey, 2007). The drug's mode of action is not completely understood at present, but previous research indicates PZQ acts through Ca^{2+}-dependent processes (Wolde Mussie et al., 1982). Furthermore, PZQ activates a schistosome transient receptor potential channel *Sm.*$TRPM_{PZQ}$ supporting its Ca^{2+}-dependent action (Park et al., 2019). With the emergence of PZQ-resistant strains, there is an urgent need for next generation anti-schistosomal therapies and alternative preventative strategies.

Cercaria, the infective stage of *Schistosoma*, emerges from the intermediate snail host and searches for a mammalian host in freshwater. Its muscular tail allows it to swim quickly through water while seeking its host. When it comes into contact with mammalian skin, its tail beats violently while proteases secreted by the acetabular gland facilitate penetration. Thus, the motility of cercariae is necessary for infection. Previous research in our laboratory identified a natural compound, Schistosome Paralysis Factor (SPF), that can paralyze cercariae (Gao et al., 2019). This compound successfully prevented cercariae from infecting mice at nanomolar concentrations. Structurally related compounds, ht-13-A and its analog ht-13-A-pr also exhibited

similar effects. However, the target(s) of these compounds remains unknown. We hypothesized that the potential target is an aminergic G protein-coupled receptor (GPCR) based on the following evidence: 1) SPF contains a serotonin backbone; 2) ht-13-A was reported to bind to several human serotonin receptors (Kamigauchi and Yasui, 2000); and 3) neurotransmitters such as serotonin and dopamine have been implicated in schistosome motility (El-Shehabi et al., 2012; Patocka et al., 2014; Ribeiro et al., 2012; Taft et al., 2010).

The GPCR superfamily constitutes one of the largest families of membrane-spanning proteins in metazoans and has essential roles in mediating most physiological responses to hormones, neurotransmitters, and environmental stimulants (Rosenbaum et al., 2009). Due to their extensive functions in physiological processes, over 30% of marketed pharmaceuticals act on GPCRs (Wise et al., 2002). Over 100 GPCRs were identified in *Schistosoma mansoni*, representing all major families, including rhodopsin, glutamate, adhesion, secretin and frizzled (Zamanian et al., 2011). Among these, 24 are potentially aminergic GPCRs based upon sequence homology. One serotonin, two dopamine and one histamine GPCR have been deorphanized in *S. mansoni* (El-Shehabi et al., 2012; Hamdan et al., 2002; Patocka et al., 2014; Taman and Ribeiro, 2009). Acting on GPCRs, these biogenic amines serve as neurotransmitters and have been implicated in parasite motor activity, life-stage transformation, host attachment, feeding, and reproduction (Ribeiro et al., 2012).

In this study, we screened a neurotransmitter library for compounds resulting in cercaria motility defects. This library has been successfully used for receptor deorphanization and behavioral screens in other model organisms (Copmans et al., 2016; Decker et al., 2017). It consists of 661

drugs related to adrenergic, dopaminergic, serotonergic, opioid, cholinergic, histaminergic, glutamatergic, GABAergic, and purinergic receptors. At all concentrations tested, the dopamine library had the most hits that phenocopied the SPF paralytic effect on cercaria. This suggests that SPF and its analogs could act through a dopamine signaling pathway. To test this hypothesis, we utilized a luciferase-based cell assay (Beets et al., 2012; Rizzuto et al., 1992; Saberi et al., 2016; Stables et al., 1997) to determine that cercaria-paralytic compounds can activate a schistosome dopamine receptor. Furthermore, we showed that Ht-13 analogs can activate a broad selection of human dopamine and serotonin receptors using the same cell assay.

3.3 Result and Discussion

Activating dopamine signaling inhibits cercaria motility

To determine whether and which neurotransmitters are involved in cercaria motility, we screened through a neurotransmitter library consisting of 83 adrenergic, 80 dopaminergic, 79 serotonergic, 74 opioid, 67 cholinergic, 41 histaminergic, 109 glutamatergic, 56 GABAergic, and 72 purinergic receptor-related drugs. The majority of these drugs are receptor ligands (agonist/antagonist) except for some inhibitors of either transporters or enzymes involved in transmitter biosynthetic pathways. We treated cercariae with each drug and recorded their phenotypes at three time points (10, 30, and 60 mins). Among all drug treatments, several motility defects were observed: 1) paralyzed, cercariae were completely paralyzed and sank to the bottom of the dish (which resembles SPF treatment); 2) wriggling, cercariae constantly beat their tails in place but failed to propel themselves through water; 3) paralyzed with occasional wriggling; 4) shaking, abnormal shaking sometimes with spinning movement; 5) partial phenotype, majority of cercariae exhibit paralyzed/wriggling phenotype with a few outliers. Drugs that resulted in any of these phenotypes were considered a hit.

The primary screen at 10 μM concentration, revealed 2 adrenergic (2.4%), 33 dopaminergic (41.3%), 9 serotonergic (11.3%), 1 opioid (1.4%), 6 cholinergic (9.0%), 2 histaminergic (4.9%), 0 glutamatergic (0%), 1 GABAergic (1.8%), and 1 purinergic receptor drug hits (1.4%) (percentage = number of drug hits/number of drugs in each category) (Appendix 2). However, subsequent tests of these drug hits at 1 μM showed that only 7 drugs from the dopaminergic library and 2 drugs from the cholinergic library still produced motility phenotypes. Interestingly, all dopaminergic drug hits at 1 μM were dopamine receptor agonists, suggesting that activating

dopamine signaling inhibits cercaria motor activity. Although at 1 μM and 10 μM, dopamine itself did not paralyze cercariae, partial paralysis was observed at 100 μM, suggesting that either dopamine was not as potent as these drug hits, or excess dopamine was taken up and sequestered by dopamine transporters while dopaminergic drug hits failed to be taken up. This is also supported by the evidence that dopamine reuptake inhibitors (GBR 12783, GBR 12935, and GBR 13069, at 10 μM) paralyzed cercariae, potentially by blocking dopamine transporters and increasing extracellular dopamine concentration (Appendix 2).

Among the 9 drug hits at 1 μM, 3 dopaminergic drug hits (3'-Fluorobenzylspiperone, (+)-UH 232, and (±)-SKF-82958) and 2 cholinergic drug hits ((±)-Vesamicol and Arecoline) exhibited only mild phenotypes, such as wriggling and partial paralysis, whereas 4 of the dopaminergic drug hits (apomorphine, lisuride, pergolide and 6-bromo-APB) completely paralyzed cercariae, albeit with different kinetics. Apomorphine and pergolide completely paralyzed cercaria throughout the duration of the experiment. In contrast, some of the lisuride-treated cercariae recovered 30 mins after initial complete paralysis. Upon 6-bromo-APB treatment, cercariae were not completely paralyzed until the end of the experiment (1 hr post-exposure) (Appendix 2).

To further quantify the dosage-dependent paralytic effects of these 4 drugs over time, we performed a cercarial paralysis assay as previously described at 0 nM, 2.5 nM, 250 nM and 2.5 μM (Gao et al., 2019). As expected, pergolide and apomorphine were more effective than lisuride and 6-bromo-APB (Fig 3.1). At the end of the experiment, 2.5 μM pergolide and apomorphine paralyzed about 90% of cercaria, whereas lisuride and 6-bromo-APB only paralyzed 60-70%. Although the paralytic effect of pergolide and apomorphine were not as

potent as SPF, which completely paralyzed cercaria at 250 nM, their competency was comparable to that of Ht-13-A (Chapter 3). Comparing the chemical structures of these compounds, we found that three (apomorphine, lisuride, and pergolide) of the four drugs contain a tetracyclic structure similar to SPF and ht-13 analogs (Fig 3.1). Lisuride and pergolide, especially, are both ergot derivatives. This suggests that these drugs could potentially target the same molecule and that the tetracyclic chemical scaffold could be important for ligand binding. The fact that most paralysis phenotypes are observed in the dopamine library and that dopaminergic drug hits share a similar chemical structure with SPF, led us to postulate that SPF might act through schistosome dopamine receptors.

Smp_202470 is a dopamine receptor

To test whether the mechanism of cercaria paralysis is through dopamine receptor, the most straightforward way is to knock down receptor expression by RNA interference (RNAi). However, an efficient RNAi protocol has yet to be established in cercariae because they have a very short lifespan and it is technically challenging to deliver double-stranded RNA when cercariae are developing in snail tissue. Thus, we decided to utilize a luciferase-based cell assay called AequoZen assay (PerkinElmer)(Beets et al., 2012; Rizzuto et al., 1992; Saberi et al., 2016; Stables et al., 1997). We obtained a CHO-K1 cell line that stably expresses a mitochondrially targeted aequorin and promiscuous $G_{\alpha 16}$ which couples to a wide range of GPCRs. Upon ligand activation, Ca^{2+} is released into the cytosol and sensed by aequorin. The luminescence produced by aequorin is then immediately captured by a plate reader. According to sequence homology based on *S. mansoni* genome v7, there are five dopamine (Smp_043290, Smp_127310, Smp_150180, Smp_202470, and Smp_315720) and four serotonin (Smp_126730, Smp_149770,

Smp_197700 and Smp_245850) receptor candidates. Among these nine receptors, one serotonin receptor Sm5HTR (Smp_126730) and two dopamine receptors SmD2 and SmGPR-3 (Smp_127310 and Smp_043290) have been deorphanized (El-Shehabi et al., 2012; Patocka et al., 2014; Taman and Ribeiro, 2009).

Previously published RNA sequencing data revealed the expression levels of each dopamine and serotonin receptor transcript in cercaria. Smp_202470 is expressed at 28.1 transcripts per million (TPM), which is highest among all of these receptor-encoding transcripts (S3.1 Table)(Protasio et al., 2012). Smp_202470 encodes a predicted protein of 563 amino acids. Hydropathy analysis (transmembrane protein topology with a hidden Markov model, TMHMM) identified seven predicted transmembrane domains, consistent with the typical GPCR topology (S3.1 Fig)(Finn et al., 2011). Similar to SmD2, we also found a highly conserved aspartate residue in TM3 (position 123) and three serine residues in TM5 (positions 220, 221, and 224) that interact with a protonated amine group and hydroxyl group, respectively (Cox et al., 1992; Mansour et al., 1992; Taman and Ribeiro, 2009). We cloned a full-length Smp_202470 transcript from cercaria cDNA and inserted it into pcDNA3.1(-). The sequence was then confirmed by Sanger sequencing to be identical to the predicted sequence. To determine ligand binding, we transiently expressed receptor in AequoZen cells using Lipofectamine LTX (Invitrogen). To test ligand specificity, we treated transfected cells with different biogenic amines, including acetylcholine, dopamine, histamine, octopamine, serotonin, and tyramine. Smp_202470 was specifically activated by dopamine, suggesting that Smp_202470 is a dopamine receptor (Fig 3.2A). To compare with Smp_202470 and as proof of principle, we also cloned a previously published schistosome serotonin receptor Sm5HTR (Smp_126730), and performed the same assay. As

expected, Smp_126730 was activated specifically by serotonin (Fig 3.2B). Next, we tested the dose responses of Smp_202470 and Smp_126730 to varying concentrations of dopamine and serotonin, respectively. The EC_{50} value was calculated to be 507 nM and 21.5 μM, respectively (Fig 3.2C and 2D).

Cercaria-paralytic compounds can activate schistosome dopamine receptor

Since chemical synthesis of SPF has not been achieved, we were unable to test SPF binding to Smp_204070 and Smp_126730. However, we had enough quantities to test the binding of dopamine drug hits (apomorphine, lisuride and pergolide) and chemically synthesizable Ht-13 analogs (Ht-13-A and Ht-13-A-pr), which phenocopied SPF. As a comparison, we also included Ht-13-B, which shares a similar structure but does not paralyze cercaria. While pergolide, Ht-13-A and Ht-13-A-pr activated Smp_202470 at 10 μM, none of the tested compounds activated Smp_126730 (Fig 3.2A and 2B). Consistent with its lack of paralytic effect on cercaria, Ht-13-B did not efficiently activate Smp_202470 (Fig 3.2A). These results provide evidence that these compounds might work through dopamine receptors. However, several questions need to be addressed before making a definitive statement: 1) Do cercaria-paralytic compounds affect other dopamine/serotonin receptors? 2) Do these compounds inhibit serotonin receptors instead of activating them? 3) Could paralysis be due to the drugs affecting both dopamine and serotonin receptors? Future experiments will also explore more serotonin receptors and test if Ht-13 analogs act as antagonists using the same cell assays.

Cercaria-paralytic compounds can activate various human dopamine and serotonin receptors

While testing how various paralytic compounds affect other schistosome receptors, we also wanted to establish a pharmacological profile for cercaria-paralytic compounds in relation to human dopamine and serotonin receptors. Such a profile will not only provide more evidence for the paralysis mechanism but may also give insights to future drug development. There are 14 distinct serotonin receptors in the human genome, which are further classified into 7 families. Except HTR3, which is a ligand-gated channel, the rest are GPCRs. On the other hand, there are 5 dopamine receptors in humans and all of them are GPCRs. We cloned all 13 serotonin and 5 dopamine receptors and expressed them in AequoZen cells. So far, we identified 7 serotonin and 3 dopamine receptors that are compatible with this assay (Fig 3.3 and 3.4). All of them were selectively activated by their corresponding endogenous ligand. We next tested dopaminergic drug hits (apomorphine, lisuride and pergolide) and ht-13 analogs (Ht-13-A, Ht-13-B, Ht-13-A-pr) at 10 μM on each receptor. All receptors were activated by one or more compounds at 10 μM (Fig 3.3 and 3.4), except for HTR4, which was not activated by any of the tested compounds. Although apomorphine, lisuride and pergolide are anti-parkinson agents that target dopamine receptors, they are also known to broadly activate various serotonin and adrenergic receptors (Cussac et al., 2008; Kimura et al., 1991; Kvernmo et al., 2008; Lam, 2000). As expected, both lisuride and pergolide exhibited broad activation in our assays.

Interestingly, broad activation of human dopamine and serotonin receptors was also observed with Ht-13-A and Ht-13-A-pr (Fig 3.3 and 3.4). Ht-13-A, especially, was more potent than serotonin on HTR6. In contrast, Ht-13-B, only activated type 1 and type 6 serotonin receptors to

a moderate extent. For human dopamine receptors, activation by Ht-13 analogs was more selective compared to serotonin receptors. DRD2 was only activated by Ht-13-A-pr, while DRD5 was only activated by Ht-13-A (Fig 3.4B and 3.4C).

Dopamine and serotonin signaling are generally associated with neurological diseases. For example, migraine is often associated with low serotonin levels in the brain. The first-line acute therapy for migraine, triptans, are agonists for HTR1B and HTR1D (Negro et al., 2018). Lower levels of serotonin were also observed in patients with Alzheimer's disease. Treatment includes selective serotonin reuptake inhibitors, which increase extracellular serotonin levels (Meltzer et al., 1998; Whitford, 1986). On the other hand, Parkinson's disease is caused by dopaminergic neuron degeneration. Current therapy involves levodopa which can cross the blood-brain barrier and get converted to dopamine in brain (Tambasco et al., 2018). However, for patients with suboptimal responses to levodopa, dopamine agonists such as pergolide are used to increase dopamine levels in the brain (Ahlskog and Muenter, 1988). Since Ht-13 analogs activated human dopamine and serotonin receptors, they could be promising drug candidates for neurological diseases.

3.4 Conclusion

S. mansoni lives as a parasite in the snail *Biomphalaria glabrata*, whereas *R. rotatoria* lives as a commensal on the snail's shell (Fig 3.5). Previous work identified a tetracyclic alkaloid, SPF, which is released by *R. rotatoria* and can paralyze cercariae. In this work, we explored the mechanism of the SPF-induced paralysis. We identified several dopaminergic compounds that phenocopy the paralytic effect of SPF on cercaria, suggesting that SPF potentially acts through dopamine signaling. In addition, we show that Smp_202470 is a schistosome dopamine receptor. Using a luciferase-based cell assay, we showed that dopamine receptor Smp_202470 is activated by cercarial-paralytic compounds, supporting our hypothesis that dopamine signaling plays a role in cercaria paralysis. However, additional experiments need to be done to decipher the mechanism of paralysis, including investigating other schistosome dopamine and serotonin receptors. Given the evidence that Ht-13 analogs activate a broad range of human serotonin receptors, it is still possible that cercarial-paralytic compounds can affect schistosome serotonin receptors other than Smp_126730. If this is the case, the paralysis phenotype could result from affecting both types of receptors.

