Targeting Schistosome Cholinesterases

for Vaccine & Drug Development

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Publications by the candidate relevant to the thesis

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Abstract

The nervous system of schistosomes has been successfully targeted by anthelmintic drugs but the use of many of these has discontinued because of toxic side effects and so there is a need to better understand key neuronal processes at a molecular level to develop safer and more effective intervention strategies that target this vital system. Cholinesterases - acetylcholinesterases (AChE)s and butyrylcholinesterases (BChE)s - are key enzymes that play a pivotal role in the nervous system of schistosomes by regulating neurotransmission through acetylcholine hydrolysis and, accordingly, are an example of such an intervention target.

The first results chapter (chapter two) of this thesis investigated the anti-schistosome efficacy of polypyridylruthenium (II) complexes and showed they were active against all intra-mammalian stages of *S. mansoni*. Two compounds, $Rubb_{12}$-tri and $Rubb_{7}$-tnl, which were among the most potent in their ability to kill schistosomula and adult worms and inhibit egg hatching *in vitro*, were assessed for their efficacy in a mouse model of schistosomiasis using 5 consecutive daily i.v. doses of 2 mg/kg ($Rubb_{12}$-tri) and 10 mg/kg ($Rubb_{7}$-tnl). Mice treated with $Rubb_{12}$-tri showed an average 42% reduction ($P = 0.009$), over two independent trials, in adult worm burden. Liver egg burdens were not significantly decreased in either drug-treated group but ova from both of these groups showed significant decreases in hatching ability ($Rubb_{12}$-tri - 68%, $Rubb_{7}$-tnl - 56%) and were significantly morphologically altered ($Rubb_{12}$-tri - 62% abnormal, $Rubb_{7}$-tnl - 35% abnormal). I hypothesize that the drugs exerted their activity, at least partially, through inhibition of both neuronal and tegumental acetylcholinesterases (AChEs), as worms treated *in vitro*

showed significant decreases in activity of these enzymes. Further, treated parasites exhibited a significantly decreased ability to uptake glucose, significantly depleted glycogen stores and withered tubercules (a site of glycogen storage), implying drug-mediated interference in this nutrient acquisition pathway.

Chapter three of this thesis provided the first comprehensive molecular characterization of three *S. mansoni* cholinesterases (*Sm*ChEs), designated as *Sm*AChE1, *Sm*BChE1 and *Sm*AChE3, which were identified from the interrogation of the now wholly annotated *S. mansoni* genome. Anti-*Sm*ChE antibodies localized the proteins to the tegument and neuromusculature of adults and schistosomula and developmental expression profiling differed among the molecules, suggestive of functions extending beyond traditional cholinergic signaling for each of them. I also reported the presence of ChE activity in parasite ES products for the first time and proteomically identified the molecules responsible (*Sm*AChE1 and *Sm*BChE1). Functional recombinant versions of the three SmChEs were produced in *Pichia pastoris* and enzyme nomenclature (AChE or BChE) was verified based on substrate preference. Lastly, in the first characterization study of a BChE from helminths, evidence is provided that *Sm*BChE1 may act as a bio-scavenger of AChE inhibitors as the addition of recombinant *Sm*BChE1 to parasite cultures mitigated the effect of the anti-schistosomal AChE inhibitor dichlorvos whereas *Sm*BChE1-silenced parasites displayed increased sensitivity to dichlorvos.

*Sm*ChEs were further characterized by RNAi-based experiments in chapter four of this thesis. RNAi-mediated silencing of individual *Sm*ChEs, or simultaneous silencing of all three *Sm*ChEs, significantly suppressed transcript and protein expression levels and AChE activity in parasites. In a dissection of the hypothesis that tegumental AChE mediates exogenous glucose scavenging by the parasite, I showed that RNAi-mediated knockdown of *Sm*AChE1 and *Sm*AChE3, but not *Sm*BChE1, significantly reduced glucose uptake by schistosomes. Parasite survivability *in vitro* and *in vivo* was significantly impaired with the silencing of SmChEs, either individually or in combination, attesting to the essentiality of these molecules.

Chapter five of this thesis explored the vaccine potential of *Sm*ChEs. When treated *in vitro* with anti-*Sm*ChE IgG, parasites displayed significantly decreased ChE activity, which eventually resulted in death. Vaccination with individual SmChEs, or a combination of all three SmChEs, significantly reduced worm burdens (28% - 38%, averaged across two independent trials) compared to controls. Liver egg burdens were significantly decreased for all mice across both trials (13% - 46%) except those vaccinated with *Sm*AChE1 in trial 1. Egg viability, as determined by egg hatching from liver homogenates, was significantly reduced in the groups vaccinated with the *Sm*ChE cocktail (40%) and SmAChE3 (46%). Surviving worms from each vaccinated group were significantly stunted and depleted of glycogen stores, compared to controls.

In conclusion, this thesis has identified the burgeoning potential of a new class of anti-schistosome drugs that, at least in part, target the nervous system of the parasite and provided

a comprehensive characterization of a family of ChEs from *S. mansoni*, giving compelling evidence for the essentiality of the proteins and their utility as intervention targets against schistosomiasis.

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List of Abbreviations

°C	degrees Celsius
2YT	2X YT (two times yeast and tryptone)
AA	Antibiotic/Antimycotic
ABTS	2,2'-Azino-Bis (3-Ethylbenziazoline-6-Sulfonic Acid)
ACh	acetylcholine
AChE	acetylcholinesterase
AcSCh	acetylthiocholine
ADCC	antibody-dependent cellular cytotoxicity
Alum	aluminium hydroxide
AOX	Alcohol Oxidase
AP	Alkaline phosphatase
bb_n	bis[4(4'-methyl-2,2'-bipyridyl)]-1,n-alkane (n = number)
BCA	Bicinchoninic acid
BCh	butrylcholine
BcSCh	butyrylthiocholine
BLAST	Basic Local Alignment Search Tool
BMGY	Buffered Glycerol-complex medium
BMMY	Buffered Methanol-complex medium
BSA	Bovine serum albumin
cDNA	complementary Deoxyribonucleic acid

ChE	cholinesterase
CNS	central nervous system
CO_2	carbon dioxide
DALYs	Disability Adjusted Life Years
DDVP	Dichlorvos
DEPC	Diethyl pyrocarbonate
DIR	drug-induced resistance
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DTNB	5'-dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EC_{50}	Concentration required to kill 50% of parasites
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ES	Excretory-secretory
FCS	Fetal calf serum
g	relative centrifugal force
g	gram
HRP	horseradish peroxidase
i.v.	Intravenous
IC_{50}	Concentration required to inhibit 50% of enzyme activity

IgG	Immunoglobulin class G
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilodalton
L	litre
m	milli
M	molar
mAChRs	muscarinic acetylcholine receptors
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionisation-Time Of Flight Mass Spectrometry
MDA	mass drug administration
MTT	3-(3,4-Dimethylthiazol-2yl)-5-diphenyl tetrazolium bromide
n	nano
nAChRs	nicotinic acetylcholine receptors
NCBI	National Centre for Biotechnology Information
OD	optical density
ORF	open reading frame
OXA	Oxamniquine
PAGE	polyacrylamide gel electrophoresis
PAS	peripheral anionic site
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.05% Tween 20

PCR	Polymerase chain reaction
PDE	Phosphodiesterase
pH	power of hydrogen
pNPP	p-Nitrophenyl phosphate
PNS	peripheral nervous system
PRiMA	Proline-Rich Membrane Anchor
PZQ	Praziquantel
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	revolution per minute
RT-PCR	Reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SEM	Scanning electron microscopy
SGTP	schistosome glucose transporters
siRNA	small interfering RNA
TAC	tris (p-aminophenyl) carbonium salts
WHO	World Health Organization
YPD	Yeast extract/Peptone/Dextrose
YPDS	Yeast extract/Peptone/Dextrose/sorbitol
µ	micro

CHAPTER 1

Introduction and Literature Review

1.1 Introduction

1.1.1 Schistosomiasis

Schistosomiasis, also known as Bilharzia, is one of the neglected tropical diseases caused by trematodes of the genus *Schistosoma* (Webster et al. 2006). Schistosomes, a digenetic trematode (flatworm) are blood-dwelling flukes which inhabit the host vasculature, producing eggs which cause the majority of disease manifestations resulting from infection (McManus et al. 2008). While there are over twenty species of *Schistosoma*, the three species of most public health importance are *Schistosoma mansoni*, which causes hepatic schistosomiasis; *Schistosoma haematobium*, causing urogenital schistosomiasis; and *Schistosoma japonicum*, which is the causative agent of hepato-intestinal schistosomiasis. Although less common and geographically restricted to a few areas, *Schistosoma intercalatum,* and *Schistosoma mekongi* are also medically important (Olveda et al. 2013).

1.1.2 Epidemiology and geographic distribution

Schistosomiasis is a common cause of morbidity and mortality worldwide and described as second only to malaria in its debilitating effects when disease burden is measured with reference to Disability Adjusted Life Years (DALYs). For this reason, the World Health Organization (WHO) prioritized schistosomiasis for focused global intervention (Hotez et al. 2007).

Schistosomes infect approximately 200 million people in over 78 countries, and more than 750 million people are at risk of acquiring the infection in endemic areas (Gryseels et al. 2006; Hotez

et al. 2010). The majority of cases (90%) occur in Africa (Utzinger et al. 2009). Globally, it is estimated that 120 million infected people are clinically symptomatic, with 20 million manifesting severe symptoms from the disease (Colley et al. 2014). Schistosomiasis contributes to more than 200, 000 deaths annually, with DALYs, lost to the disease potentially as high as 70 million (King et al. 2008). Infection with schistosomes can also inflict massive health and socio-economic burden on endemic areas with illness leading to disability and sluggish physical and mental development in children (Hotez et al. 2007).

The geographic distribution of the three dominant *Schistosoma* species in humans is attributed to the presence of a suitable intermediate host – the freshwater snail. *S. mansoni* and *S. haematobium* are endemic in Sub-Saharan Africa and the Arabian Peninsula, with the former also present in South America. *S. japonicum* is endemic in the swampland and lake regions of China, the Philippines, and Indonesia (Fig 1.1) (Gryseels et al. 2006).

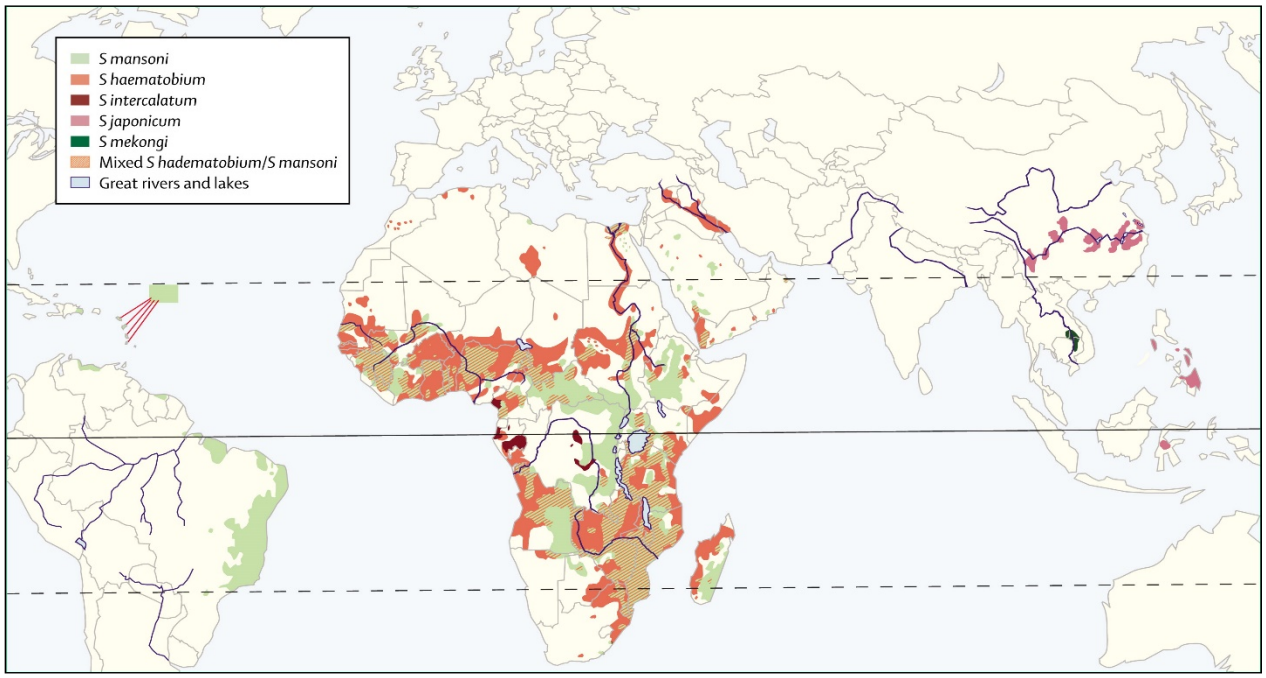


Fig 1.1. Geographic distribution of the common schistosome species infecting humans

(Gryseels et al. 2006).

1.1.3 Transmission

Endemic schistosomiasis transmissions have been reported in 52 countries of three continents with moderate to high transmission rates (Colley et al.). This highlights the need for preventative measures that improve the lives of millions of people living in these regions. The disease is highly prevalent in poor rural communities where there is poor access to safe drinking water and adequate sanitation as transmission usually occurs when water sources are contaminated with feces containing parasite eggs, which hatch in stagnant or slow-moving fresh water. *Schistosoma* transmission is highest among individuals that have frequent contact with stagnant water (fishers, farmers using irrigation, women fetching water), with the most vulnerable members of

the population being pregnant women and children, who if not treated, suffer chronic health consequences into later life (Hotez et al. 2007; McManus et al. 2008).

1.1.4 Lifecycle

It is notoriously difficult to study Schistosoma biology as the parasite has a complex life cycle involving two host; a definitive vertebrate host in which adult male and female worms grow and sexual reproduction follows, and a species-specific intermediate host, freshwater snails of the genus *Bulinus* (*S. haematobium*), *Biomphalaria* (*S. mansoni*), *Oncomelania* (*S. japonicum*) in which they multiply asexually (Utzinger et al. 2009). This multi-stage lifecycle is tightly regulated, apparently by strict control of stage-specific gene expression (Anderson et al. 2016). However, the mechanisms regulating vital gene expression in schistosomes are still poorly understood.

Eggs are excreted in feces or urine of the definitive hosts. Upon reaching fresh water, the deposited eggs hatch into free-swimming miracidia in response to changes in osmotic pressure, light and a decrease in temperature, reviewed elsewhere (Xu et al. 1990). Miracidia demonstrate host-seeking behaviors in response to chemosensory cues. It then penetrates the intermediate host, a process which is initiated by signals from macromolecular glycol-fatty acid conjugates found on the snail surface (Kalbe et al. 2004). Parasites within the snail develop into sporocysts and then cercariae (Collins et al. 2011). Sporocysts of *S. mansoni* possess a birth pore with acetylcholinesterase (AChE) activity and nervous and muscular structures that could enable release of the cercariae by actively opening and closing the pore activity (Driguez et al. 2016).

AChE activity in sporocysts has also been hypothesized to be involved in chronobiological mechanisms (Driguez et al. 2016). It is worth mentioning that the nervous system of *Schistosoma* larvae was primarily revealed using AChE staining (Bruckner et al. 1974)

Snails shed cercariae into their aqueous environment that penetrate the skin of a definitive host, causing them to undertake transformation into schistosomula. These larvae migrate through the dermal layers of the skin and then lung capillaries over a number of days to eventually reside in the vasculature of the liver, intestines or bladder, depending on the species (Ross et al. 2002). In these sites, mature worms pair at 5-6 weeks post-infection and spend the rest of their life (an average of 3-5 years) inside the host in the cupola. Female worms produce eggs, which enter the intestinal lumen or bladder to be voided in the feces or urine. Excreted eggs are fully embryonated and hatch only when they come in contact with bodies of fresh water to continue the cycle (Ross et al. 2002)

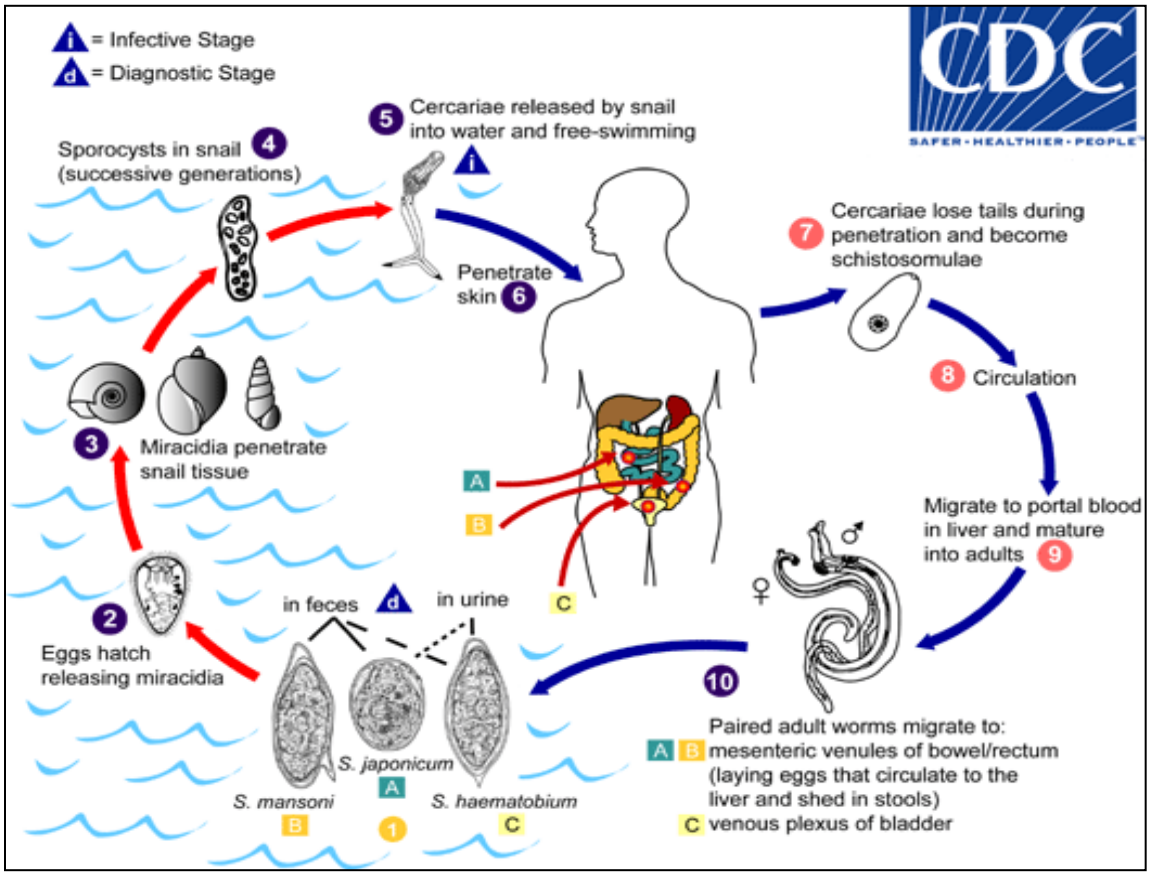


Fig 1.2. The life cycle of the schistosome parasite
(http://www.cdc.gov/parasites/schistosomiasis/biology.html).

1.1.5 Pathology and clinical manifestation

Schistosoma infection can be acute or chronic. Typically, parasites inside human tissues induce an immunological response that causes local and systemic pathological effects ranging from anemia, delayed growth and cognitive development, and malnutrition to organ-specific effects such as fibrosis of the liver and urogenital cancer (Gray et al. 2011).

Cercarial dermatitis (swimmer's itch) is the first stage of *Schistosoma* infection and is characterized by fever and a maculopapular rash at the site of skin penetration (Ross et al. 2001; Ross et al. 2002). The second stage of disease – acute schistosomiasis (Katayama fever) - is due to a host inflammatory response against the release of highly antigenic eggs. Acute infection usually occurs in children or young adults with no past exposure to the disease (Gryseels et al. 2006). It occurs within a couple of months after cercarial penetration with common symptoms including, fever, generalized myalgia, bloody diarrhea, and hepatosplenomegaly. The final stage - chronic schistosomiasis - arises mostly because of the host's unregulated immune response to entrapped eggs in tissues. It can present months to years after exposure, making diagnosis difficult. Excretory-secretory (ES) products released from the eggs deposited in different host tissues induce a localized inflammatory reaction arising from a T-helper 2 (Th2) type immune response, characterized by the formation of granuloma around the egg (Ashton et al. 2001). Prolonged years of granuloma formation due to massive infection results in extensive hepatic and intestinal fibrosis and collagen deposition that is the primary pathology of chronic schistosomiasis (Pearce et al. 2002; Sandor et al. 2003).

1.1.6 Chemotherapeutic intervention and challenges

Currently, safe and effective treatment of human schistosomiasis relies on the administration of a single drug, Praziquantel (PZQ) at an oral dose of 40 mg/kg (Trainor-Moss et al. 2016). It has comparatively few side effects and is effective against the adult stage of all common species of schistosomes pathogenic to humans, thus advantageous for the control of mixed infections

(Tchuem Tchuenté et al. 2013), but it does not affect eggs or juvenile stages (Gryseels et al. 2006) and does not provide absolute protection from reinfection. The precise mechanism of action of the drug remains unclear. However, experimental work in mice has demonstrated that PZQ treatment disrupts the tegument (worm surface), exposing tegumental antigens to the host immune system (Mehlhorn et al. 1981) which results in quantitative alteration of host-parasite-specific immune responses (Mutapi et al. 2007). Interestingly, in a subset of the population, PZQ-mediated modification of the immune response confers a degree of resistance to re-infection – drug-induced resistance (DIR) (Pearson et al. 2015).

Overall, in endemic areas where the risk of re-infection is high, preventive chemotherapy, or mass drug administration (MDA) using PZQ is the mainstay of control (Mduluza et al. 2001). This periodic MDA is however unsustainable as a stand-alone control measure and ideally, should be integrated with additional intervention strategies. Besides, there is considerable concern about the development of PZQ resistance (Botros et al. 2005). This scenario underscores the increasing need for the development of novel and inexpensive drugs against schistosomiasis.

Yet another obstacle to early intervention is the lack of accurate diagnostic tools that can distinguish between active and late infections. Current diagnosis of *Schistosoma* infection relies upon microscopic detection of species-characteristic eggs in stool samples, or urine (in the case of *S. haematobium*). When prevalence and intensity of infection are decreased after chemotherapy, however, microscopic methods become less sensitive (van der Werf et al. 2003).

Immunological approaches for the detection of *Schistosoma* antibodies or antigens (e.g., circulating cathodic and anodic antigen) (Kremsner et al. 1994) offer increased sensitivity but decreased specificity and difficulties in obtaining a genuinely quantitative diagnosis are inherent problems. PCR-based diagnostics have been developed to overcome these issues, but the need for specialized equipment and training to conduct these tests in endemic settings is a disadvantage (Utzinger et al. 2015).

1.1.7 A vaccine for schistosomiasis?

The current schistosomiasis control strategy that relies on periodic MDA of PZQ, is logistically difficult, expensive and difficult to achieve in our lifetime (Fenwick et al. 2016). The development of an anti-schistosome vaccine and its integrated use with sustainable MDA strategies, therefore, is an essential tool for the global fight against schistosomiasis.

The feasibility of a schistosomiasis vaccine has been demonstrated in a series of proof-of-concept studies where laboratory animals - initially mice (Dean et al. 1983) and, later, non-human primates (Kariuki et al. 2004) - were immunized with radiation-attenuated cercariae and found to be >80% resistant to a subsequent experimental schistosomal challenge. While the acquisition of protective immunity to human schistosomiasis, either naturally occurring or drug-induced, is well documented (Correa-Oliveira et al. 2000; Black et al. 2010), it can be slow in maturing and is not widespread throughout endemic populations. In order to augment this resistance and

stimulate anti-schistosomal immunity, it is crucial that an antigen threshold is reached (Wilson et al. 2014) to perturb parasite pathogenesis through immune intervention.

Currently, vaccination strategies are focused on targeting the intra-mammalian stages of the parasite, particularly, the migrating schistosomula (widely considered most susceptible to immune attack) and adult female (to prevent the release of tissue-destructive and pathology-inducing eggs). Further, proteins of the tegument and ES fraction of these parasitic stages are arguably the most important groups of molecules to target for vaccine antigen identification due to their role in numerous fundamental interactions with the host and accessibility to the host immune system (Loukas et al. 2007). Most approaches have concentrated on identifying protective antigens from these stages and delivering these recombinant constructs or purified extracts in various formulations (McManus et al. 2008). The availability of comprehensively annotated genomes for all human schistosome species (Berriman et al. 2009; Young et al. 2012) and the publication of juvenile and adult tegumental and ES proteomic studies (Gaze et al. 2014; Pearson et al. 2015; Sotillo et al. 2015; Driguez et al. 2016) have provided researchers with a goldmine of bioinformatic and evidence-based information regarding the protein composition of these “sites of vaccine potential”, significantly facilitating the rational selection of vaccine candidate antigens.

Table 1. 1. Current lead vaccine candidates for schistosomiasis

Feature	*Candidate antigen*				
	SmTSP-2	**Sm-p80**	**Sh28GST**	**Sm14**	**Sm29**
Known function of the antigen	Molecular organization of membranes (Tran et al. 2010)	Renewal/recycling of the parasite surface (Siddiqui et al. 1993)	Detoxification of ROI (Vibanco-Perez et al. 1998)	FABP for lipid uptake from the host (Moser et al. 1991)	ND
Expressed by all human stage	Yes (Smyth et al. 2003)	Yes (Siddiqui et al. 1993)	Yes (Walker et al. 1993)	Yes (Brito et al. 2002)	Adults and schistosomula (Cardoso et al. 2008)
Essential for survival	Yes[§] (Tran et al. 2010)	ND[*]	ND	Yes[§§] (Furlong 1991)	ND
Cross-species protection	ND	Yes(Karmakar et al. 2014)	Yes (Boulanger et al. 1995)	Yes (Tendler et al. 1996)	ND
Located on tegument	Yes (Braschi et al. 2006; Tran et al. 2006)	Yes (Siddiqui et al. 1993)	Yes (Porchet et al. 1994)	Yes (Brito et al. 2002)	Yes (Cardoso et al. 2008)
antigenic polymorphism	Conserved (Cupit et al. 2011)	ND	Conserved (Trottein et al. 1992)	Polymorphic(Ramos et al. 2003)	ND

Antibodies inhibit parasite growth *in vitro*	ND	Yes, ADCC** (Torben et al. 2012)	Yes, ADCC (Riveau et al. 1998)	ND	ND
Evidence of protection in animal models	Mice (Tran et al. 2006)	Primates (Ahmad et al. 2011)	Primates (Boulanger et al. 1999)	Mice (Tendler et al. 1996)	Mice (Cardoso et al. 2008; Alves et al. 2015)
Cross-reactivity with vertebrate proteins	ND	No (Siddiqui et al. 1993)	No (Trottein et al. 1992; Hughes 1993)	ND	ND
Antibody recognition by resistant humans	Yes (Gaze et al. 2014)	Yes (Gaze et al. 2014; Pearson et al. 2015)	ND	Yes (Gaze et al. 2014)	Yes (Gaze et al. 2014)
Clinical trial status	Phase I (Merrifield et al. 2016)	Preclinical (Merrifield et al. 2016)	Phase III (Mo et al. 2014)	Phase I (Merrifield et al. 2016)	Preclinical (Merrifield et al. 2016)

**antibody-dependent cell cytotoxicity, *not determined, § determined by RNAi, §§ determined from biochemical studies

The upsurge in the availability of "omics-based" information has recently paved the way for the use of protein array technology as a vaccine antigen discovery tool. This technology permits the profiling of an individual's immune response to the entire immunome (the protein subset of an organism that is capable of inducing an immune response in a natural infection) of a pathogen and comparing the differences in antibody profiles between susceptible and resistant endemic populations allows the identification of antigens involved in a protective immune response (Driguez et al. 2015). In schistosome research, protein array studies have contributed significantly to our understanding of the human immune responses to schistosomiasis and allowed the identification of numerous vaccine candidate antigens, both previously documented (validating protein arrays as a vaccine antigen discovery tool) and novel (Gaze et al. 2014; Pearson et al. 2015; Driguez et al. 2016). Indeed, one of the antigens identified from two independent schistosome protein array was acetylcholinesterase (AChE) (Pearson et al. 2015; Driguez et al. 2016), which has provided the impetus for the study herein.

1.1.8 Acetylcholine, acetylcholinesterase and butyrylcholinesterase

Signaling in the nervous system is mediated through the interaction of neurotransmitters (for example, biogenic amines, neuroactive amino acids and gasses and acetylcholine (ACh)) with their cognate receptors located at the neuromuscular junction (Hu et al. 2011). In the synapse of the axon terminal of a motor neuron and the motor end plate, for instance, ACh is loaded into vesicles and transported across the synapse via a pH gradient. Following synaptic vesicle fusion and neurotransmitter release, the ACh disperses within the synaptic cleft and activates

muscarinic (mAChRs) or nicotinic acetylcholine receptors (nAChRs), located on post-synaptic cells (Albuquerque et al. 2009). Stimulation of nicotinic receptors produces a rapid, excitatory effect and the result of muscarinic receptor stimulation is slower and can be excitatory or inhibitory, depending on the stimuli. Once the appropriate effector function of the AChR has been achieved, unbound ACh must be regulated or controlled in order to avoid prolonged contraction or relaxation (Nishimura et al. 2010).

The action of unbound ACh is terminated by single step enzymatic hydrolysis of the neurotransmitter into acetate and choline by acetylcholinesterase (AChE). The hydrolysis of ACh is initiated when the acyl group of ACh is initially transferred to the active-site serine of AChE, after which a water nucleophile attacks this ester, removing acetate and completing the hydrolysis. AChE is an efficient enzyme, capable of hydrolyzing ACh within 100 milliseconds of reaching the active site and catalyzing the breakdown of up to 10,000 ACh molecules per second (Dvir et al. 2010). After hydrolysis, most of the choline is transported back into the presynaptic nerve ending by high-affinity choline transporter and is then available for the synthesis of additional ACh (Day et al. 1996; Pax et al. 1996; Halton 1997) (Fig 1.3).

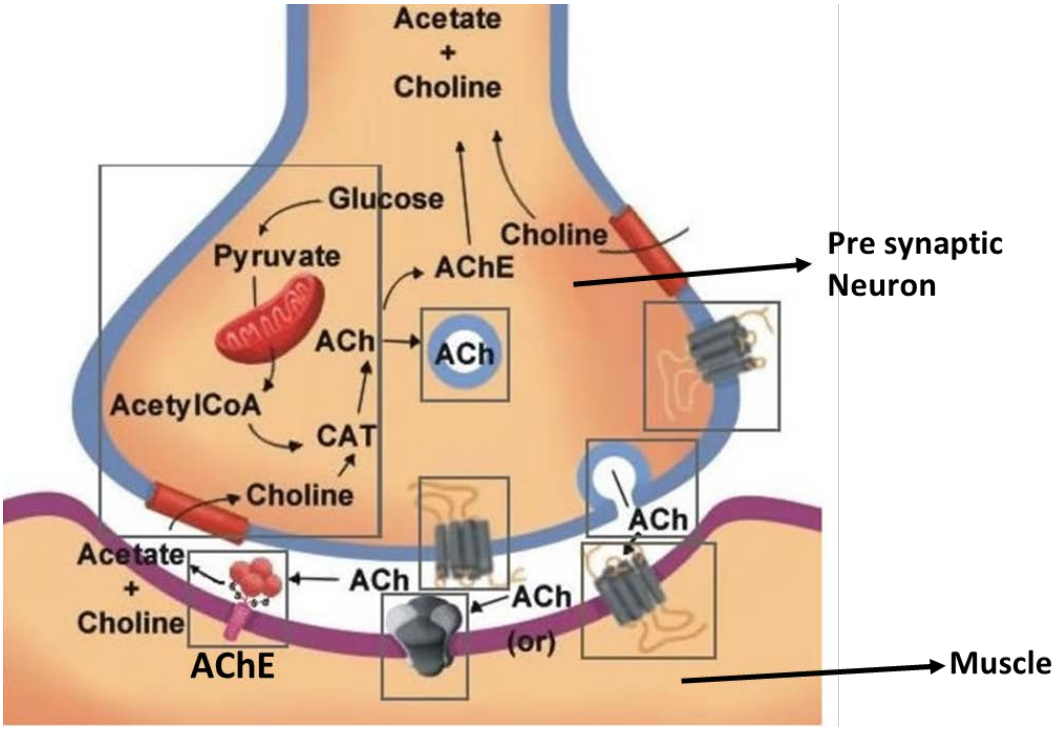


Fig 1.3. The neurotransmission routes at the neuromuscular junction.
Modified from http://neuroscience.uth.tmc.edu/s1/chapter11.html#synthesis

Butyrylcholinesterase (BChE) is also a cholinesterase (ChE) domain-containing protein with features similar to AChE (Barker et al. 1966; Rumjanek 1987; Camacho et al. 1995; Bentley et al. 2004) and is present in extra-neuronal tissues such as liver, lung and plasma. Enzymatically, BChE is also known as a pseudocholinesterase as it is less substrate specific for ACh than AChE and preferentially hydrolyzes butyrylcholine BCh (Mack et al. 2000), however, its physiological function is not known since humans lacking BChE activity do not show any pathology (Massoulie 2002). Since BChE presents as a soluble form in the circulation of mammals, is suggested to serve as a safeguard against the diffusion of excess ACh and orally ingested toxins into the bloodstream because of its broad specificity to different substrates (Allon et al. 1998; Huang et al. 2007;

Lockridge 2015). In addition, it could also serve as a competitive inhibitor of orally ingested compounds toxic to AChE, including plant alkaloids such as cocaine (Massoulie 2002; Zhan et al. 2003). Previous studies, in schistosomes, demonstrated the presence of pseudocholinesterase (nonspecific cholinesterase activity) (Bueding 1952; Fripp 1967; Bruckner et al. 1974; Camacho et al. 1994); however, there is no evidence so far to support the existence of BChE in schistosomes.

1.1.9 Molecular diversity of AChE

Based on studies in vertebrates, AChE exists in multiple molecular forms based on differences in the carboxy-terminal region that define oligomerization state and membrane anchoring (Massoulie 2002). Catalytic subunits may be assembled into asymmetric collagen-tailed or globular molecules or remain as monomers (Massoulie 2002). The C-termini of $AChE_H$ isoforms are hydrophobic and glycophosphotidyl (GPI) anchored to cell membranes and the cysteine in this region forms an intramolecular disulfide bond between monomers to form a dimer. The $AChE_S$ form of AChE has a truncated C-terminus and remains soluble and monomeric. Thirdly, $AChE_T$ (tailed) proteins are collagen-tailed forms that associate with the Proline-Rich Membrane Anchor (PRiMA) protein on cell membranes which operate to organize $AChE_T$ into tetramers (Pezzementi et al. 2012).

1.1.10 The nervous system of schistosomes

The Platyhelminthes (flatworms), to which *Schistosoma* belong, are the most primitive organisms on earth to have developed a bilateral nervous system (Baguñà et al. 1978). Schistosomes are acoelomate parasites that have no circulatory system or endocrine glands and so, in the absence of a body cavity and circulating body fluid, lack the capacity for classical endocrine cellular communication. Thus, the only mechanism for long distance signal transduction in schistosomes for the coordination of such critical processes as movement, metabolism and reproduction, is performed by the nervous system (Halton et al. 1996; Ribeiro et al. 2010).

The schistosome nervous system is comprised of two main interconnected components: the central nervous system (CNS) and peripheral nervous system (PNS). The CNS consists of a bi-lobed cephalic ganglion and several pairs of longitudinal nerve cords that run along the entire length of the worm (Reuter et al. 1996). The longitudinal nerve cords are connected via a network of circular regularly spaced connecting bands of nerve tissue (Reuter et al. 1996; Ribeiro et al. 2010). The PNS consists of smaller nerve cords and nerve plexuses (large meshed networks of neurons) that supply the worm's body structure, muscles and organs (Halton et al. 1996; Ribeiro et al. 2005).

There are two distinct nerve plexuses in schistosomes: the sub-muscular plexus, which innervates the body wall musculature and the oral and 9 ventral suckers (Mair et al. 2000); and the sub-tegumental plexus, which is associated with the innervation of sensory structures, such as

papillae (Gobert et al. 2003) in the male gynocophoric canal and on the tegument of the worm (Mair et al. 2004). Cholinergic neurons, nerve cells responsible for the neurotransmission of ACh, are found throughout the entire schistosome nervous system, as evidenced by histochemical staining for AChE (Fripp 1967). Further, nAChRs have been identified on the tegument of *S. mansoni* (Camacho et al. 1995) and both the body wall muscles and the surface of *S. haematobium* (Bentley et al. 2004).

1.1.11 Acetylcholine in schistosomes

While ACh has an excitatory effect on muscle contraction and motility for most flatworms (Pax et al. 1996), it acts as an inhibitory neurotransmitter in schistosomes. This fact is documented by studies showing the dose-dependent induction of flaccid paralysis of extracted muscle fiber and whole worms when they are treated with ACh (Barker et al. 1966). The exact mechanism by which this neurotransmitter inhibits muscle contraction in flatworm, however, remains unknown (Day et al. 1996).

In addition to the neuromuscular function of ACh, the neurotransmitter has a role in the uptake of exogenous glucose; an idea first postulated with the finding that nAChRs were concentrated on the male dorsal tegument (Camacho et al. 1995), a major nutrient absorptive surface for the worm pair (Rumjanek 1987). Evidence for this relationship was provided with the discovery that glucose uptake was increased in schistosomes *in vitro* with the addition of blood-equivalent concentrations of ACh and ablated with specific agonists of nAChRs (Camacho et al. 1995). The

proposed mechanism behind this uptake system is that binding of ACh to tegumental nAChRs influences glucose transport through the modulation of tegumental schistosome glucose transporters (SGTP), such as SGTP1 and SGTP4 (Skelly et al. 1996) which are also present on the surface of this parasite (Jones et al. 2002).

1.1.12 Acetylcholinesterase in schistosomes

AChE activity was first demonstrated in adult schistosomes in 1952 (Bueding 1952) and later confirmed in other stages of the schistosome lifecycle (Espinoza et al. 1991; Espinoza et al. 1991; Tarrab-Hazdai et al. 1991; Camacho et al. 1994), both biochemically and using schistosome AChE-specific antibodies. Initially the enzyme was characterized by biochemical purification from worm extracts using affinity chromatography methods employing competitive inhibitors of ACh (Goldlust et al. 1986) and, more recently, recombinant AChEs have been expressed from different schistosome species (Jones et al. 2002; Bentley et al. 2003; Bentley et al. 2005; You et al. 2016). The involvement of AChE in controlling the motor activity of schistosomes was shown by demonstrating that AChE induced paralysis in worms while the administration of eserine, an AChE inhibitor, caused a concentration-dependent longitudinal muscle relaxation in the parasite (Pax et al. 1981).

Schistosome AChE is associated with the neuro-musculature, and tegument of the parasite (Levi-Schaffer et al. 1984). In *S. mansoni* schistosomula, AChE was found to be highly enriched in the tegument (350 fold) compared to the rest of the worm (Arnon et al. 1987). The enzyme

predominantly exists in a dimeric globular form (Camacho et al. 1994) and is reportedly attached to the tegument and muscle via a covalently linked GPI anchor (Arnon et al. 1987). More recent sequence comparison of schistosome AChE with the vertebrate enzyme, however, has revealed unique C-terminii and it has been suggested that schistosome AChE may associate with another protein which is GPI-anchored itself (Bentley et al. 2003). Different levels of AChE activity has been reported among different species of *Schistosoma* and this is due to the differing amounts of the enzyme in their teguments (*S. haematobium* and *S. bovis*, respectively, have 20 and 6.9 times the amount of tegumental AChE activity than *S. mansoni*) (Camacho et al. 1994).

It should be noted that only one AChE-encoding gene from *Schistosoma* has been described over a decade ago (Bentley et al. 2003; Bentley et al. 2005). However, interrogation of the S*. mansoni* and *S. haematobium* genomes (Berriman et al. 2009; Young et al. 2012) has revealed multiple separate genes. In *S. mansoni,* three paralogs (*Sm*AChE1 (Smp_154600), *Sm*BChE1 (Smp_125350) and *Sm*AChE3 (Smp_136690)) have been identified (http://www.geneDB.org) of comparable length encoding AChEs. Previous studies on the biochemically purified material, therefore, would have been unable to attribute enzyme characteristics to anyone AChE protein. Accordingly, it is the aim of the studies proposed herein to explore the function and interplay of all three *Sm*ChEs from *S. mansoni*.

1.1.13 Non-neuronal functions of helminth acetylcholinesterase

It is now widely accepted that AChE can perform multiple biological functions due to its presence in multiple cell types and sub-cellular locations (Santos et al. 2007). In helminths, neuronal AChE appears to perform a "traditional" cholinergic activity while surface-associated or secreted AChE has roles in non-neuronal functions such as nutrient acquisition, parasite adhesion to host tissue and immune modulation (Arnon et al. 1999; Selkirk et al. 2005).

1.1.13.1 Helminth AChE and glucose uptake

Glucose is a primary and essential nutritional source used by schistosomes to fuel their growth and fecundity and, while the parasite is capable of glycolysis and glycogenesis, the intra-mammalian stages of the worm rely heavily on the consumption of exogenous glucose (You et al. 2014). The role of AChE in host glucose uptake by schistosomes is suggested to limit the interaction of host ACh with tegumental nAChR (Camacho et al. 1995), presumably decreasing the amount of glucose uptake through surface glucose transporters (Jones et al. 2002). The contribution of tegumental AChE to this system is evidenced by the ablation of glucose uptake with a membrane-impermeable inhibitor of AChE, which has the same result as the administration of an excess of ACh (Jones et al. 2002). The specific mechanism by which nAChRs mediate glucose transport remains unknown.

1.1.13.2 Helminth AChE and adhesion

Further to the observation that some adhesion proteins shared significant sequence homology with ChE domains (Scholl et al. 2003), there is now a growing body of evidence that AChE plays a non-classical role as an adhesion protein (Soreq et al. 2001; Silman et al. 2005). Examples of these ChE-like adhesion molecules include glutactin, neurotactin, gliotactin from *Drosophila* and the mammalian neuroligins (Botti et al. 1998). These proteins are absent of hydrolase activity as they lack a complete catalytic triad, but their ChE-like domain has high sequence similarity with AChE and acts as a protein-protein interaction motif, suggesting that AChE itself may function in a similar manner (Darboux et al. 1996). Further, cholinesterase-like adhesion molecules possess a typical electrostatic motif of negative potential around a zone that is homologous to the active-site gorge (Peripheral anionic site) of AChE (Felder et al. 1997). Electrostatic interactions can facilitate target recognition through a protein-complex formation, further lending support to an adhesive function for AChE (Botti et al. 1998).

The possible role of schistosomal AChE in facilitating adhesion has not been explored. Two hypotheses are that the tegumental AChE of schistosomes may aid in adsorption of host molecules onto the outer surface of the parasite, one of many immune-evasive strategies employed by the worm (Clegg et al. 1971) or assist in a vascular attachment via an adhesive interaction (Wyszomirska et al. 2005). *In vivo* treatment with anti-schistosomal compounds and AChE inhibitors such as tris (p-aminophenyl) carbonium salts (TAC) (Bueding et al. 1967), results

in a shift of the worms from the mesenteries to the liver which may occur due to an inability of the parasite to attach to the vasculature.

1.1.13.3 Helminth AChE and immune responses

Acetylcholinesterase secreted by some nematodes are reported to inhibit local ulceration in the stomach by hydrolyzing ACh, which is known to stimulate gastric acid secretion (Pritchard 1993), but this was not corroborated by experimental evidences and It has been indirectly shown that the nematode *N. brasiliensis* employs parasite-derived AChE to alter the host cytokine environment to inhibit M2 macrophage recruitment, a condition favorable to worm survival (Vaux et al. 2016). Also, lysosomes are secreted by phagocytic cells during phagocytosis and the circulating ACh is suggested to enhance the release of lysosomal enzymes by selectively increasing of intracellular levels of cGMP (Ignarro et al. 1973; Ignarro et al. 1974). It is, therefore, possible that AChE secreted by helminths may interfere with the process by hydrolyzing ACh before it reaches its target immunological cells, thus reducing inflammation in the surroundings where the parasites reside and preventing efficient expulsion. Circulating ACh also plays a role in the upregulation of intestinal helminth killing by antibody-dependent cellular cytotoxicity (ADCC) through the mediation of leukocyte cGMP levels (Gale et al. 1974) and so helminth-derived AChE may interfere with this process through the hydrolysis of ACh.

1.1.13.4 Helminth AChE and host pathogenicity

Several studies on nematodes have implicated parasite-derived AChE in the pathogenesis of helminth infection. It has been suggested that AChE may provide acetate and choline precursors for helminth metabolism (Pritchard 1993). It was also suggested that AChE is involved in controlling the permeability of parasites as observed in the presence of AChE inhibitors (Schwabe et al. 1961).

1.1.13.5 Helminth AChE and apoptosis

There is data to strongly suggest that AChE is a vital component in the common pathway leading to apoptosis as more than 40 different types of cells have demonstrated AChE expression during and after the induction of apoptosis by different stimuli (Zhang et al. 2012). It is proposed that AChE play a key role in the formation of the apoptosome as silencing of the enzyme ablated the interaction between apoptotic protease-activating factor-1 and cytochrome c (Park et al. 2004). While there is no research showing the involvement of parasite-derived AChE in the apoptosis of helminth cells, the schistosome apoptosis pathway, which is active throughout the parasite's life cycle, is similar to that in mammals (Lee et al. 2011; Han et al. 2013).

1.1.14 Schistosoma AChE as a vaccine target?

There are several indications to support the usefulness of schistosomal AChE as a vaccine target, in line with the criteria used for Table 1.1. Firstly, AChE has been immunolocalized to the tegument of schistosomula and adult worms (Camacho et al. 1995; Espinoza et al. 1995), a result

consistent with the detection of AChE activity in these membranes (Camacho et al. 1994), suggesting that the enzyme is accessible to immune attack. Secondly, a panel of monoclonal (Espinoza et al. 1995) and polyclonal (Espinoza et al. 1991; Espinoza et al. 1991; Tarrab-Hazdai et al. 1991) anti-schistosomal antibodies were able to interact with the surface localized enzyme on intact schistosomula and produce almost total complement-dependent killing of the parasite. Thirdly, some of the same monoclonal antibodies and polyclonal anti-schistosomal AChE antibodies showed no cross-reactivity against human AChE (Espinoza et al. 1991; Espinoza et al. 1991; Tarrab-Hazdai et al. 1991; Espinoza et al. 1995), indicating that a vaccine safe for human use could possibly be designed. Lastly, recent protein array studies have detected considerably high levels of circulating antibodies to schistosomal AChE in humans exhibiting resistance and low pathology to schistosomiasis, suggesting an involvement of these antibodies in a protective anti-schistosomal response (Pearson et al. 2015; Driguez et al. 2016). Recently, a study in *S. japonicum* has shown that a recombinant AChE is capable of generating a protective immune response that resulted in 33% male worm burden and 73% mature intestinal egg reduction, respectively, in a mice model of schistosomiasis (You et al. 2018).

1.1.15 AChE inhibitors

Acetylcholinesterase is expressed in all vertebrate and invertebrate animals as a key enzyme of ACh-mediated neurotransmission (Lang et al. 2012) and, owing to its critical role in organism survival; AChE is a sensitive target for both natural and synthetic inhibitors, including organophosphates (Fig 1.4). AChE inhibitors are present in the venom of many animals and have been employed in a variety of uses including insecticides and as chemical weapons (Silman I 2000).

In clinical medicine, certain medical disorders such as dementia, myasthenia gravis, and glaucoma can be directly related to a dysfunction of the cholinergic system and thus treated through modulation of the cholinergic function using AChE inhibitors (Čolović et al. 2013).

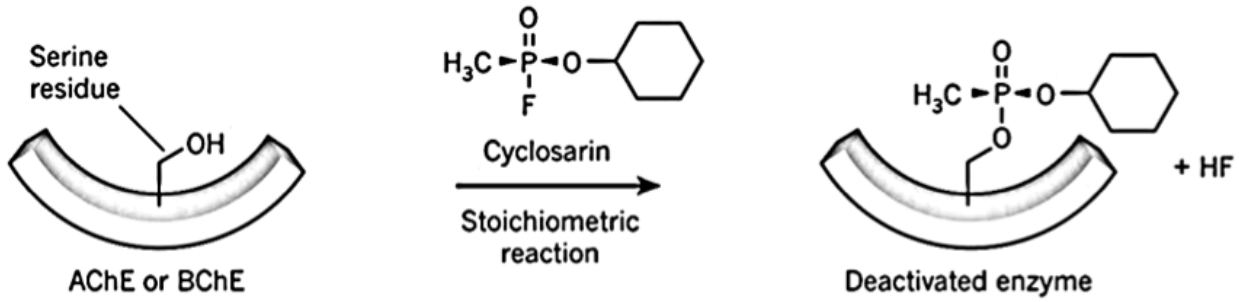


Fig 1.4. AChE inhibition by organophosphates. Depicted is the interaction of AChE with sarin nerve gas. The phosphate group of the inhibitor binds to the carboxyl-ester group of the active site serine and, in this form, the enzyme is no longer available for its next physiological function.

1.1.15.1 AChE inhibitors as anti-schistosomals

Schistosoma AChE is the target of several anti-parasitic drugs, including hycanthone (Hillman et al. 1975) metrifonate (Bueding et al. 1972; Feldmeier et al. 1982), phosphonium salts and phosphoranes (Levi-Schaffer et al. 1984) and TAC (Bueding et al. 1967). Historically, some of these drugs were widely used for the treatment of schistosomiasis but have either been withdrawn from the market or are restricted in their use due to medical, operational, and economic criteria (Reich et al. 2001).

Metrifonate is an organophosphate compound initially used as an insecticide (Lorenz et al. 1955). It has been shown to be effective against *S. mansoni* and *S. haematobium in vitro* (Bueding et al. 1972) but is more effective against *S. haematobium* (Davis et al. 1969; Feldmeier et al. 1982). It has been suggested that *S. haematobium* may be more susceptible to metrifonate than *S. mansoni* because of approximately 20 times much higher levels of AChE activity in its tegument (Camacho et al. 1994; Abdi 1995). Metrifonate was withdrawn from the market and further development in 1998 because of issues with toxicity (Lopez-Arrieta et al. 2006) and it is no longer on the WHO model list of essential drugs (Kramer et al. 2014).

Hycanthone (a metabolite of the anti-schistosomal compound lucanthone) was also one of the most widely used drugs both *S. mansoni* and *S. haematobium* (Rosi et al. 1965) and has been shown to stimulate the motor activity of the parasite through the inhibition of AChE. The drug is commercially discontinued because of toxicity to the host (Roquis et al. 2014) and the development of drug resistance (Cioli et al. 1989).

Oxamniquine (OXA) is structurally similar to hycanthone. In addition to possessing AChE inhibitory activity which increases parasite motility upon treatment (Hillman et al. 1975), it is closely associated with inhibition of the nucleic acid metabolism of the worm (Abdi 1995). Resistance to OXA has limited it use (Cioli et al. 1989) and, while this has been attributed to a mutation in a sulfotransferase, no link between resistance and AChE mutation was reported (Valentim et al. 2013). Although highly effective, albeit only against *S. mansoni* (Organization 1993), OXA has more side-effects and is more expensive than PZQ and so is being phased out of the world market (Beck et al. 2001). Studies aimed at creating safer and cheaper OXA derivatives are currently underway (Valentim et al. 2013).

Another group of anticholinergic drugs studied for their efficacy against *Schistosoma* was phosphonium salts and TAC (Bueding et al. 1967; Levi-Schaffer et al. 1984). The effect of these compounds and their derivatives were described to have substantial effects on the catalytic activity of AChE and the viability of the various life stages of *S. mansoni* worms (Bueding et al. 1967; Levi-Schaffer et al. 1984).

1.1.16 Metal-based drugs in medicine

Traditionally, the use of organic compounds in the field of medicinal chemistry and pharmaceutics has been the primary choice of any medical research (Guo et al. 1999). More recently, metal-based complexes have gained great interest as potential therapeutic agents, particularly following the remarkable accidental success of the anti-cancer drug cisplatin (Cohen 2007). The principle is that inert transition metal complexes, such as ruthenium can replace organic compounds in the targeting

of the active sites of enzymes (Debreczeni et al. 2006). One of the main advantages of these metal complexes is that they can be more readily prepared because they can be constructed from smaller, simpler, and more synthetically accessible components (Bregman et al. 2006).

1.1.16.1 The use of metal-based drugs to combat infectious disease

Encouraged by the success of many metal-based compounds as anti-tumor and anti-arthritic agents, researchers began to investigate their activity against protozoan parasites (Navarro et al. 2010) and bacteria (Li et al. 2015). Some transition metal ions have been used to form coordination complexes with the anti-malarial drug chloroquine (Navarro et al. 2010). Treatment of experimental rodent malaria with ruthenium complexes of chloroquine was demonstrated to be more effective than the parent drug, reducing the parasitemia levels by 94% compared to 55% at an equivalent concentration without producing acute toxicity (Sanchez-Delgado et al. 1996).

There are limited examples of metal complexes that have activity against helminths. The gold complex auranofin has been shown to have anti-schistosomal properties, killing parasites *in vitro* at a concentration of 5 µM and reducing worm burdens by 60% upon treatment of *S. mans*oni-infected mice with 6mg/kg doses of auranofin (twice daily for 9 days beginning at seven weeks post-infection) (Kuntz et al. 2007). Auranofin is also documented to kill *Taenia crassiceps* metacestodes in the culture at an LD_{50} of 3.8 µM. In the case of both flatworms, there is evidence that the gold complex exerts its activity by inhibiting the unique platyhelminth redox enzyme thioredoxin-glutathione reductase (Kuntz et al. 2007; Bonilla et al. 2008; Angelucci et al. 2009; Martinez-Gonzalez et al. 2010).

1.1.16.2 Ruthenium complexes as antimicrobials

From all the transition metals, ruthenium (Ru) is at the forefront of several critical areas of science, including chemotherapy, and some ruthenium (II) polypyridyl complexes have shown significant biological activity (Keene et al. 2009; Li et al. 2015) in this regard. This is partly due to the fact that Ru complexes have relatively low toxicity, are cost-effective compared to other transition metals, and have established synthetic chemistry for establishing octahedral coordination geometries (Meggers 2009). Also, they exert their biological effect by strongly binding to biological ligands with similar exchange kinetics to platinum complexes (Li et al. 2015). Further, the photophysical properties of Ru complexes facilitate the cellular accumulation and localization studies by the use of conventional techniques such as confocal microscopy and flow cytometry (Keene et al. 2009; Gill et al. 2012).

The antibacterial potential of Ru complexes was documented some six decades ago by Dwyer and co-workers (Dwyer et al. 1952) and there have been numerous recent studies documenting the efficacy of Ru complexes against a variety of different microbial pathogens (Li et al. 2011; Pandrala et al. 2013; Gorle et al. 2014).

One of the advantages of using Ru complexes as antimicrobial is that they are significantly less toxic to human cells compared to prokaryotic cells. The positively charged Ru complexes are suggested to selectively target bacterial cells over eukaryotic cells due to the presence of predominantly negative charges on the surface of the bacterial membrane and cell wall (Silhavy et al. 2010). Further, the

overall neutral charge in the outer membrane leaflet of eukaryotic cells (Mason et al. 2007) creates a significantly reduced capacity for electrostatic interaction with the Ru complexes (Gorle et al. 2016).

In eukaryotes, Ru complexes have been shown to target and inhibit enzymes such as AChE (Vyas et al. 2014). These cationic Ru complexes are speculated to bind through a combination of electrostatic and hydrophobic interactions at the peripheral anionic (PAS) site of AChE which is located at the gate of the enzyme's catalytic gorge (Bourne et al. 2005; Meggers 2009) and increasing level of AChE inhibition have been achieved by making modifications at the periphery of the Ru polypyridine scaffold (Mulcahy et al. 2008).

In this project, I will be evaluating the inhibition of schistosome AChE by Ru complexes and exploring the anti-parasitic effects of this interaction with a view to developing new chemotherapeutics to combat schistosomiasis.

CHAPTER 2

Polypyridylruthenium (II) complexes exert anti-schistosome activity through inhibition of parasite acetylcholinesterases.

This chapter has been published in its entirety as:

(Sundaraneedi et al. 2017)

2.1 Introduction

More than half a billion people are at risk of contracting schistosomiasis (bilharzia), a neglected tropical parasitic disease that is endemic in more than 75 countries in Africa, Asia, and South America, where over 200 million individuals are infected and approximately 280,000 die every year (Gryseels et al. 2006; Hotez et al. 2010; Hotez et al. 2014). Schistosomiasis is caused by infection with blood flukes of the genus *Schistosoma*, and disease results from the deposition of eggs in host tissues, leading to granuloma formation that can cause fibrosis, portal hypertension, and even death (Hams et al. 2013).

Despite this considerable disease burden, to date, there are no vaccines against schistosomiasis (Molehin et al. 2016). Praziquantel (PZQ) remains the only effective frontline drug to treat the parasite despite its shortcomings, which include ineffectiveness against juvenile stages of the worm (Vale et al. 2017), poor efficacy in treating pre-patent infections (Seto et al. 2011), reports of reduced efficacy in field studies (Wang et al. 2012) and the inevitable risk of the development of resistant strains in response to periodic mass drug administration (Bergquist et al. 2017). Since there are no alternative effective drug treatments for these parasites, new therapies are urgently required.