On the other hand, since most dopaminergic drug hits are anti-Parkinson agents that activate human dopamine receptors, it raises the question whether SPF and Ht-13 analogs could do the same. We evaluated Ht-13 analogs' effects on several human dopamine and serotonin receptors and found various degrees of activation. Thus, the novel tetracyclic chemical scaffold shared by SPF and Ht-13 analogs might provide a new path to drug development for neurological diseases.

3.5 Material and Methods

Neurotransmitter Drug screen.

Cercaria for drug screen were obtained and maintained as described (Gao et al., 2019). The stock concentration of each Neurotransmitter library (Enzo Life Science) was 10 mM (except Opioid peptides are 1 mM) in a 96-well format. Working stocks at 100 μM and 10 μM were generated by serial dilutions of each library. In a clear 96-well plate (Costar), 90 μL of artificial pond water containing ~50 cercariae were transferred into each well. 10 μL of each drug were added using multi-channel pipette to achieve 10 μM and 1μM final concentrations. Cercaria motility was observed using a stereomicroscope (Leica MZ125), and phenotypes were noted at 10, 30, and 60 min time points. Only drugs that exhibited effects at 10 μM were tested further at 1 μM. To show dose responses, we performed cercarial paralysis assay as previous described (Gao et al., 2019).

GPCR cloning.

Cercaria mRNA was extracted using TRIzol (Invitrogen) with extra freeze and thaw steps. TRIzol containing cercariae was frozen in dry ice and broken down into a slurry using a motorized pestle. This step was repeated several times for better lysis and homogenization. cDNA was generated using an iScript cDNA synthesis kit (Bio-Rad). Each schistosome GPCR was cloned from cercaria cDNA using primers designed for Gibson Assembly (S3.2 Table). Human serotonergic and dopaminergic GPCRs were subcloned from publicly deposited plasmids obtained from Addgene (Kroeze et al., 2015). Each PCR product was then Gibson assembled into EcoRV cut pcDNA3.1(-) plasmid (Invitrogen). Inserted sequence in each plasmid was confirmed by Sanger sequencing.

Cell culture and transfection.

AequoZen cells (ES-000-A24), which are CHO-K1 cells stably expressing the mitochondrially targeted Aequorin and the promiscuous $G_{\alpha 16}$, were obtained from PerkinElmer Inc. Cells were cultured in Ham's F-12K (Kaighn's) Media (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 250 μg/ml Zeocin as a monolayer at 37°C in a humidified atmosphere with 5% CO_2. Antibiotics were removed before transfection and cell assay. Transient transfection was performed according to the manufacturer's protocol using Lipofectamine LTX reagent (Invitrogen).

Reagents for cell assay.

Assay buffer: DMEM/HAM's F12 with HEPES, without phenol red (Invitrogen) supplemented with 0.1% sterilized BSA. Coelenterazine h stock solution: 500 μM coelenterazine h (Promega) in methanol. Digitonin stock solution: 50 mM digitonin in DMSO. All drugs were kept at 100 mM in DMSO as stock solutions except dopamine which was made fresh prior to each experiment due to fast oxidation. For cell assays, each drug was serially diluted in assay buffer to reach a 2x concentration. Apomorphine and lisuride were purchased from Tocris Bioscience. Pergolide and neurotransmitters were purchased from Sigma-Aldrich. Ht-13-A, Ht-13-B and Ht-13-A-pr were synthesized by GL Chemtec International according to published protocols (Tao et al., 2016).

Calcium-based cell assay.

Cell assays were performed according to the manufacturer's protocol with minor modifications. Briefly, one-day post-transfection cells were trypsinized (Gibco) for 5 mins, recovered by

centrifugation, and resuspended in assay buffer at a concentration of 3×10^5 cells/mL. Coelenterazine h was added at a final concentration at 5 μM to the cell suspension. Cells were gently agitated for 4 hrs in dark, diluted to 1×10^5 cells/mL in assay buffer and then agitated for another hour. In a 96-well solid white plate (Corning), 50 μL of drugs were added in triplicate. Using injector 1 of the plate reader (Synergy H1, BioTek), 50 μL of cells (~5000 cells) were injected per well and light emission was immediately recorded for 20 sec. Using injector 2, 20 μL of 600 μM digitonin (diluted in assay buffer) was injected at a final concentration of 100 μM per well and light emission was immediately record for another 20 sec. This luminescence readout from digitonin treatment represents total receptor-independent cellular calcium response, which is proportional to cell number. Light emission from each drug treatment was normalized to its corresponding digitonin treatment value to account for variable cell numbers in each well, which provided the final relative light emission (RLU) value.

3.6 Figures

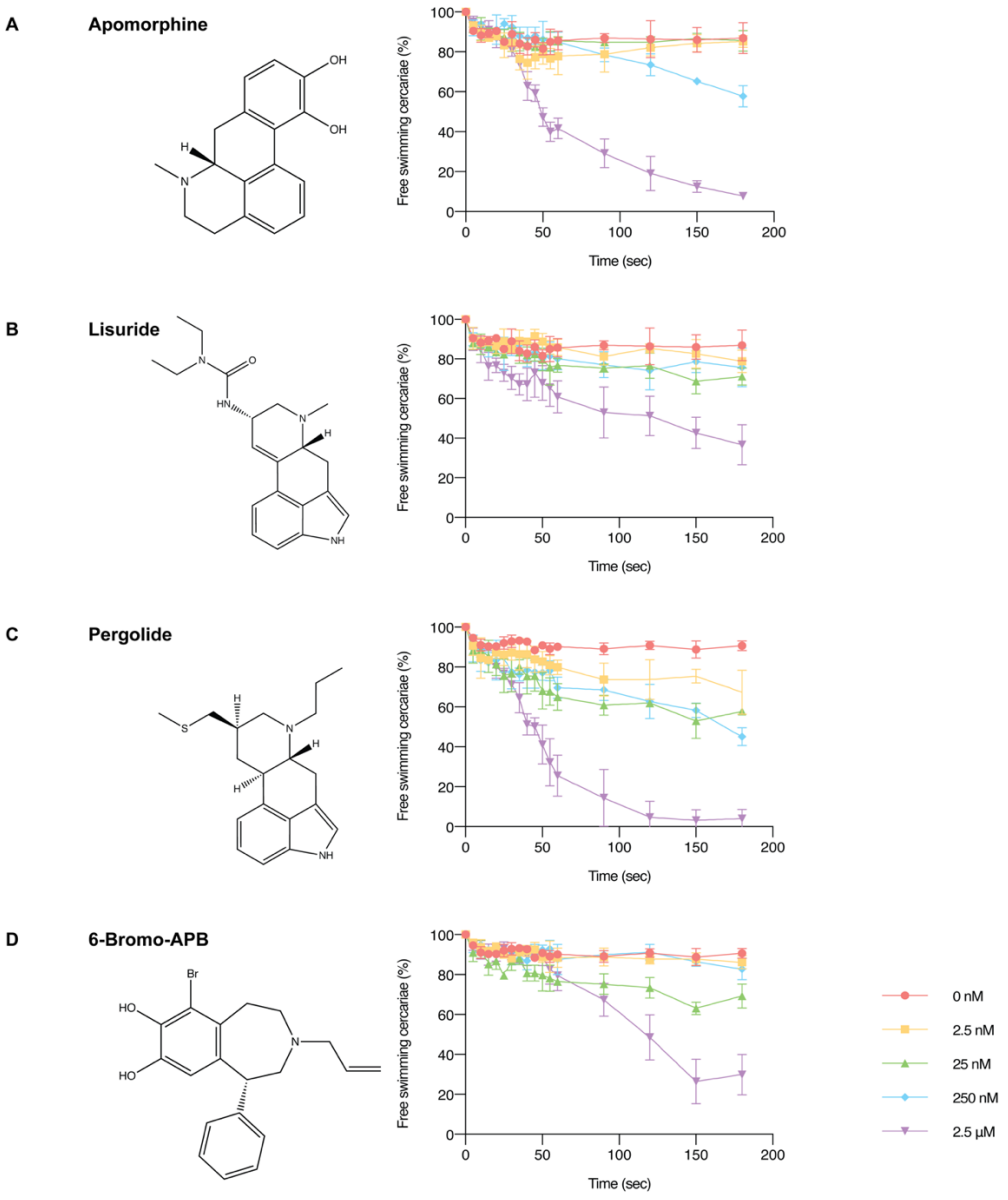


Fig 3.1. Cercarial motility assays and chemical structures for dopaminergic drug hits. (A-D), Percentage of cercariae (~50) continuing to swim over three minutes after addition of each compound at specified final concentrations. Triplicates were performed. Data are mean ± S.D.

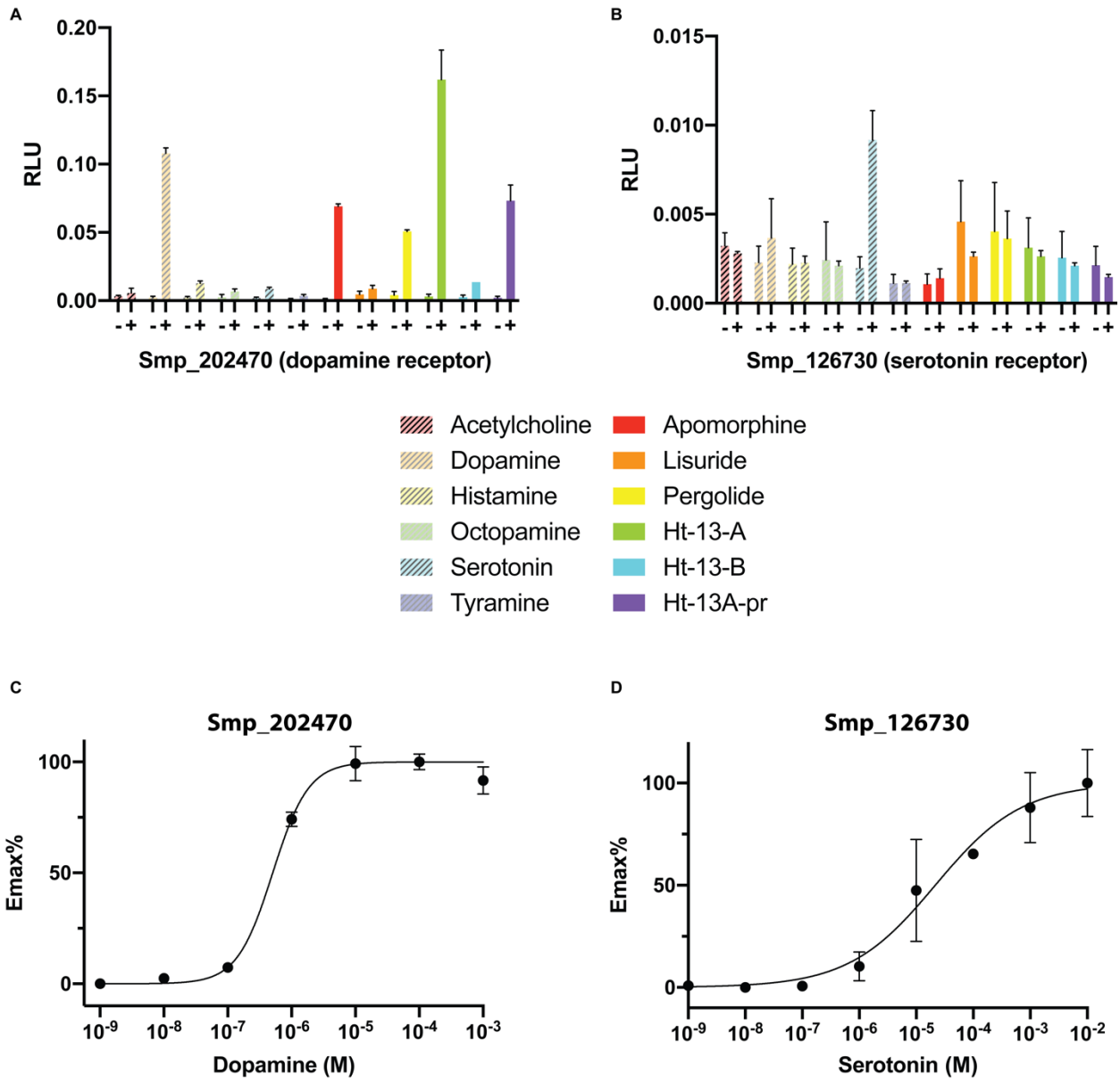


Fig 3.2. Smp_202470 is a dopamine receptor and can be activated by cercaria-paralytic compounds.

(A-B), Normalized relative luminescence unit (RLU) upon 10 μM drug treatment. "-" represents cells transfected with empty vector. "+" represents cells transfected with receptor. (C-D) Dose-response curve of Smp_202470 and Smp_126730. All data are normalized to maximum RLU. Triplicates were performed. Data are mean ± S.D.

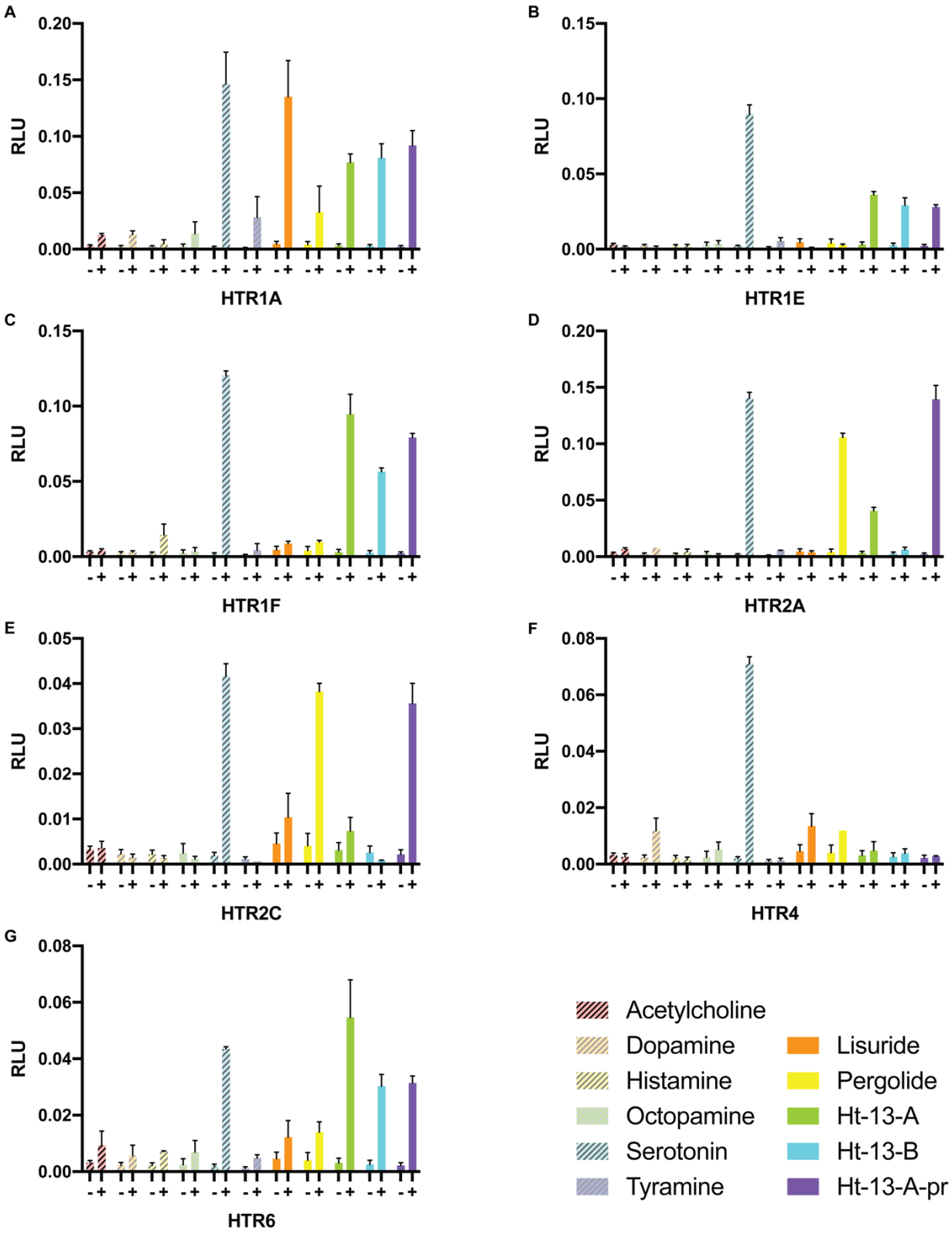


Fig 3.3. Cercaria-paralytic compounds can activate various human serotonin receptors.

(A-G), Normalized relative luminescence unit (RLU) upon 10 μM drug treatment. “-” represents cells transfected with empty vector. “+” represents cells transfected with receptor. Triplicates were performed. Data are mean ± S.D.

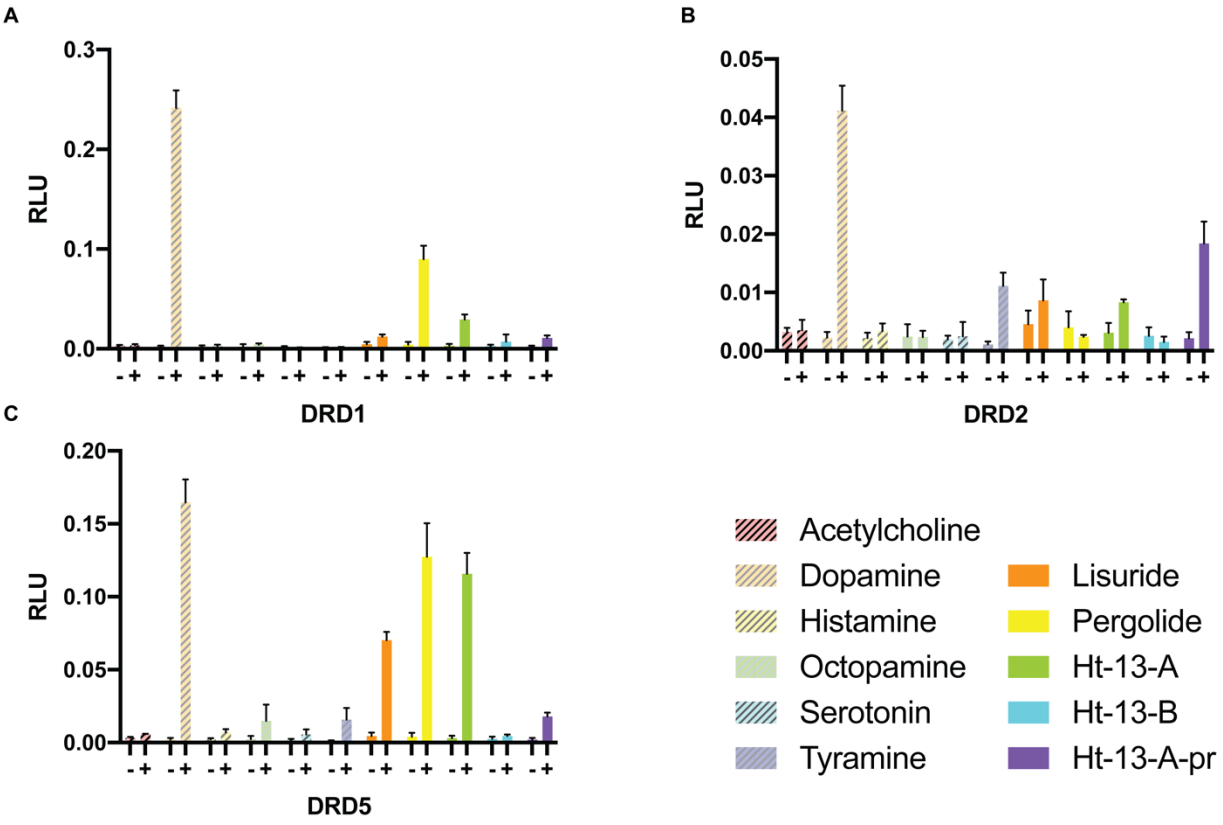


Fig 3.4. Cercaria-paralytic compounds can activate various human dopamine receptors. (A-G), Normalized relative luminescence unit (RLU) upon 10 μM drug treatment. "-" represents cells transfected with empty vector. "+" represents cells transfected with receptor. Triplicates were performed. Data are mean ± S.D.

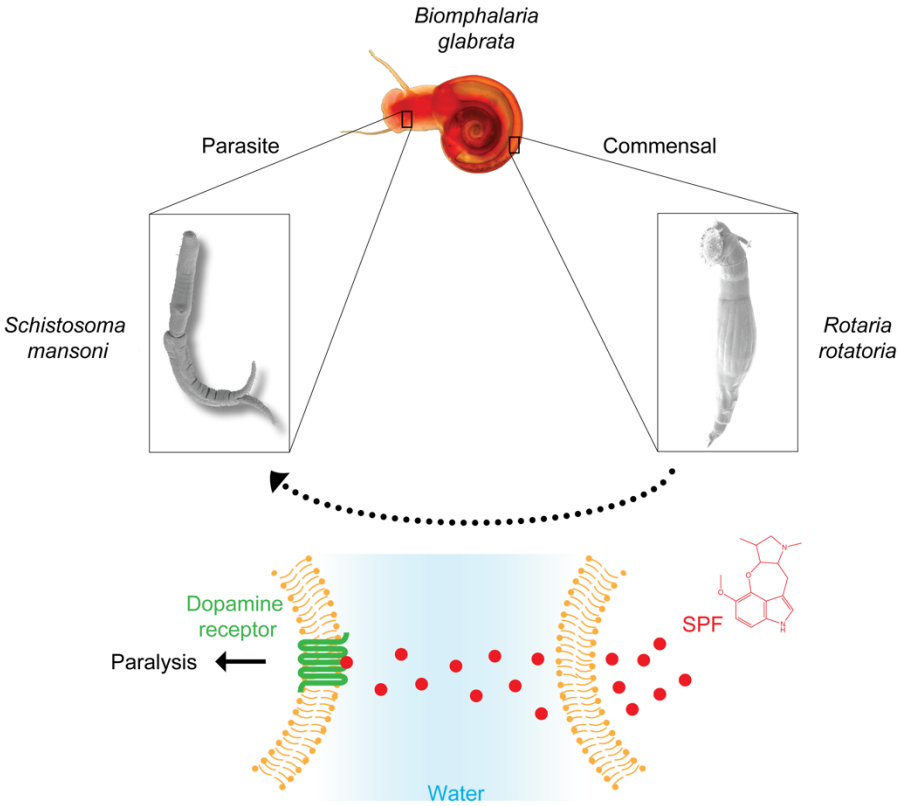


Fig 3.5. Schematic model depicting the mechanism of SPF-induced cercarial paralysis.

In the microenvironment of a snail (*B. glabrata*), commensal *R. rotatoria* produces and releases SPF in water. SPF paralyzes cercaria by activating its dopamine receptor. Schematic model is not to scale.