Among potential targets for chemotherapy are acetylcholinesterases (AChEs). These enzymes catalyze the rapid breakdown of the neurotransmitter acetylcholine (ACh) in both central and peripheral nervous systems of eukaryotic organisms, and so control neuronal function (Massoulie

et al. 1993). In addition to controlling cholinergic synapses, the enzyme is present in large amounts on the tegument of schistosomes (Tarrab-Hazdai et al. 1984), where it has been implicated to play a role in the regulation of host glucose uptake by the parasite by limiting the interaction of host ACh with tegumental nicotinic ACh receptors (nAChRs) (Camacho et al. 1995). These receptors are associated both spatially and temporally with surface AChE expression and are concentrated on the tegument (Camacho et al. 1995), the primary site of glucose uptake (Skelly et al. 2014). Evidence for this relationship is shown by the ablation of glucose uptake with a membrane-impermeable inhibitor of AChE (which has the same result as the administration of an excess of ACh) or specific agonists of nAChRs. The interaction of ACh with tegumental nAChRs is thought to decrease the amount of glucose uptake through surface glucose transporters but the specific mechanism for this is not known (Camacho et al. 1995; Camacho et al. 1995).

Concerning its termination of synaptic transmission, inhibition of AChE produces an excess accumulation of ACh and overstimulation of its receptors, causing uncoordinated neuromuscular function that often results in death due to respiratory paralysis (Thapa et al. 2017). As such, AChE inhibitors are widely used as pesticides (Kwong 2002) and anthelmintics (Orhan 2013). Indeed, metrifonate, an organophosphate AChE inhibitor initially used as an insecticide has also been used for the treatment of schistosomiasis until it was withdrawn from the market and further development because of off-target toxicity (Kramer et al. 2014).

In addition to organophosphates, mono-nucleated chemical complexes of the transition metal ruthenium have been shown to target and inhibit enzymes such as AChE (Vyas et al. 2014), and there are numerous recent studies documenting the efficacy of polypyridylruthenium(II) complexes against a variety of different microbial pathogens (Li et al. 2011; Pandrala et al. 2013; Gorle et al. 2014). Unlike their organophosphate counterparts, ruthenium complexes are speculated to exert their inhibitory effects through a combination of electrostatic and hydrophobic interactions at the peripheral anionic (PAS) site of AChE, which is located at the gate of the enzyme's catalytic gorge (Bourne et al. 2005), and not through direct interaction with the active site. Ruthenium complexes are thought to be less toxic to human cells than small-molecule inhibitors because of this mode of inhibition and also because the overall neutral charge in the outer membrane leaflet of eukaryotic cells (Mason et al. 2007) creates a greatly reduced capacity for electrostatic interaction with the metal compounds (Gorle et al. 2016).

Results of this study demonstrate the AChE-inhibitory action of two mononuclear and a series of di-, tri- and tetra-nuclear polypyridylruthenium(II) complexes linked by the bis[4(4'-methyl-2,2'-bipyridyl)]-1,n-alkane ligand ("bb_n"; n = 7, 10, 12 and 16) against extracts of *Schistosoma mansoni* (*Sm*AChE) and *Schistosoma haematobium* (*Sh*AChE) and both adult and juvenile *S. mansoni* parasites *in vitro*. Results also provide evidence consistent with the capacity of these complexes to disrupt the parasite's glucose uptake ability through the inhibition of tegumental AChE, a cholinergic pathway unique to schistosomes (Camacho et al. 1995; Camacho et al. 1995). Finally, experimental results show the *in vivo* efficacy of two ruthenium complexes in a mouse model of

schistosomiasis, providing evidence that drugs based on these compounds could be a valuable addition to the chemotherapeutic arsenal against this debilitating disease.

2.2 Methods

2.2.1 Ethics Statement

The James Cook University (JCU), animal ethics committee, approved all experimental work involving animals (ethics approval number A2271). Mice were raised in cages in the JCU quarantine facility for the duration of the experiments in compliance with the 2007 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the 2001 Queensland Animal Care and Protection Act. All reasonable efforts were made to minimize animal suffering.

2.2.2 Nomenclature and preparation of ruthenium complexes

[Ru(phen)2(Me2bpy)]2+ and the mononuclear (Rubbn-mono), dinuclear (Rubbn-di), trinuclear (Rubbn-tri), tetranuclear linear (Rubbn-tl) and tetranuclear non-linear (Rubbn-tnl) polypyridylruthenium(II) complexes (Fig 8) were synthesized using the appropriate bis[4'-(4-methyl-2,2'-bipyridyl)]-1,n-alkane bridging ligand (bb_n) as previously described (Gorle et al. 2014). Compounds were dissolved in H_2O at stock concentrations of 1 mM.

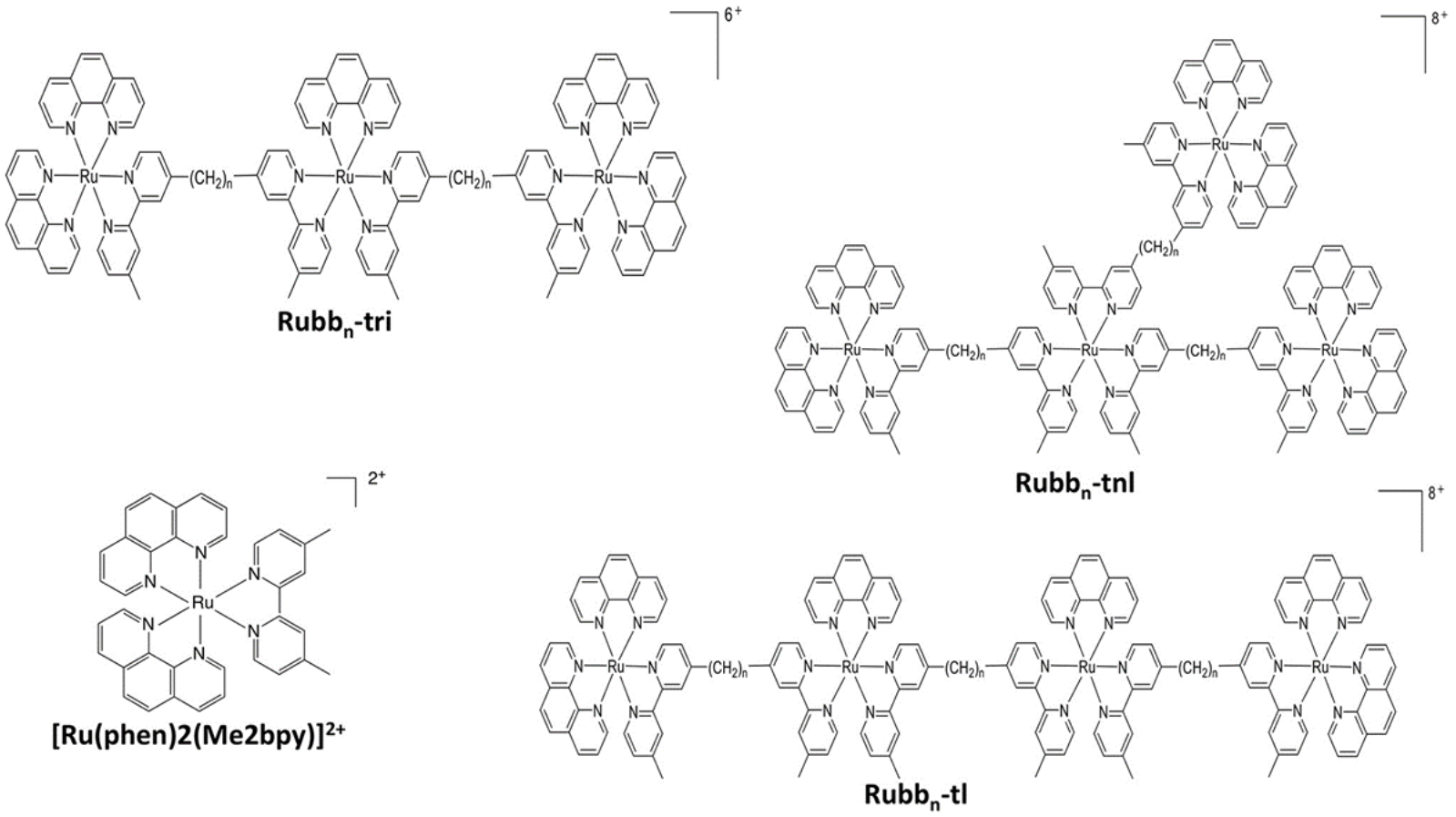


Fig 2.1. The kinetically inert tri-nuclear (Rubb$_n$-tri), linear tetra-nuclear Rubb$_n$-tl and non-linear tetra-nuclear Rubb$_n$-tnl) ruthenium (II) complexes.

2.2.3 Parasite extract preparation

S. mansoni cercariae were shed from infected *Biomphalaria glabrata* snails (Biomedical Research Facility, MD, USA) by exposure to light at 28°C for 2 hours. Cercariae were used to infect 6-8 week old male BALB/c mice (Animal Resources Centre, WA, Australia) by tail penetration (180 *S. mansoni* cercariae/mouse) and adults were harvested by vascular perfusion at 7 weeks post-infection (Lewis et al. 1986). *S. mansoni* eggs were purified from infected mouse livers as previously described (Dalton et al. 1997) and were used in the xWORM egg hatching assay or to make soluble egg antigen (SEA) (Tucker et al. 2013). For experiments involving schistosomula, *S. mansoni* cercariae were mechanically transformed as previously described (Tucker et al. 2013). To make PBS-soluble extracts, *S. mansoni* adult worms were homogenized in PBS (50 μl/worm pair) at 4°C using a TissueLyser II (Qiagen) and the supernatant collected by centrifugation at 15,000 x*g* for 60 min at 4°C. Triton X-100-soluble extracts of *S. mansoni* and *S. haematobium* were made in the same way except worms were lysed in buffer containing 1% Triton X-100, 40 mM Tris-HCl, pH 7.4. Protein concentration was determined using the Pierce BCA Protein Assay kit (Thermofisher), aliquoted and stored at -80°C until use.

2.2.4 Enzyme activity in parasite extracts and inhibition assays

*Sm*AChE or *Sh*AChE, *S. mansoni* nucleotide pyrophosphatase-phosphodiesterase 5 (*Sm*NPP5) and *S. mansoni* alkaline phosphatase (*Sm*AP) activity in Triton X-100-soluble adult worm extracts and *Sm*AChE activity in SEA were determined in a Polarstar Omega microplate reader (200 μl final volume in 96-well plates). *Sm*AChE and *Sh*AChE activity was determined using the Ellman method

(Ellman et al. 1961); extracts were serially diluted (20 - 5 μg) in AChE assay buffer (0.1 M sodium phosphate, pH 7.4), 2 mM acetylthiocholine (ACh) and 0.5 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) were added and absorbance was measured at 405 nm every 10 min for 5 h at 37°C. For *Sm*AChE and *Sh*AChE inhibition assays, parasite extracts equal to a specific activity of 0.55 nmol/min/well were diluted in AChE assay buffer to a final volume of 170 μl and pre-incubated with ruthenium complexes (10 nM – 100 μM) for 20 min at RT. ACh and DTNB were added at 2 mM and 0.5 mM, respectively and absorbance was measured at 405 nm every 10 min for 5 h at 37°C. *Sm*NPP5 activity (Rofatto et al. 2009) was measured by serially diluting extracts in *Sm*NPP5 assay buffer (50 mM Tris-HCl, pH 8.9, 120 mM NaCl, 5 mM KCl, 60 mM glucose), adding 0.5 mM p-nitrophenyl thymidine 5'-monophosphate (p-Nph-5'-TMP) and reading the absorbance (405 nm) every 10 min for 5 h at 37°C. For *Sm*NPP5 inhibition assays, parasite extract equal to 32 nmol/min/well was diluted in *Sm*NPP5 assay buffer to a final volume of 180 μl and pre-incubated with ruthenium complexes (10 nM – 100 μM) for 20 min at RT. A substrate (p-Nph-5'-TMP) was added to 0.5 mM and absorbance was measured at 405 nm every 10 min for 5 h at 37°C. *Sm*AP activity (Cesari et al. 1981) was measured by serially diluting extracts in AP assay buffer (0.1 M glycine, pH 10.4, 1 mM $MgCl_2$, 1 mM $ZnCl_2$), adding 2 mM p-nitrophenyl phosphate (pNPP) and measuring absorbance at 405 nm every 2 min for 1 h at 37°C. For *Sm*AP inhibition assays, parasite extract equal to 1 nmol/min/well was diluted in AP assay buffer to a final volume of 180 μl and pre-incubated with ruthenium complexes (10 nM – 100 μM) for 20 min at RT. A substrate (pNPP) was added to 2 mM and absorbance was measured at 405 nm every 2 min for 1 h at 37°C. For all kinetic enzyme assays, specific activity was calculated using the initial velocity of the reaction.

For all assays, inhibition for each sample was calculated relative to the negative control (reactions without ruthenium complexes) and reactions were performed in duplicate with data presented as the average ± SEM.

2.2.5 Effect of ruthenium complexes against larval *S. mansoni* parasites

Newly-transformed schistosomula (100 parasites) were cultured (37°C, 5% CO_2) in 200 µl of Basch medium supplemented with 4× antibiotic/antimycotic (AA - 200 units/ml penicillin, 200 µg/ml streptomycin and 0.5 µg/ml amphotericin B) in a 96 well plate in the presence of a series of serially-diluted ruthenium complexes (100 µM – 10 nM). After 48 h, parasite viability was assessed microscopically by Trypan Blue exclusion staining as previously described (Wangchuk et al. 2016). Six of the most effective compounds were tested again for their larvacidal efficacy (100 schistosomula per treatment); this time at concentrations of 200, 100, 50, 25, 12.5 and 6.25 µM. Experiments were performed in duplicate with IC_{50} data presented as the average ± SEM.

2.2.6 Effect of ruthenium complexes against adult *S. mansoni* parasites

Five pairs of adult worms were cultured in 2 ml of Basch medium supplemented with 4x AA in a 24 well plate in the presence of ruthenium complexes (50 µM). Control worms were treated with an equal amount of H_2O. Worms were cultured at 37°C and 5% CO_2 for 7 days, monitored every 24 h for motility by microscopic examination and considered dead if no movement was seen. The most effective ruthenium complexes (5 compounds) were tested in duplicate (five pairs of worms

each) at 10, 50 and 100 μM. Data are presented as the average of each duplicate experiment ± SEM.

2.2.7 Effect of ruthenium complexes on *S. mansoni* egg hatching and development

Egg hatching was evaluated by the xWORM egg hatching assay (Rinaldi et al. 2015). Ova (5,000 per well, 200 μl reaction volume) were incubated in 0.1x PBS, pH 7.2, containing ruthenium complexes (50 μM) and induced to hatch under bright light at RT for 16 h. The motility of the miracidia released from the hatched eggs was monitored every 15 s and the motility index was calculated as described (Rinaldi et al. 2015). Experiments were performed in triplicate with control reactions containing no ruthenium compounds. To investigate the effects of ruthenium complexes on egg development, triplicate sets of five pairs of adult *S. mansoni* worms were cultured in Basch media with or without 5 μM $Rubb_{12}$-tri, for 72 h. The eggs released into the media were counted and misshapen, malformed or immature eggs (Pellegrino et al. 1962) were scored as "abnormally developed". Egg hatching and morphology data is presented as the average of each triplicate experiment ± SEM.

2.2.8 Assessment of enzyme inhibitory effects induced by treatment of worms with ruthenium complexes

Freshly perfused worms were cultured in the presence of sub-lethal concentrations (5 μM) of $Rubb_{12}$-tri or $Rubb_{16}$-tnl - two ruthenium compounds determined to be most effective in terms of their combined activity against schistosomula, adult worms and eggs – in Basch medium at

37°C and 5% CO_2. To measure surface *Sm*AChE or *Sm*AP activity, 5 pairs of worms (preliminary experiments by us showed this number of parasites was sufficient to accurately measure surface enzyme activity) were transferred to either AChE assay buffer (0.1 M sodium phosphate, pH 7.4, 2 mM ACh, 0.5 mM DTNB) or AP assay buffer (0.1 M glycine, pH 10.4, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 2mM p-nitrophenyl phosphate). Surface enzyme activities were quantified by measuring the absorbance at 405 nm after incubation for 60 min (*Sm*AChE) or 30 min (*Sm*AP). Activity was measured from triplicate sets of parasites (5 pairs of worms) and each assay was technically replicated three times. For each enzyme assay, activities of drug-treated parasites were expressed relative to worms cultured without ruthenium complexes (negative controls). To measure somatic *Sm*AChE activity, PBS-soluble extracts were made from worms used for surface *Sm*AChE activity (triplicate sets of five pairs of worms) and then assayed in triplicate as described above. Data are presented as the average of each triplicate biological and technical experiment ± SEM.

2.2.9 Effect of glucose uptake and glycogen storage on worms treated with ruthenium complexes

Five pairs of freshly-perfused worms were cultured in the presence of sub-lethal concentrations (5 µM) of $Rubb_{12}$-tri or $Rubb_{16}$-tnl in DMEM (1 mg/ml glucose). Media (50 µl) from each experiment was collected after 24 h and the amount of glucose was quantified using a colorimetric glucose assay kit (Sigma) according to the manufacturer's instructions. Media was collected from triplicate sets of parasites (five pairs of worms) and each assay was replicated 3

times. Glucose levels were expressed relative to media collected from worms which received no drug (negative control). Data is presented as the average of each triplicate biological and technical experiment ± SEM. To measure the glycogen content of worms treated with ruthenium drugs, Triton X-100-soluble extracts were made and assayed for glycogen in a modified procedure described by Gomez- Lechon et al. (Gómez-Lechón et al. 1996). Briefly, 0.2 M sodium acetate, pH 4.8, was added to 30 µg parasite extract and 50 µl glucoamylase (10 U/ml) to make a reaction volume of 150 µl. The mixture was incubated at 40°C for 2 h with shaking at 100 rpm, 40 µl added to a new microplate with 10 µl 0.25 M NaOH and the amount of glucose quantified using the colorimetric glucose assay kit. Extracts were made from triplicate sets of parasites (five pairs of worms) and assays were performed three times. Data is presented as the average of each triplicate biological and technical experiment ± SEM.

2.2.10 Scanning electron microscopy

Control parasites and worms treated with 5 µM $Rubb_{12}$-tri were prepared for scanning electron microscopy by fixation in 3% glutaraldehyde followed by successive dehydration for 15 min each in a graded ethanol series (100%, 90%, 80%, 70%, 60%, 50%), 1:1 ethanol:hexamethyldisilizane (HMDS) and, finally, 100% HMDS. Dehydrated worms were covered and left overnight in a fume hood to allow the HMDS to evaporate then mounted on an aluminum stub, sputtered with gold and visualized using a JEOL JSM scanning electron microscope operating at 10 kV. Images were acquired digitally using Semaphore software.

2.2.11 Cytotoxicity assays

Cytotoxicity assays were performed using the mitochondrial-dependent reduction of 3-(3,4-dimethylthiazol-2yl)-5-diphenyl tetrazolium bromide (MTT) to formazan as described by Pandrala (Pandrala et al. 2015). The human bile duct cell line H69 was cultured in 96-well microtiter plates containing 0.1 ml of growth factor-supplemented media (DMEM/F12 with high glucose (4 mg/ml), 10% FCS, 1×AA, 25 µg/ml adenine, 5 µg/ml insulin, 1 µg/ml epinephrine, 8.3 µg/ml holo-transferrin, 0.62 µg/ml hydrocortisone, 13.6 ng/ml triiodo-1-thyronine (T3) and 10 ng/ml epithelial growth factor (EGF)) (Smout et al. 2015) to a cell density of 5,000 per well at 37°C in 5% CO_2. Cell viability was assessed after continuous exposure to a concentration series (50, 25, 10, 5, 1, 0.5 and 0.1 µM) of $Rubb_{12}$-tri, $Rubb_{16}$-tnl, PZQ or Dichlorvos (DDVP) – a metabolite of the anti-schistosome AChE inhibitor metrifonate - for 72 h. The amount of reduced MTT to formazan within the cells was quantified by measuring the absorbance at 550 nm. Data are the average of six replicate experiments $\pm$ SEM.

2.2.12 Tolerability study

In order to determine the maximum tolerated dose of $Rubb_{12}$-tri and $Rubb_{7}$-tnl to be administered to mice infected with *S. mansoni*, five intravenous (i.v.) doses (tail vein) of $Rubb_{12}$-tri and $Rubb_{7}$-tnl were given to groups of three male BALB/c mice (6-8 weeks) daily for five consecutive days. The doses ranged from 0.25 to 10 mg/kg in PBS and were administered in a volume of 30 µl. Animals were closely monitored for adverse clinical signs throughout the study and mice showing adverse effects were euthanized using CO_2 asphyxiation. The highest dose that

did not cause any adverse clinical signs was considered to be the maximum tolerated dose (MTD) for five consecutive daily doses.

2.2.13 *In vivo* efficacy of ruthenium complexes

Groups of 8 male BALB/c mice (6-8 weeks) were infected with *S. mansoni* cercariae as described above. At 35 days post-infection, groups were given five consecutive daily i.v. doses (tail vein - 30 µl) of the MTD of either $Rubb_{12}$-tri (2 mg/kg in PBS) or $Rubb_7$-tnl (10 mg/kg in PBS). PBS was similarly administered to the control group. Two independent trials were performed. Parasites were harvested by vascular perfusion at 49 days post-infection and the average worm burden per mouse for each group of mice (trial 1 PBS control – n = 8 mice, trial 1 $Rubb_{12}$-tri-treated – n = 8 mice, trial 1 $Rubb_7$-tnl-treated – n = 6 mice, trial 2 PBS control – n = 7 mice, trial 2 $Rubb_{12}$-tri-treated – n = 7 mice, trial 2 $Rubb_7$-tnl-treated – n = 8 mice) was calculated. Data is presented as a combination of the two independent trials $\pm$ SE. Livers from each group (trial 1 PBS control – n = 8 mice, trial 1 $Rubb_{12}$-tri-treated – n = 8 mice, trial 1 $Rubb_7$-tnl-treated – n = 6 mice, trial 2 PBS control – n = 7 mice, trial 2 $Rubb_{12}$-tri-treated – n = 7 mice, trial 2 $Rubb_7$-tnl-treated – n = 8 mice) were collected, halved and weighed, with one half digested with 5% KOH to determine liver eggs per gram of tissue (EPG) as previously described (Tran et al. 2006). The other half of each liver was pooled according to group, homogenized in H_2O and placed in identical foil-covered volumetric flasks under bright light to hatch eggs released from the livers. After 1 h, the number of miracidia in 10 x 50 µl aliquots of H_2O (sampled from the extreme top of each flask) were counted. The amount of eggs in each flask at the start of the hatching experiment was

determined by liver EPG calculations, allowing the egg hatching index of each group to be calculated by expressing the hatched eggs (miracidia) as a percentage of the total eggs. Data presented is for trial 1 only and represents the average of ten counts ± SEM. To assess fitness of parasites recovered from mice treated with ruthenium complexes compared to controls, worms recovered from each group were pooled and five pairs were assayed for surface enzyme activity (*Sm*AChE, *Sm*NPP5 and *Sm*AP) or glucose uptake ability as described above. Somatic *Sm*AChE activity was determined from homogenates made from the worms assayed for surface *Sm*AChE activity, also as described above. Each assay was technically replicated three times and data is presented as the average of triplicate technical replicates of both trials combined ± SEM. To compare eggs released from parasites recovered from mice treated with ruthenium complexes and controls, triplicate sets of worms (five pairs) from each pool were incubated in Basch media at 37°C in 5% CO_2 for 72 h. The number of eggs released were counted and scored on the basis of development and morphology (Pellegrino et al. 1962) by visualization under a FITC filter on a Zeiss AxioImager-M1 fluorescent microscope. Data presented is for trial 2 and is the average of triplicate experiments ± SEM.

2.2.14 Statistical analyses

Statistical analyses were performed using GraphPad Prism 7. Inhibition curves and IC_{50} values were generated using sigmoidal dose-response (variable slope) with a non-linear fit model. One-way ANOVA with Dunn's multiple comparisons was used to determine significance (*p*), which was set at 0.05. In the case where only two groups were compared, student's *t* test was used.

2.3 Results

2.3.1 Inhibition of AChE in schistosome extracts by ruthenium complexes

A series of ruthenium complexes of different nuclearity (mono-, di-, tri- and tetra-linear and tetra-nonlinear) and with different chain lengths in the linking ligand (bb_7, bb_{10}, bb_{12}, bb_{16}) were screened (1 µM) for AChE inhibitory activity in Triton X-100-soluble extracts from adult *S. mansoni* and *S. haematobium* and *S. mansoni* soluble egg antigen (SEA) (Table 2.1). In *S. mansoni* extracts, all tri- and tetra-nuclear complexes inhibited AChE activity by 70-90% and 7 of the 13 compounds had IC_{50} values ≤ 1 µM. A dose-response curve and Lineweaver-Burk plot is shown for the most potent of these complexes ($Rubb_{12}$-tri, IC_{50} = 0.3 µM) (Fig 2.2).

Table 2. 1. Inhibition of acetylcholinesterase (AChE) activity in adult *S. mansoni* and *S. haematobium* Triton X-100-soluble extracts and *S. mansoni* soluble egg extract (SEA) by a series of ruthenium complexes.

Compound	*S. mansoni* extract		*S. haematobium* extract		*S. mansoni* SEA	
	AChE Inhibition (%)[a, b]	IC_{50} (µM)[b]	AChE Inhibition (%)[a, b]	IC_{50} (µM)[b]	AChE Inhibition (%)[a, b]	IC_{50} (µM)[b]
$Ru(phen)_2(Me_2bpy)$	9.3 ± 1.8	67.0 ± 11.6	13.3 ± 1.8	90.2 ± 1.2	66.5 ± 5.8	ND
$Rubb_{12}$-mono	11.1 ± 0.8	18.9 ± 2.8	52.2 ± 0.5	1.1 ± 0.1	73.5 ± 4.9	ND
$Rubb_{12}$-di	73.9 ± 0.5	1.6 ± 0.3	20.9 ± 0.2	16.8 ± 0.4	94.2 ± 0.8	ND
$Rubb_7$-tri	84.3 ± 4.5	0.4 ± 0.0	37.2 ± 0.3	0.4 ± 0.0	77.5 ± 0.6	ND
$Rubb_{10}$-tri	78.7 ± 0.7	0.5 ± 0.0	73.6 ± 2.0	3.0 ± 0.0	91.3 ± 0.3	ND
$Rubb_{12}$-tri	89.4 ± 0.4	0.3 ± 0.0	87.6 ± 0.4	0.7 ± 0.0	96.3 ± 1.4	0.2 ± 0.0
$Rubb_{16}$-tri	46.8 ± 1.1	1.9 ± 0.0	31.6 ± 0.5	18.0 ± 0.1	89.7 ± 0.1	ND
$Rubb_7$-tl	73.4 ± 1.5	1.0 ± 0.0	24.8 ± 0.3	36.4 ± 4.4	78.7 ± 8.0	ND
$Rubb_{12}$-tl	89.9 ± 1.6	0.3 ± 0.0	55.4 ± 2.6	1.0 ± 0.1	97.2 ± 0.6	0.2 ± 0.0
$Rubb_{16}$-tl	76.3 ± 1.6	2.4 ± 0.1	59.3 ± 0.1	1.9 ± 0.1	90.8 ± 0.2	ND
$Rubb_7$-tnl	84.0 ± 0.6	0.9 ± 0.1	19.5 ± 0.3	ND	98.1 ± 0.3	ND
$Rubb_{12}$-tnl	80.0 ± 0.1	0.4 ± 0.0	75.9 ± 2.5	3.2 ± 0.0	92.7 ± 0.3	0.3 ± 0.0
$Rubb_{16}$-tnl	73.0 ± 0.8	2.3 ± 0.1	51.1 ± 6.0	ND	89.6 ± 0.1	ND

[a]inhibition of *Sm*-AChE activity assessed at 1µM.

[b]data represents the mean of duplicate experiments ± SEM

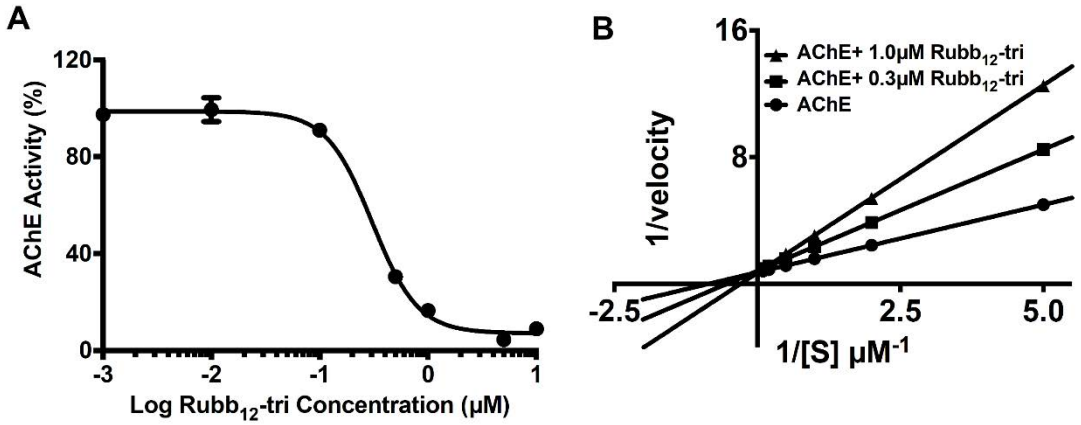


Fig 2.2. Effect of ruthenium complexes on *Sm*AChE activity in adult *S. mansoni* extracts.