3.7 Supplementary materials

```
# Smp_202470 Length: 563
# Smp_202470 Number of predicted TMHs:  7
# Smp_202470 Exp number of AAs in TMHs: 156.26547
# Smp_202470 Exp number, first 60 AAs:  23.05735
# Smp_202470 Total prob of N-in:        0.00074
# Smp_202470 POSSIBLE N-term signal sequence
Smp_202470      TMHMM2.0        outside     1    35
Smp_202470      TMHMM2.0        TMhelix     36   58
Smp_202470      TMHMM2.0        inside      59   70
Smp_202470      TMHMM2.0        TMhelix     71   93
Smp_202470      TMHMM2.0        outside     94   123
Smp_202470      TMHMM2.0        TMhelix     124  146
Smp_202470      TMHMM2.0        inside      147  165
Smp_202470      TMHMM2.0        TMhelix     166  188
Smp_202470      TMHMM2.0        outside     189  214
Smp_202470      TMHMM2.0        TMhelix     215  237
Smp_202470      TMHMM2.0        inside      238  428
Smp_202470      TMHMM2.0        TMhelix     429  448
Smp_202470      TMHMM2.0        outside     449  462
Smp_202470      TMHMM2.0        TMhelix     463  485
Smp_202470      TMHMM2.0        inside      486  563
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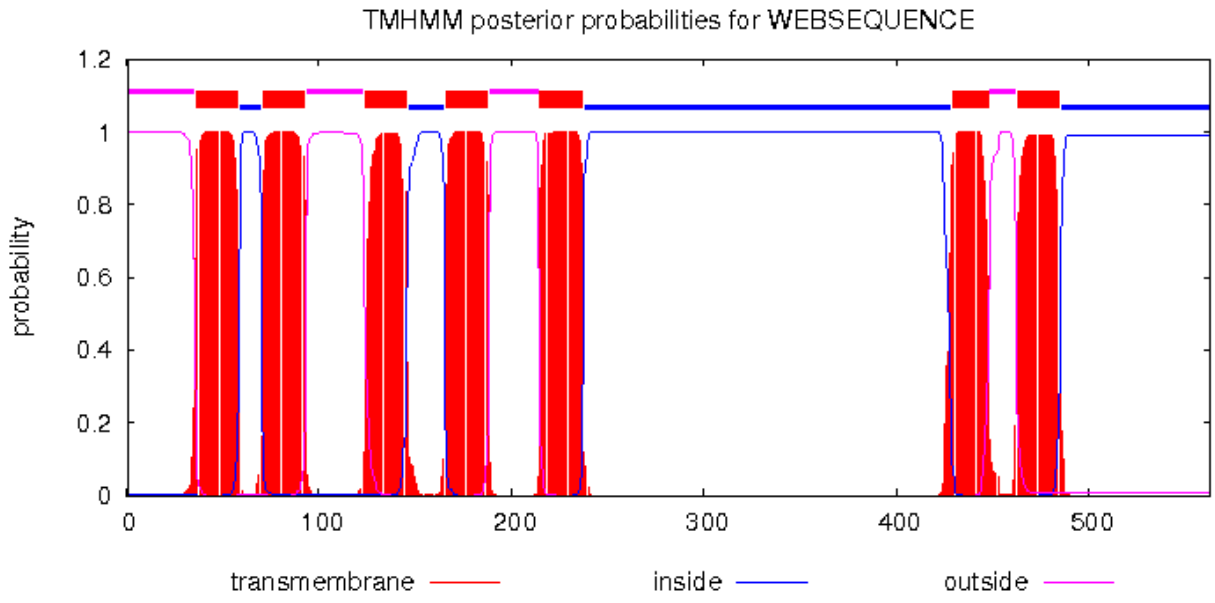

S3.1 Fig. Hydropathy analysis revealed seven transmembrane domains in Smp_202470.

GPCR	Type	TPM
Smp_126730	Serotonin	2.4
Smp_149770	Serotonin	3.1
Smp_197700	Serotonin	0.2
Smp_245850	Serotonin	8.1
Smp_043290	Dopamine	0
Smp_127310	Dopamine	12.2
Smp_150180	Dopamine	1.7
Smp_202470	Dopamine	28.1
Smp_315720	Dopamine	3.3

S3.1 Table. TPM values for *S. mansoni* dopamine and serotonin receptors.

Data obtained from published work (Protasio et al., 2012).

Gene	Primer	Sequence
Drd1	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGCGCACATTGAATACTTC
Drd1	Reverse	TGTGGTGGAATTCTGCAGATTCAAGTTGGATGTTGACCGTTCT
Drd2	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGGATCCTCTGAATCTGTCATG
Drd2	Reverse	TGTGGTGGAATTCTGCAGATTCAACAGTGGAGAATCTTCAGAAATG
Drd3	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGGCATCTCTGAGCCAGC
Drd3	Reverse	TGTGGTGGAATTCTGCAGATTCAGCAAGACAGGATCTTGAGGA
Drd4	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGGGTAATCGAAGCACTGC
Drd4	Reverse	TGTGGTGGAATTCTGCAGATTCAACAGCAAGCCCGGAGAG
Drd5	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGTTGCCACCAGGGAGC
Drd5	Reverse	TGTGGTGGAATTCTGCAGATTCAGTGAAATCCGTTGGGCGTAA
HTR1A	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGGACGTGCTGAGTCCC
HTR1A	Reverse	TGTGGTGGAATTCTGCAGATTCATTGTCTACAGAACTTGCACTTAATTATC
HTR1B	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGGAGGAGCCTGGCGC
HTR1B	Reverse	TGTGGTGGAATTCTGCAGATTCATGATGTACACTTGAACCGAATCA
HTR1D	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGAGTCCTCTTAACCAGAGTG
HTR1D	Reverse	TGTGGTGGAATTCTGCAGATTCAAGAGGCCTTTCTAAAAGGGAC
HTR1E	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGAATATAACTAACTGCACTACAGAGG
HTR1E	Reverse	TGTGGTGGAATTCTGCAGATTCATGTATGCTCTCTACAGCGTATAAG
HTR1F	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGGATTTTCTGAATTCCTCTGAC
HTR1F	Reverse	TGTGGTGGAATTCTGCAGATTCAACACCTGCAGCGAACAAG
HTR2A	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGGATATCTTGTGCGAGGAG
HTR2A	Reverse	TGTGGTGGAATTCTGCAGATTCAAACGCAGGAGACTTTCTCG
HTR2B	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGGCCCTGTCTTATCGGG
HTR2B	Reverse	TGTGGTGGAATTCTGCAGATTCACACATAGGAGACCTGCTCCT
HTR2C	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGGTTAATCTTCGAAACGCG
HTR2C	Reverse	TGTGGTGGAATTCTGCAGATTCACACTGATGATATCCGCTCACTAAC
HTR4	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGGATAAGCTGGATGCTAACG
HTR4	Reverse	TGTGGTGGAATTCTGCAGATTCAAGTATCTGAAGGTTGAGCCG
HTR5	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGGACCTGCCCGTCAAC
HTR5	Reverse	TGTGGTGGAATTCTGCAGATTCAATGCTGCCGTGAAAAGAAATTT
HTR6	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGGTGCCCGAGCCGG
HTR6	Reverse	TGTGGTGGAATTCTGCAGATTCAATTGGTTGGTATACCCAGAGGAT
HTR7	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGATGGACGTTAACAGCTCTG
HTR7	Reverse	TGTGGTGGAATTCTGCAGATTCAGTCATGGATCATTACCTTCTTCTCA
Smp_126730	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGTGGTATATTCCAACAAAACATGAA
Smp_126730	Reverse	TGTGGTGGAATTCTGCAGATTTAAGACTTCCCTAACCATCTTGAAG
Smp_202470	Forward	CGGCCGCCACTGTGCTGGATGCCACCATGAACACTGAGAACTATCTAAATG
Smp_202470	Reverse	TGTGGTGGAATTCTGCAGATCTATGGTGAAGATGGCGATT

S3.2 Table. Cloning primers for Gibson assembly.

Chapter 4: Future directions

4.1 Paralysis mechanism

The main question we have been trying to address is whether SPF and Ht-13 analogs act through schistosome dopamine/serotonin G protein-coupled receptors (GPCRs). Preliminary results (Chapter 3) identified a dopamine GPCR (Smp_202470) that was activated by Ht-13 analogs. Thus, one immediate future direction is to express all other schistosome dopamine (Smp_043290, Smp_127310, Smp_150180, and Smp_315720) GPCRs in AequoZen cells (PerkinElmer) and perform cell assays. Furthermore, since Ht-13 analogs activated human serotonin receptors, we wanted to thoroughly investigate schistosome serotonin receptors by performing the same cell assay. Together, these assays will provide a comprehensive understanding of the interactions between Ht-13 analogs and schistosome dopamine/serotonin receptors. However, a successful cell assay requires several key steps, including: 1) a known full-length sequence for each receptor; 2) an adequate transient expression of the receptor in CHO-K1 cells; 3) proper folding and location of the receptor; and 4) receptor compatibility with $G_{\alpha 16}$ or $G_{\alpha q}$. To acquire a full-length sequence for the receptor, RNA Ligase-Mediated Rapid Amplification of cDNA Ends (RLM-RACE) can be performed. Trying different promotors and using codon optimization may be necessary to ensure protein expression in this heterologous expression system. To detect proper receptor localization upon expression, we can introduce a small epitope tag at the C- or N-terminus of the receptor and use an antibody specific to the epitope for immunostaining. GFP tagging may serve as an alternative to visualize the receptor's localization to the membrane, but the relatively large size of GFP might interrupt receptor folding, trafficking and function. Although we might be able to detect improperly localized receptors, it would be difficult to re-orient them in the correct position. A possible solution could

be adding a signal peptide to the N-terminus of the receptor that directs the trafficking to the plasma membrane (Quitterer et al., 2011). In case the receptor is not compatible with $G_{\alpha 16}$ or $G_{\alpha q}$ in the AequoZen system, we can use a GloSensor assay (Promega) for receptors that are compatible with $G_{\alpha s}$ or $G_{\alpha I}$ which detects cAMP production from GPCR signaling as previously described (Chan et al., 2016). An enzyme-linked immunosorbent assay (ELISA) using commercially available cAMP detection kit (e.g., Abcam) could also be used as an alternative strategy. This assay could also be performed on drug-treated cercariae to directly answer the question if cAMP is the downstream signal transducer *in vivo*.

To understand the mechanism at a cellular level, we need to know where the targeted receptors are expressed. The paralysis phenotype suggests that these receptors are most likely expressed in muscles or neurons which directly control motility. We are currently troubleshooting the RNA *in situ* hybridization protocol to detect receptor mRNA in cercaria. Meanwhile, we are generating antibodies against Smp_202470 and Smp_126730 to detect protein expression. Membrane localization is expected for these receptors. Together, using both RNA in situ hybridization and immunostaining of receptors using antibodies will enable us to determine the expression patterns of receptors in cercarial tissue/cell types, which will further help us dissect the mechanism of paralysis.

4.2 Biosynthetic pathway of schistosome paralysis factor (SPF)

SPF is a naturally occurring alkaloid produced by *Rotaria rotatoria*. SPF's chemical structure was determined using 2D NMR (Gao et al., 2019). Chemical synthesis of SPF is essential for validating the structure and biological activity as well as providing material for cell assays. Although the chemical synthesis of Ht-13 analogs has been published, the same scheme was not successful with SPF. While our collaborator who specializes in organic chemistry continues to work on SPF synthesis, we could begin to decipher the biosynthetic pathway of SPF. One possible approach could be to compare the genome/transcriptome of *Rotaria rotatoria* to a closely related *Rotaria* species that does not produce SPF. Then we can search for genes that are related to known alkaloid-producing enzymes. According to a previous report in the Ht-13 analogs patent, these compounds were produced by bacteria *Streptomyces* (Kamigauchi and Yasui, 2000). However, the species of the *Streptomyces* was not disclosed in the patent so we are currently unable to directly work on it. However, since rotifers are known to have horizontal gene transfer (HGT), it is possible that *R. rotatoria* obtained these enzymes through HGT from bacteria (Eyres et al., 2015). We can possibly search for HGT features (e.g., no introns) in the *R. rotatoria* genome to look for these enzymes. The function of enzyme candidates can be tested using RNA interference (RNAi). Although an established protocol has not been developed, soaking rotifers with double-stranded RNA (dsRNA) or feeding with dsRNA-expressing bacteria will be a promising starting point.

4.3 SPF's effect on other life-cycle stages of schistosomes

Schistosomes have a complicated life cycle that involves multiple body plans. Despite the differences in movement between stages, overall motility remains a key component for schistosomes to complete their whole life cycle. Therefore, SPF's paralysis effect on cercaria gives a window of opportunity to determine if such signaling is conserved throughout the life cycle and thus can be used to target other stages of the parasite. Schistosomula, juveniles, and adult parasites are the intra-mammalian stages that require robust motility to move from the skin to blood vessels and migrate against blood flow (Nation et al., 2020). Blocking their movements will stop the development of the disease. Furthermore, the only chemotherapy currently available, praziquantel (PZQ), fails to eliminate the juvenile stage (Caffrey, 2007). Thus, drugs that inhibit juvenile motility could be complementary to PZQ and make treatment more efficient. To test whether dopamine or serotonin signaling is also playing a role in these stages, we could treat them with SPF, Ht-13 analogs and also screen through drug libraries. However, since Basch media (Basch, 1981), which contains serotonin, was used to culture these stages, such drug-treatment experiments should be carried out in serotonin-free Basch media to avoid interference.