Concentration-dependent inhibition of *Sm*AChE activity in *S. mansoni* adult extracts when treated with Rubb12-tri, a representative member of the ruthenium complexes tested, as determined by Ellman assay. (**A**) Dose-response curve of Rubb12-tri. (**B**) Lineweaver-Burk inhibition plot of *Sm*AChE activity in *S. mansoni* adult extracts in the presence of Rubb12-tri. Data represent the average of triplicate experiments ± SEM.

Interestingly, a different pattern of inhibition by the ruthenium complexes was observed against *Sh*AChE activity with the IC_{50} values being considerably more varied than was observed for *Sm*AChE activity, and not all compounds showed correlated potency between the two species. In addition, there was more variability among the tri- and tetra-nuclear complexes with the more lipophilic complexes (e.g. $Rubb_{10}$-tri and $Rubb_{12}$-tri) having stronger inhibitory activity. $Rubb_{12}$-mono, $Rubb_{12}$-tri and $Rubb_{12}$-tnl showed greater activity and the IC_{50} values of these complexes were less than 1 μM. Overall, ruthenium compounds displayed a similar pattern of inhibition

against *Sm*AChE activity in *S. mansoni* egg versus adult extracts, although most complexes showed a stronger inhibitory capacity towards *Sm*AChE activity in SEA with three compounds ($Rubb_{12}$-tri, $Rubb_{12}$-tl and $Rubb_{7}$-tnl) achieving >95% inhibition.

In order to examine the selectivity for *Sm*AChE, the series of ruthenium complexes was screened (10 µM) against *S. mansoni* extract for inhibition of two major tegumental enzymes –*Sm*NPP5 and *Sm*AP. None of the compounds strongly inhibited activity of either enzyme at 10 µM, a tenfold higher concentration than was used for the *Sm*AChE inhibition assays (Table 2.2).

Table 2.2. Inhibition of SmNPP-5 and alkaline phosphatase activity in adult *S. mansoni* Triton X-100-soluble extracts by a series of ruthenium complexes.

Compound	SmNPP-5 Inhibition (%)[a]	AP Inhibition (%)[a]
Ru(phen)2(Me2bpy)	2	3
Rubb12-mono	15	2
Rubb12-di	1	1
Rubb7-tri	9	1
Rubb10-tri	12	8
Rubb12-tri	2	4
Rubb16-tri	15	6
Rubb7-tl	13	2
Rubb12-tl	3	2
Rubb16-tl	26	1
Rubb7-tnl	13	1
Rubb12-tnl	24	1

[a]inhibition of enzyme activities assessed at 10 µM.

2.3.2 *In vitro* effect of ruthenium complexes on larval *S. mansoni* parasites

The entire series of Ru complexes were screened for their larvacidal activity against *S. mansoni* schistosomula with IC_{50} values calculated for the most effective compounds in a separate experiment. (Table 2.3).

Table 2.3. Potency of selected ruthenium complexes against *S. mansoni* schistosomula after 48 h treatment.

Compound	IC_{50} (µM)[1]
$Rubb_{12}$-tri	45.1 ± 4.8
$Rubb_{12}$-tl	68.5 ± 3.6
$Rubb_{12}$-tnl	42.8 ± 1.2
$Rubb_{7}$-tl	81.3 ± 0.6
$Rubb_{7}$-tnl	30.3 ± 2.0
$Rubb_{16}$-tnl	27.3 ± 0.4

[1]data represents the mean of duplicate experiments ± SEM

2.3.3 *In vitro* effect of ruthenium complexes on adult *S. mansoni* parasites

S. mansoni worms were cultured in the presence of 50 µM of each ruthenium complex to investigate their effectiveness in killing adult parasites, which was assessed by lack of motility. The effects of selected compounds on worm survival is shown in Fig 2.3A. Similar to the enzyme inhibition in parasite extracts, the tri-and tetra-nuclear complexes were the most effective

compared to the mono- and di-nuclear complexes. The killing ability increased with the increasing number of ruthenium centers in the complex, and the tetra-nonlinear complexes were more active in comparison with their linear counterparts. As with the schistosomula killing experiment, the most effective compounds (five) were tested again, this time at concentrations of 100 µM, 50 µM and 10 µM (Fig 2.3B). All tri-and tetra-nuclear complexes (10 µM) killed 100% of the parasites in six days. Treatment with the ruthenium complexes induced significant changes in the gross morphology of the parasites (Fig 2.3C). In particular, a tight coiling of the treated worms was observed.

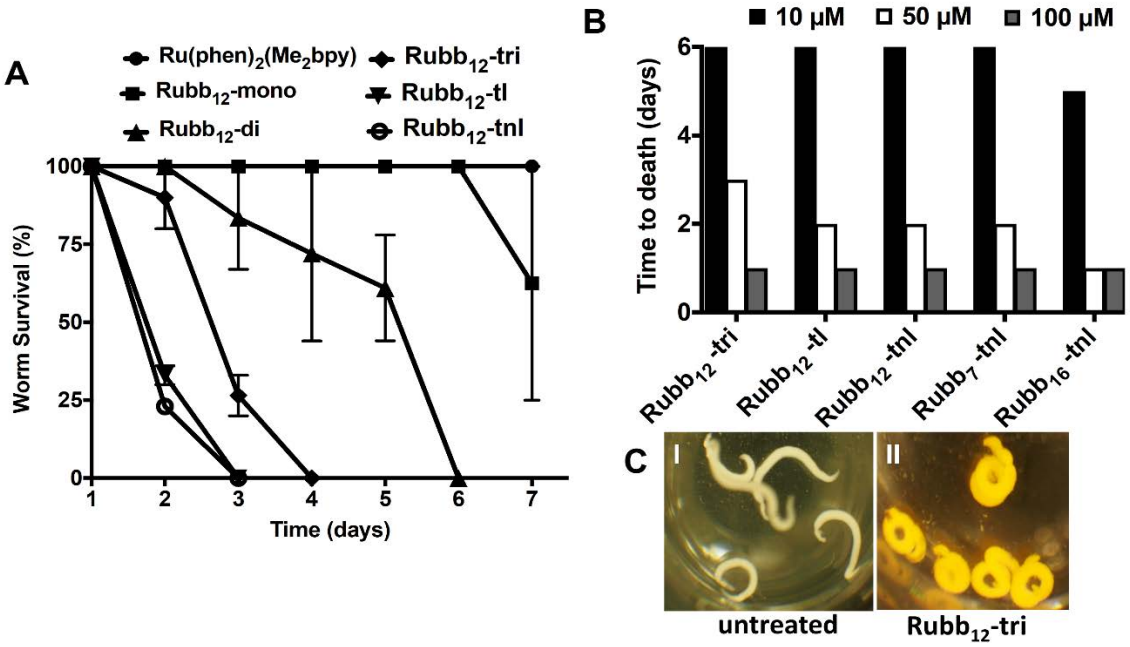


Fig 2.3. Activity of ruthenium complexes against adult *S. mansoni* worms. (A) Selective representation of survival of parasites (cultured in Basch media) after treatment with Ru complexes (50 µM). Data represents the average of duplicate experiments ± SEM. **(B)** Survival of parasites (cultured in Basch media) after treatment with various concentrations of the most potent ruthenium complexes determined from the screening experiment. Data represents the average of

duplicate experiments ± SEM. **(C)** Alteration in general morphology of adult *S. mansoni* worms caused by ruthenium complexes. I: control parasites; II: parasites treated with $Rubb_{12}$-tri.

2.3.4 *In vitro* effect of ruthenium complexes on *S. mansoni* egg hatching and development

S. mansoni egg hatching in the presence (50 µM) of ruthenium complexes was investigated by measuring the motility index of hatched miracidia from eggs using the xWORM assay (Fig 2.4A). Significant reduction in hatching/motility was observed for 9/13 compounds tested. $Rubb_{12}$-tnl was the most effective, reducing the motility index by 67% ($P < 0.0001$). To analyze the effect of ruthenium complexes on egg development, the eggs released from worms incubated for 3 days in the presence of 5 µM $Rubb_{12}$-tri were scored for morphology. Eggs released from treated worms were abnormally developed (immature or misshapen) compared to controls (Fig 2.4B; $P < 0.01$).

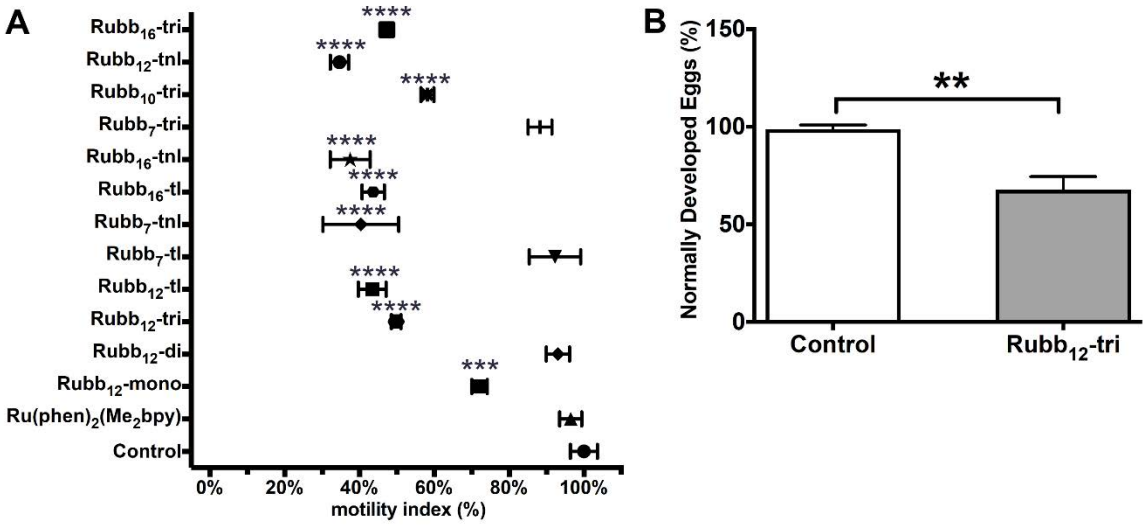


Fig 2.4. Inhibition of *S. mansoni* egg hatching and effect on egg development by ruthenium complexes. (A) Graph representing the percentage of *S. mansoni* eggs hatched (motility index) in the presence of various ruthenium complexes (50 µM) as determined by the x-WORM motility assay. Data represents the average of triplicate experiments ± SEM. **(B)** Triplicate sets of five pairs of adult *S. mansoni* worms were cultured in Basch media with or without 5 µM $Rubb_{12}$-tri for 72 h. The eggs released into the media were counted and those that were misshapen or immature were scored as "abnormally developed". Graph shows the difference in percentage of normally developed eggs between treated and control groups and data represents the average of triplicate experiments ± SEM. Differences in egg hatching were measured by ANOVA and differences in egg development by t test. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$,****$P \leq 0.0001$.

2.3.5 Mechanism of anti-schistosome action of $Rubb_{12}$-tri and $Rubb_{16}$-tnl

Adult worms were cultured in the presence of sub-lethal concentrations (5 µM) of $Rubb_{12}$-tri or $Rubb_{16}$-tnl - the two ruthenium compounds deemed to be most effective at killing adult parasites – for 24 h and then examined for changes in surface and somatic *Sm*AChE activity and glucose uptake (given this pathway can be ablated by an organophosphorus AChE inhibitor). Treated worms showed significantly decreased levels of surface and somatic *Sm*AChE activity in the presence of each complex (Fig 2.5A and 2.5B) however, consistent with enzyme inhibition experiments using parasite extracts, AP activity was not significantly affected (Fig 2.5C).

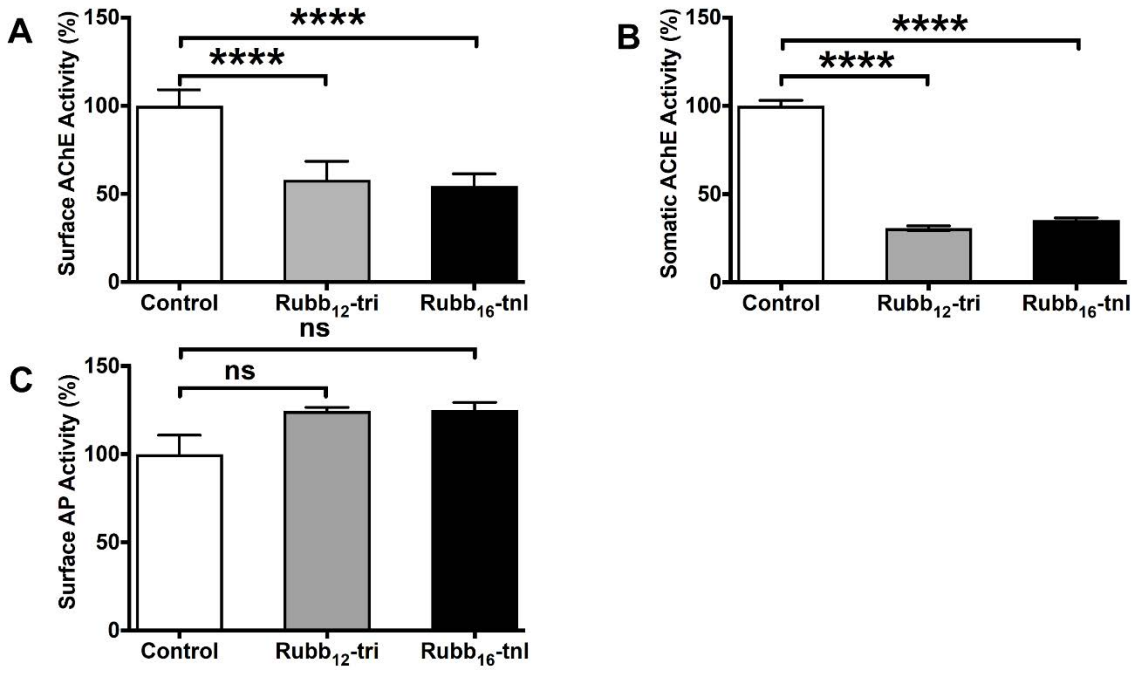


Fig 2.5. Action of $Rubb_{12}$-tri and $Rubb_{16}$-tnl on adult *Sm*AChE and *Sm*AP activity.

Five pairs of worms were cultured in Basch media for 24 h in the presence of a sub-lethal dose (5 µM) of $Rubb_{12}$-tri or $Rubb_{16}$-tnl and then incubated in AChE assay buffer or AP assay buffer. PBS extracts were then made from equal amounts of control and treated worms and 30 µg of each

extract was used to determine somatic *Sm*AChE activity by the Ellman method. **(A)** Surface *Sm*AChE **(B)** somatic *Sm*AChE and **(C)** surface *Sm*AP activity of control worms and worms treated with $Rubb_{12}$-tri or $Rubb_{16}$-tnl. For all assays, data are the average of triplicate biological and technical experiments ± SEM. Differences were measured by ANOVA. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, **** $P \leq 0.0001$.

The amount of glucose in the media of parasites treated with either complex was significantly higher than control worms (Fig 2.6A), suggestive of impaired glucose uptake in the presence of ruthenium compounds. Consistent with these results, extracts of treated parasites had a significantly lower glycogen content compared to controls (Fig 2.6B). Moreover, scanning electron micrographs of the tegument of male parasites treated with $Rubb_{12}$-tri or $Rubb_{16}$-tnl showed the dorsal tubercules (a site of glycogen storage (Bueding et al. 1967)) to be withered and flattened (Fig 2.6C).

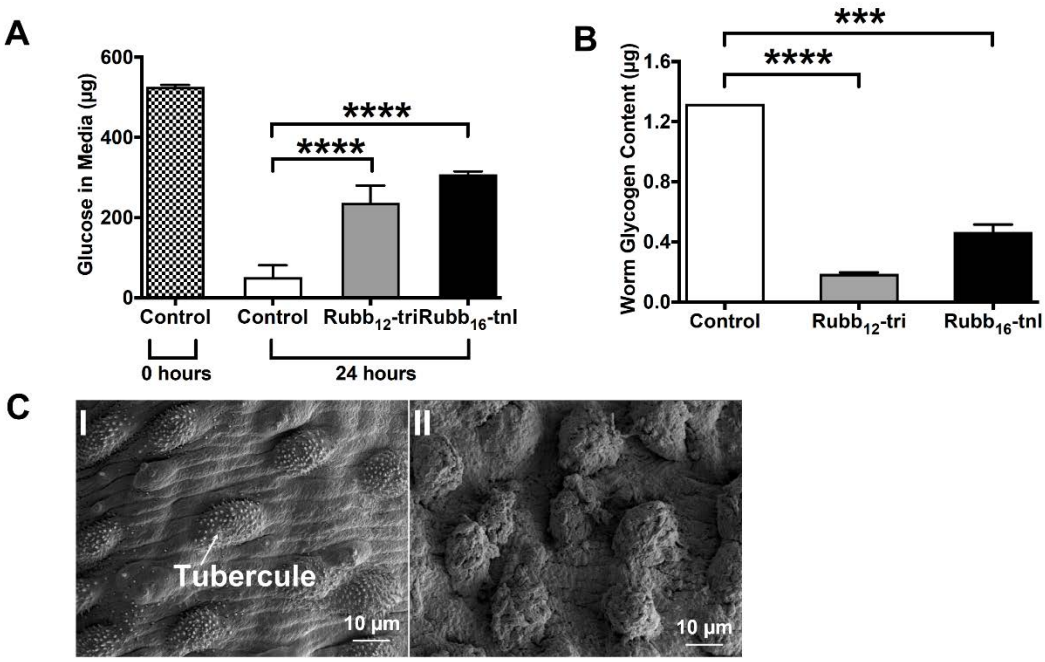


Fig 2.6. Effect of $Rubb_{12}$-tri and $Rubb_{16}$-tnl on adult *S. mansoni* glucose uptake and storage ability. Worms were cultured in Basch media for 24 h in the presence of a sub-lethal dose (5 µM) of $Rubb_{12}$-tri or $Rubb_{16}$-tnl. Worms were then incubated for 24 h in DMEM containing 1 mg/ml glucose. PBS extracts were then made from equal amounts of control and treated worms and 30 µg of each extract was used to determine the glycogen content of the worms using a modified glucose oxidase assay. **(A)** Amount of glucose in media collected from control and treated *S. mansoni* worms. **(B)** Levels of glycogen in extracts made from control and treated *S. mansoni* worms. For all assays, data are the average of triplicate biological and technical experiments ± SEM. Differences were measured by ANOVA. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, **** $P \leq 0.0001$. **(C)** Scanning electron micrographs of adult male *S. mansoni* worm tegument after incubation with 5 µM $Rubb_{12}$–tri; (I) intact tubercles of control worms; (II) withered tubercules of treated worms.

2.3.6 Toxicity of $Rubb_{12}$-tri and $Rubb_7$-tnl

The toxicity of $Rubb_{12}$-tri and $Rubb_7$-tnl, two of the ruthenium complexes shown to have high *in vitro* efficacy against all schistosome stages tested, was assessed against human bile duct cells and in male BALB/c mice (6-8 weeks) before investigating their *in vivo* efficacy in a mouse model of schistosomiasis. PZQ and DDVP - a metabolite of the anti-schistosome AChE inhibitor metrifonate – were included in the study for comparison. When $Rubb_{12}$-tri and $Rubb_7$-tnl were used at 5 μM, a concentration where they significantly inhibited surface and somatic *Sm*AChE activity and glucose uptake in adult worms, cell viability was 42% and 73% for $Rubb_{12}$-tri and $Rubb_7$-tnl, respectively. The EC_{50} values of $Rubb_{12}$-tri and $Rubb_7$-tnl were calculated as 3.489 ± 0.532 μM and 6.829 ± 0.625 μM, respectively. DDVP was highly toxic to the cells, killing 100% of the cells even at 0.1 μM (Fig 2.7). To determine the MTD in mice, $Rubb_{12}$-tri or $Rubb_7$-tnl was administered to groups of male BALB/c mice (6-8 weeks). $Rubb_7$-tnl did not show any toxicity even after five consecutive daily injections (the proposed dosage frequency of the *in vivo* drug efficacy study) of 10 mg/kg (mice were adversely affected at doses of 20 mg/kg) and so the MTD of $Rubb_7$-tnl was considered to be at least 10 mg/kg. The MTD of $Rubb_{12}$-tri, using the same dosage frequency, was determined to be 2 mg/kg (mice were adversely affected at doses of 4 mg/kg). If 100% bioavailability is assumed due to i.v. administration, the host bloodstream concentrations of $Rubb_7$-tnl and $Rubb_{12}$-tri at the MTD can be approximated at 12 μM and 49 μM, respectively.

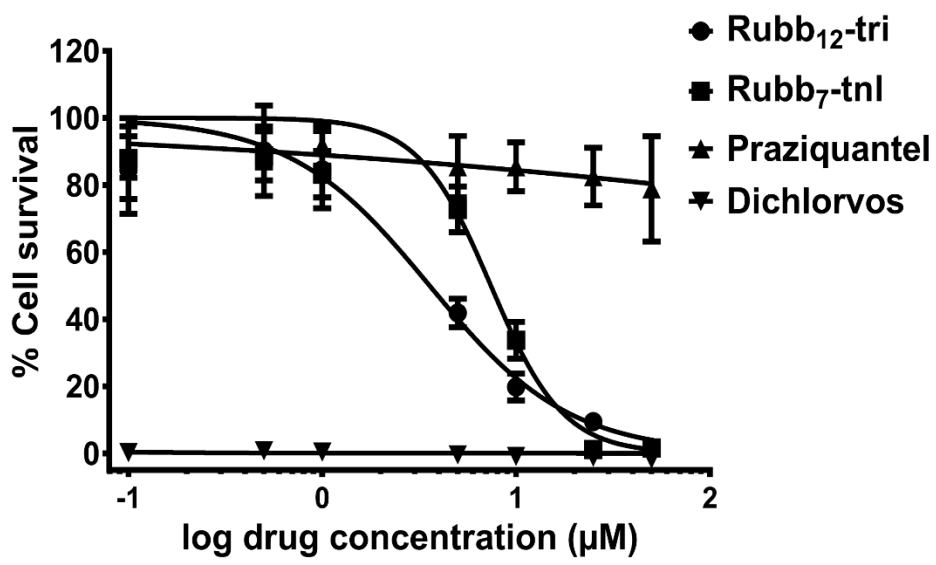


Fig 2.7. Cytotoxicity of ruthenium complexes.

Toxicity against human bile duct cells (H69) after 72 h incubation with $Rubb_{12}$-tri or $Rubb_7$-tnl, PZQ and DDVP – an organophosphate AChE inhibitor - as determined by the MTT cell viability assay. Data are the average of six replicate experiments ± SEM.

2.3.7 *In vivo* efficacy of $Rubb_{12}$-tri and $Rubb_7$-tnl

Over two independent trials, a significant reduction in worm burden (42%, $P = 0.009$) was seen in mice treated with $Rubb_{12}$-tri compared to controls whereas a non-significant trend towards decreased worm burden was observed in $Rubb_7$-tnl-treated mice (Fig 2.8A). Surface *Sm*AChE activity was decreased in worms collected from mice treated with ruthenium complexes but only reached significance for $Rubb_{12}$-tri-treated animals (Fig 2.8B). Surface *Sm*NPP5, surface *Sm*AP, somatic *Sm*AChE and glucose uptake activity was not significantly different.

Although there was no decrease in parasite egg burden (as determined by recovery of ova from the liver), the viability of these eggs from trial 1 was examined and highly significant ($P < 0.0001$) reductions in hatching capability (68% and 56%) were observed for the $Rubb_{12}$-tri- and $Rubb_{7}$-tnl-treated mice, respectively (Fig 2.8C). Egg hatching viability was not determined for trial 2. Moreover, eggs released from worms recovered from treated mice (trial 2) were significantly different ($P < 0.0001$) in terms of their development (immature, misshapen, eggshell malformation) compared to those from parasites recovered from control mice (Fig 2.8D and 2.8E). Morphology of eggs released from worms recovered from mice in trial 1 was not determined.

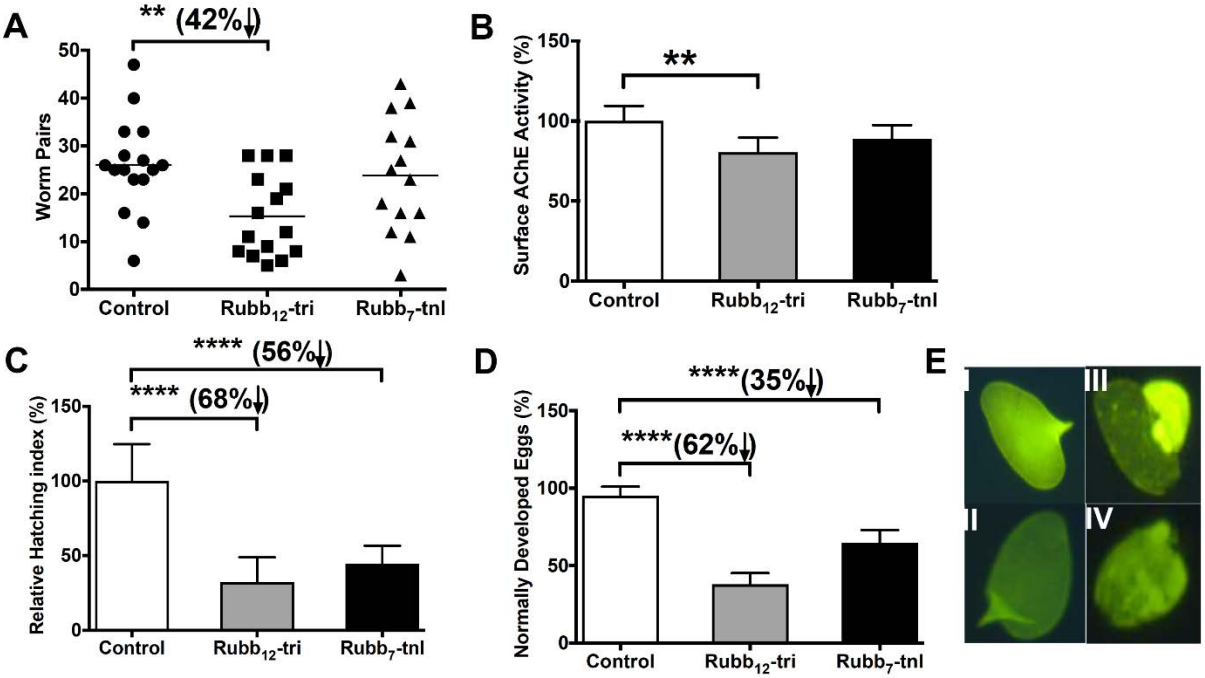


Fig 2.8. *In vivo* effect of Rubb$_{12}$-tri and Rubb$_7$-tnl on *S. mansoni*-infected mice.

(A) Effect of Rubb$_{12}$-tri and Rubb$_7$-tnl on adult worm burden. Symbols represent data from individual mice and are the combination of two independent trials (trial 1 PBS control – n = 8 mice, trial 1 Rubb$_{12}$-tri-treated – n = 8 mice, trial 1 Rubb$_7$-tnl-treated – n = 6 mice, trial 2 PBS control – n = 7 mice, trial 2 Rubb$_{12}$-tri-treated – n = 7 mice, trial 2 Rubb$_7$-tnl-treated – n = 8 mice). **(B)** Surface *Sm*AChE activity of worms recovered from control and treated mice. Data are the average of triplicate technical assays ± SEM on extracts made from worms (five pairs) pooled from each group of each of the two trials. **(C)** Hatching viability of eggs obtained from the pooled livers of control and treated mice from trial 1 (PBS control – n = 8, Rubb$_{12}$-tri-treated – n = 8, Rubb$_7$-tnl-treated – n = 6). Data are the average of ten replicate counts ± SEM of hatched miracidia. **(D)** Eggs were harvested from triplicate sets of worms (five pairs) from a pool of each group of trial 2 (PBS control – n = 7 mice, Rubb$_{12}$-tri-treated - n = 7 mice, Rubb$_7$-tnl-treated – n = 8 mice) after culturing the

parasites for 24 h in Basch media and the percentage of mature, morphologically "normal" eggs released from worms recovered from control and treated mice was assessed. Data are the average counts ± SEM of eggs released from triplicate sets (five pairs) of worms from trial 2. Differences were measured by ANOVA. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, **** $P \leq 0.0001$. (E) Auto-fluorescence images (20×) of eggs released from worms recovered from (I and II) control and (III and IV) treated mice.

2.4 Discussion

Control of schistosomiasis, a neglected tropical disease which affects over 200 million people, relies on periodic treatment with single drug, PZQ, a strategy that is unsustainable in its current form (Seto et al. 2011; Wang et al. 2012; Vale et al. 2017). As no new anti-schistosome drug (or any anti-parasitic) has been registered in the last decade (Pedrique et al. 2013), the need for additional therapeutic compounds has become unquestionable and has driven research efforts towards the discovery of alternative anti-schistosome chemotherapies, including those derived from natural products (Wangchuk et al. 2016) and metal-based compounds (Kuntz et al. 2007). Accordingly, this study has described the anti-schistosome efficacy of a series of mono- and multi-nucleated metal-based compounds (ruthenium complexes) which exert their action through the inhibition of AChE, an enzyme pivotal to the control of worm neuromuscular function and implicated in the mediation of host glucose scavenging (Camacho et al. 1995; Arnon et al. 1999).

It has been previously shown that mononuclear ruthenium complexes inhibit AChE (*E. electricus*) by a non-competitive or mixed mode of inhibition (Vyas et al. 2014). However, in the present study, the trinuclear complex ($Rubb_{12}$-tri) displayed a competitive mode of inhibition in the kinetic experiments. The mononuclear polypyridylruthenium(II) complexes are thought to interact with the peripheral anionic site (PAS) of AChE located at the rim of the active-center gorge through a combination of electrostatic and hydrophobic interactions (Meggers 2009). The tri- and tetra-nuclear complexes showed greater activity compared to mononuclear complexes, presumably due to the presence of the flexibly-linked multiple metal centers which may provide more interactions (electrostatic and hydrophobic) with the PAS, or each individual center may contribute nonspecific additional points of contact. The activity of the ruthenium complexes varied in extracts made from different life stages (adult extracts and SEA) and various species of the parasite which is most likely due to differences in enzyme orthologs and the existence of multiple paralogs at least in *S. mansoni* (Chapter 3) which are present in different life stages and species (Arnon et al. 1999).

Encouraged by the activity of ruthenium complexes against parasite extracts, selected compounds were tested against all three intra-mammalian stages of the parasite *in vitro* and found similar trends in anti-parasite activity as was seen for extracts; i.e., the tri- and tetra-nucleated complexes were more effective against each stage of the parasite than the mono- and di-nuclear compounds. Three of the most effective compounds in terms of their combined activity against *S. mansoni* extracts and all intra-mammalian stages were $Rubb_7$-tnl, $Rubb_{12}$-tri

and $Rubb_{16}$-tnl. Further, $Rubb_{12}$-tri was also the most effective at inhibiting AChE activity in *S. haematobium* extracts; availability of material prevented us from doing any experiments on live parasites or eggs but I believe that that the similar trends observed between *S. mansoni* anti-parasitic activity and extract activity hold true for *S. haematobium* and ruthenium complexes such as $Rubb_{12}$-tri would display potent anti-schistosome activity against this species. Further, *S. haematobium* has higher levels of tegumental AChE than *S. mansoni*, which makes the parasite more sensitive to AChE inhibitors (Camacho et al. 1994) and might render this species more vulnerable to ruthenium drugs.

Any differences observed between the AChE-inhibitory ability and anti-schistosome effect of ruthenium complexes could be due to the target of these drugs not solely being AChE. While I showed that ruthenium complexes did not have any inhibitory effects on the major tegumental enzymes *Sm*NPP5 and *Sm*AP, these drugs have been documented to act as dual inhibitors of telomerase and topoisomerase (Liao et al. 2015), thioredoxin reductase (Luo et al. 2014) and protein and lipid kinases (Meggers 2009). There are numerous reports in the literature documenting the development of drug resistance in parasites due to mutation (for example, benzamidazole resistance in nematodes due to single nucleotide polymorphisms in β-tubulin (Von Samson-Himmelstjerna et al. 2007) and mutation of a schistosome sulfotransferase resulting in resistance to oxamniquine (Valentim et al. 2013), and so the use of a drug that is directed against multiple molecular targets may decrease the chance of resistance evolving.

Visually, the effects of the compounds were most pronounced against adult worms, which became immobile and coiled when incubated with ruthenium complexes, possibly due to paralysis induced by *Sm*AChE inhibition and cholinergic accumulation, effects similarly seen in schistosomes treated with other inhibitors of *Sm*AChE (Bueding et al. 1972; Hillman et al. 1975; Pax et al. 1981). This observation should be treated with caution, however, as other drugs, such as PZQ (which is not a *Sm*AChE inhibitor), induce the same morphological changes. Additional evidence of the mechanistic effects of ruthenium complexes on schistosomes manifested in the reduced glucose uptake observed in drug-treated worms, potentially a consequence of inhibiting the tegumental *Sm*AChE-mediated regulation of host glucose scavenging, a pathway unique to schistosomes (Camacho et al. 1995). Further confirmation of this inhibition was evidenced by significantly depleted glycogen stores (quantified in parasite extracts and observed by the withering of male tubercules – a site of glycogen storage (Bueding et al. 1967)) in these parasites, an effect seen in worms recovered from mice treated with carbamate-based AChE inhibitors (Bueding et al. 1967). In another example of tegument-mediated glucose regulation, previous work by You and colleagues (You et al. 2010) has shown that inhibition of schistosome insulin receptor activity significantly decreased glucose uptake by the parasite. It would be interesting to explore any relationship that existed between these two regulatory mechanisms, a possibility given the alternative is to imagine the evolution of two mechanistically distinct pathways of glucose regulation. Additionally, a combination chemotherapeutic strategy could be developed using drugs which target different aspects of schistosome glucose uptake.

Two of the ruthenium complexes which was considered most effective *in vitro* ($Rubb_{12}$-tri and $Rubb_{7}$-tnl) were tested for cytotoxicity before assessment of their *in vivo* efficacy in a mouse model of schistosomiasis. $Rubb_{16}$-tnl, even though effective *in vitro*, was not included in the cytotoxicity assay or the *in vivo* study as earlier work by us has shown that ruthenium complexes with longer chain lengths are more toxic to eukaryotic cells (Gorle et al. 2016). Both ruthenium complexes tested, $Rubb_{7}$-tnl and $Rubb_{12}$-tri, exhibited lower cytotoxicity against eukaryotic cells (H69) compared to DDVP, an organophosphorus AChE inhibitor and previously licensed, but now withdrawn anti-schistosome drug. Further, studies comparing AChE from schistosomes and higher eukaryotes (Jones et al. 2002; Bentley et al. 2003) reveal differences in functionally important amino acid residues (human AChE shares 33-36% primary sequence homology across all schistosome AChEs) with the active site serine conserved across species. It was previously shown that DDVP covalently binds to the active site serine and reduces the AChE activity in eukaryotes (e.g., in rat forebrain, erythrocytes and plasma) (Hinz et al. 1996; Jann 1998). By contrast, it was considered that the ruthenium complexes might be relatively less toxic to mice than DDVP due to the differential binding to AChE. The results of the MTD study, where both the ruthenium complexes were well tolerated by mice, supported this argument.

$Rubb_{12}$-tri and $Rubb_{7}$-tnl both showed promising *in vivo* efficacy at doses which were equivalent to lethal *in vitro* concentrations yet well tolerated in mice, with $Rubb_{12}$-tri-treated mice showing a significant reduction in worm burden and recovered worms displaying a small but significant decrease in tegumental AChE activity, providing evidence that the anti-schistosome effect may

be partially due to AChE inhibition. Studies by us on di-nuclear ruthenium complexes have shown the compounds to have a short serum half-life (Li et al. 2017), and the different *in vivo* efficacies of each complex in this study may be attributed to differences in the pharmacokinetic/pharmacodynamic (PK/PD) properties of $Rubb_{12}$-tri and $Rubb_{7}$-tnl. Although these experiments have yet to be performed, the differences exhibited between these two compounds in the cytotoxicity assay and tolerability study suggests that their PK/PD are not the same. Despite no significant reduction in egg burden in both trials, ova recovered from both $Rubb_{12}$-tri- and $Rubb_{7}$-tnl-treated mice had significantly reduced hatching ability and were morphologically abnormal compared to controls, in agreement with *in vitro* data. That there was no decrease in egg burden in light of a reduced worm load in either treated group was surprising, but this did result in an increase in the number of eggs per female in these groups, compared to controls. To explain these observations, I postulate that treatment with ruthenium drugs may stimulate schistosome reproductive tract motility (AChE inhibitors have been shown to stimulate gastrointestinal motility in various organisms (De Giorgio et al. 2004; McNamara et al. 2008)), resulting in premature release of under-developed eggs, and have a direct effect on egg formation, resulting in abnormally developed eggs (studies in ticks have shown that treatment with AChE inhibitors effect ova development (Perez-Gonzalez et al. 2014; Prado-Ochoa et al. 2014)). Another possible factor contributing to abnormal egg development is that the worms are under-nourished due an impaired glucose uptake ability (albeit an effect that could only measure *in vitro*) resulting from ruthenium drug treatment and unable to meet the energetically demanding task of producing normally developed ova. There is considerable interest in the use

of agents that show ovicidal activity or affect oviposition to control schistosomiasis due to their ability to block transmission of the disease. In this regard, ruthenium complexes offer a potential advantage over PZQ in that it is only effective against mature worms and so cannot be used to interrupt disease transmission, as evidenced by high rates of re-infection in PZQ-treated endemic populations (Webster et al. 2013).

So far, this is the first report detailing the anti-parasitic activity of ruthenium complexes and this work has identified some lead anti-schistosome compounds. The modular nature of ruthenium complexes makes it possible to synthesize these compounds to target specific enzymes, so future work will involve tailoring Ru complexes to increase their selectivity and potency. Finally, these complexes could be administered in combination with PZQ, overcoming the limitations of current monotherapy and augmenting existing schistosomiasis control initiatives.

CHAPTER 3

Characterization of novel cholinesterase paralogs of *Schistosoma mansoni*

3.1 Introduction

The functioning of the nervous system is a tightly regulated process controlled through multiple catalytic and non-catalytic signaling molecules. Among the catalytic molecules, cholinesterases (ChEs) play a pivotal role in regulating the signaling activity of the nervous system. There are two major types of ChEs, acetylcholinesterase (AChE) and pseudocholinesterase, or butyrylcholinesterase (BChE), and they can be distinguished both kinetically and pharmacologically (Girard et al. 2007). AChE selectively hydrolyzes the neurotransmitter acetylcholine (ACh) to maintain neurotransmitter homeostasis (You et al. 2017) while the main role of BChE is widely accepted to be the detoxification of organophosphorus esters which are inhibitors of AChE (Lockridge 2015). ChEs are generally believed to be functionally redundant in cholinergic signaling with the main differences between paralogs lying in their spatial and temporal expression as well as non-cholinergic functionality (Soreq et al. 2001; Silman et al. 2005).

While AChE activity has been previously reported in different schistosomes (Arnon et al. 1999), "pre-genomic" studies have documented only one AChE-encoding gene in each species (Bentley et al. 2003; Bentley et al. 2005). Moreover, to the best of our knowledge, genes encoding proteins with BChE activity have not been previously described in schistosomes or any other helminth. Interrogation of the now fully annotated *S. mansoni* genome (Berriman et al. 2009) has revealed three different *Sm*ChE paralogs; however, their individual contributions to ChE function remain unknown.

Traditionally, parasite ChEs have been regarded solely as neurotransmitter terminators; however, there is increasing data to suggest that these enzymes play a variety of roles within the cells that extend beyond this cholinergic function (Soreq et al. 2001; Silman et al. 2005; Paraoanu et al. 2008; Zhang et al. 2012). In schistosomes, the most prominent among these is in the uptake of glucose by the parasite from the external environment (Camacho et al. 1995). One of the proposed mechanisms is by limiting the interaction of host ACh with tegumental nicotinic ACh receptors (nAChRs) thus availing surplus glucose (Camacho et al. 1995; Camacho et al. 1995). The nAChRs are also associated both spatially and temporally with surface AChE expression and are concentrated on the tegument (Camacho et al. 1995), the primary site of glucose uptake (Skelly et al. 2014).

Many intestinal nematodes secrete AChE (Rathaur et al. 1987; Lawrence et al. 1993; Hussein et al. 2002; Hussein et al. 2002; Selkirk et al. 2005; Selkirk et al. 2005; Hewitson et al. 2013), which play a vital role in both cholinergic and non-cholinergic activities. For instance, emerging evidence suggests that AChE promote parasite survival not only by inhibiting the host cholinergic signaling resulting in inhibition of muscle contraction (Selkirk et al. 2005) but also by altering host cytokine profiles which suppress the development of M2 macrophages (Vaux et al. 2016). Despite this breadth of literature in nematodes, there has been no documentation of secreted AChE activity from schistosomes.

In this chapter, two novel ChEs from *S. mansoni* (*Sm*ChEs): *Sm*BChE1 and *Sm*AChE3 have been identified and functionally characterized, in addition to further characterizing the only previously identified AChE-encoding gene from *S. mansoni* (Bentley et al. 2003; Bentley et al. 2005), which I have termed *Sm*AChE1. In a first for schistosomes, *Sm*ChE activity in ES (excretory and secretory) products of the parasite have also been documented and partially characterized. Finally, evidence is provided that the novel *Sm*BChE1 may act as a competitive binder of AChE inhibitors to detoxify the effects of these molecules.

3.2 Material and Methods

3.2.1 Ethics statement

All experimental procedures reported in the study were approved by the James Cook University (JCU) animal ethics committee (ethics approval numbers A2271 and A2391). Mice were maintained in cages in the university's quarantine facility (Q2152) for the duration of the experiments. The study protocols were in accordance with the 2007 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the 2001 Queensland Animal Care and Protection Act.

3.2.2 Parasite maintenance, culture and ES collection

Biomphalaria glabrata snails infected with *S. mansoni* (NMRI strain) were obtained from the Biomedical Research Institute (BRI). Cercariae were shed from infected snails through exposure to light at 28°C for 1.5 h and were mechanically transformed into schistosomula (Ramalho-Pinto

et al. 1974). To obtain adult *S. mansoni* worms, 6-8 week old male BALB/c mice (Animal Resource Centre, WA) were infected with 180 cercariae via tail penetration and adults were harvested by vascular perfusion at 7-8 weeks post-infection (Lewis et al. 1986). Both adult worms and schistosomula were cultured (10 adult pairs/ml and 2000 schistosomula/ml, respectively, at 37°C and 5% CO_2 in serum-free modified Basch medium (Basch 1981) supplemented with 4× antibiotic/antimycotic (AA - 200 units/ml penicillin, 200 µg/ml streptomycin and 0.5 µg/ml amphotericin B) in 6 well plates. Media, containing ES products, was initially collected after 24 h (adults) and 3 h (schistosomula) and then harvested and replenished daily and stored at -80°C. Media was thawed when needed, pooled if required, concentrated through Amicon centrifugation filters (Sigma) with a 3 kDa molecular weight cutoff (MWCO) and buffer exchanged into phosphate buffered saline, pH 7.4 (PBS). Concentrated ES products were then quantified using the Pierce BCA™ Protein Assay kit (Thermofisher), aliquoted and stored at -80°C. To collect cercarial ES, freshly-shed cercariae were incubated in H_2O (4000/ml) at 25°C for 3 h. H_2O was filtered through Whatman filter paper (11 µm) to remove cercariae and ES products were concentrated, quantified and stored as for adult and schistosomula ES.

3.2.3 Parasite extract preparation

To make PBS-soluble extracts, worms were homogenized in PBS (50 µl/adult worm pair or 50 µl/1000 schistosomula) at 4°C using a TissueLyser II (Qiagen), homogenates incubated overnight with mixing at 4°C and the supernatant collected by centrifugation at 15,000 *xg* for 1 h at 4°C. Triton X-100-soluble extracts were made from the PBS-insoluble pellets by resuspension in 1%

Triton X-100, 40 mM Tris-HCl, pH 7.4, mixing overnight at 4°C and the supernatant collected by centrifugation at 15,000 *xg* for 1 h at 4°C. Tegument extraction was achieved using a combination of freeze/thaw/vortex technique (Sotillo et al. 2015). In brief, parasites were slowly thawed on ice, washed in TBS (10 mM Tris/HCl, 0.84% NaCl, pH 7.4) and incubated for 5 min on ice in 10 mM Tris/HCl, pH 7.4 followed by vortexing five times with 1-s bursts. *Subsequently, t*he tegumental extract was pelleted at 1000 x*g* for 30 min and solubilized (3x) in 200 µl of 0.1% (w/v) SDS, 1% (v/v) Triton X-100 in 40 mM Tris, pH 7.4 with pelleting at 15,000 x*g* between each wash. Protein concentration was determined using the Pierce BCA Protein Assay kit (Thermofisher), aliquoted and stored at -80°C until use.

3.2.4 Bioinformatics studies

Based on Pfam analysis (search = cholinesterase) of the *S. mansoni* genome (http://www.geneDB.org/Homepage/Smansoni), three *Sm*ChE paralogs (*Sm*AChE1 - Smp_154600, *Sm*BChE1 - Smp_125350 and *Sm*AChE3 - Smp_136690) were identified. Homologous ChE sequences from other species were identified using BLASTP. (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the resulting sequences were used to generate a multiple sequence alignment using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

MEGA 7 was used to generate a neighbor-joining tree with the Poisson correction distance method and a bootstrap test of 1000 replicates (Kumar et al. 2016). Structure-homology 3D models of *Sm*ChEs were generated using the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). For structure visualization and catalytic triad analyses, the Accelrys Discovery Studio (Accelerys Inc.) and UCSF Chimera MatchMaker ver. 1.4 (University of California) software packages were utilized.

3.2.5 Developmental expression analysis of *Sm*ChE genes by real-time qPCR

RNA from miracidia, sporocyst, cercariae, adult male worms, adult female worms, and eggs were obtained from BRI. Schistosomula were cultured as described in section 3.2.2, harvested (1000 parasites) after either 3 h, 24 h, 3 or 5 days, washed three times in PBS and stored at -80°C until use. Total RNA extraction from schistosomula stages was performed using the Trizol (Thermofisher) reagent according to manufacturer's instructions. After air-drying, RNA pellets were re-suspended in 12 µl DEPC water. Concentration and purity of RNA was determined using an ND2000 Nanodrop spectrophotometer (Thermofisher). Synthesis of cDNA was carried out using 1 µg of total RNA using Superscript-III-Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Finally, cDNA was quantified, diluted to 50 ng/µl, aliquoted and stored at -20°C.

Real-time qPCR primers for each *Sm*ChE (Supplementary Table 3.1) were designed using Primer3 (http://frodo.wi.mit.edu/). *Sm*COX1 was selected as an internal control to normalize relative

*Sm*ChE gene expression (Long et al. 2016). Each qPCR (1 µl (50 ng) of cDNA, 5 µl of 2x SYBR green master mix (Bioline), 1µl (5 pmol/µl) each of forward and reverse primers and 2 µl of nuclease-free water) was run in a Rotor-Gene Q thermal cycler (Qiagen) using 40 cycles of 95°C for 10 seconds, 50-55°C for 15 seconds and 72°C for 20 seconds. Stage-specific *Sm*ChE gene expression levels were normalized against *Sm*COX1 gene expression using the comparative $2^{-\Delta\Delta CT}$ method (Schmittgen et al. 2008). All results represent the average of five independent experiments with data presented as mean ± SEM.

3.2.6 Cloning and expression of *Sm*ChE gene fragments in *E. coli*

Complete ORFs for *Sm*AChE1, *Sm*BChE1 and *Sm*AChE3 were synthesized by GENEWIZ, (https://www.genewiz.com). Attempts to express full-length sequences in *E. coli* were unsuccessful, so primer sets incorporating *Nde*I (forward primer) and *Xho*I restriction enzyme sites (reverse primer) were designed (Supplementary Table 3.1) to amplify partial, non-conserved regions of each *Sm*ChE (p*Sm*ChE), which might prove more amenable to expression. Sequences for each p*Sm*ChE were amplified from each full-length template by PCR, amplicons digested by *Nde*I and *Xho*I, ligated into the similarly-digested pET41a expression vector (Novagen) and transformed into *E. coli* DH5α (Thermofisher) on LB plates supplemented with 50 µg/ml kanamycin (LB_{kan}). Colonies were screened by PCR and positive transformants were cultured in 5 ml LB_{kan} overnight at 37°C and 200 rpm. Plasmids were extracted from each culture by miniprep and recombination confirmed by *Nde*I/*Xho*I digestion and sequencing using T7 promoter and T7 terminator primers. Plasmids selected for expression were transformed into *E. coli* BL21(DE3)

(Thermofisher) and positive colonies seeded into 2x5 ml LB_{kan} and incubated overnight at 37°C and 200 rpm. Overnight starter cultures were diluted 1:100 in 1L of $2YT_{kan}$ broth and cultured at 37°C and 200 rpm. Cells were induced by 1 mM Isopropyl beta-D-1-thiogalactopyranoside (IPTG) when the OD_{600} of the cultures reached 1 and further incubated at 37°C for 24 h. Finally, cultures were harvested by centrifugation (8000 *xg* for 20 min at 4°C), re-suspended in 50 ml lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, and 40 mM imidazole) and stored at -80°C.

3.2.7 Purification of p*Sm*ChEs

Pilot expression experiments showed each p*Sm*ChE to be produced in insoluble inclusion bodies (IBs). Cell pellets were lysed by three freeze-thaw cycles at -80°C and 42°C followed by sonication on ice (10 x 5 s pulses [70% amplitude] with 30 s rest periods in between each pulse) with a Qsonica Sonicator. Triton X-100 was added to each lysate at a final concentration of 3% and incubated for 1 h at 4°C with end-over-end mixing. Insoluble material (containing p*Sm*ChEs) was pelleted by centrifugation at 20,000 *xg* for 20 min at 4°C. The supernatant was discarded, and IBs were washed twice by resuspension in 30 ml of lysis buffer followed by centrifugation at 20,000 *xg* for 20 min at 4°C. IBs were then solubilized sequentially by resuspension in 25 ml lysis buffers containing either 2 M, 4 M or 8 M urea, end-over-end mixing overnight at 4°C and centrifugation at 20,000 *xg* for 20 min at 4°C. Finally, supernatant containing solubilized IBs were diluted 1:4 in lysis buffer containing 8M urea and filtered through a 0.22 µm membrane (Millipore). Solubilized IBs were purified by immobilized metal affinity chromatography (IMAC) by loading onto a prepacked 1 ml His-Trap HP column (GE Healthcare) equilibrated with lysis buffer containing 8M

urea at a flow rate of 1 ml/min using an AKTA-pure-25 FPLC (GE Healthcare). After washing with 20 ml lysis buffer containing 8M urea, bound His-tagged proteins were eluted using the same buffer with a stepwise gradient of 50-250 mM imidazole (50 mM steps). Fractions containing p*Sm*ChEs (as determined by SDS-PAGE) were pooled and concentrated using Amicon Ultra-15 centrifugal devices with a 3 kDa MWCO (Sigma) and quantified using the Pierce BCA Protein Assay kit (Thermofisher). The final concentration of each p*Sm*ChE was adjusted to 1 mg/ml) and proteins were aliquoted and stored at -80°C.

3.2.8 Generation of anti-*Sm*ChE antisera and purification of IgG

Three groups of five male BALB/c mice (6-week-old) were intraperitoneally immunized with either p*Sm*AChE1, p*Sm*BChE1 or p*Sm*AChE3 subunits (50 µg/mouse). Antigens were mixed with an equal volume of Imject alum adjuvant (Thermofisher) and administered three times, two weeks apart. Two weeks after the final immunization, mice were sacrificed and blood was collected via cardiac puncture. Blood from all mice in each group was pooled and serum was separated by centrifugation after clotting and stored at −20°C. Polyclonal antibodies were purified from mouse sera by using Protein A Sepharose-4B (Thermofisher) according to manufacturer's instructions. Serum from naïve mice was similarly processed.

3.2.9 Immunolocalization using anti-*Sm*ChE antisera

Adult worm sections: Freshly perfused adult *S. mansoni* and *S. haematobium* worms were fixed in 4% paraformaldehyde, embedded in paraffin and sections (7 µm thick) were cut in a cryostat.

Following deparaffinization in xylene and rehydration in an ethanol series, antigen retrieval was performed by boiling the slides in 10 mM sodium citrate, pH 6.0, for 40 min followed by a solution of 10 mM Tris, 1 mM EDTA, 0.05% Tween, pH 9.0, for 20 min. All sections were then blocked with 10% heat-inactivated goat serum for 1 h RT. After washing 3 times with PBST, sections were incubated with anti-*Sm*AChE1, anti-*Sm*BChE1, anti-*Sm*AChE3, naïve sera (negative control), *S. mansoni* or *S. haematobium* infected sera (positive controls) (1:50 in PBST) overnight at 4°C and then washed again (3x5 min each). Finally, the sections were incubated with Goat-anti-mouse IgG-alexafluor647 (Sigma) (1:200 in PBST) for 1 h in the dark at RT. After a final washing step, slides were mounted with coverslips in Entellan mounting medium (Millipore). Fluorescence and bright-field microscopy were performed with an AxioImager M1 fluorescence microscope (Zeiss) using 10x and 20x objectives.

Live schistosomula: *In vitro*-cultured living cercariae and schistosomula (3 h, 24 h, 3 and 5 days old) larvae were harvested and washed with PBS and then blocked with PBST/10% heat-inactivated goat serum for 30 min at RT. Following three washes, the larvae were incubated with anti-*Sm*AChE1, anti-*Sm*BChE1, anti-*Sm*AChE3 or naïve serum (negative control) (1:100 in PBST) overnight at 4°C. Parasites were washed again before incubation with Goat-anti-mouse IgG-alexafluor647 (Sigma) (1: 200 in PBST) for 1 h in the dark at RT, followed by 3 washes. Finally, schistosomes were fixed in 4% paraformaldehyde and transferred to a microscopic slide for fluorescence microscopy using an AxioImager M1 fluorescence microscope (Zeiss).

3.2.10 Cloning and expression of full-length *Sm*ChEs in *P. pastoris*

Specific primer sets containing flanking *EcoR*I and *Xba*I sites were designed to amplify full-length sequences (minus the signal peptide) of *Sm*AChE1, *Sm*BChE1 and *Sm*AChE3 (f*Sm*ChEs). Amplicons were then digested and cloned in-frame with the α-factor secretion signal peptide into the C-terminal 6XHis tagged pPICZαA expression vector (Invitrogen). The constructs were then transformed into *E. coli* DH5α cells (Thermofisher), plated onto LB agar plates supplemented with 25 µg/ml of zeocin (LB_{zeo}) and incubated overnight at 37˚C. Recombinant colonies were confirmed by colony PCR and used to inoculate 5 ml LB_{zeo} which were grown overnight at 37˚C and 200 rpm. Plasmids were extracted by mini-prep and recombination verified by both restriction digestion and sequencing.

Recombinant plasmids (20 µg) were linearized with *Pme*I (f*Sm*AChE1 & f*Sm*BChE1) and *Sac*I (f*Sm*AChE3) for 3 h at 37°C, purified by ethanol precipitation and resuspended in 15 µl of H_2O. Linearized vectors were electroporated according to manufacturer's instructions into *P. pastoris* X-33 cells (Thermofisher) in 2 mm cuvettes (2 ms, 2000V, 25 µF, 200 Ω, square wave pulse), using a Gene Pulser Xcell (Bio-Rad), plated onto YPDS agar plates containing 100 µg/ml zeocin, covered in foil and incubated for 3 days at 30°C. Resultant colonies were then picked and patched onto YPDS agar containing 2 mg/ml zeocin and plates incubated at 30°C until colonies were visible. Integration of the recombinant cassette into the *P. pastoris* chromosome was confirmed by colony PCR. For each integrated *Sm*ChE, ten PCR-positive colonies were selected for pilot expression studies and each seeded into 3 ml of BMGY in 50 ml falcon tubes and incubated

overnight at 30°C with shaking at 250 rpm. Cells were pelleted (5000 *xg* for 20 min at RT), re-suspended in 6 ml BMMY to induce protein expression and incubated at 30°C with shaking at 250 rpm. Methanol was added to 0.5% at 24, 48 and 72 h after media replacement to maintain protein induction. At these timepoints, 500 µl samples of culture media were taken and analyzed for protein expression by standard Western blotting techniques using an anti-His antibody and *Sm*ChE-specific polyclonal antibodies (generated in section 3.2.8).

The highest-expressing *P. pastoris* culture (determined from the pilot expression experiments) of each f*Sm*ChE was plated onto YPD agar supplemented with 50 µg/ml zeocin and incubated for 2 days at 30°C. A single colony was used to inoculate 5 ml BMGY media (supplemented with 50 ug/ml zeocin) which was grown overnight at 30°C and 250 rpm. The entire culture was then used to inoculate 250 ml of BMGY in a 2L baffled flask and incubation was continued for 24 h. Cells were pelleted at 5000 *xg* for 20 min at RT, re-suspended in 1L of BMMY and split between 2 x 2L baffled flasks, which were incubated with shaking (250 rpm) at 30°C for 72 h. Methanol was added to a final concentration of 0.5% (2.5 ml/flask) every 24 h. Culture medium containing the secreted f*Sm*ChE proteins were harvested by centrifugation (5000 *xg* for 20 min at RT) and filtered through a 0.22 µm membrane filter (Millipore). Recombinant proteins were purified by IMAC using the AKTA-pure-25 FPLC (GE Healthcare). Briefly, culture medium was loaded onto a 5 ml His-excel column, pre-equilibrated with binding buffer (50 mM PBS pH 7.4, 300 mM NaCl), washed with 20 column volumes of binding buffer and then eluted with binding buffer containing a linear imidazole gradient (20 to 500 mM). The purity of fractions within the main peak was

analyzed by standard SDS-and fractions of appropriate purity were pooled, concentrated and buffer exchanged into PBS using Amicon Ultra-15 centrifugal devices with a 3 kDa MWCO (Millipore) and quantified using the Pierce BCA Protein Assay kit (Thermofisher). The final concentration of each f*Sm*ChE was adjusted to 1 mg/ml) and proteins were aliquoted and stored at -80°C. Finally, the recombinant proteins were confirmed by SDS-PAGE and Western blotting with anti-His (anti-His-HRP) (Invitrogen) and anti-*Sm*ChEs antibodies (generated in section 3.2.8).