The miracidium is the other freshwater stage of the schistosome life cycle. Unlike cercariae, miracidia swim with cilia and infect snails. Although no motility defects were observed when miracidia were directly treated with SPF, a reduced transformation efficiency was noticed during *in vitro* miracidium-to-sporocyst transformation (Fig 4.1). 2.5 μM SPF completely blocked transformation. Miracidia kept swimming in sporocyst media without any sign of transforming (e.g., releasing ciliated plates). To determine if dopamine or serotonin signaling plays a role in miracidium motility, we screened through the same dopamine and serotonin libraries used for

cercaria, which consists of 80 dopaminergic and 79 serotonergic drugs. At 1 μM, we found ten dopaminergic and two serotonergic drugs causing motility defects including paralysis, spinning and swimming with occasional spinning (Table 4.1). Among the ten dopaminergic drug hits, six were dopamine reuptake inhibitors. Interestingly, the two serotonergic drug hits shared the same diphenylmethane group with these dopamine reuptake inhibitors (Fig 4.2). This suggests that these two compounds are potentially targeting dopamine transporters in schistosomes even though they are characterized as serotonergic drugs. Although none of these drug hits overlapped with cercaria drug hits at the same concentration tested, the same dopamine reuptake inhibitors paralyzed cercaria at a higher concentration (10 μM). This suggests that the balance between dopamine transporters and GPCRs may differ between cercariae and miracidia or that dopamine transporters in cercaria are less accessible than miracidia. Despite such differences, increasing extracellular dopamine concentration reduces both cercaria and miracidia motility.

4.4 Other questions

This project was inspired by an important observation made about 40 years ago that rotifer infestation on snails reduced cercaria quality and quantity (Stirewalt and Lewis, 1981). Over the past years, we have made significant discoveries that begin to elucidate the molecular basis of this phenomenon. However, there are still many interesting questions that have yet to be addressed. For example, why is *R. rotatoria* making SPF? Is the paralysis of cercaria directed specifically at schistosomes or is it just a side effect? If it is a side effect, is SPF released to communicate within the same species or to repel other competing rotifer species? Is GPCR signaling also involved in such communication or competition? Can we use SPF and/or related compounds (e.g., Ht-13 analogs) to prevent schistosome infection? Can SPF and Ht-13 analogs be developed into neurological (e.g., Parkinson's) disease drugs? Can we chemically modify SPF and Ht-13 analogs to synthesize more potent derivatives? Can SPF and Ht-13 analogs paralyze other trematodes or other flatworms? How conserved is the signaling pathway involved in paralysis? Answering these questions will provide invaluable contributions to multiple fields such as pharmacology, chemical ecology, cell biology and neurobiology.

4.5 Materials and Methods

Culture media

All culture media is made with previously published recipes (Ivanchenko et al., 1999; Mann et al., 2010). Artificial pond water: 0.125 mg/L $FeCl_3 \bullet 6H_2O$, 32.25 mg/L $CaCl_2 \bullet 2H_2O$, 25 mg/L $MgSO_4 \bullet 7H_2O$, 42.5 mg/L KH_2PO_4, 1.875 mg/L $(NH_4)_2SO_4$, pH 7.2. Medium F: 1X BME vitamins, 1X BME amino acids, 6 mg/L serine, 2.9 mg/L proline, 2.4 mg/L L-alanine, 2.8 mg/L aspartic acid, 4.7 mg/L glutamic acid, 2.4 mg/L glycine, 2.4 mg/L b-alanine, 40 mg/L malic acid, 30 mg/L ketoglutaric acid, 10 mg/L succinic acid, 5 mg/L fumaric acid, 10 mg/L citric acid, 70 mg/L Na2HPO4, 0.53 g/L CaCl2 .2H2O, 0.15 g/ L KCl, 0.45 g/L MgSO4 .7H2O, 1.5 g/L NaCl, 4.5 g/L galactose, 1 g/L glucose, 25 mM HEPES, pH 7. Sporocyst culture medium: 10% heat inactivated FBS, 23.5% Medium F, 23.5% DMEM/F12, 10% Schneider's Drosophila Medium, 2 g/L lactalbumin hydrolysate, 0.6 g/L galactose, 55 mM 2-mercaptoethanol, 0.005% Chemically defined lipid (Invitrogen).

Obtaining *S. mansoni* miracidia

Infected livers were obtained from mice that were 7 weeks-post infection and then minced with razor blades. Minced livers were then transferred into a 500 ml or 1L flask. Artificial pond water was slowly added to flask all the way to the top. A bright light source was place at the top of the flask while the rest of the flask was wrapped with aluminum foil. Hatched miracidia were collected from the surface, cleaned and then concentrated with homemade 20 μm baskets.

Miracidia-to-sporocyst transformation assay

Miracidia were filtered out from artificial pond water and cultured in sporocyst culture medium. Roughly 200 miracidia were transferred into each well of a 24-well plate. SPF was diluted in sporocyst media and added to reach the final concentration indicated. The number of transformed miracidia was counted after 24 hrs incubation. Transformed miracidia appeared on the bottom of the dish as a smooth sporocyst shape lacking cilia plates, while untransformed miracidia kept swimming.

Miracidia drug screen.

Dopamine and serotonin libraries (Enzo Life Science) were diluted to 10 μM with artificial pond water. In a 96-well plate, 90 μL artificial pond water containing ~50 miracidia was transferred into each well. Then 10 μL of each drug was added for a final concentration of 1 μM. Motility defects were noted at 10, 30 and 60 mins time points.

4.6 Figures

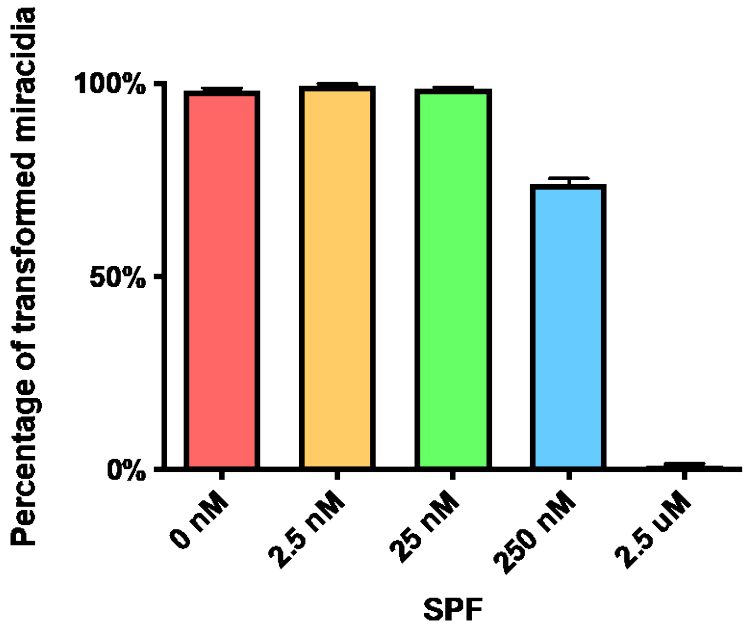

Fig 4.1. SPF inhibits in vitro miracidium-to-sporocyst transformation efficiency in a dose-dependent manner.

Percentage of transformed miracidia was shown after 24 hrs incubation in presence of SPF. Triplicates were performed. Data are mean ± S.D.

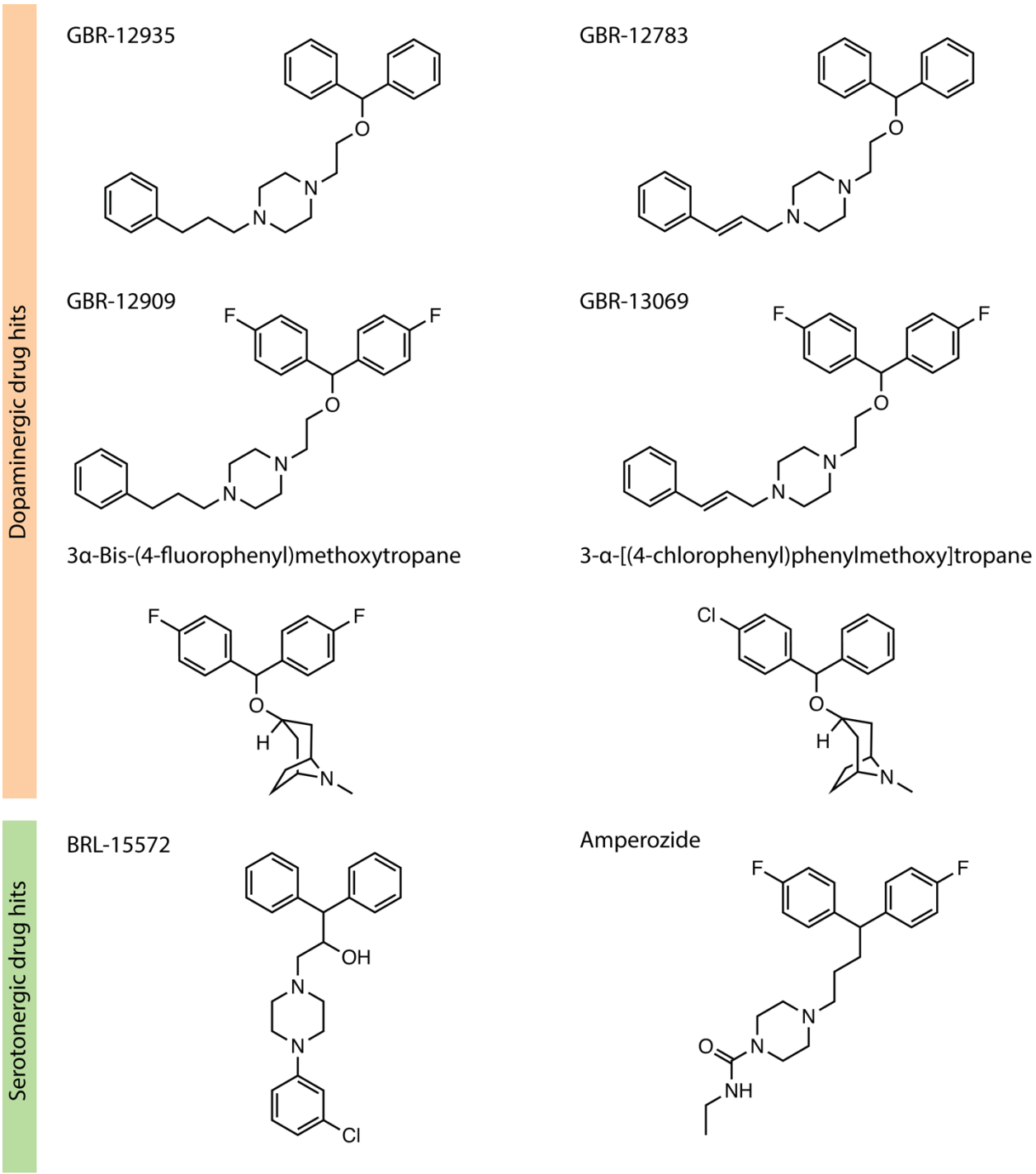

Fig 4.2. Dopaminergic and serotonergic drug hits share similar chemical structure.

CAS	Name	Activity	1 μM 10 mins	1 μM 30 mins	1 μM 60 mins
193611-72-2	BRL 15572·HCl	Serotonin 5-HT 1D antagonist.	Shaking/ spinning in the bottom	Shaking/spinning in the bottom	Shaking/ spinning in the bottom (some recovered)
75558-90-6	Amperozide·HCl	High affinity for 5-HT 2 receptors and low affinity for D2 receptors	Shaking/ spinning in the bottom	Shaking/spinning in the bottom	Shaking/ spinning in the bottom (some recovered)
67469-78-7	GBR-12909·2HCl	Dopamine uptake inhibitor	No	Shaking/spinning in the bottom	Not moving at all
130-61-0	Thioridazine·HCl	Dopamine D4 antagonist	Swimming with occasional spinning	Swimming with occasional spinning	Swimming with occasional spinning
69-09-0	Chlorpromazine·HCl	Dopamine antagonist	Swimming with occasional spinning	No	No
1135278-61-3	3'-Fluorobenzylspiperone maleate	D2 receptor ligand	Swimming with occasional spinning	No	No
202646-03-5	3α-Bis-(4-fluorophenyl)methoxytropane·HCl	Dopamine uptake inhibitor	Swimming with occasional spinning	Swimming with occasional spinning	Swimming with occasional spinning
14008-79-8	3-α-[(4-chlorophenyl)phenylmethoxy]tropane·HCl	Dopamine uptake inhibitor	No	Swimming with occasional spinning	Swimming with occasional spinning
67469-57-2	GBR 12783·2HCl	Dopamine uptake inhibitor	Shaking/ spinning in the bottom	Shaking/spinning in the bottom	Not moving at all
76778-22-8	GBR 12935·2HCl	Dopamine uptake inhibitor	Shaking/ spinning in the bottom	Shaking/spinning in the bottom	Not moving at all
77862-93-2	GBR 13069·2HCl	Dopamine uptake inhibitor	Shaking/ spinning in the bottom	Shaking/spinning in the bottom	Not moving at all
71787-90-1	(±)-2-(N-phenylethyl-N-propyl)amino-5-hydroxytetralin·HCl	D2 dopamine agonist	Swimming with occasional spinning	Swimming with occasional spinning	Swimming with occasional spinning

Table 4.1. Serotoninergic and dopaminergic drug hits on miracidia.

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Appendix 1: Supplementary data for Chapter 2

Table 5.1. Raw data for mouse infection experiments in Fig 2.4.

Number of adult parasites recovered (male/female), liver weight, spleen weight, and egg counts per liver section were reported for each experiment.

Date	Mouse ID	Drug	Conc. (nM)	Male	Female	Whole Liver Weight (g)	Spleen Weight (g)	Liver section	Liver Area (mm^2)	Egg counts	Egg/Area (/mm^2)
1/16/18	A01	Control	0	34	21	2.51	0.3102	1	28.562	47	1.65
1/16/18	A01	Control	0	34	21	2.51	0.3102	2	24.858	37	1.49
1/16/18	A01	Control	0	34	21	2.51	0.3102	3	28.214	36	1.28
1/16/18	A01	Control	0	34	21	2.51	0.3102	4	14.892	31	2.08
1/16/18	A01	Control	0	34	21	2.51	0.3102	5	30.969	36	1.16
1/16/18	A01	Control	0	34	21	2.51	0.3102	6	29.029	40	1.38
1/16/18	A02	Control	0	70	29	3.5902	0.7271	1	31.544	31	0.98
1/16/18	A02	Control	0	70	29	3.5902	0.7271	2	47.443	57	1.20
1/16/18	A02	Control	0	70	29	3.5902	0.7271	3	38.979	35	0.90
1/16/18	A02	Control	0	70	29	3.5902	0.7271	4	26.964	31	1.15
1/16/18	A02	Control	0	70	29	3.5902	0.7271	5	44.378	58	1.31
1/16/18	A03	Control	0	49	41	3.1607	0.4473	1	39.515	53	1.34
1/16/18	A03	Control	0	49	41	3.1607	0.4473	2	35.025	47	1.34
1/16/18	A03	Control	0	49	41	3.1607	0.4473	3	39.814	46	1.16
1/16/18	A03	Control	0	49	41	3.1607	0.4473	4	37.241	60	1.61
1/16/18	A03	Control	0	49	41	3.1607	0.4473	5	31.27	48	1.54
1/16/18	B01	Ht-13-A	250	30	22	2.9259	0.382	1	28.6	19	0.66
1/16/18	B01	Ht-13-A	250	30	22	2.9259	0.382	2	27.31	15	0.55
1/16/18	B01	Ht-13-A	250	30	22	2.9259	0.382	3	34.773	23	0.66
1/16/18	B01	Ht-13-A	250	30	22	2.9259	0.382	4	33.446	26	0.78
1/16/18	B01	Ht-13-A	250	30	22	2.9259	0.382	5	24.523	13	0.53
1/16/18	B01	Ht-13-A	250	30	22	2.9259	0.382	6	38.935	19	0.49
1/16/18	B02	Ht-13-A	250	30	18	2.5076	0.3379	1	24.903	16	0.64
1/16/18	B02	Ht-13-A	250	30	18	2.5076	0.3379	2	33.488	27	0.81
1/16/18	B02	Ht-13-A	250	30	18	2.5076	0.3379	3	35.753	29	0.81
1/16/18	B02	Ht-13-A	250	30	18	2.5076	0.3379	4	33.373	20	0.60
1/16/18	B02	Ht-13-A	250	30	18	2.5076	0.3379	5	17.73	21	1.18
1/16/18	B02	Ht-13-A	250	30	18	2.5076	0.3379	6	27.558	27	0.98
1/16/18	B03	Ht-13-A	250	12	12	2.4301	0.3305	1	28.355	51	1.80

1/16/18	B03	Ht-13-A	250	12	12	2.4301	0.3305	2	29.678	54	1.82
1/16/18	B03	Ht-13-A	250	12	12	2.4301	0.3305	3	31.637	57	1.80
1/16/18	B03	Ht-13-A	250	12	12	2.4301	0.3305	4	34.341	59	1.72
1/16/18	B03	Ht-13-A	250	12	12	2.4301	0.3305	5	36.354	42	1.16
1/16/18	C01	Ht-13-A	2500	47	35	2.4597	0.2695	1	35.232	12	0.34
1/16/18	C01	Ht-13-A	2500	47	35	2.4597	0.2695	2	32.68	9	0.28
1/16/18	C01	Ht-13-A	2500	47	35	2.4597	0.2695	3	17.35	7	0.40
1/16/18	C01	Ht-13-A	2500	47	35	2.4597	0.2695	4	43.196	30	0.69
1/16/18	C01	Ht-13-A	2500	47	35	2.4597	0.2695	5	34.872	12	0.34
1/16/18	C01	Ht-13-A	2500	47	35	2.4597	0.2695	6	35.668	22	0.62
1/16/18	C02	Ht-13-A	2500	44	23	2.8903	0.4041	1	35.525	15	0.42
1/16/18	C02	Ht-13-A	2500	44	23	2.8903	0.4041	2	28.682	22	0.77
1/16/18	C02	Ht-13-A	2500	44	23	2.8903	0.4041	3	36.539	21	0.57
1/16/18	C02	Ht-13-A	2500	44	23	2.8903	0.4041	4	19.367	18	0.93
1/16/18	C02	Ht-13-A	2500	44	23	2.8903	0.4041	5	38.812	23	0.59
1/16/18	C02	Ht-13-A	2500	44	23	2.8903	0.4041	6	39.161	38	0.97
1/16/18	C03	Ht-13-A	2500	25	9	3.1326	0.3709	1	28.723	15	0.52
1/16/18	C03	Ht-13-A	2500	25	9	3.1326	0.3709	2	32.513	11	0.34
1/16/18	C03	Ht-13-A	2500	25	9	3.1326	0.3709	3	38.946	12	0.31
1/16/18	C03	Ht-13-A	2500	25	9	3.1326	0.3709	4	44.107	10	0.23
1/16/18	C03	Ht-13-A	2500	25	9	3.1326	0.3709	5	18.658	6	0.32
1/16/18	C03	Ht-13-A	2500	25	9	3.1326	0.3709	6	42.922	8	0.19
1/16/18	D01	Ht-13-A	25000	8	5	2.2077	0.2293	1	30.521	6	0.20
1/16/18	D01	Ht-13-A	25000	8	5	2.2077	0.2293	2	30.949	10	0.32
1/16/18	D01	Ht-13-A	25000	8	5	2.2077	0.2293	3	29.074	6	0.21
1/16/18	D01	Ht-13-A	25000	8	5	2.2077	0.2293	4	31.264	4	0.13
1/16/18	D01	Ht-13-A	25000	8	5	2.2077	0.2293	5	22.779	5	0.22
1/16/18	D02	Ht-13-A	25000	1	0	2.1986	0.1937	1	31.2	1	0.03
1/16/18	D02	Ht-13-A	25000	1	0	2.1986	0.1937	2	32.401	0	0.00
1/16/18	D02	Ht-13-A	25000	1	0	2.1986	0.1937	3	30.443	2	0.07
1/16/18	D02	Ht-13-A	25000	1	0	2.1986	0.1937	4	15.723	3	0.19
1/16/18	D02	Ht-13-A	25000	1	0	2.1986	0.1937	5	15.398	2	0.13
1/16/18	D02	Ht-13-A	25000	1	0	2.1986	0.1937	6	31.81	4	0.13
1/16/18	D03	Ht-13-A	25000	2	1	2.4603	0.2525	1	20.103	1	0.05
1/16/18	D03	Ht-13-A	25000	2	1	2.4603	0.2525	2	16.786	3	0.18
1/16/18	D03	Ht-13-A	25000	2	1	2.4603	0.2525	3	31.95	3	0.09
1/16/18	D03	Ht-13-A	25000	2	1	2.4603	0.2525	4	32.809	1	0.03
1/16/18	D03	Ht-13-A	25000	2	1	2.4603	0.2525	5	28.269	2	0.07
1/16/18	D03	Ht-13-A	25000	2	1	2.4603	0.2525	6	26.689	0	0.00

Date	Mouse ID	Drug	Conc. (nM)	Male	Female	Whole Liver Weight (g)	Spleen Weight (g)	Liver section	Liver Area (mm^2)	Egg counts	Egg/Area (/mm^2)
3/1/18	A01	Control	0	83	65	2.8955	0.505	1	68.87	171	2.48
3/1/18	A01	Control	0	83	65	2.8955	0.505	2	69.281	163	2.35
3/1/18	A01	Control	0	83	65	2.8955	0.505	3	49.663	102	2.05
3/1/18	A01	Control	0	83	65	2.8955	0.505	4	35.426	103	2.91
3/1/18	A02	Control	0	105	77	3.2277	0.6214	1	69	170	2.46
3/1/18	A02	Control	0	105	77	3.2277	0.6214	2	66.152	157	2.37
3/1/18	A02	Control	0	105	77	3.2277	0.6214	3	62.306	162	2.60
3/1/18	A02	Control	0	105	77	3.2277	0.6214	4	28.232	84	2.98
3/1/18	A03	Control	0	65	59	2.9908	0.6193	1	65.818	129	1.96
3/1/18	A03	Control	0	65	59	2.9908	0.6193	2	60.892	111	1.82
3/1/18	A03	Control	0	65	59	2.9908	0.6193	3	60.892	98	1.61
3/1/18	A03	Control	0	65	59	2.9908	0.6193	4	22.047	61	2.77
3/1/18	B01	Ht-13-A-pr	250	66	43	2.55	0.381	1	38.866	49	1.26
3/1/18	B01	Ht-13-A-pr	250	66	43	2.55	0.381	2	21.352	46	2.15
3/1/18	B01	Ht-13-A-pr	250	66	43	2.55	0.381	3	44.683	79	1.77
3/1/18	B01	Ht-13-A-pr	250	66	43	2.55	0.381	4	47.167	86	1.82
3/1/18	B02	Ht-13-A-pr	250	83	54	3.521	0.505	1	81.905	153	1.87
3/1/18	B02	Ht-13-A-pr	250	83	54	3.521	0.505	2	72.468	98	1.35
3/1/18	B02	Ht-13-A-pr	250	83	54	3.521	0.505	3	83.883	132	1.57
3/1/18	B02	Ht-13-A-pr	250	83	54	3.521	0.505	4	41.547	83	2.00
3/1/18	B03	Ht-13-A-pr	250	63	38	2.8406	0.3654	1	66.612	112	1.68
3/1/18	B03	Ht-13-A-pr	250	63	38	2.8406	0.3654	2	18.936	40	2.11
3/1/18	B03	Ht-13-A-pr	250	63	38	2.8406	0.3654	3	56.223	92	1.64
3/1/18	B03	Ht-13-A-pr	250	63	38	2.8406	0.3654	4	50.286	85	1.69
3/1/18	C01	Ht-13-A-pr	2500	48	35	2.9276	0.4176	1	66.79	42	0.63
3/1/18	C01	Ht-13-A-pr	2500	48	35	2.9276	0.4176	2	65.204	55	0.84
3/1/18	C01	Ht-13-A-pr	2500	48	35	2.9276	0.4176	3	37.639	25	0.66
3/1/18	C01	Ht-13-A-pr	2500	48	35	2.9276	0.4176	4	34.795	31	0.89
3/1/18	C02	Ht-13-A-pr	2500	64	23	3.2035	0.5126	1	56.965	83	1.46
3/1/18	C02	Ht-13-A-pr	2500	64	23	3.2035	0.5126	2	77.661	94	1.21
3/1/18	C02	Ht-13-A-pr	2500	64	23	3.2035	0.5126	3	70.545	82	1.16

3/1/18	C02	Ht-13-A-pr	2500	64	23	3.2035	0.5126	4	33.774	48	1.42
3/1/18	C03	Ht-13-A-pr	2500	30	9	2.5846	0.3224	1	58.948	53	0.90
3/1/18	C03	Ht-13-A-pr	2500	30	9	2.5846	0.3224	2	50.914	55	1.08
3/1/18	C03	Ht-13-A-pr	2500	30	9	2.5846	0.3224	3	61.73	78	1.26
3/1/18	C03	Ht-13-A-pr	2500	30	9	2.5846	0.3224	4	33.486	31	0.93
3/1/18	D01	Ht-13-A-pr	25000	5	0	1.9457	0.2142	1	39.701	1	0.03
3/1/18	D01	Ht-13-A-pr	25000	5	0	1.9457	0.2142	2	46.333	1	0.02
3/1/18	D01	Ht-13-A-pr	25000	5	0	1.9457	0.2142	3	47.927	0	0.00
3/1/18	D01	Ht-13-A-pr	25000	5	0	1.9457	0.2142	4	17.094	2	0.12
3/1/18	D02	Ht-13-A-pr	25000	1	0	2.1557	0.2762	1	29.866	0	0.00
3/1/18	D02	Ht-13-A-pr	25000	1	0	2.1557	0.2762	2	53.444	0	0.00
3/1/18	D02	Ht-13-A-pr	25000	1	0	2.1557	0.2762	3	58.189	0	0.00
3/1/18	D02	Ht-13-A-pr	25000	1	0	2.1557	0.2762	4	23.627	0	0.00
3/1/18	D03	Ht-13-A-pr	25000	2	0	1.5747	0.1318	1	32.349	0	0.00
3/1/18	D03	Ht-13-A-pr	25000	2	0	1.5747	0.1318	2	46.053	0	0.00
3/1/18	D03	Ht-13-A-pr	25000	2	0	1.5747	0.1318	3	45.881	0	0.00
3/1/18	D03	Ht-13-A-pr	25000	2	0	1.5747	0.1318	4	21.618	0	0.00

Date	Mouse ID	Drug	Conc. (nM)	Male	Female	Whole Liver Weight (g)	Spleen Weight (g)	Liver section	Liver Area (mm^2)	Egg counts	Egg/Area (/mm^2)
3/15/18	A01	Control	0	38	42	2.705	0.3951	1	63.423	75	1.18
3/15/18	A01	Control	0	38	42	2.705	0.3951	2	49.539	55	1.11
3/15/18	A01	Control	0	38	42	2.705	0.3951	3	42.945	52	1.21
3/15/18	A01	Control	0	38	42	2.705	0.3951	4	36.261	55	1.52
3/15/18	A02	Control	0	47	38	3.402	0.3761	1	59.26	56	0.95
3/15/18	A02	Control	0	47	38	3.402	0.3761	2	50.893	49	0.96
3/15/18	A02	Control	0	47	38	3.402	0.3761	3	70.79	73	1.03
3/15/18	A02	Control	0	47	38	3.402	0.3761	4	54.21	51	0.94
3/15/18	A03	Control	0	84	72	3.441	0.5931	1	41.584	84	2.02
3/15/18	A03	Control	0	84	72	3.441	0.5931	2	60.526	104	1.72
3/15/18	A03	Control	0	84	72	3.441	0.5931	3	44.577	109	2.45
3/15/18	A03	Control	0	84	72	3.441	0.5931	4	37.964	75	1.98
3/15/18	B01	SPF	25	50	39	2.958	0.3261	1	57.691	50	0.87
3/15/18	B01	SPF	25	50	39	2.958	0.3261	2	51.058	51	1.00
3/15/18	B01	SPF	25	50	39	2.958	0.3261	3	55.191	76	1.38
3/15/18	B01	SPF	25	50	39	2.958	0.3261	4	25.835	33	1.28
3/15/18	B02	SPF	25	18	15	2.829	0.308	1	34.507	20	0.58
3/15/18	B02	SPF	25	18	15	2.829	0.308	2	58.887	24	0.41
3/15/18	B02	SPF	25	18	15	2.829	0.308	3	49.245	24	0.49
3/15/18	B02	SPF	25	18	15	2.829	0.308	4	56.253	23	0.41
3/15/18	B03	SPF	25	33	33	3.298	0.462	1	84.648	62	0.73
3/15/18	B03	SPF	25	33	33	3.298	0.462	2	75.425	52	0.69
3/15/18	B03	SPF	25	33	33	3.298	0.462	3	65.016	46	0.71
3/15/18	B03	SPF	25	33	33	3.298	0.462	4	33.821	18	0.53
3/15/18	C01	SPF	250	0	0	2.308	0.17	1	53.125	0	0.00
3/15/18	C01	SPF	250	0	0	2.308	0.17	2	36.086	0	0.00
3/15/18	C01	SPF	250	0	0	2.308	0.17	3	49.445	0	0.00
3/15/18	C01	SPF	250	0	0	2.308	0.17	4	23.228	0	0.00
3/15/18	C02	SPF	250	0	0	2.607	0.2182	1	27.86	0	0.00
3/15/18	C02	SPF	250	0	0	2.607	0.2182	2	58.902	0	0.00
3/15/18	C02	SPF	250	0	0	2.607	0.2182	3	43.155	0	0.00
3/15/18	C02	SPF	250	0	0	2.607	0.2182	4	60.49	0	0.00
3/15/18	C03	SPF	250	0	0	2.685	0.1576	1	56.954	0	0.00
3/15/18	C03	SPF	250	0	0	2.685	0.1576	2	60.65	0	0.00
3/15/18	C03	SPF	250	0	0	2.685	0.1576	3	37.196	0	0.00
3/15/18	C03	SPF	250	0	0	2.685	0.1576	4	31.902	0	0.00
3/15/18	D01	SPF	2500	0	0	2.503	0.116	1	56.443	0	0.00

3/15/18	D01	SPF	2500	0	0	2.503	0.116	2	60.904	0	0.00
3/15/18	D01	SPF	2500	0	0	2.503	0.116	3	44.227	0	0.00
3/15/18	D01	SPF	2500	0	0	2.503	0.116	4	16.011	0	0.00
3/15/18	D02	SPF	2500	0	0	1.931	0.0836	1	25.972	0	0.00
3/15/18	D02	SPF	2500	0	0	1.931	0.0836	2	41.228	0	0.00
3/15/18	D02	SPF	2500	0	0	1.931	0.0836	3	40.736	0	0.00
3/15/18	D02	SPF	2500	0	0	1.931	0.0836	4	26.375	0	0.00
3/15/18	D03	SPF	2500	0	0	1.833	0.0957	1	40.953	0	0.00
3/15/18	D03	SPF	2500	0	0	1.833	0.0957	2	45.986	0	0.00
3/15/18	D03	SPF	2500	0	0	1.833	0.0957	3	41.965	0	0.00
3/15/18	D03	SPF	2500	0	0	1.833	0.0957	4	41.063	0	0.00
3/15/18	E01	Ht-13-A	25000	0	0	3.151	0.261	1	62.657	0	0.00
3/15/18	E01	Ht-13-A	25000	0	0	3.151	0.261	2	67.563	0	0.00
3/15/18	E01	Ht-13-A	25000	0	0	3.151	0.261	3	56.631	0	0.00
3/15/18	E01	Ht-13-A	25000	0	0	3.151	0.261	4	30.779	0	0.00
3/15/18	E02	Ht-13-A	25000	0	0	2.419	0.168	1	56.392	0	0.00
3/15/18	E02	Ht-13-A	25000	0	0	2.419	0.168	2	51.483	0	0.00
3/15/18	E02	Ht-13-A	25000	0	0	2.419	0.168	3	56.658	0	0.00
3/15/18	E02	Ht-13-A	25000	0	0	2.419	0.168	4	21.538	0	0.00
3/15/18	E03	Ht-13-A	25000	0	0	1.852	0.156	1	53.766	0	0.00
3/15/18	E03	Ht-13-A	25000	0	0	1.852	0.156	2	41.242	0	0.00
3/15/18	E03	Ht-13-A	25000	0	0	1.852	0.156	3	35.521	0	0.00
3/15/18	E03	Ht-13-A	25000	0	0	1.852	0.156	4	17.165	0	0.00

Date	Mouse ID	Drug	Conc. (nM)	Male	Female	Whole Liver Weight (g)	Spleen Weight (g)	Liver section	Liver Area (mm^2)	Egg counts	Egg/Area (/mm^2)
3/29/18	A01	Control	0	30	20	2.682	0.368	1	50.49	39	0.77
3/29/18	A01	Control	0	30	20	2.682	0.368	2	64.646	36	0.56
3/29/18	A01	Control	0	30	20	2.682	0.368	3	57.296	32	0.56
3/29/18	A01	Control	0	30	20	2.682	0.368	4	44.467	26	0.58
3/29/18	A01	Control	0	30	20	2.682	0.368	5	29.023	22	0.76
3/29/18	A02	Control	0	54	20	2.502	0.332	1	59.321	54	0.91
3/29/18	A02	Control	0	54	20	2.502	0.332	2	59.341	57	0.96
3/29/18	A02	Control	0	54	20	2.502	0.332	3	38.927	35	0.90
3/29/18	A02	Control	0	54	20	2.502	0.332	4	40.217	44	1.09
3/29/18	A02	Control	0	54	20	2.502	0.332	5	22.428	32	1.43
3/29/18	A03	Control	0	45	17	2.975	0.372	1	60.236	52	0.86
3/29/18	A03	Control	0	45	17	2.975	0.372	2	64.499	59	0.91
3/29/18	A03	Control	0	45	17	2.975	0.372	3	45.039	43	0.95
3/29/18	A03	Control	0	45	17	2.975	0.372	4	54.55	53	0.97
3/29/18	A03	Control	0	45	17	2.975	0.372	5	33.848	46	1.36
3/29/18	A03	Control	0	45	17	2.975	0.372	6	15.891	18	1.13
3/29/18	B01	SPF	25	24	9	3.017	0.367	1	59.257	53	0.89
3/29/18	B01	SPF	25	24	9	3.017	0.367	2	58.413	59	1.01
3/29/18	B01	SPF	25	24	9	3.017	0.367	3	61.945	43	0.69
3/29/18	B01	SPF	25	24	9	3.017	0.367	4	40.955	30	0.73
3/29/18	B01	SPF	25	24	9	3.017	0.367	5	30.971	28	0.90
3/29/18	B02	SPF	25	26	14	2.449	0.334	1	44.292	22	0.50
3/29/18	B02	SPF	25	26	14	2.449	0.334	2	60.394	39	0.65
3/29/18	B02	SPF	25	26	14	2.449	0.334	3	58.496	47	0.80
3/29/18	B02	SPF	25	26	14	2.449	0.334	4	27.652	27	0.98
3/29/18	B03	SPF	25	31	18	2.923	0.302	1	63.733	58	0.91
3/29/18	B03	SPF	25	31	18	2.923	0.302	2	64.943	59	0.91
3/29/18	B03	SPF	25	31	18	2.923	0.302	3	50.954	43	0.84
3/29/18	B03	SPF	25	31	18	2.923	0.302	4	51.679	40	0.77
3/29/18	B03	SPF	25	31	18	2.923	0.302	5	24.003	23	0.96
3/29/18	C01	SPF	250	0	0	1.956	0.121	1	49.532	0	0.00
3/29/18	C01	SPF	250	0	0	1.956	0.121	2	50.549	0	0.00
3/29/18	C01	SPF	250	0	0	1.956	0.121	3	37.748	0	0.00
3/29/18	C01	SPF	250	0	0	1.956	0.121	4	21.869	0	0.00
3/29/18	C02	SPF	250	0	0	2.602	0.144	1	43.835	0	0.00
3/29/18	C02	SPF	250	0	0	2.602	0.144	2	57.654	0	0.00
3/29/18	C02	SPF	250	0	0	2.602	0.144	3	47.709	0	0.00

3/29/18	C02	SPF	250	0	0	2.602	0.144	4	23.615	0	0.00
3/29/18	C02	SPF	250	0	0	2.602	0.144	5	21.266	0	0.00
3/29/18	C03	SPF	250	0	0	1.967	0.151	1	46.829	1	0.02
3/29/18	C03	SPF	250	0	0	1.967	0.151	2	33.728	1	0.03
3/29/18	C03	SPF	250	0	0	1.967	0.151	3	38.259	2	0.05
3/29/18	C03	SPF	250	0	0	1.967	0.151	4	29.078	1	0.03
3/29/18	D01	SPF	2500	0	0	2.078	0.126	1	50.065	0	0.00
3/29/18	D01	SPF	2500	0	0	2.078	0.126	2	39.582	0	0.00
3/29/18	D01	SPF	2500	0	0	2.078	0.126	3	51.315	0	0.00
3/29/18	D01	SPF	2500	0	0	2.078	0.126	4	24.588	0	0.00
3/29/18	D02	SPF	2500	0	0	2.521	0.139	1	61.378	0	0.00
3/29/18	D02	SPF	2500	0	0	2.521	0.139	2	44.918	0	0.00
3/29/18	D02	SPF	2500	0	0	2.521	0.139	3	48.441	0	0.00
3/29/18	D02	SPF	2500	0	0	2.521	0.139	4	61.222	0	0.00
3/29/18	D02	SPF	2500	0	0	2.521	0.139	5	21.541	0	0.00
3/29/18	D03	SPF	2500	0	0	2.617	0.15	1	55.833	0	0.00
3/29/18	D03	SPF	2500	0	0	2.617	0.15	2	58.168	0	0.00
3/29/18	D03	SPF	2500	0	0	2.617	0.15	3	35.283	0	0.00
3/29/18	D03	SPF	2500	0	0	2.617	0.15	4	37.909	0	0.00
3/29/18	D03	SPF	2500	0	0	2.617	0.15	5	19.143	0	0.00
3/29/18	E01	Ht-13-A-pr	25	44	23	2.226	0.279	1	47.914	68	1.42
3/29/18	E01	Ht-13-A-pr	25	44	23	2.226	0.279	2	48.766	53	1.09
3/29/18	E01	Ht-13-A-pr	25	44	23	2.226	0.279	3	35.555	42	1.18