3.2.11 *Sm*ChE Enzyme Assays

Activity of f*Sm*ChEs, extracts and ES samples was determined by the Ellman method (Ellman et al. 1961); modified for use with 96 well microplates. Samples (parasite extracts, ES and f*Sm*ChEs) were diluted in assay buffer (0.1M sodium phosphate, pH 7.4), and 2 mM acetylthiocholine (AcSCh) or butyrylthiocholine (BcSCh) (Sigma) and 0.5 mM 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) (Sigma) was added. The absorbance increase was monitored every 5 min at 405 nm in a Polarstar Omega microplate reader (BMG Labtech). Specific activity was calculated using the initial velocity of the reaction and extinction coefficient of 13260 M^{-1} cm^{-1} for TNB. To investigate sensitivity of parasite ES products to AChE inhibitors, 25 µg of adult ES was pre-treated with 1 µM Dichlorvos (DDVP) - active metabolite of the organophosphorus AChE inhibitor metrifonate - for 20 min at RT before measuring activity. Kinetic parameters of f*Sm*ChEs were characterized by measuring enzyme activity at differing substrate concentrations and plotting enzyme activity [V] vs. substrate concentration [S]. The Km ([S] at 1/2 Vmax) was calculated using the Michaelis Menton equation. Substrate concentration was plotted against reaction rate to check whether

the enzymes obeyed Michaelis–Menten kinetics and Km and Vmax were determined from a Lineweaver–Burk plot. Enzyme assays with inhibitors were performed as above except that f*Sm*ChEs in assay buffer were pre-treated with 1 µM DDVP, in the case of f*Sm*AChE1 and f*Sm*AChE3, or (1 mM) iso-OMPA – a membrane-impermeable specific BChE inhibitor, in the case of f*Sm*BChE1, for another 20 min at RT. Experiments were performed in triplicate with data presented as the mean ± SEM.

3.2.12 Purification of secreted *Sm*ChEs from adult *S. mansoni* ES products

Affinity chromatography using edrophonium chloride-sepharose was used to purify *Sm*AChE from *S. mansoni* based on the method of (Hodgson et al. 1983). Briefly, 1 g of epoxy-activated sepharose 6B beads were washed with distilled H_2O, the slurry centrifuged at 814 *xg* for 5 min and the pellet gently resuspended in 50 mM sodium phosphate, pH 8.0, containing 200 mM edrophonium chloride (1:2 ratio of sepharose:edrophonium chloride). The pH of the solution was adjusted to 10.0 and coupling of edrophonium with the sepharose was facilitated by incubating the mixture overnight with shaking at 50°C. The gel was then washed sequentially with 10 volumes each of 100 mM sodium acetate, pH 4.5, 12 mM sodium borate, pH 10.0, and distilled H_2O and finally resuspended in distilled H_2O to generate a 1 ml gel slurry. The gel slurry was packed into a chromatography column (10 cm long, 1 cm diameter) and equilibrated by gravity flow at 4°C with 10 column volumes (CV) of equilibration buffer (50 mM phosphate buffer, pH 8.0). Approximately 20 ml of ES from adult *S. mansoni* (concentrated through a 10Kda MWCO centrifugal filter from a starting volume of 500 ml of media, harvested each day for 7 days from

100 pairs of adult worms and buffer exchanged into equilibration buffer) was added to the column followed by washes with 20 CV of equilibration buffer and 20 CV of equilibration buffer containing 500 mM NaCl. Bound *Sm*ChE was then eluted with 10 CV of equilibration buffer containing 500 mM NaCl and 20 mM edrophonium chloride. The eluate was concentrated and buffer exchanged into PBS using a 10Kda MWCO centrifugal filter (edrophonium chloride is an AChE inhibitor and would interfere with subsequent activity assays) and resolved by 10% SDS-PAGE to check purity and facilitate identification by mass spectrometry.

3.2.13 Mass spectrometric analysis of purified, secretory *Sm*ChE

Bands of interest were manually excised from the gel, washed with 50% acetonitrile and dried under vacuum at 30°C. Cysteine residues were, then, reduced with 20 mM DTT for 1 h at 65°C followed by alkylation with 50 mM iodoacetamide for 40 min at 37°C in the dark. In-gel trypsin digestion was performed at 37°C overnight with 0.8 ng of trypsin in trypsin reaction buffer (40 mM ammonium bicarbonate, 9% acetonitrile). The supernatant was removed to a fresh Eppendorf tube and stored at 4°C, and the remaining peptides were further extracted from the gel pieces by incubation with 0.1% trifluoracetic acid (TFA) at 37°C for 45 min. The newly extracted supernatant was combined with the previously collected supernatant, then dried under vacuum. Prior to the matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analysis, peptides were concentrated and desalted using ZipTips (Millipore) following manufacturer's instructions.

Tryptic peptides were re-dissolved in 10 µl 5% formic acid and 6 µl was injected onto a 50 mm 300 µm C18 trap column (Agilent Technologies) followed by initial wash step by Buffer A (5% (v/v) ACN, 0.1% (v/v) formic acid) for 5 min at 30 µl/min. Peptides were eluted at a flow rate of 0.3 µl/min onto an analytical nano HPLC column (15 cm x 75 µm 300SBC18, 3.5 µm, Agilent Technologies). The eluted peptides were then separated by a 55-min gradient of buffer B (90/10 acetonitrile/ 0.1% formic acid) 1-40% followed by a 5 min steeper gradient from 40-80%. The mass spectrometer (ABSciex 5600 Triple Tof) operated in data-dependent acquisition mode, in which full scan TOF-MS data was acquired over the range of 350-1400 m/z, and over the range of 80-1400 m/z for product-ion observed in the TOF-MS scan exceeding a threshold of 100 counts and a charge state of +2 to +5. Analyst 1.6.1 (ABSCIEX) software was used for data acquisition and analysis.

For protein identification, a database was built using the *S. mansoni* genome v5.0 (http://www.genedb.org/Homepage/Smansoni) with the common repository of adventitious proteins (cRAP, http://www.thegpm.org/crap/) appended to it. Mascot v.2.5.1 (Matrix Science) was used for database search. Carbamidomethylation of Cys was set as a fixed modification and oxidation of Met and deamidation of Asn and Gln were set as variable modifications. MS and MS/MS tolerance were set at 10 ppm and 0.1 Da, respectively and only proteins with at least two unique peptides (each composed of at least seven amino acid residues) identified were considered reliably identified and used for analysis.

3.2.14 Bio-scavenging of organophosphorus esters by *Sm*BChE1

To test the hypothesis that *Sm*BChE1 may play a role in the bio-scavenging of AChE-inhibitory organophosphates (OPs), I first sought to determine whether inhibition of BChE activity would potentiate the AChE-inhibitory and anti-schistosomal effects of OPs. Schistosomula extracts (20 µg) were diluted in assay buffer, pre-incubated with iso-OMPA (1 or 2 mM) for 20 min at RT and then 1 µM (DDVP) for another 20 min at RT (180 µl final volume). ACh (2 mM) and DTNB (0.5 mM) were then added and the absorbance increase monitored every 5 min at 405 nm in a Polarstar Omega microplate reader (BMG Labtech). Extracts untreated with iso-OMPA with or without DDVP treatment were used as controls. Experiments were performed in triplicate with data presented as the mean ± SEM.

The same experiments were performed on live schistosomula using either an inhibitor- or RNAi-based approach. For the inhibitor-based experiment, 24 h schistosomula (1000/treatment in 1 ml serum-free Basch medium supplemented with 4XAA) were pretreated with iso-OMPA at the non-lethal concentration of 100 µM and, 1 h after iso-OMPA treatment, schistosomula were treated with 1 µM DDVP and cultured for 5 h at 37°C in 5% CO_2. Parasites untreated with iso-OMPA but treated with DDVP were used as controls. For the RNAi-based experiment, 24 h schistosomula (1500/100 µl serum-free Basch medium supplemented with 4XAA) were electroporated with either 10 µg of a short interfering RNA duplex (siRNA) (Supplementary Table 4.1) targeting *Sm*BChE1 or firefly luciferase (irrelevant siRNA control) ((Tran et al. 2010) in Genepulser 4 mm electroporation cuvettes (Bio-Rad) using a Bio-Rad Gene Pulser Xcell (single 20

ms pulse - 125V, 25 µF capacitance, 200 resistance, square wave electroporation) at RT. Parasites were added to 24 well plates containing 1 ml pre-warmed, serum-free Basch medium (supplemented with 4XAA) and incubated (37°C, 5% CO_2) for 3 days before being treated with 1 µM DDVP and cultured for a further 5 h. For both inhibitor- and RNAi-based experiments, schistosomula viability was determined using Trypan Blue staining and data is presented as the mean ± SEM of two biological and three technical replicates.

In a reverse testing of the bio-scavenging hypothesis, I sought to determine whether addition ofsCHIcould mitigate the effects of DDVP. Ten micrograms of f*Sm*BChE1 was pre-incubated with 1 µM DDVP in AChE assay buffer (170 µl final volume) for 20 min at RT. Schistosomula extracts (20 µg), ACh (2 mM) and DTNB (0.5 mM) were then added and the absorbance increase monitored every 5 min at 405 nm in a Polarstar Omega microplate reader (BMG Labtech). Reactions without f*Sm*BChE or without DDVP were used as controls. Experiments were performed in triplicate with data presented as the mean ± SEM. Again, the same experiments were performed on live schistosomula. After the pre-treatment of different amounts of f*Sm*BChE (10, 5, and 2.5 µg) with 1 µM DDVP in 500 µl serum-free Basch medium supplemented with 4XAA, twenty-four hour schistosomula (1000/treatment in 500 µl serum-free Basch medium supplemented with 4XAA) were added, incubated (37°C , 5% CO_2) for 24 hours and then parasite viability measured by Trypan Blue Staining. Experiments where a similarly expressed and purified but irrelevant protein (*Sm*TSP2) was used instead of f*Sm*BChE, and schistosomula cultured in

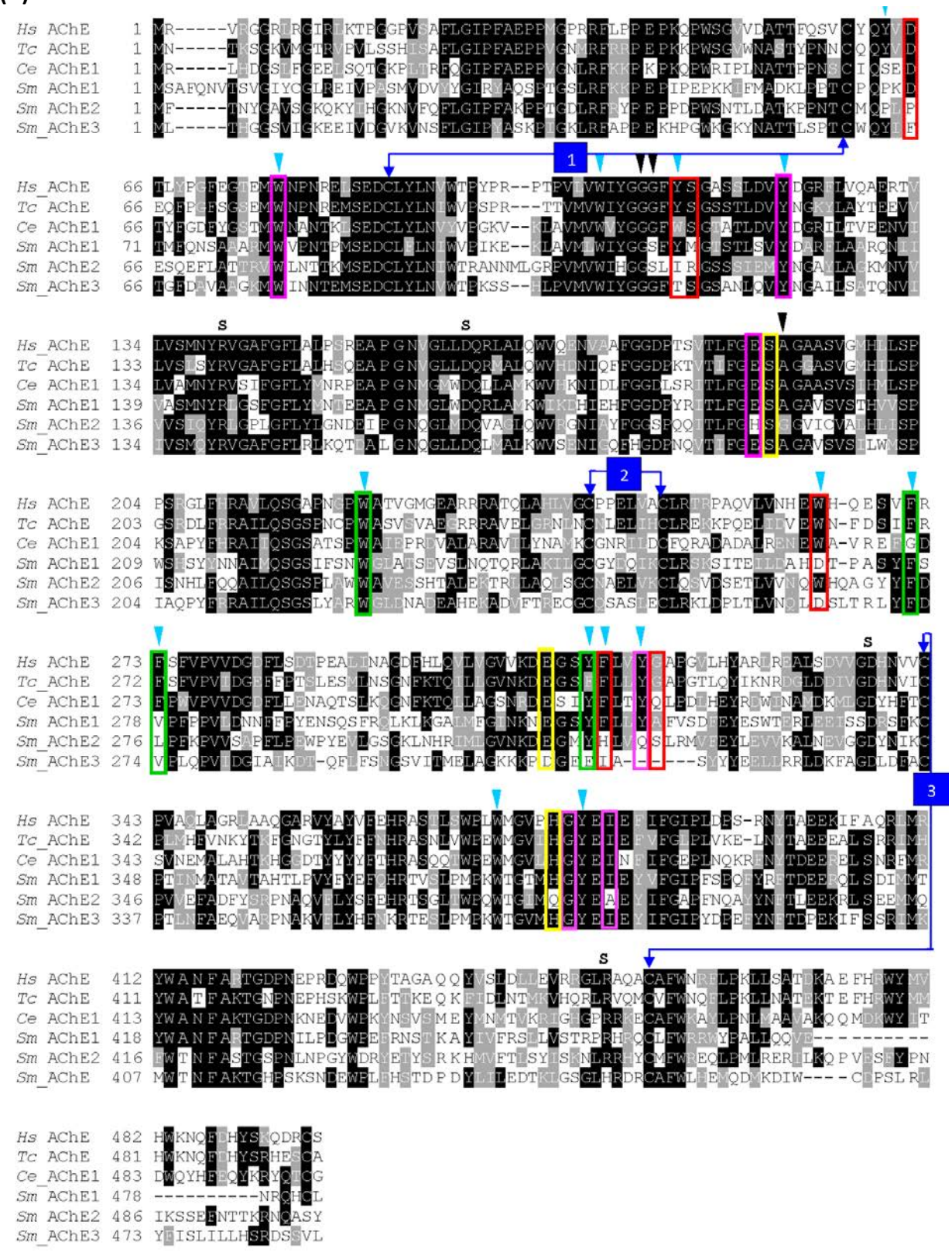
media alone, were used as controls. Data is presented as the mean ± SEM of two biological and three technical replicates.

3.3 Results

3.3.1 Identification of novel genes encoding ChE proteins in *S. mansoni*

Three putative ChE paralogs were identified from interrogation of the *S. mansoni* genome: *Sm*AChE1 (Smp_154600), *Sm*BChE1 (Smp_125350) and *Sm*AhE3 (Smp_136690). The predicted *Sm*ChEs were then aligned with characterized AChE enzymes from *Homo sapiens*, the electric eel *Torpedo californica*, and the nematode *Caenorhabditis elegans* (Fig 3.1A). Homology analysis of amino acid sequences revealed that the *Sm*AChE1*, Sm*BChE1*,* and *Sm*AChE3 share (32-35%) sequence identity and (49-52%) sequence similarity. Further, all *Sm*ChEs have 36-40% amino acid identity with *H. sapiens* and *T. californica* AChE. All identified *Sm*ChEs had ChE-specific characteristics, including a catalytic triad with an active site serine, which is required for ester hydrolysis (Dvir et al. 2010). Interestingly, the His residue of the catalytic triad of *Sm*BChE1 appears to have been substituted for Gln, a change consistent among all the BChE1 homologs shown for other Platyhelminthes, but not nematode or model organism BChE1 sequences (Fig 3.1B).

(A)



(B)

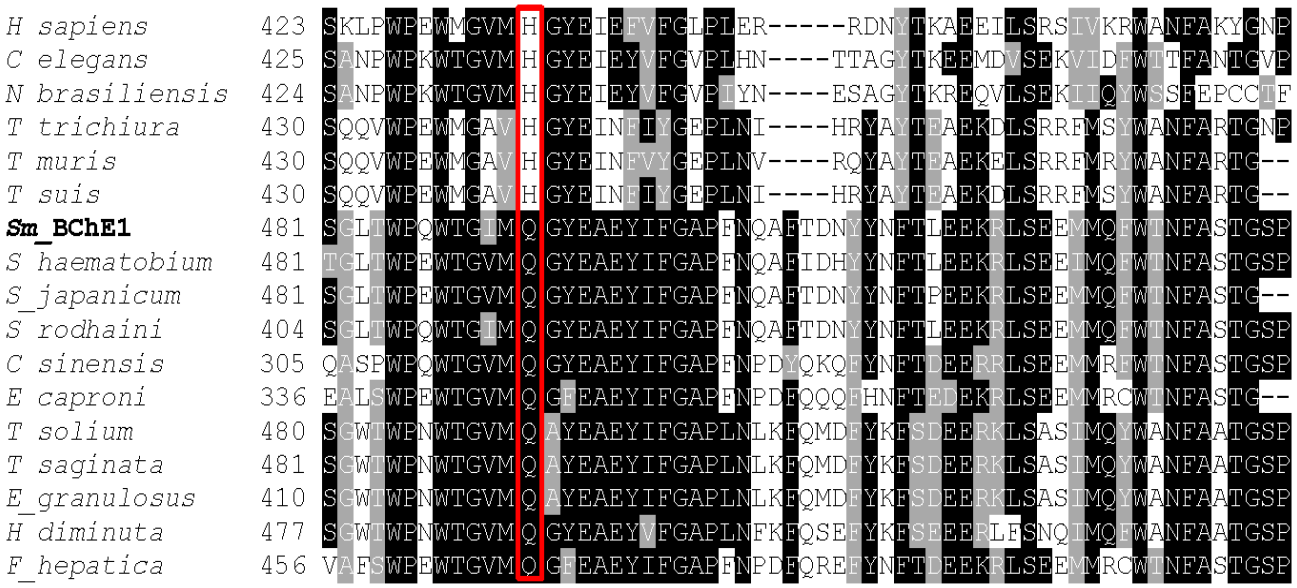
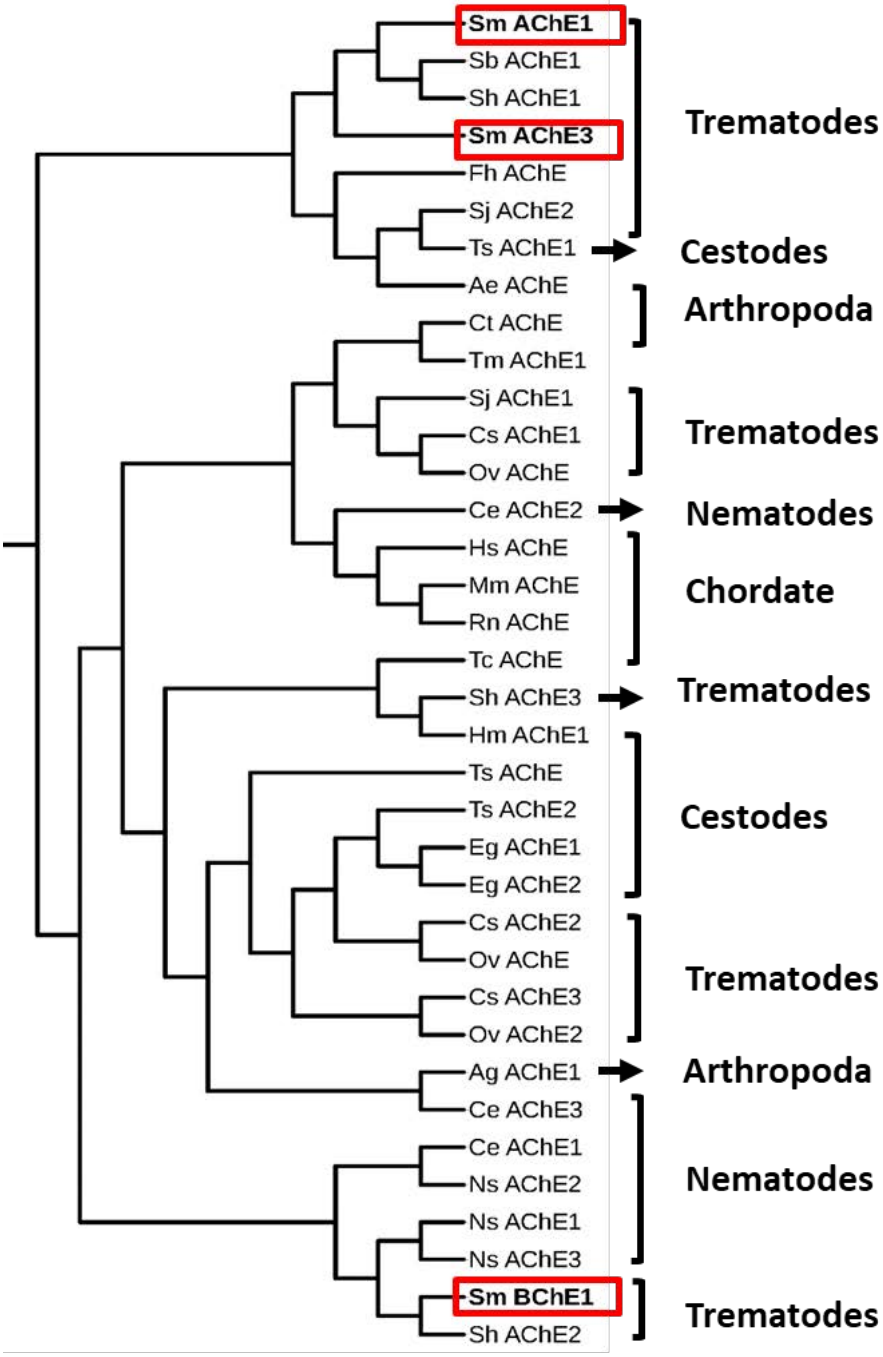

H sapiens 423 SKLPWPEWMGVMHGYEIEFVFGLPLER-----RDNYTKAEEILSRSIVKRWANFAKYGNP
C elegans 425 SANPWPKWTGVMHGYEIEYVFGVPLHN----TTAGYTKEEMDVSEKVIDFWTTFANTGVP
N brasiliensis 424 SANPWPKWTGVMHGYEIEYVFGVPIYN----ESAGYTKREQVLSEKIIQYWSSFEPCCTF
T trichiura 430 SQQVWPEWMGAVHGYEINFIYGEPLNI----HRYAYTEAEKDLSRREMSYWANFARTGNP
T muris 430 SQQVWPEWMGAVHGYEINFVYGEPLNV----RQYAYTEAEKELSRREMRYWANFARTG--
T suis 430 SQQVWPEWMGAVHGYEINFIYGEPLNI----HRYAYTEAEKDLSRREMSYWANFARTG--
Sm_BChE1 481 SGLTWPQWTGIMQGYEAEYIFGAPFNQAFTDNYYNFTLEEKRLSEEMMQFWTNFASTGSP
S haematobium 481 TGLTWPEWTGVMQGYEAEYIFGAPFNQAFIDHYYNFTLEEKRLSEEIMQFWTNFASTGSP
S japanicum 481 SGLTWPEWTGVMQGYEAEYIFGAPFNQAFTDNYYNFTPEEKRLSEEMMQFWTNFASTG--
S rodhaini 404 SGLTWPQWTGIMQGYEAEYIFGAPFNQAFTDNYYNFTLEEKRLSEEMMQFWTNFASTGSP
C sinensis 305 QASPWPQWTGVMQGYEAEYIFGAPFNPDYQKQFYNFTDEERRLSEEMMRFWTNFASTGSP
E caproni 336 EALSWPEWTGVMQGFEAEYIFGAPFNPDFQQQFHNFTEDEKRLSEEMMRCWTNFASTG--
T solium 480 SGWTWPNWTGVMQAYEAEYIFGAPLNLKFQMDFYKFSDEERKLSASIMQYWANFAATGSP
T saginata 481 SGWTWPNWTGVMQAYEAEYIFGAPLNLKFQMDFYKFSDEERKLSASIMQYWANFAATGSP
E granulosus 410 SGWTWPNWTGVMQAYEAEYIFGAPLNLKFQMDFYKFSDEERKLSASIMQYWANFAATGSP
H diminuta 477 SGWTWPNWTGVMQGYEAEYVFGAPLNFKFQSEFYKFSEEERLFSNQIMQFWANFAATGSP
F hepatica 456 VAFSWPEWTGVMQGFEAEYIFGAPFNPDFQREFYNFTDEEKRLSEEMMRCWTNFASTGSP

Fig 3.1. The amino acid sequence alignment of ChEs from *S. mansoni* and other species. (A) ▼= the 14 aromatic rings, ⬇ = Oxyanion holes, S= salt bridges, Red box= PAS, Yellow box= Catalytic triad, Green box= Acyl Binding Pocket, Numbered Arrow =3 disulphide bonds and Magenta box=Anionic site. *H. Sapiens* (NP000656), *T. Californica* (CAA27169), *C. elegans* (NP510660), *Sm*AChE1 (Smp_154600), *Sm*BChE1 (Smp_125350), *Sm*AChE3 (Smp_136690). **(B)** Sequence alignment of the *Sm*BChE1 homologous sequences in other helminths. Accession numbers of sequences: *Schistosoma mansoni* (*Sm*BChE1 – Smp_125350), *Schistosoma rodhaini* (SROB_0000329201), *Schistosoma haematobium* (KGB33101), *Schistosoma japonicum* (Sjp_0015690), *Clonorchis sinensis (*csin111679), *Echinostoma caproni* (ECPE_0000670801), *Fasciola hepatica* (PIS83327.1), *Hymenolepis diminuta* (HDID_0000005301), *Echinococcus granulosus* (EGR_07475.1), *Taenia solium* (TsM_000234300), *Taenia saginata* (TSAs00071g07627m00001), *Trichuris muris* (TMUE_3000012587), *Trichuris trichiura* (TTRE_0000364501), *Trichuris suis (*M514_03850), *Nippostrongylus brasiliensis* (NBR_0000102801), *Caenorhabditis elegans* (Y48B6A.8.1) and *Homo sapiens* (P06276).

A phylogenetic tree of the alignment (Fig 3.2A) shows that *Sm*ChEs were clustered into three distinct branches, with *Sm*ChE1 being quite phylogenetically distinct from *Sm*BChE1 and *Sm*AChE3. In addition, each *Sm*ChE was grouped together with closely related flatworms, including other *Schistosoma* species. Importantly, as shown in the sequence alignment, *Sm*ChEs are divergent from human host homologs. Fig 3.2B shows that reflective of the catalytic triad residue differences in Fig.1B, trematode BChEs are phylogenetically divergent from nematode and human BChEs.

A 3D model of the three *Sm*ChEs was constructed by homology modeling with AChE from model organisms (*H. sapiens* and *T. calfornica* (Fig 3.3A-C). The overall fold model of all *Sm*ChEs exhibited folding characteristic of the functional globular enzymes as most of the α-helical and β-stranded sheets were tightly aligned. Each predicted *Sm*ChE structure consisted of a ChE catalytic domain but, although the core architecture of the catalytic gorges was well aligned, regions that are associated with substrate specificity and catalytic efficiency were disparate, in agreement with the sequence alignment. In particular, the catalytic triad of *Sm*BChE1 was predicted to be Ser-Gln-Glu instead of the canonical Ser-His-Glu present in the other two paralogs.