3/29/18	E01	Ht-13-A-pr	25	44	23	2.226	0.279	4	26.868	44	1.64
3/29/18	E01	Ht-13-A-pr	25	44	23	2.226	0.279	5	15.229	23	1.51
3/29/18	E02	Ht-13-A-pr	25	33	24	2.365	0.291	1	57.58	47	0.82
3/29/18	E02	Ht-13-A-pr	25	33	24	2.365	0.291	2	58.507	54	0.92
3/29/18	E02	Ht-13-A-pr	25	33	24	2.365	0.291	3	45.376	53	1.17
3/29/18	E02	Ht-13-A-pr	25	33	24	2.365	0.291	4	34.394	31	0.90
3/29/18	E02	Ht-13-A-pr	25	33	24	2.365	0.291	5	22.581	40	1.77
3/29/18	E03	Ht-13-A-pr	25	29	20	2.728	0.516	1	57.923	63	1.09
3/29/18	E03	Ht-13-A-pr	25	29	20	2.728	0.516	2	50.632	50	0.99
3/29/18	E03	Ht-13-A-pr	25	29	20	2.728	0.516	3	50.541	47	0.93
3/29/18	E03	Ht-13-A-pr	25	29	20	2.728	0.516	4	39.872	38	0.95
3/29/18	E03	Ht-13-A-pr	25	29	20	2.728	0.516	5	26.043	30	1.15

3/29/18	E04	Ht-13-A-pr	25	26	12	3.223	0.455	1	61.489	36	0.59
3/29/18	E04	Ht-13-A-pr	25	26	12	3.223	0.455	2	62.918	32	0.51
3/29/18	E04	Ht-13-A-pr	25	26	12	3.223	0.455	3	38.209	21	0.55
3/29/18	E04	Ht-13-A-pr	25	26	12	3.223	0.455	4	55.946	24	0.43
3/29/18	E04	Ht-13-A-pr	25	26	12	3.223	0.455	5	34.584	25	0.72
3/29/18	E05	Ht-13-A-pr	25	33	17	2.46	0.367	1	57.079	60	1.05
3/29/18	E05	Ht-13-A-pr	25	33	17	2.46	0.367	2	47.924	31	0.65
3/29/18	E05	Ht-13-A-pr	25	33	17	2.46	0.367	3	35.45	30	0.85
3/29/18	E05	Ht-13-A-pr	25	33	17	2.46	0.367	4	35.534	33	0.93
3/29/18	E05	Ht-13-A-pr	25	33	17	2.46	0.367	5	25.417	23	0.90
3/29/18	E06	Ht-13-A-pr	25	29	16	3.246	0.518	1	57.503	39	0.68
3/29/18	E06	Ht-13-A-pr	25	29	16	3.246	0.518	2	60.127	32	0.53
3/29/18	E06	Ht-13-A-pr	25	29	16	3.246	0.518	3	61.598	44	0.71
3/29/18	E06	Ht-13-A-pr	25	29	16	3.246	0.518	4	29.494	22	0.75
3/29/18	F01	Ht-13-A-pr	250	39	16	2.548	0.356	1	43.854	33	0.75
3/29/18	F01	Ht-13-A-pr	250	39	16	2.548	0.356	2	56.887	38	0.67
3/29/18	F01	Ht-13-A-pr	250	39	16	2.548	0.356	3	57.103	47	0.82
3/29/18	F01	Ht-13-A-pr	250	39	16	2.548	0.356	4	41.822	28	0.67
3/29/18	F01	Ht-13-A-pr	250	39	16	2.548	0.356	5	17.762	17	0.96
3/29/18	F02	Ht-13-A-pr	250	34	24	2.914	0.434	1	57.717	34	0.59
3/29/18	F02	Ht-13-A-pr	250	34	24	2.914	0.434	2	44.433	24	0.54
3/29/18	F02	Ht-13-A-pr	250	34	24	2.914	0.434	3	60.951	41	0.67
3/29/18	F02	Ht-13-A-pr	250	34	24	2.914	0.434	4	40.732	35	0.86
3/29/18	F02	Ht-13-A-pr	250	34	24	2.914	0.434	5	36.297	40	1.10
3/29/18	F03	Ht-13-A-pr	250	25	14	2.444	/	1	42.18	22	0.52
3/29/18	F03	Ht-13-A-pr	250	25	14	2.444	/	2	55.745	35	0.63
3/29/18	F03	Ht-13-A-pr	250	25	14	2.444	/	3	41.363	24	0.58
3/29/18	F03	Ht-13-A-pr	250	25	14	2.444	/	4	41.363	23	0.56
3/29/18	F03	Ht-13-A-pr	250	25	14	2.444	/	5	15.837	10	0.63
3/29/18	G01	Ht-13-A-pr	2500	20	13	3.214	0.319	1	62.164	27	0.43
3/29/18	G01	Ht-13-A-pr	2500	20	13	3.214	0.319	2	51.472	21	0.41
3/29/18	G01	Ht-13-A-pr	2500	20	13	3.214	0.319	3	66.395	22	0.33

3/29/18	G01	Ht-13-A-pr	2500	20	13	3.214	0.319	4	45.156	18	0.40
3/29/18	G01	Ht-13-A-pr	2500	20	13	3.214	0.319	5	20.415	9	0.44
3/29/18	G02	Ht-13-A-pr	2500	30	16	2.848	0.469	1	58.092	43	0.74
3/29/18	G02	Ht-13-A-pr	2500	30	16	2.848	0.469	2	61.947	41	0.66
3/29/18	G02	Ht-13-A-pr	2500	30	16	2.848	0.469	3	48.851	40	0.82
3/29/18	G02	Ht-13-A-pr	2500	30	16	2.848	0.469	4	36.696	19	0.52
3/29/18	G02	Ht-13-A-pr	2500	30	16	2.848	0.469	5	30.581	25	0.82
3/29/18	G03	Ht-13-A-pr	2500	24	9	2.543	0.343	1	33.793	11	0.33
3/29/18	G03	Ht-13-A-pr	2500	24	9	2.543	0.343	2	61.167	13	0.21
3/29/18	G03	Ht-13-A-pr	2500	24	9	2.543	0.343	3	43.293	8	0.18
3/29/18	G03	Ht-13-A-pr	2500	24	9	2.543	0.343	4	60.435	14	0.23
3/29/18	G03	Ht-13-A-pr	2500	24	9	2.543	0.343	5	21.845	9	0.41
3/29/18	H01	Ht-13-A-pr	25000	2	2	1.886	0.196	1	43.589	4	0.09
3/29/18	H01	Ht-13-A-pr	25000	2	2	1.886	0.196	2	46.899	5	0.11
3/29/18	H01	Ht-13-A-pr	25000	2	2	1.886	0.196	3	32.389	2	0.06
3/29/18	H01	Ht-13-A-pr	25000	2	2	1.886	0.196	4	21.468	5	0.23
3/29/18	H02	Ht-13-A-pr	25000	1	0	2.673	0.196	1	54.29	0	0.00
3/29/18	H02	Ht-13-A-pr	25000	1	0	2.673	0.196	2	59.717	0	0.00
3/29/18	H02	Ht-13-A-pr	25000	1	0	2.673	0.196	3	43.641	0	0.00
3/29/18	H02	Ht-13-A-pr	25000	1	0	2.673	0.196	4	31.176	0	0.00
3/29/18	H02	Ht-13-A-pr	25000	1	0	2.673	0.196	5	21.843	0	0.00
3/29/18	H03	Ht-13-A-pr	25000	7	2	2.169	0.192	1	48.149	1	0.02
3/29/18	H03	Ht-13-A-pr	25000	7	2	2.169	0.192	2	50.811	0	0.00
3/29/18	H03	Ht-13-A-pr	25000	7	2	2.169	0.192	3	31.777	0	0.00
3/29/18	H03	Ht-13-A-pr	25000	7	2	2.169	0.192	4	39.467	0	0.00
3/29/18	H03	Ht-13-A-pr	25000	7	2	2.169	0.192	5	16.14	0	0.00
3/29/18	I01	Ht-13-A	25	65	34	3.12	0.556	1	61.713	73	1.18
3/29/18	I01	Ht-13-A	25	65	34	3.12	0.556	2	69.306	104	1.50
3/29/18	I01	Ht-13-A	25	65	34	3.12	0.556	3	62.903	85	1.35
3/29/18	I01	Ht-13-A	25	65	34	3.12	0.556	4	45.149	63	1.40
3/29/18	I01	Ht-13-A	25	65	34	3.12	0.556	5	32.772	42	1.28
3/29/18	I02	Ht-13-A	25	54	19	3.005	0.347	1	63.479	35	0.55
3/29/18	I02	Ht-13-A	25	54	19	3.005	0.347	2	58.162	40	0.69

3/29/18	I02	Ht-13-A	25	54	19	3.005	0.347	3	31.999	16	0.50
3/29/18	I02	Ht-13-A	25	54	19	3.005	0.347	4	23.943	19	0.79
3/29/18	I03	Ht-13-A	25	52	29	3.137	0.542	1	64.128	34	0.53
3/29/18	I03	Ht-13-A	25	52	29	3.137	0.542	2	51.663	40	0.77
3/29/18	I03	Ht-13-A	25	52	29	3.137	0.542	3	44.967	30	0.67
3/29/18	I03	Ht-13-A	25	52	29	3.137	0.542	4	60.645	50	0.82
3/29/18	I03	Ht-13-A	25	52	29	3.137	0.542	5	31.913	37	1.16
3/29/18	I04	Ht-13-A	25	28	20	2.459	0.401	1	41.416	36	0.87
3/29/18	I04	Ht-13-A	25	28	20	2.459	0.401	2	61.962	64	1.03
3/29/18	I04	Ht-13-A	25	28	20	2.459	0.401	3	58.535	84	1.44
3/29/18	I04	Ht-13-A	25	28	20	2.459	0.401	4	26.593	39	1.47
3/29/18	I05	Ht-13-A	25	27	17	2.275	0.237	1	44.903	30	0.67
3/29/18	I05	Ht-13-A	25	27	17	2.275	0.237	2	53.617	42	0.78
3/29/18	I05	Ht-13-A	25	27	17	2.275	0.237	3	34.587	18	0.52
3/29/18	I05	Ht-13-A	25	27	17	2.275	0.237	4	37.179	30	0.81
3/29/18	I05	Ht-13-A	25	27	17	2.275	0.237	5	18.388	14	0.76
3/29/18	I06	Ht-13-A	25	55	37	2.976	0.356	1	60.488	63	1.04
3/29/18	I06	Ht-13-A	25	55	37	2.976	0.356	2	48.902	50	1.02
3/29/18	I06	Ht-13-A	25	55	37	2.976	0.356	3	61.306	78	1.27
3/29/18	I06	Ht-13-A	25	55	37	2.976	0.356	4	45.04	47	1.04
3/29/18	I06	Ht-13-A	25	55	37	2.976	0.356	5	16.531	20	1.21
3/29/18	J01	Ht-13-A	250	35	11	2.114	0.246	1	59.905	27	0.45
3/29/18	J01	Ht-13-A	250	35	11	2.114	0.246	2	59.18	27	0.46
3/29/18	J01	Ht-13-A	250	35	11	2.114	0.246	3	41.389	25	0.60
3/29/18	J01	Ht-13-A	250	35	11	2.114	0.246	4	37.737	28	0.74
3/29/18	J01	Ht-13-A	250	35	11	2.114	0.246	5	12.466	14	1.12
3/29/18	J02	Ht-13-A	250	37	22	3.134	0.356	1	62.941	42	0.67
3/29/18	J02	Ht-13-A	250	37	22	3.134	0.356	2	65.214	46	0.71
3/29/18	J02	Ht-13-A	250	37	22	3.134	0.356	3	45.689	31	0.68
3/29/18	J02	Ht-13-A	250	37	22	3.134	0.356	4	33.223	28	0.84
3/29/18	J03	Ht-13-A	250	28	11	2.797	0.439	1	47.034	22	0.47
3/29/18	J03	Ht-13-A	250	28	11	2.797	0.439	2	57.225	20	0.35
3/29/18	J03	Ht-13-A	250	28	11	2.797	0.439	3	43.732	29	0.66
3/29/18	J03	Ht-13-A	250	28	11	2.797	0.439	4	39.143	29	0.74
3/29/18	K01	Ht-13-A	2500	27	8	2.089	0.261	1	49.032	13	0.27
3/29/18	K01	Ht-13-A	2500	27	8	2.089	0.261	2	48.232	8	0.17
3/29/18	K01	Ht-13-A	2500	27	8	2.089	0.261	3	38.188	6	0.16
3/29/18	K01	Ht-13-A	2500	27	8	2.089	0.261	4	21.303	7	0.33
3/29/18	K02	Ht-13-A	2500	28	17	2.889	0.459	1	57.174	20	0.35

3/29/18	K02	Ht-13-A	2500	28	17	2.889	0.459	2	59.138	27	0.46
3/29/18	K02	Ht-13-A	2500	28	17	2.889	0.459	3	36.821	18	0.49
3/29/18	K02	Ht-13-A	2500	28	17	2.889	0.459	4	40.016	26	0.65
3/29/18	K02	Ht-13-A	2500	28	17	2.889	0.459	5	18.023	14	0.78
3/29/18	K03	Ht-13-A	2500	18	10	2.651	0.354	1	59.347	11	0.19
3/29/18	K03	Ht-13-A	2500	18	10	2.651	0.354	2	59.085	12	0.20
3/29/18	K03	Ht-13-A	2500	18	10	2.651	0.354	3	42.602	10	0.23
3/29/18	K03	Ht-13-A	2500	18	10	2.651	0.354	4	32.948	9	0.27
3/29/18	L01	Ht-13-A	25000	2	0	2.532	0.2	1	27.609	0	0.00
3/29/18	L01	Ht-13-A	25000	2	0	2.532	0.2	2	51.427	0	0.00
3/29/18	L01	Ht-13-A	25000	2	0	2.532	0.2	3	56.074	0	0.00
3/29/18	L01	Ht-13-A	25000	2	0	2.532	0.2	4	49.984	0	0.00
3/29/18	L02	Ht-13-A	25000	0	1	2.564	/	1	51.143	0	0.00
3/29/18	L02	Ht-13-A	25000	0	1	2.564	/	2	40.363	0	0.00
3/29/18	L02	Ht-13-A	25000	0	1	2.564	/	3	51.711	0	0.00
3/29/18	L02	Ht-13-A	25000	0	1	2.564	/	4	47.076	0	0.00
3/29/18	L03	Ht-13-A	25000	1	0	2.583	0.168	1	58.122	0	0.00
3/29/18	L03	Ht-13-A	25000	1	0	2.583	0.168	2	56.403	0	0.00
3/29/18	L03	Ht-13-A	25000	1	0	2.583	0.168	3	45.537	0	0.00
3/29/18	L03	Ht-13-A	25000	1	0	2.583	0.168	4	35.19	0	0.00

Table 5.2. Raw data for stringent mouse infection experiments in S2.8 Fig.

Number of adult parasites recovered (male/female), liver weight, spleen weight, and egg counts per liver section were reported for each experiment.

Date	Mouse ID	Drug	Conc. (nM)	Male	Female	Whole Liver Weight (g)	Spleen Weight (g)	Liver section	Liver Area (mm^2)	Egg counts	Egg/Area (/mm^2)
5/2/18	A01	Control	0	18	8	3.482	0.46	1	62.533	25	0.40
5/2/18	A01	Control	0	18	8	3.482	0.46	2	51.386	23	0.45
5/2/18	A01	Control	0	18	8	3.482	0.46	3	52.854	29	0.55
5/2/18	A01	Control	0	18	8	3.482	0.46	4	36.08	21	0.58
5/2/18	A02	Control	0	12	10	4.304	0.648	1	88.962	63	0.71
5/2/18	A02	Control	0	12	10	4.304	0.648	2	84.495	48	0.57
5/2/18	A02	Control	0	12	10	4.304	0.648	3	65.721	42	0.64
5/2/18	A02	Control	0	12	10	4.304	0.648	4	40.943	27	0.66
5/2/18	A03	Control	0	20	13	3.753	0.85	1	68.933	32	0.46
5/2/18	A03	Control	0	20	13	3.753	0.85	2	55.666	20	0.36
5/2/18	A03	Control	0	20	13	3.753	0.85	3	68.713	26	0.38
5/2/18	A03	Control	0	20	13	3.753	0.85	4	39.585	21	0.53
5/2/18	A04	Control	0	14	10	3.541	0.499	1	39.485	18	0.46
5/2/18	A04	Control	0	14	10	3.541	0.499	2	69.497	24	0.35
5/2/18	A04	Control	0	14	10	3.541	0.499	3	60.621	27	0.45
5/2/18	A04	Control	0	14	10	3.541	0.499	4	54.687	31	0.57
5/2/18	A05	Control	0	19	16	5.241	1.005	1	88.718	60	0.68
5/2/18	A05	Control	0	19	16	5.241	1.005	2	96.182	64	0.67
5/2/18	A05	Control	0	19	16	5.241	1.005	3	72.544	42	0.58
5/2/18	A05	Control	0	19	16	5.241	1.005	4	18.775	19	1.01
5/2/18	A05	Control	0	19	16	5.241	1.005	5	62.878	39	0.62
5/2/18	A06	Control	0	19	12	3.443	0.602	1	15.415	20	1.30
5/2/18	A06	Control	0	19	12	3.443	0.602	2	39.741	29	0.73
5/2/18	A06	Control	0	19	12	3.443	0.602	3	52.305	25	0.48
5/2/18	A06	Control	0	19	12	3.443	0.602	4	49.325	55	1.12
5/2/18	A06	Control	0	19	12	3.443	0.602	5	55.461	57	1.03
5/2/18	A07	Control	0	21	11	3.636	0.524	1	46.911	35	0.75
5/2/18	A07	Control	0	21	11	3.636	0.524	2	72.462	27	0.37
5/2/18	A07	Control	0	21	11	3.636	0.524	3	65.29	40	0.61
5/2/18	A07	Control	0	21	11	3.636	0.524	4	58.593	38	0.65
5/2/18	A08	Control	0	16	11	3.65	0.691	1	66.826	37	0.55
5/2/18	A08	Control	0	16	11	3.65	0.691	2	61.751	30	0.49

5/2/18	A08	Control	0	16	11	3.65	0.691	3	75.94	31	0.41
5/2/18	A08	Control	0	16	11	3.65	0.691	4	43.211	24	0.56
5/2/18	B01	SPF	2500	0	0	2.377	0.154	1	23.105	0	0.00
5/2/18	B01	SPF	2500	0	0	2.377	0.154	2	21.31	0	0.00
5/2/18	B01	SPF	2500	0	0	2.377	0.154	3	46.527	0	0.00
5/2/18	B01	SPF	2500	0	0	2.377	0.154	4	39.399	0	0.00
5/2/18	B02	SPF	2500	0	0	1.491	0.121	1	27.607	0	0.00
5/2/18	B02	SPF	2500	0	0	1.491	0.121	2	33.566	0	0.00
5/2/18	B02	SPF	2500	0	0	1.491	0.121	3	30.196	0	0.00
5/2/18	B03	SPF	2500	0	0	2.094	0.128	1	44.787	0	0.00
5/2/18	B03	SPF	2500	0	0	2.094	0.128	2	47.02	0	0.00
5/2/18	B03	SPF	2500	0	0	2.094	0.128	3	34.936	0	0.00
5/2/18	B03	SPF	2500	0	0	2.094	0.128	4	21.43	0	0.00
5/2/18	B04	SPF	2500	0	0	2.448	0.165	1	19.452	0	0.00
5/2/18	B04	SPF	2500	0	0	2.448	0.165	2	36.77	0	0.00
5/2/18	B04	SPF	2500	0	0	2.448	0.165	3	46.207	0	0.00
5/2/18	B04	SPF	2500	0	0	2.448	0.165	4	39.301	0	0.00
5/2/18	B05	SPF	2500	0	0	1.475	0.089	1	13.651	0	0.00
5/2/18	B05	SPF	2500	0	0	1.475	0.089	2	27.139	0	0.00
5/2/18	B05	SPF	2500	0	0	1.475	0.089	3	34.939	0	0.00
5/2/18	B05	SPF	2500	0	0	1.475	0.089	4	32.081	0	0.00
5/2/18	B06	SPF	2500	0	0	1.984	0.155	1	36.944	0	0.00
5/2/18	B06	SPF	2500	0	0	1.984	0.155	2	40.445	0	0.00
5/2/18	B06	SPF	2500	0	0	1.984	0.155	3	47.534	0	0.00
5/2/18	B06	SPF	2500	0	0	1.984	0.155	4	14.945	0	0.00
5/2/18	B07	SPF	2500	0	0	2.588	0.204	1	42.766	0	0.00
5/2/18	B07	SPF	2500	0	0	2.588	0.204	2	43.959	0	0.00
5/2/18	B07	SPF	2500	0	0	2.588	0.204	3	57.705	0	0.00
5/2/18	B07	SPF	2500	0	0	2.588	0.204	4	32.43	0	0.00
5/2/18	C01	Ht-13-A-pr	2500	7	1	1.954	0.213	1	17.496	0	0.00
5/2/18	C01	Ht-13-A-pr	2500	7	1	1.954	0.213	2	40.122	3	0.07
5/2/18	C01	Ht-13-A-pr	2500	7	1	1.954	0.213	3	49.097	2	0.04
5/2/18	C01	Ht-13-A-pr	2500	7	1	1.954	0.213	4	39.664	0	0.00
5/2/18	C02	Ht-13-A-pr	2500	1	0	2.483	0.242	1	26.442	0	0.00
5/2/18	C02	Ht-13-A-pr	2500	1	0	2.483	0.242	2	49.634	0	0.00
5/2/18	C02	Ht-13-A-pr	2500	1	0	2.483	0.242	3	47.406	0	0.00
5/2/18	C02	Ht-13-A-pr	2500	1	0	2.483	0.242	4	36.789	0	0.00

5/2/18	C03	Ht-13-A-pr	2500	0	0	2.466	0.219	1	53.603	0	0.00
5/2/18	C03	Ht-13-A-pr	2500	0	0	2.466	0.219	2	48.816	0	0.00
5/2/18	C03	Ht-13-A-pr	2500	0	0	2.466	0.219	3	26.58	0	0.00
5/2/18	C03	Ht-13-A-pr	2500	0	0	2.466	0.219	4	25.899	0	0.00
5/2/18	C04	Ht-13-A-pr	2500	3	2	2.202	0.26	1	50.099	13	0.26
5/2/18	C04	Ht-13-A-pr	2500	3	2	2.202	0.26	2	37.982	10	0.26
5/2/18	C04	Ht-13-A-pr	2500	3	2	2.202	0.26	3	48.146	10	0.21
5/2/18	C04	Ht-13-A-pr	2500	3	2	2.202	0.26	4	18.532	7	0.38
5/2/18	C05	Ht-13-A-pr	2500	5	3	2.511	0.411	1	19.372	3	0.15
5/2/18	C05	Ht-13-A-pr	2500	5	3	2.511	0.411	2	46.58	3	0.06
5/2/18	C05	Ht-13-A-pr	2500	5	3	2.511	0.411	3	35.327	8	0.23
5/2/18	C05	Ht-13-A-pr	2500	5	3	2.511	0.411	4	46.605	5	0.11
5/2/18	C06	Ht-13-A-pr	2500	0	0	1.741	0.126	1	49.957	0	0.00
5/2/18	C06	Ht-13-A-pr	2500	0	0	1.741	0.126	2	46.005	0	0.00
5/2/18	C06	Ht-13-A-pr	2500	0	0	1.741	0.126	3	26.182	0	0.00
5/2/18	C06	Ht-13-A-pr	2500	0	0	1.741	0.126	4	17.331	0	0.00
5/2/18	C07	Ht-13-A-pr	2500	0	0	1.604	0.158	1	35.4	0	0.00
5/2/18	C07	Ht-13-A-pr	2500	0	0	1.604	0.158	2	36.347	0	0.00
5/2/18	C07	Ht-13-A-pr	2500	0	0	1.604	0.158	3	40.433	0	0.00
5/2/18	C07	Ht-13-A-pr	2500	0	0	1.604	0.158	4	17.939	0	0.00
5/2/18	D01	Ht-13-A	2500	8	3	2.172	0.259	1	38.172	2	0.05
5/2/18	D01	Ht-13-A	2500	8	3	2.172	0.259	2	31.155	7	0.22
5/2/18	D01	Ht-13-A	2500	8	3	2.172	0.259	3	31.997	6	0.19
5/2/18	D01	Ht-13-A	2500	8	3	2.172	0.259	4	26.236	5	0.19
5/2/18	D02	Ht-13-A	2500	2	2	4.326	0.479	1	61.746	21	0.34
5/2/18	D02	Ht-13-A	2500	2	2	4.326	0.479	2	63.851	13	0.20
5/2/18	D02	Ht-13-A	2500	2	2	4.326	0.479	3	51.045	11	0.22
5/2/18	D02	Ht-13-A	2500	2	2	4.326	0.479	4	32.559	9	0.28
5/2/18	D03	Ht-13-A	2500	0	0	2.033	0.149	1	47.025	0	0.00
5/2/18	D03	Ht-13-A	2500	0	0	2.033	0.149	2	47.555	0	0.00
5/2/18	D03	Ht-13-A	2500	0	0	2.033	0.149	3	32.143	0	0.00
5/2/18	D03	Ht-13-A	2500	0	0	2.033	0.149	4	25.619	0	0.00
5/2/18	D04	Ht-13-A	2500	4	3	2.409	0.311	1	55.632	6	0.11
5/2/18	D04	Ht-13-A	2500	4	3	2.409	0.311	2	48.178	7	0.15

5/2/18	D04	Ht-13-A	2500	4	3	2.409	0.311	3	46.768	3	0.06
5/2/18	D04	Ht-13-A	2500	4	3	2.409	0.311	4	21.357	3	0.14
5/2/18	D05	Ht-13-A	2500	2	0	1.748	0.171	1	16.832	0	0.00
5/2/18	D05	Ht-13-A	2500	2	0	1.748	0.171	2	41.803	0	0.00
5/2/18	D05	Ht-13-A	2500	2	0	1.748	0.171	3	38.53	0	0.00
5/2/18	D05	Ht-13-A	2500	2	0	1.748	0.171	4	43.626	0	0.00
5/2/18	D06	Ht-13-A	2500	0	0	2.346	0.195	1	57.338	0	0.00
5/2/18	D06	Ht-13-A	2500	0	0	2.346	0.195	2	39.817	0	0.00
5/2/18	D06	Ht-13-A	2500	0	0	2.346	0.195	3	55.969	0	0.00
5/2/18	D06	Ht-13-A	2500	0	0	2.346	0.195	4	27.065	0	0.00
5/2/18	D07	Ht-13-A	2500	7	1	2.809	0.273	1	23.27	1	0.04
5/2/18	D07	Ht-13-A	2500	7	1	2.809	0.273	2	53.144	4	0.08
5/2/18	D07	Ht-13-A	2500	7	1	2.809	0.273	3	82.545	6	0.07
5/2/18	D07	Ht-13-A	2500	7	1	2.809	0.273	4	52.404	5	0.10
5/2/18	D07	Ht-13-A	2500	7	1	2.809	0.273	5	55.701	8	0.14

Appendix 2: Supplementary data for Chapter 3

Table 6.1. Cercaria motility phenotypes upon drug treatments.

Each background color represents a different libaray.

CAS	Name	Activity	10 μM 10 mins	10 μM 30 mins	10 μM 60 mins	1 μM 10 mins	1 μM 30 mins	1 μM 60 mins
1166-34-3	Cinanserin·HCl	Serotonin 5-HT 2 antagonist.	No	Partial paralyzed	No	N/D	N/D	N/D
19728-88-2	Methiothepin maleate	Serotonin 5-HT 1 / 5-HT 2 antagonist. 5-HT autoreceptor antagonist.	Paralyzed with occasional wriggling	Paralyzed with occasional wriggling	Paralyzed with occasional wriggling	No	No	No
110429-49-8	Paroxetine·HCl	Highly potent and selective serotonin (5-HT) uptake inhibitor	Partial paralyzed	No	No	N/D	N/D	N/D
77372-73-7	6-Nitroquipazine maleate	Serotonin uptake inhibitor.	Paralyzed with occasional wriggling	Paralyzed with occasional wriggling	No	N/D	N/D	N/D
	N-Chlorophenyl-N-hexylpiperazine	0	Wriggling	Wriggling	Wriggling	No	No	No
193611-72-2	BRL 15572·HCl	Serotonin 5-HT 1D antagonist.	Paralyzed with occasional wriggling	Paralyzed	Paralyzed	No	No	No
176912-51-2	Metergoline phenylmethyl ester	Serotonin 5-HT 1 / 5-HT 2 antagonist; activity at 5-HT 1D.	No	Wriggling	Paralyzed with occasional wriggling	No	No	No
17321-77-6	Clomipramine·HCl	Serotonin uptake inhibitor.	Wriggling	Wriggling	Wriggling	No	No	No
57432-61-8	Methylergometrine maleate	Serotonin 5-HT 1 / 2 antagonist.	No	Partial paralyzed	No	N/D	N/D	N/D
22260-51-1	Bromocriptine mesylate	Dopamine agonist	No	No	Paralyzed	No	No	No
2058-52-8	Clothiapine	Dopamine D4 and D2 antagonist	Wriggling	Wriggling	Wriggling	No	No	No

CAS	Name	Activity	10 μM 10 mins	10 μM 30 mins	10 μM 60 mins	1 μM 10 mins	1 μM 30 mins	1 μM 60 mins
67469-78-7	GBR-12909·2HCl	Dopamine uptake inhibitor	Paralyzed	Paralyzed	Paralyzed	No	No	No
130-61-0	Thioridazine·HCl	Dopamine D4 antagonist	Paralyzed	Paralyzed	Paralyzed	No	No	No
314-19-2	(R)-(-)-Apomorphine·HCl	Dopamine agonist	Paralyzed	Paralyzed	Paralyzed	Paralyzed	Paralyzed	Paralyzed
69-09-0	Chlorpromazine·HCl	Dopamine antagonist	Wriggling	Wriggling	Wriggling	No	No	No
2709-56-0	Flupentixol	Dopmaine antagonist	Wriggling	Wriggling	Wriggling	No	No	No
146-56-5	Fluphenazine·2HCl	Dopamine antagonist	No	Wriggling	Wriggling	No	No	No
19875-60-6	Lisuride maleate	Dopamine D2 agonist, dopamine D1 antagonist	Paralyzed	Partial paralyzed	Wriggling	Paralyzed	Partial paralyzed	Partial paralyzed
66104-23-2	Pergolide mesylate	Dopamine agonist	Paralyzed with occasional wriggling	Paralyzed with occasional wriggling	Paralyzed with occasional wriggling	Paralyzed	Paralyzed	Paralyzed
100999-26-6	CY 208-243	Selective D1 agonist	No	No	Partial paralyzed	No	No	No
52-86-8	Haloperidol	Dopamine antagonist	No	Wriggling	Wriggling	No	No	No
1135278-61-3	3'-Fluorobenzylspiperone maleate	D2 receptor ligand	Paralyzed	Paralyzed	Paralyzed	Wriggling	Wriggling	No
1217473-50-1	(+)-UH 232 maleate	D3 dopamine agonist	No	Paralyzed	Paralyzed with occasional wriggling	No	Partial paralyzed	No
24140-98-5	2-Chloro-11-(4-methylpiperazino)dibenz[B,F]oxepin maleate	D4 receptor ligand	Paralyzed with occasional wriggling	No	No	N/D	N/D	N/D
170856-41-4	PNU 96415E	High affinity dopamine D4 receptor ligand	Wriggling	Wriggling	Wriggling (transforming-like)	No	No	No
156337-32-5	L-741,742·HCl	D4 dopamine antagonist	Paralyzed with occasional wriggling	Paralyzed	Paralyzed	No	No	No

CAS	Name	Activity	10 μM 10 mins	10 μM 30 mins	10 μM 60 mins	1 μM 10 mins	1 μM 30 mins	1 μM 60 mins
81409-90-7	Cabergoline	Selective D2 like dopamine receptor agonist	No	No	Partial paralyzed	No	No	No
14008-79-8	3-α-[(4-chlorophenyl)phenylmethoxy]tropane·HCl	Dopamine uptake inhibitor	No	Wriggling	Wriggling	No	No	No
67469-57-2	GBR 12783·2HCl	Dopamine uptake inhibitor	Paralyzed	Paralyzed	Paralyzed	No	No	No
76778-22-8	GBR 12935·2HCl	Dopamine uptake inhibitor	Paralyzed	Paralyzed	Paralyzed	No	No	No
77862-93-2	GBR 13069·2HCl	Dopamine uptake inhibitor	Paralyzed	Paralyzed	Paralyzed	No	No	No
440-17-5	Trifluoperazine·2HCl	D2 dopamine antagonist	Paralyzed	Paralyzed	Paralyzed	No	No	No
1021868-80-3	L-750,667·3HCl	D4 dopamine antagonist	Wriggling	Wriggling	Wriggling	No	No	No
145307-34-2	R(+)-6-BROMO-APB·HBr	D1 dopamine agonist	Wriggling	Wriggling	Wriggling	No	Partial paralyzed	Paralyzed
	A-77636·HCl·H2O	D1 dopamine agonist	No	Partial paralyzed	Paralyzed with occasional wriggling	No	No	No
129-51-1	Ergonovine maleate	Dopamine partial agonist	No	Paralyzed	Paralyzed	No	No	No
80751-65-1	(±)-SKF-82958·HBr	D1 dopamine agonist	Wriggling	Wriggling	Paralyzed with occasional wriggling	No	No	Partial paralyzed
65273-66-7	Dipropyldopamine·HBr	Dopamine agonist	Paralyzed	Paralyzed	Paralyzed	No	No	No
77630-01-4	R(-)-2,10,11-Trihydroxyaporphine·HBr	Dopamine agonist	No	No	Partial paralyzed	No	No	No
77630-02-5	R(-)-2,10,11-Trihydroxy-N-propyl-noraporphine·HBr	D2 dopamine agonist	Paralyzed	Paralyzed	Paralyzed	No	No	No

CAS	Name	Activity	10 µM 10 mins	10 µM 30 mins	10 µM 60 mins	1 µM 10 mins	1 µM 30 mins	1 µM 60 mins
71787-90-1	(±)-2-(N-phenylethyl-N-propyl)amino-5-hydroxytetralin·HCl	D2 dopamine agonist	Wriggling	Wriggling	Wriggling (transforming-like)	No	No	No
138982-67-9	Ziprasidone·HCl·H2O	Dopamine D2 antagonist	Wriggling	Wriggling	Wriggling	No	No	No
64-47-1	Physostigmine hemisulfate	Cholinesterase inhibitor.	No	No	Partial paralyzed	No	No	No
120447-62-3	(±)-Vesamicol·HCl	Acetylcholine storage inhibitor.	Wriggling, stretching and clumping up	Wriggling, stretching and clumping up	Wriggling, stretching and clumping up	No	Wriggling	Wriggling
148152-66-3	(±)-Epibatidine	Nicotinic agonist.	Partial wriggling	Swim in place, more active than normal	Swim in place, more active than normal	No	No	No
115066-04-1	2-(α-Naphthoyl)-ethyltrimethylammonium iodide	Choline acetyltransferase inhibitor.	No	Slower tail beating	Clumping up and shaking in the bottom	No	No	No
300-08-3	Arecoline·HBr	Muscarinic agonist.	Paralyzed	Paralyzed	Paralyzed	Wriggling	Partial paralyzed	Partial paralyzed
52358-55-1	Delcorine	Nicotinic antagonist.	No	More active than normal	Shaking/ spinning in the bottom	No	No	No
68844-77-9	Astemizole	H1 Histamine antagonist.	Shaking/ spinning in the bottom (partial)	Shaking/ spinning in the bottom (partial)	Shaking/ spinning in the bottom (partial)	No	No	No
66357-59-3	Ranitidine·HCl	H2 histamine antagonist.	Abnormal shakes when swimming	Abnormal shakes when swimming	Abnormal shakes when swimming	No	No	No
169505-93-5	RS 17053·HCl	Alpha 1A adrenergic antagonist.	Wriggling on surface	Paralyzed on surface	Decapitated	No	No	No
67339-62-2	ARC 239·2HCl	Alpha 2B adrenergic antagonist.	Wriggling	Wriggling	Wriggling	No	No	No
174422-5	Riluzole·HCl	GABA uptake inhibitor.	Paralyzed with occasional wriggling	Paralyzed with occasional wriggling	Paralyzed with occasional wriggling	No	No	No

CAS	Name	Activity	10 μM 10 mins	10 μM 30 mins	10 μM 60 mins	1 μM 10 mins	1 μM 30 mins	1 μM 60 mins
80206-91-3	8-(p-Sulfophenyl)theophylline	Adenosine antagonist	Wriggling	Partial wriggling	Partial wriggling	No	No	No
207572-62-1	4-phenyl-1-(4-phenylbutyl)piperidine maleate	Sigma ligand.	Paralyzed with occasional wriggling	Paralyzed	Paralyzed with a lot decapitation	No	No	No