(A)


Sm AChE1
Sb AChE1
Sh AChE1
Sm AChE3
Fh AChE
Sj AChE2
Ts AChE1
Ae AChE
Ct AChE
Tm AChE1
Sj AChE1
Cs AChE1
Ov AChE
Ce AChE2
Hs AChE
Mm AChE
Rn AChE
Tc AChE
Sh AChE3
Hm AChE1
Ts AChE
Ts AChE2
Eg AChE1
Eg AChE2
Cs AChE2
Ov AChE
Cs AChE3
Ov AChE2
Ag AChE1
Ce AChE3
Ce AChE1
Ns AChE2
Ns AChE1
Ns AChE3
Sm BChE1
Sh AChE2
Trematodes
Cestodes
Arthropoda
Trematodes
Nematodes
Chordate
Trematodes
Cestodes
Trematodes
Arthropoda
Nematodes
Trematodes

(B)

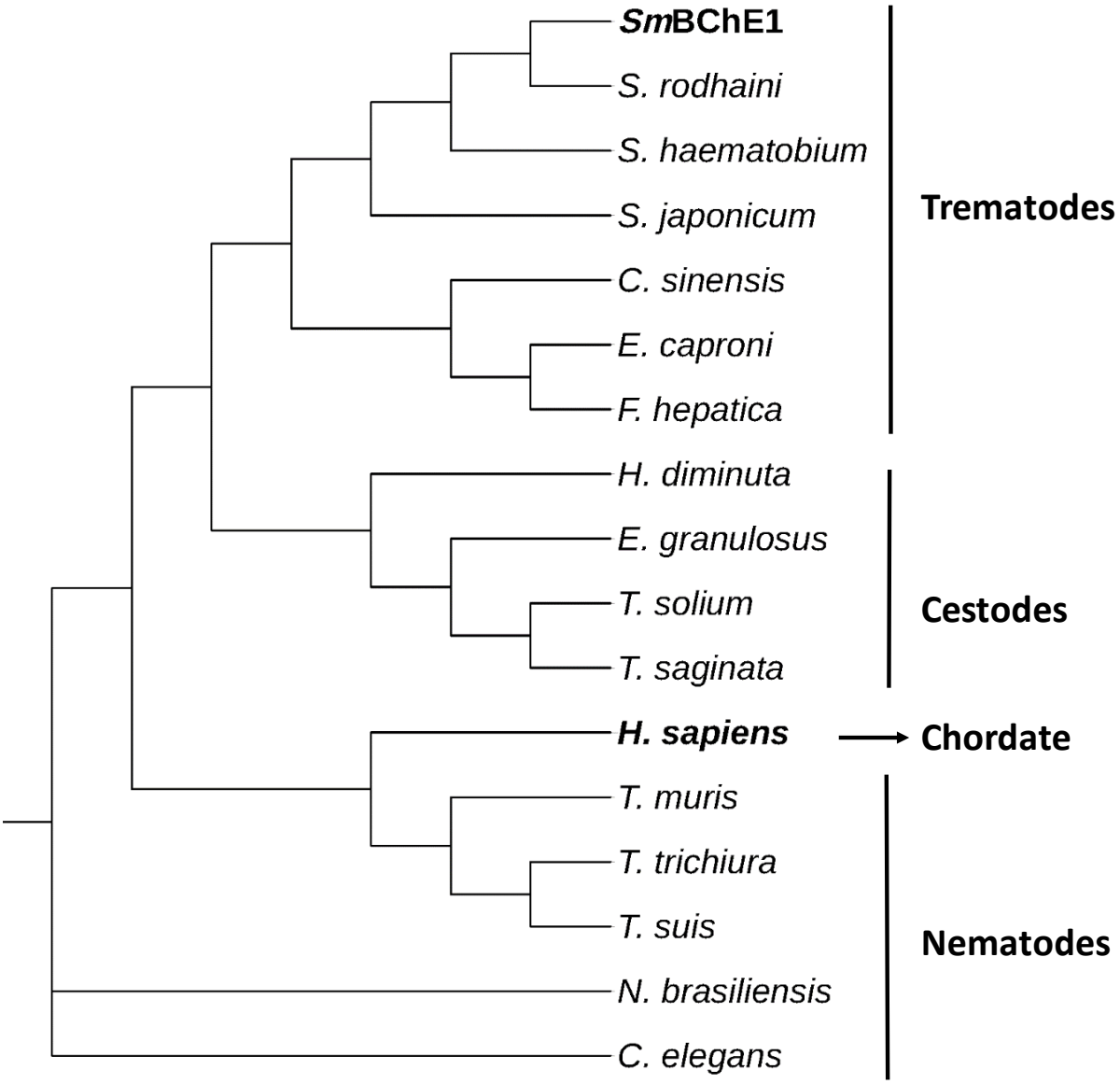

*Sm*BChE1
S. rodhaini
S. haematobium
S. japonicum
C. sinensis
E. caproni
F. hepatica
H. diminuta
E. granulosus
T. solium
T. saginata
H. sapiens
T. muris
T. trichiura
T. suis
N. brasiliensis
C. elegans
Trematodes
Cestodes
Chordate
Nematodes

Fig 3.2. Phylogenetic of AChEs. (A) The three *Sm*ChEs are indicated by bold font inside a red box. Relationship between *Sm*ChEs and other invertebrate and vertebrate species. Evolutionary history was inferred using the Neighbor-Joining method and the phylogenetic tree was generated using a ClustalW alignment. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated, making for a total of 236 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016). Accession numbers: *Schistosoma mansoni* (Sm_AChE1 - Smp_154600, Sm_BChE1 - Smp_125350, Sm_AChE3 - Smp_136690)*; Schistosoma bovis* (Sb_AChE1 - AAQ14323); *Schistosoma haematobium (*Sh_AChE1 - AAQ14322, Sh_AChE2 - KGB33101, Sh_AChE3 - KGB33661)*; Schistosoma japonicum* (Sj_AChE1 - ANH56887, Sj_AChE2 - Sjp0045440.1); *Fasciola hepatica* (Fh_AChE - THD22977.1); *Clonorchis sinensis* (Cs_AChE1 - GAA52478, Cs_AChE2 - GAA53463, Cs_AChE3 - GAA27255); *Opisthorchis viverrini* (Ov_AChE - XP009170845, Ov_AChE - XP009168237, Ov_AChE - XP009170760); *Echinococcus granulosus (*Eg_AChE1 - JN662938, Eg_AChE2 - EgG000732400)*; Hymenolepis microstoma (*Hm_AChE1 - LK053025)*; Taenia solium (*Ts_AChE1 - TsM000234300, Ts_AChE - TsM001220100, Ts_AChE - TsM000001700)*; Anopheles gambiae (*Ag_AChE1 - AGM16375)*; Aedes aegypti (*Ae_AChE - AAB35001)*; Culex tritaeniorhynchus (*Ct_AChE - BAD06210)*; Caenorhabditis elegans (*Ce_AChE1 - NP510660, Ce_AChE2 - NP491141, Ce_AChE3 - NP496963)*; Trichuris muris (*Tm_AChE1 - TMUEs0033000600)*; Nippostrongylus brasiliensis (*Nb_AChE1 - AAK44221, Nb_AChE2 - AAC05785, Nb_AChE3 - AAK44221)*; Homo sapiens (*Hs_AChE - NP000656)*; Torpedo californica (*Tc_AChE - CAA27169)*; Danio rerio (*Dr_AChE - NP571921)*; Mus musculus (*Mm_AChE - CAA39867)*;*

*Rattus norvegicus (*Rn_AChE - NP742006). **(B)** Phylogenetic analysis of *Sm*BChE1 and its human and other helminth homologs. The phylogenetic tree was built using the maximum likelihood method with *Sm*BChE1 and the top 16 similar helminths cholinesterase protein sequences identified from the BLASTp search, as well as one known Human BChE protein. Accession numbers of sequences: *Schistosoma mansoni* (*Sm*BChE1 – Smp_125350), *Schistosoma rodhaini* (SROB_0000329201), *Schistosoma haematobium* (KGB33101), *Schistosoma japonicum* (Sjp_0015690), *Clonorchis sinensis (*csin111679), *Echinostoma caproni* (ECPE_0000670801), *Fasciola hepatica* (PIS83327.1), *Hymenolepis diminuta* (HDID_0000005301), *Echinococcus granulosus* (EGR_07475.1), *Taenia solium* (TsM_000234300), *Taenia saginata* (TSAs00071g07627m00001), *Trichuris muris* (TMUE_3000012587), *Trichuris trichiura* (TTRE_0000364501), *Trichuris suis (*M514_03850), *Nippostrongylus brasiliensis* (NBR_0000102801), *Caenorhabditis elegans* (Y48B6A.8.1) and *Homo sapiens* (P06276).

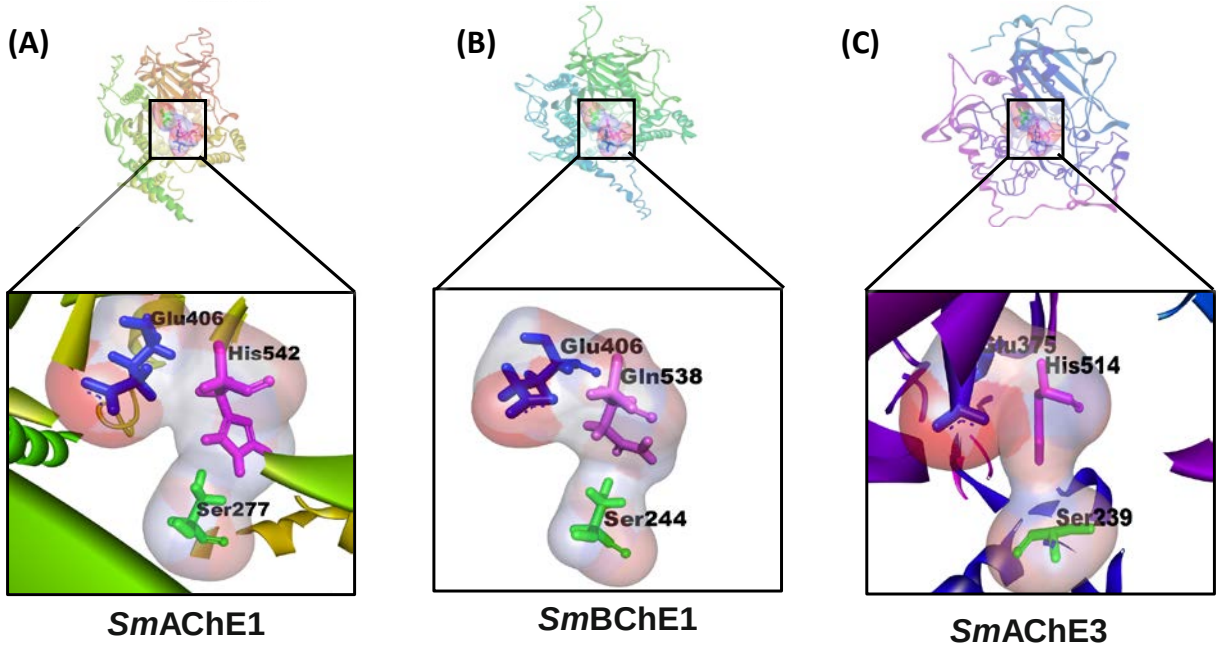


Fig 3.3. Magnified view of 3D models showing the catalytic triads of *Sm*AChE1, *Sm*BChE1, and *Sm*AChE3. The amino acid residues of the catalytic triad of each paralog are magnified and their position number is given according to *Torpedo* AChE numbering: *Sm*AChE1 (Ser277, Gln538, Glu406), *Sm*BChE1 (Ser244, Gln538, Glu406), and *Sm*AChE3 (Ser239, His514, Glu375).

3.3.2 Developmental expression analysis of *Sm*ChE genes

Gene expression patterns of the three *Sm*ChE paralogs across different developmental stages were measured using semi-quantitative qPCR (Fig 3.4A-C) and this data was used to generate a comparative expression heat map of all three genes (Fig 3.4D). While all *Sm*ChE developmental expression patterns were variable, the transcript levels of all the three genes were relatively lower in cercariae compared to the other developmental stages. Overall, the transcript levels of *Sm*AChE1 and *Sm*AChE3 genes in most life stages were higher as compared to the *Sm*BChE1 transcript. In adult worms, *Sm*AChE1 was expressed at higher levels, specifically in male parasites

followed by sporocysts. Eggs and sporocysts exhibited ubiquitous expression of *Sm*BChE1 and *Sm*AChE3, respectively.

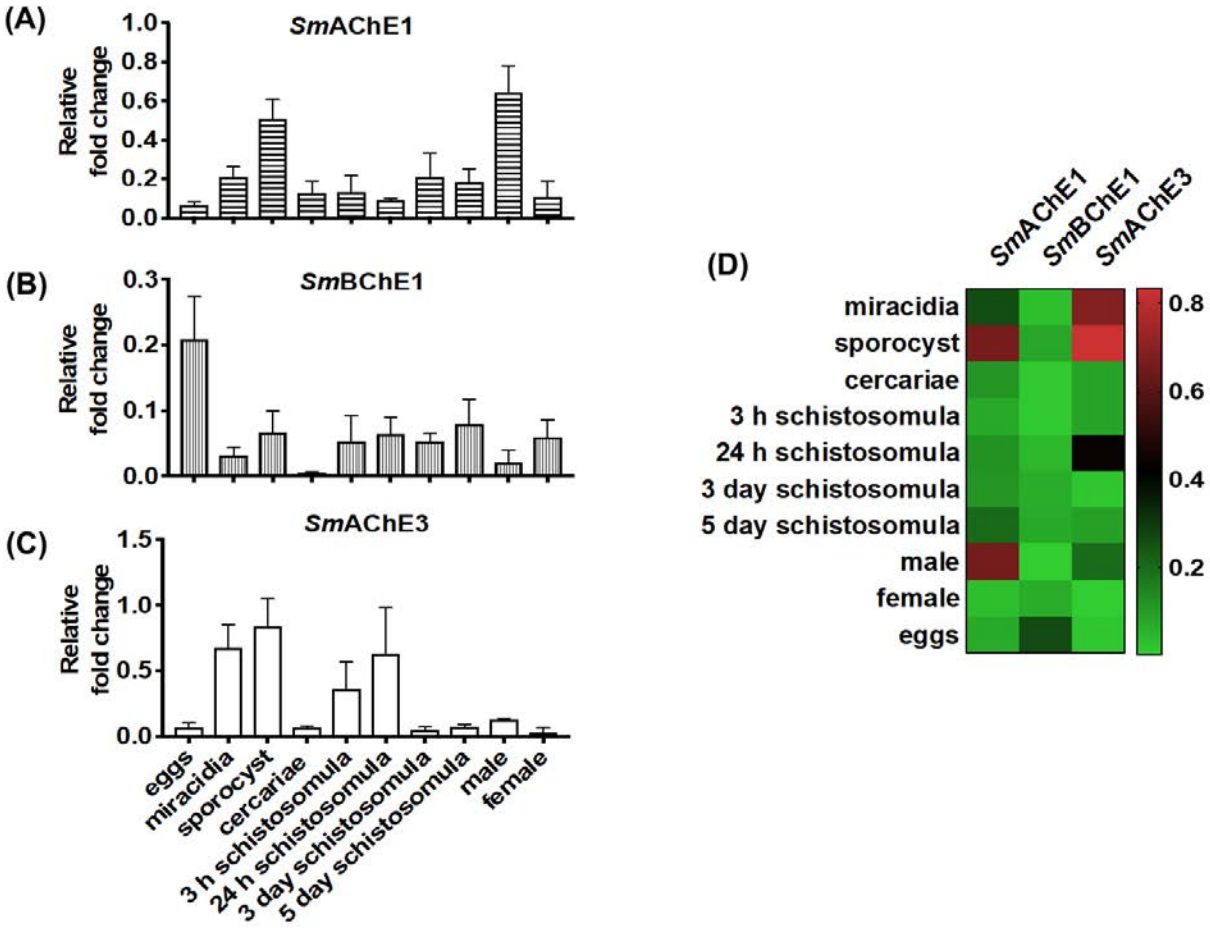


Fig 3.4. Expression profiles of *Sm*AChE1, *Sm*BChE1, and *Sm*AChE3.

The expression of *Sm*AChE1 **(A)**, *Sm*ABChE1 **(B)**, and *Sm*AChE3 **(C)** genes at different developmental stages of *S. mansoni* as quantified by qPCR analysis. **(D)** The heat map shows the comparative expression pattern of the paralogs in each developmental stage. Data are presented as mean ± SEM of five independent experiments and are normalized to the *Sm*COX1 housekeeping gene.

3.3.3 Cloning and protein expression of *Sm*ChEs in bacteria

Partial ORFs selected from highly polymorphic regions of each paralog were cloned and expressed in *E. coli* BL21 (DE3) cells. Recombinant proteins of expected size were purified from inclusion bodies using IMAC under denaturing conditions. Final yields were: p*Sm*AChE1 (25 kDa) – 6.0 mg/L, p*Sm*BChE1 (23 kDa) - 16 mg/L and p*Sm*AChE3 (25 kDa) – 3.5 mg/L.

3.3.4 Immunolocalization of *Sm*ChEs

To gain insight into the anatomical sites of expression of ChE proteins in S. *mansoni*, *Sm*ChEs were immunolocalized in whole juvenile and sectioned adult parasites. In adults, and consistent with their predicted cholinergic function, all *Sm*ChEs were expressed throughout the worms' internal structures (presumably localizing to the neuromusculature) and on their surface. *Sm*AChE1 was the least uniformly distributed of all *Sm*ChEs, localizing mostly to the tegument (Fig 3.5A). Additionally, anti-*Sm*ChE antibodies were able to detect homologous ChEs in adult *S. haematobium* sections. *Sm*ChE proteins were detected in all stages of larval development tested and, as was the case with adult worms, localized to the tegument.

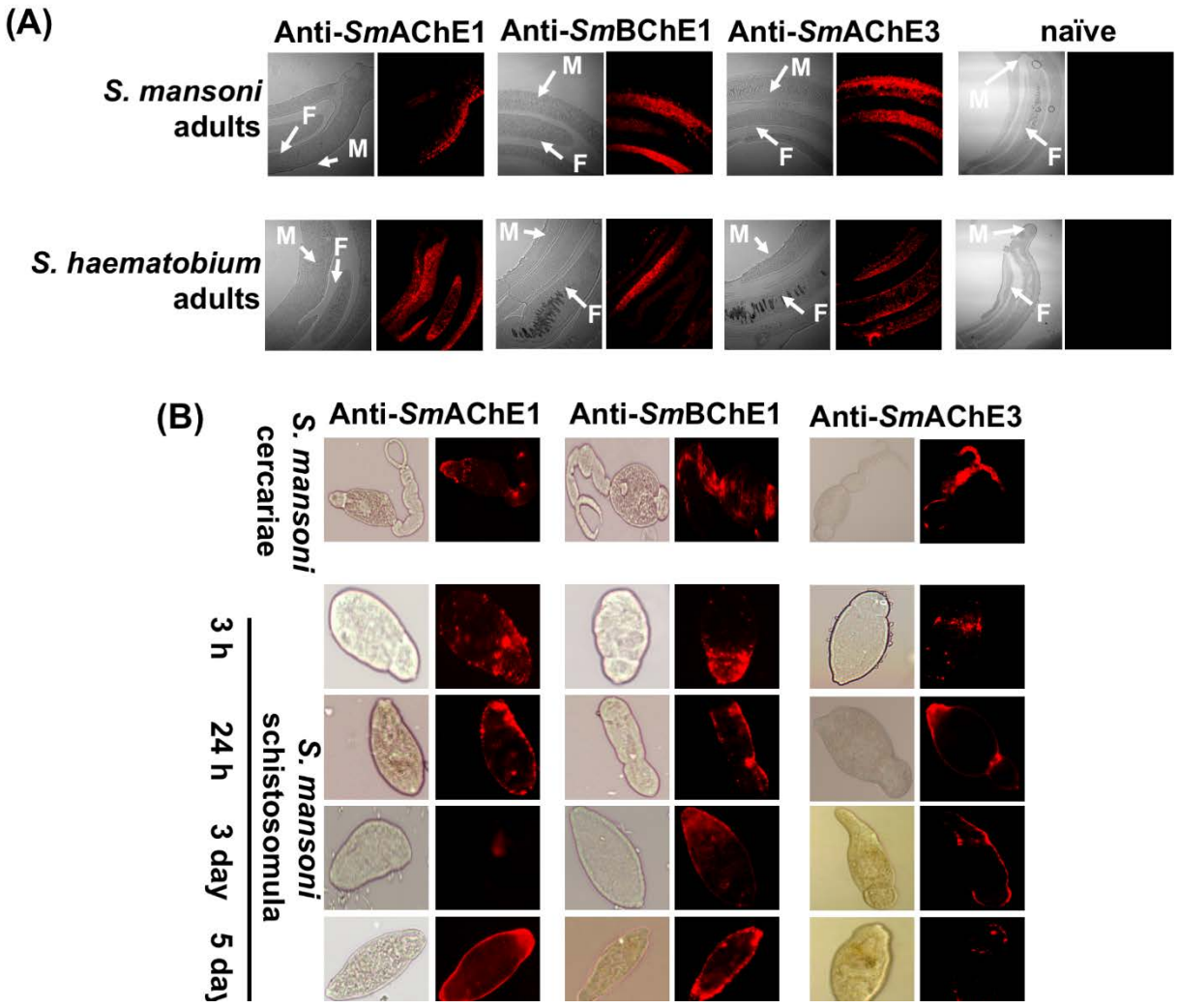


Fig 3.5. Immunofluorescent localization of *Sm*ChEs.

Fluorescence and brightfield images of (**A**) Male (M) and female (F) *S. mansoni* and S*. haematobium* adult worm sections. **(B)** Live, fixed cercariae, and schistosomula at 3 h, 24 h, 3 days and 5 days after transformation. Both adult sections and juvenile parasites were labeled with either anti-*Sm*AChE1, anti-*Sm*BChE1 or anti-*Sm*AChE3 primary antibody (1:100 in PBST) followed by goat-anti-mouse IgG-alexafluor647) (1:200 in PBST). Naive mouse sera was used as a negative control.

3.3.5 Expression and ChE activity of f*Sm*ChEs

Soluble, functionally active proteins were expressed in *P. pastoris* purified via IMAC. Both f*Sm*AChE1 and f*Sm*AChE3 demonstrated significantly stronger hydrolase activity when AcSCh was used as a substrate, compared to f*Sm*BChE1. Conversely, f*Sm*BChE1 hydrolyzed BcSCh to significantly higher levels compared to f*Sm*AChE1 and f*Sm*AChE3. Both classes of f*Sm*ChEs showed significantly higher activity against their preferred substrate (Fig 3.6B). All paralogs exhibited Michaelis–Menten kinetics (Table 3.1) when hydrolyzing their designated substrate. In addition, preferred substrate activity of both f*Sm*AChE1 and f*Sm*AChE3 were inhibited by DDVP (Fig 3.6C), an AChE inhibitor that forms an irreversible covalent bond with the catalytic serine, but iso-OMPA, a specific inhibitor of BChE, only inhibited *Sm*BChE1 activity (Fig 3.6D).

Table 3. 1. *K*m and *V*max f*Sm*ChEs as estimated by Michaelis-Menten equation

***Sm*ChE paralog**	**Vmax (nmol/min/mg)**	**Km (mM)**
*fSm*AChE1	5.57 ± 0.54	5.83 ± 1.62
*fSm*AChE3	5.59 ± 1.37	10.87 ± 6.17
*fSm*BChE1	1.7 ± 0.09	34.38 ± 12.71

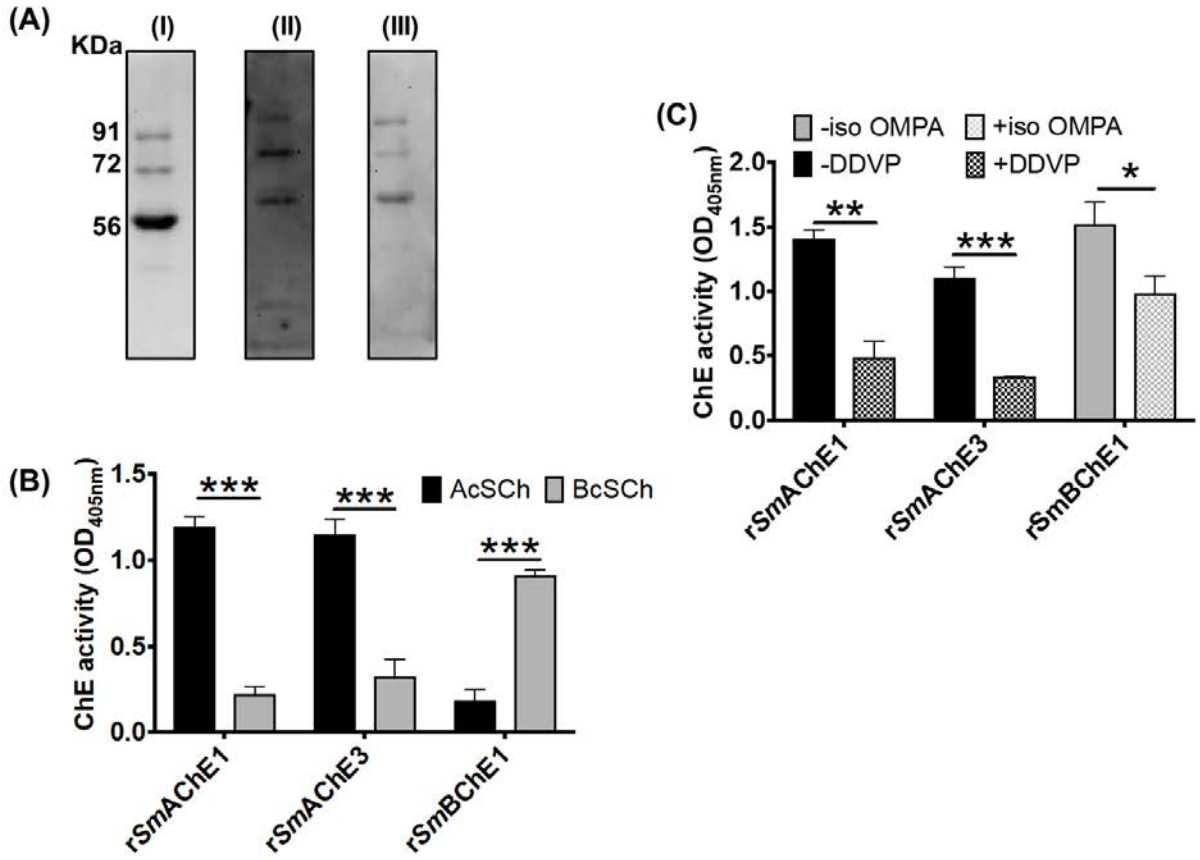


Fig 3.6. Enzymatic activity of f*Sm*ChEs. (**A**) Western Blot analysis of purified recombinant f*Sm*AChE1, f*Sm*BChE1, and f*Sm*AChE3 expressed in *P. pastoris.* Each f*Sm*ChE was probed with its cognate p*Sm*ChE antibody (**I**) f*Sm*AChE1 probed with anti-*Sm*AChE1 mice antisera, (**II**) f*Sm*BChE1 probed with anti-*Sm*BChE1 (**III**) f*Sm*AChE3 probed with anti-*Sm*AChE3. Multiple bands observed are due to protein degradation during the purification process. **(B)** Cholinergic substrate preference (AcSCh or BcSCh) of each f*Sm*ChE. **(C)** Inhibition of f*Sm*AChE1 and f*Sm*AChE3 with DDVP (AcSCh used as a substrate) and inhibition of f*Sm*BChE1 with iso-OMPA (BcSCh used as a substrate). Data are presented as mean ± SEM of triplicate experiments and differences between groups were measured by the student's *t* test. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001.

3.3.6 BChE and secretory AChE activity in schistosomes

Although the presence of nonspecific ChE activity has long been known in schistosomes (Bueding 1952), the gene identity and its function remain unknown. Prompted by the identification of *Sm*BChE1 as a BChE based on its substrate preference and inhibition by iso-OMPA, I sought to investigate the distribution of BChE activity in juvenile and adult schistosomes. Assay of BChE activity revealed that extracts from *S. mansoni* schistosomula had higher BChE activity compared to *S. mansoni* adult worms (Fig 3.7A) and that activity was significantly greater in *S. mansoni* compared with *S. haematobium* adults (Fig 3.7B). Varied amounts of AChE activity was detected in ES from all developmental stages tested. ES products from adult males had double the AChE activity compared to adult female ES products ($P < 0.001$) while cercarial ES exhibited the highest activity (at least ten-fold more than male ES products ($P < 0.0001$) and egg ES had the lowest (Fig 3.7C). Availability of ES precluded the measurement of secretory BChE activity from the same developmental stages but, of those tested, the BChE activity in schistosomula ES products was the highest - twice as high as that of adult ($P < 0.01$) and cercarial ($P < 0.01$) ES (Fig 3.7D). *Sm*ChEs were purified from ES products of *S. mansoni* adult worms using edrophonium–sepharose affinity chromatography. Purification resulted in an activity increase of more than 200-fold relative to crude ES (Fig 3.7E). Resolution of the purified sample by SDS-PAGE resulted in a doublet with an estimated molecular weight of 70 kDa (Fig 3.7F). The identity of purified, secretory *Sm*ChEs was substantiated by in-gel LC-MS/MS spectrometry analysis with the peptide data generated used to interrogate the *S. mansoni* proteome (predicted from the *S. mansoni* genome

(http://www.genedb.org/Homepage/Smanson)). The false discovery rate was set at <1% and only proteins with at least two unique peptides having significant Mascot identification scores ($P < 0.05$) were considered. The top protein hits were identified as *Sm*AChE1 (Smp_154600) and *Sm*BChE1 (Smp_125350) with *Sm*AChE1 having a relative abundance more than 40 fold that of *Sm*BChE1 (Supplementary Table 3.2).

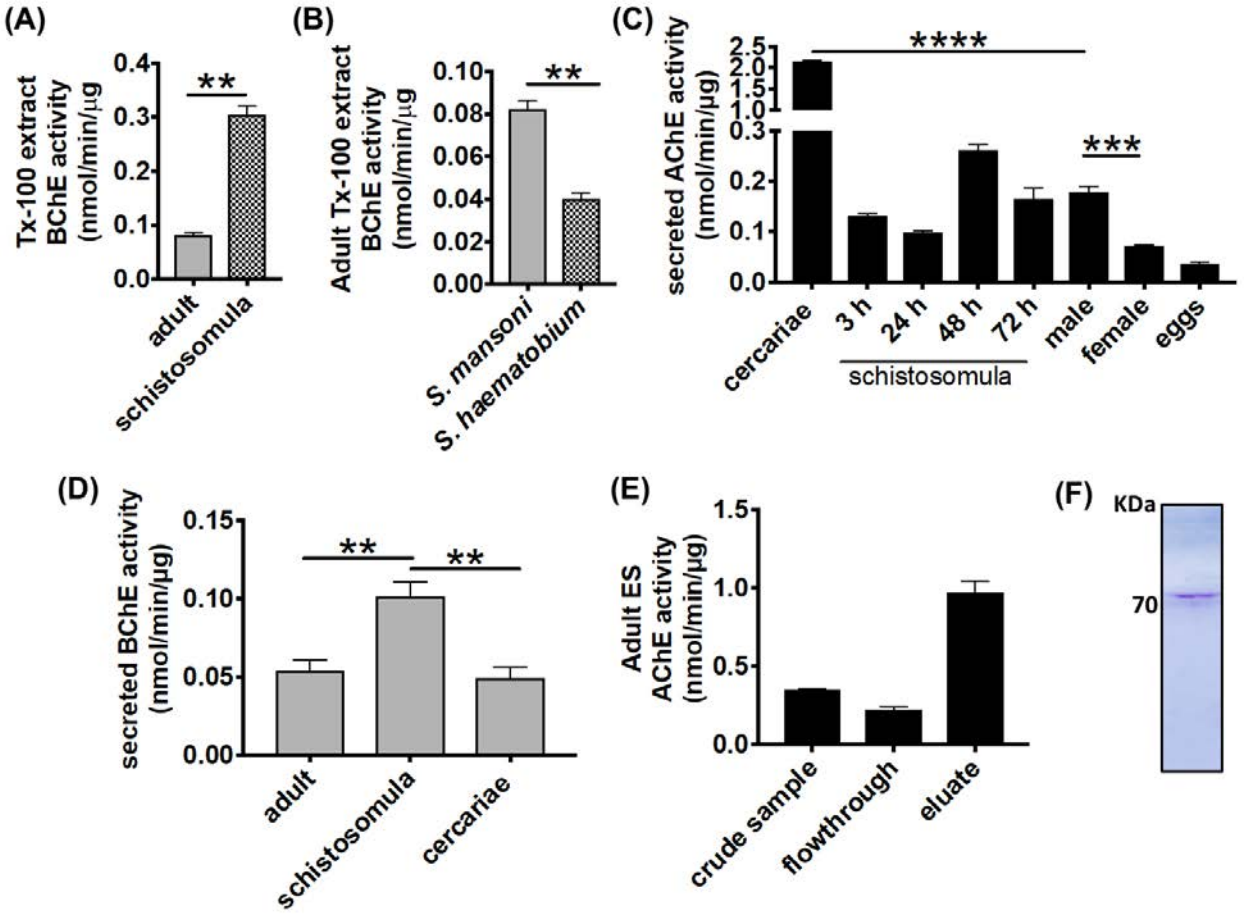


Fig 3.7. BChE and secretory AChE activity in schistosomes. (A) BChE activity in *S. mansoni* adults and schistosomula Tx-100 extracts. **(B)** BChE activity in Tx-100 extracts from *S. mansoni* and *S. haematobium*. **(C)** AChE and **(D)** BChE activity of ES products from different developmental stages of *S. mansoni*. (**E**) AChE activity and (**F**) SDS-PAGE analysis of purified, secretory *Sm*ChEs. Data are

presented as mean ± SEM of triplicate experiments and differences between groups were measured by the student's *t* test. *$P \leq 0.05$, **$P \leq 0.01$.

3.3.7 Bio-scavenging of organophosphorus esters by *Sm*BChE1

The hypothesis that *Sm*BChE may act as a molecular decoy in schistosomes and detoxify the effects of organophosphorus AChE inhibitors was examined by testing whether (a) inhibition of parasite-derived BChE potentiated the effects of DDVP (an organophosphorus AChE inhibitor) and (b) addition of exogenous BChE (f*Sm*BChE1) mitigated the effects of DDVP. Inhibition of AChE in extracts by DDVP significantly increased in the presence of increasing amounts of the BChE inhibitor, iso-OMPA (Fig 3.8A) and DDVP-mediated killing of schistosomula was significantly increased in the presence of iso-OMPA (60.9% compared with 21.8%; $P < 0.0001$) (Fig 3.8B) and in *Sm*BChE-silenced parasites (83.44% compared with 22.95%; $P < 0.0001$) (Fig 3.8C). Conversely, DDVP-induced inhibition of AChE in extracts was completely ablated in the presence of f*Sm*BChE (Fig 3.8D) and schistosomula were increasingly resistant to DDVP-mediated killing with the addition of increasing amounts of recombinant protein to the culture media (Fig 3.8E).

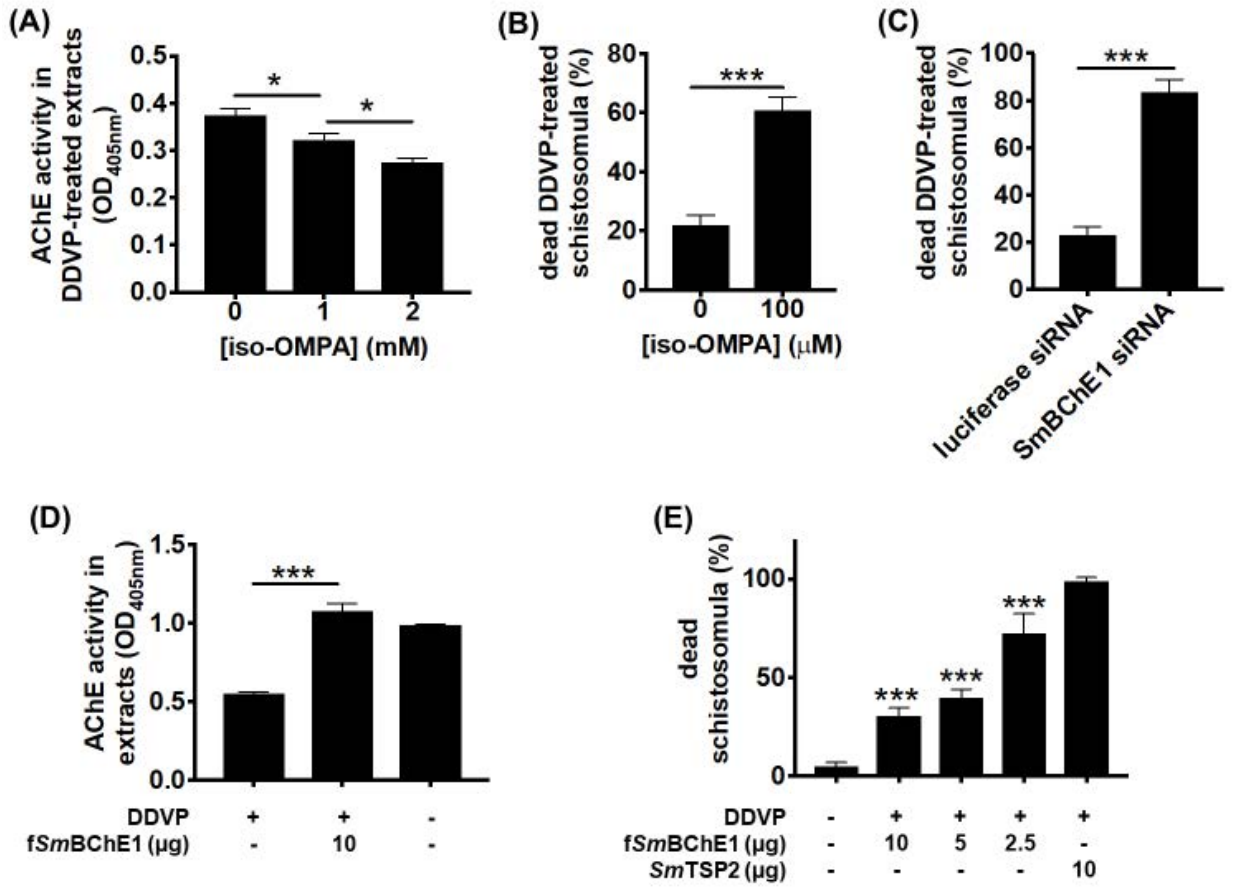


Fig 3.8. *Sm*BChE1 bio-scavenges DDVP and protects parasites against DDVP-induced effects.(**A**) Schistosomula extracts were treated with DDVP (1 µM), or pretreated with iso-OMPA (1 and 2 mM) and then DDVP, before assaying AChE activity. (**B**) Schistosomula were treated with DDVP (1 µM) or pretreated with iso-OMPA (100 µM) and then DDVP, and parasite viability measured 5 h after treatment. (**C**) *Sm*BChE1-silenced or luciferase siRNA-treated schistosomula were treated with DDVP (1 µM) and parasite viability measured 5 h after treatment. (**D**) Schistosomula extracts were pre-incubated with f*Sm*BChE1 (10 µg) then treated with DDVP (1 µM), or treated with DDVP alone, before assaying AChE activity. (E) DDVP was pre-incubated with f*Sm*BChE1 (10, 5 and 2.5 µg) or 10 µg of *Sm*TSP2 for 1 h before being used to treat schistosomula. Parasite viability was measured 24

h post-treatment. For all assays, data are the average of triplicate biological and technical experiments ± SEM and differences were measured by the student's *t* test. *$P \leq 0.05$, ***$P \leq 0.001$, ****$P \leq 0.0001$.

3.4 Discussion

Cholinesterase (ChE) activity in *S. mansoni* was first described by Bueding in 1952 (Bueding 1952) and was well characterized biochemically in the four decades succeeding this discovery. The technological limitations of this time period meant that most of the evidence for *Sm*ChEs came from whole worm studies and analyses of crude parasite extracts (reviewed in (Arnon et al. 1999)), which could not ascribe ChE activity to any particular protein. Several studies in the early 2000's characterized a single AChE from *S. mansoni* (Smp_154600 in the current gene annotation nomenclature) and its direct homolog in other species of schistosomes (Jones et al. 2002; Bentley et al. 2003; Bentley et al. 2005) but lack of a comprehensive schistosome genome annotation precluded identification of more ChE family members. Interrogation of the most recent iteration of the *S. mansoni* genome assembly has identified two additional ChE-encoding genes that are paralogs to Smp_154600 (termed *Sm*AChE1); Smp_125350 (*Sm*BChE1) and Smp_136690 (*Sm*AChE3). In this current study, I have provided a more in-depth characterization of the previously documented *Sm*AChE1 and described two novel ChEs from *S. mansoni*: *Sm*AChE3 – an AChE not reported before – and *Sm*BChE1 – a BChE which, to the best of our knowledge, has never been documented in the helminth literature.

All *Sm*ChEs share a modest level of identity which is consistent with their divergence over evolutionary time, an occurrence possibly due to a series of gene duplications as each paralog's clade is distant from one other. This divergence between *Sm*ChEs and, also, ChEs of other organisms, provides evidence for the increasing reports of non-cholinergic functions appearing for ChEs in the literature. Additionally, the relative lack of sequence identity between *Sm*ChEs and human ChEs suggests potential scope for the development of intervention strategies targeting schistosome ChEs that will not affect the host. Despite the diversity between ChEs, all enzymes analyzed would appear to be enzymatically active as they possessed a catalytic triad with an active site serine, the amino acid responsible for ester hydrolysis (Dvir et al. 2010). It is interesting to note, however, the catalytic triad His – Gln substitution in *Sm*BChE1 (and the other platyhelminth BChE1 homologs); while this change is not a hallmark of model BChEs, that it occurs within an entire parasite lineage is remarkable and will be investigated further.

The transcript levels of *Sm*ChEs varied among parasite developmental stages and this is likely a response to the differing cholinergic and cholinesterase-independent needs of the parasite throughout its lifecycle. For example, *Sm*AChE1 is expressed at a relatively higher level in adult males compared to females, probably due to the more "muscular" roles of attachment and movement orchestrated by the male compared to the female, which remains sedentary once inside gyancophoric groove of the male (Basch 1990). *Sm*BChE1 expression was the highest in eggs; there is evidence for BChE involvement in chicken embryo neurogenesis and development, independent of its enzymatic function (Mack et al. 2000), which suggests that *Sm*BChE1 could

play a role in parasite embryogenesis. The miracidia and sporocyst stages had the highest levels of *Sm*AChE3 expression, in agreement with Parker-Manuel et al (Parker-Manuel et al. 2011).

Immunolocalization of the *Sm*ChEs revealed expression in the neuromusculature and tegument to varying degrees, depending on the paralog, and is consistent with early localization experiments (Espinoza et al. 1991), although the antibodies used in those studies were raised against AChEs purified from parasite extracts and so the localization could not be attributed to a specific family member. Localization to the neuromusculature relates to the proteins' traditional cholinergic functions whereas tegumental distribution is suggestive of more non-neuronal cholinergic and/or non-cholinergic roles. Indeed, surface-expressed *Sm*AChE has been implicated in mediating glucose scavenging by the parasite as this process can be ablated by membrane-impermeable AChE inhibitors (Camacho et al. 1995; Sundaraneedi et al. 2017). Tegumental *Sm*AChE may also act to hydrolyze exogenous ACh, neutralizing its immune-mediating function to create an environment more conducive to parasite establishment (Vaux et al. 2016). The localization of these antigens to the tegument of schistosomula should also be noted since early developing schistosomula are considered most vulnerable to immune attack (Gobert et al. 2007) and so *Sm*AChE-targeted immunotherapeutics could be used effectively to vaccinate against schistosomiasis. Indeed, antibodies against *Sm*AChEs have been shown to interact with the molecules on the surface of schistosomula, resulting in complement-dependent killing of the parasite (Arnon et al. 1987).

Full-length and functional *Sm*ChEs were expressed in *P. pastoris*. *Sm*AChE1 had preferred substrate specificity for AcSCh over BcSCh, albeit at a three-fold less affinity than previously reported for *Sm*AChE1 expressed in *Xenopus laevi*s oocytes (Bentley et al. 2005). *Sm*AChE3 also had a substrate preference for AcSCh and an affinity twice that of *Sm*AChE1. Extremely low enzyme activity was observed with *Sm*BChE1 when AcSCh was used as a substrate, which significantly increased with the use of BcSCh. Although sequence alignment of *Sm*BChE1 with the other two *Sm*ChEs revealed a single amino acid substitution in the PAS (Glu – Trp), acyl binding pocket (Val – Leu) and catalytic triad (His – Gln), it was unclear whether these changes alone were enough to classify *Sm*BChE1 as a BChE. Based on the significant difference in substrate preference, however, this classification would appear valid. Cloning of a recombinant BChE from *S. mansoni* is consistent with our observations of BChE activity in parasite extracts. *S. mansoni* schistosomula exhibited significantly more activity than adults, as did *S. mansoni* compared to *S. haematobium* adults. It has been reported that *S. mansoni* is more sensitive to the BChE inhibitor, iso-OMPA, than *S. haematobium* (Camacho et al. 1994) and it may be because there is an increased BChE activity in *S. mansoni*. Indeed, this relationship has been documented between AChE and metrifonate (precursor of DDVP used in this study); *S. haematobium* is more sensitive to the inhibitor than *S. mansoni* because of the greater amount of AChE on the worm's surface (Camacho et al. 1994).

For the first time, I document the presence of secretory *Sm*ChE activity in schistosomes and AChE activity was highest in cercarial ES products. Of the intra-mammalian stages tested, AChE activity was highest in schistosomula and adults and may be acting to bind and neutralize exogenous AChE inhibitors (Mackintosh 2004) (thus protecting tegumental and somatic AChE) or host-derived ACh to mitigate the immunomodulatory effects of this molecule. Extending this hypothesis, ES products from cultured females had lower AChE activity than males and could be due to females' worm having less of a requirement for this defensive mechanism as they reside in relative shelter of the gynaecophoric canal. BChE activity was present in the ES products of adults, schistosomula and cercariae and was significantly higher in the intra-mammalian larval stage than the other two stages. The *Sm*ChE molecules present in ES were isolated by purification on edrophonium (a ChE inhibitor) sepharose and, consistent with the class of activity observed in ES, identified by mass spectrometry as *Sm*AChE1 and *Sm*BChE1; the former being forty-fold more abundant than the latter.

It is generally accepted that vertebrate BChE has a predominant role in the detoxification of ingested or inhaled drugs and poisons such as the AChE-inhibitory organophosphorus esters that constitute nerve agents and pesticides due to the binding of the enzyme to these molecules [reviewed in (Lockridge 2015)]. Inactivation of *Sm*BChE1 in parasite extracts and live schistosomula by the BChE inhibitor iso-OMPA or through RNAi-mediated silencing potentiated the effects of DDVP whereas addition of exogenous *Sm*BChE1 mitigated the effects, suggesting a similar detoxification role exists for schistosome BChE as for the vertebrate enzyme. The

localization of *Sm*BChE1 to the tegument and its presence in ES products may further support this hypothesis as the enzyme would be spatially available to interact with toxins present in the host environment and safeguarding parasite AChE against AChE inhibitors. Moreover, BChE activity is higher in *S. mansoni* than *S. haematobium*, which is more sensitive to the effects of the organophosphorus AChE inhibitor metrifonate; it has been reported that this sensitivity is due to the larger amount of tegumental AChE present in *S. haematobium* (Camacho et al. 1994), but it may also be due to the reduced amount of BChE available to detoxify the inhibitor as a similar relationship has been reported in studies which use human BChE to counter organophosphate toxicity (Saxena et al. 2006). Plasma-derived human BChE is currently in phase I clinical trial to as a nerve agent detoxifier and a recombinant human BChE mutant is being used to prevent relapse in cocaine addicts due to the enzyme's ability to hydrolyze the drug into inactive by-products (Lockridge 2015). One of the major limitations of these approaches, however, is the catalytic turnover of human BChE (Lockridge 2015) and so there is a research focus into the identification of BChE homologs from other organisms, such as *Sm*BChE1, that might act better as detoxification agents in this regard.

Inhibition of BChE in the absence of DDVP results in parasite death so a bio-scavenging role is possibly not the only function of this enzyme. Indeed, vertebrate BChE has also been shown to have roles in (1) ACh hydrolysis in situations of AChE deficiency (Boudinot et al. 2005), (2) fat metabolism by hydrolyzing the feeding stimulant peptide octanoyl ghrelin (De Vriese et al. 2004)

and (3) scavenging polyproline-rich peptides to regulate protein-protein and protein-DNA interactions (Adzhubei et al. 2013).

In summary, the work herein has identified multiple ChE paralogs in the genome of S. mansoni where previous studies, making use of the technology available at the time, attributed ChE activity to a single AChE, which I have termed *Sm*AChE1. Consistent with previous observations that ChEs are multi-faceted enzymes, I posit that the three ChE paralogs described herein may fulfil distinct neuronal and non-neuronal functions based on their anatomical and temporal expression in the parasite and its ES products and the enzymatic activity of recombinant molecules. The function of *Sm*BChE1 has been partially addressed by RNAi studies and a more comprehensive RNAi-based study of all three paralogs is described in chapter 4, which may further elucidate the function of these molecules.

CHAPTER 4

RNAi-mediated silencing of *Sm*ChE*s* affects parasite cholinergic signaling and development

4.1 Introduction

The nervous system of helminths has been long known to be a potential target for therapeutic agents (Ribeiro et al. 2012; Ribeiro et al. 2013) as it plays several **crucial** roles in parasite biology that are fundamental to survival, including coordinating motility within and outside of the host, feeding and reproduction (Kimber et al. 2005; McVeigh et al. 2005; Ribeiro et al. 2005; Sangster et al. 2005; Vermeire et al. 2005). Further, the *Schistosoma* nervous system is particularly important as this parasite lacks a body cavity and circulating body fluid (Halton et al. 1996; Ribeiro et al. 2010) and, as a result, its signaling functions are chiefly achieved via neurotransmission. The primary neurotransmitter that schistosomes utilize is acetylcholine (ACh), which allows muscle contraction. The physiological concentration of ACh, however, must be maintained otherwise it triggers paralysis (Kimber et al. 2005; McVeigh et al. 2005; Ribeiro et al. 2005).

Acetylcholinesterase (AChE) hydrolyzes excess ACh at the neuromuscular junction between the nervous system and muscles of both vertebrates and invertebrates (Soreq et al. 2001). Without the action of AChEs, uncontrolled influx of ACh causes paralysis and eventually death (Kimber et al. 2005; McVeigh et al. 2005; Ribeiro et al. 2005). Three cholinesterase (ChE) paralogs have been identified from the *S. mansoni* genome (S*m*AChE1, *Sm*BChE1, and *Sm*AChE3 – described in Chapter 3). Yet, it is not clear whether the three *Sm*ChE genes have distinct and/or overlapping functions and it remains to be determined which one of the three paralogs is an ideal therapeutic target in *Schistosoma*. The ability to inhibit the *Sm*AChE paralogs separately with gene silencing techniques may thus be valuable for understanding the distinctive functions of these molecules.

Successful application of RNA interference (RNAi) in various schistosome life stages (reviewed in (Bhardwaj et al. 2011; Da'dara et al. 2015)) highlights the potential of this tool to study the nervous system of this parasite. Indeed, in schistosomes, RNAi has been successfully used to disrupt neurotransmitter transporters (Patocka et al. 2013) as well as neuronal receptors and channels (Patocka et al. 2014; MacDonald et al. 2015). In this chapter, I describe the use of RNAi to knockdown each *Sm*ChE paralog, in isolation and combination, and examine the impact of this silencing on *Sm*ChE activity and parasite survival to assess the suitability of each *Sm*ChE as an intervention target against schistosomiasis.

4.2 Materials and Methods

4.2.1 Ethics statement

All experimental procedures reported in the study were approved by the James Cook University (JCU) animal ethics committee (ethics approval numbers A2391 and A2432). Snails and mice were maintained in the university's quarantine facilities (Q2713 and Q2152, respectively) for the duration of the experiments. The study protocols were in accordance with the 2007 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the 2001 Queensland Animal Care and Protection Act.

4.2.2 Parasite maintenance

Biomphalaria glabrata snails infected with *S. mansoni* (NMRI strain) were obtained from the Biomedical Research Institute (BRI) and were used as a source of *S. mansoni* cercariae for this

experiment. To obtain cercariae stages, 7-8 weeks old infected snails were set to shed cercariae by exposure to light at 28°C for 1.5 h (Lewis et al. 1986) and were mechanically transformed into schistosomula (Ramalho-Pinto et al. 1974), the developmental stage used for RNAi experiments.

4.2.3 siRNA Design and Synthesis

Three short interfering RNA duplexes (siRNAs) targeting each of the three identified *Sm*ChE paralogs (*Sm*AChE1 - Smp-15460, *Sm*BChE1 - Smp-125350 and *Sm*AChE3 - Smp-136690), were designed for gene-specific silencing experiments (Supplementary Table 4.1). Each siRNA was checked to avoid off-target silencing by BLAST searching the *S. mansoni* genome with each sequence. An irrelevant siRNA from firefly luciferase was selected as a negative control (Tran et al. 2010). All selected siRNAs were commercially synthesized (IDT) and oligos were suspended (1 µg/µl) using diethylpyrocarbonate (DEPC) treated water.

4.2.4 Electroporation of schistosomula with siRNA

Prior to electroporation, mechanically transformed schistosomula were cultured for 24 h (2000 schistosomula/ml, at 37°C and 5% CO_2 in modified Basch medium (Basch 1981) supplemented with 4× AA in 6 well plates. After 3 washes with PBS, schistosomula were re-suspended in modified Basch medium (3000 schistosomula/100 µl) and 3000 schistosomula were transferred into Genepulser 4 mm electroporation cuvettes (Bio-Rad) per siRNA treatment (four) and timepoints (four for each treatment – 1, 3, 5 and 7 days). Schistosomula were electroporated with 10 µg of either luciferase, *Sm*AChE1, *Sm*BChE1 or *Sm*AChE3 siRNA or a combination of all

three *Sm*ChE siRNAs (30 µg total) using a Bio-Rad Gene Pulser Xcell (single 20 ms pulse – 125 V, 25 µF capacitance, 200 Ω resistance, square wave electroporation) at RT, added to 24 well plates containing 1 ml fresh pre-warmed modified Basch medium and incubated (37°C, 5% CO_2) for 7 days. Schistosomula were harvested at each timepoint and used for either qPCR analysis (to assess transcript knockdown), protein extract preparation (to examine phenotypic knockdown) or Trypan Blue exclusion assays (to determine parasite viability). All parasite material was generated from two independent experiments.

4.2.5 Total RNA isolation, cDNA synthesis, and real-time qPCR

Schistosomula (1000 parasites) were harvested after day 1, day 3, day 5 and day 7, washed three times in PBS and stored at -80°C until use. Total RNA extraction, cDNA synthesis and RT-qPCR (triplicate assays) were performed as described in section 3.2.5.

4.2.6 Parasite extract preparation

Schistosomula (2000 parasites) were harvested from each timepoint into 300 µl 0.1% Triton X-100 and stored at -80°C until use. Parasites were homogenized on ice and mixed overnight at 4°C. Homogenates were centrifuged at 15,000 x*g* for 1 h at 4°C, supernatants collected and protein concentration determined using the Pierce BCA Protein Assay kit (Thermofisher), aliquoted and stored at -80°C until use.

4.2.7 AChE and BChE activity assay

Activity assays were performed as previously described (section 3.2.11) with 15 µg of schistosomula extract. Reactions were carried out in triplicate.

4.2.8 Determination of schistosomula viability

Schistosomula (100 parasites/replicate) were harvested at each timepoint and viability determined by Trypan Blue exclusion staining (Wangchuk et al. 2016). Briefly, schistosomula were stained with 0.16% Trypan Blue in PBS with gentle shaking for 30 min at RT and then excess stain removed by multiple washes in PBS before fixing in 10% formalin. Parasites were counted under 10x objective and the live parasites (which had not taken up stain) expressed as a percentage of total worms. Each assay was performed in triplicate.

4.2.9 Evaluation of protein expression

Western blots were performed with day 7 parasite extracts (20 µg) following standard procedures. The blots were probed with polyclonal *Sm*ChE antibodies (generated in section 3.2.8). A polyclonal anti-*Sm*-paramyosin antibody (Tran et al. 2010) was used as a loading control.

4.2.10 Glucose uptake of schistosomula treated with siRNA

Newly transformed schistosomula (5000/treatment) were incubated in complete Basch media for 5 days. Parasites were then washed several times with PBS and transferred to serum-free DMEM. At day 5, all parasites were electroporated with *Sm*ChE siRNAs (as in section 4.2.4) and transferred into fresh DMEM (1 mg/ml glucose). Media (50 µl) from each experiment was

collected 48 h post-treatment and the amount of glucose was quantified using a colorimetric glucose assay kit (Sigma) following the manufacturer's instructions. Parasite viability at this timepoint was determined by Trypan Blue exclusion and transcript levels of *Sm*ChEs, as well as the glucose transporters SGTP1 and SGTP4, were also measured. Glucose levels were normalized according to the number of parasites and expressed relative to the luciferase group. Data is the average of 2 biological and three technical replicates ± SEM.

4.2.11 Infection of mice with *Sm*ChE siRNA-treated schistosomula

One-day-old cultured schistosomula (10,000) were electroporated in 500 µl of modified basch medium with 50 µg of either luciferase, *Sm*AChE1, *Sm*BChE1 or *Sm*AChE3 siRNA or a combination of all three *Sm*ChE siRNAs (150 µg total) (as in section 4.2.4). Parasites were injected intramuscularly (1,000 each thigh) into male, 6-8 week BALB/c mouse (5 mice per group) using a 23-gauge needle. A control group of mice were similarly injected with non-electroporated schistosomula. Adult worms were perfused 20 days later to assess the number of worms that had matured and reached the mesenteries.

4.2.12 Statistical analyses

Data were reported as the means ± standard error of the mean (SEM). Statistical differences were assessed using the student's *t* test. *P* values less than 0.05 were considered statistically significant.

4.3 Results

4.2.13 *Sm*ChE transcript reduction and protein suppression

Schistosomula electroporated with *Sm*AChE1 siRNA showed respective decreases in *Sm*AChE1 mRNA levels of 55.4% ($P \leq 0.05$) and 81.3% ($P \leq 0.001$) at 1 and 7 days post-treatment, respectively, compared to the luciferase control (Fig 4.1A), while treating parasites with *Sm*BChE1 siRNA caused 32.0% ($P \leq 0.001$) and 84.5% ($P \leq 0.001$) *Sm*BChE1 mRNA suppression at day 3 and 7 after electroporation, respectively, compared to the luciferase control (Fig 4.1B). Treatment of schistosomula with *Sm*AChE3 siRNA resulted in respective decreases in *Sm*AChE3 mRNA levels of average 27.4% ($P \leq 0.001$) and 47.2% ($P \leq 0.01$) 3 and 7 days after electroporation, compared to the luciferase control (Fig 4.1C). Schistosomula electroporated with a cocktail of all three *Sm*ChE siRNAs showed decreases of all three transcript levels over time, with *Sm*AChE3 mRNA levels decreasing by an average of 90% ($P \leq 0.001$) by day 3 after treatment, compared to the luciferase control (Fig 4.1D).

Seven days after treatment with *Sm*AChE1, *Sm*BChE1 or *Sm*AChE3 siRNAs, parasites showed decreases in *Sm*AChE1, *Sm*BChE1 or *Sm*AChE3 protein expression of 73%, 59% and 46%, respectively, compared to luciferase controls (Fig 4.1E).

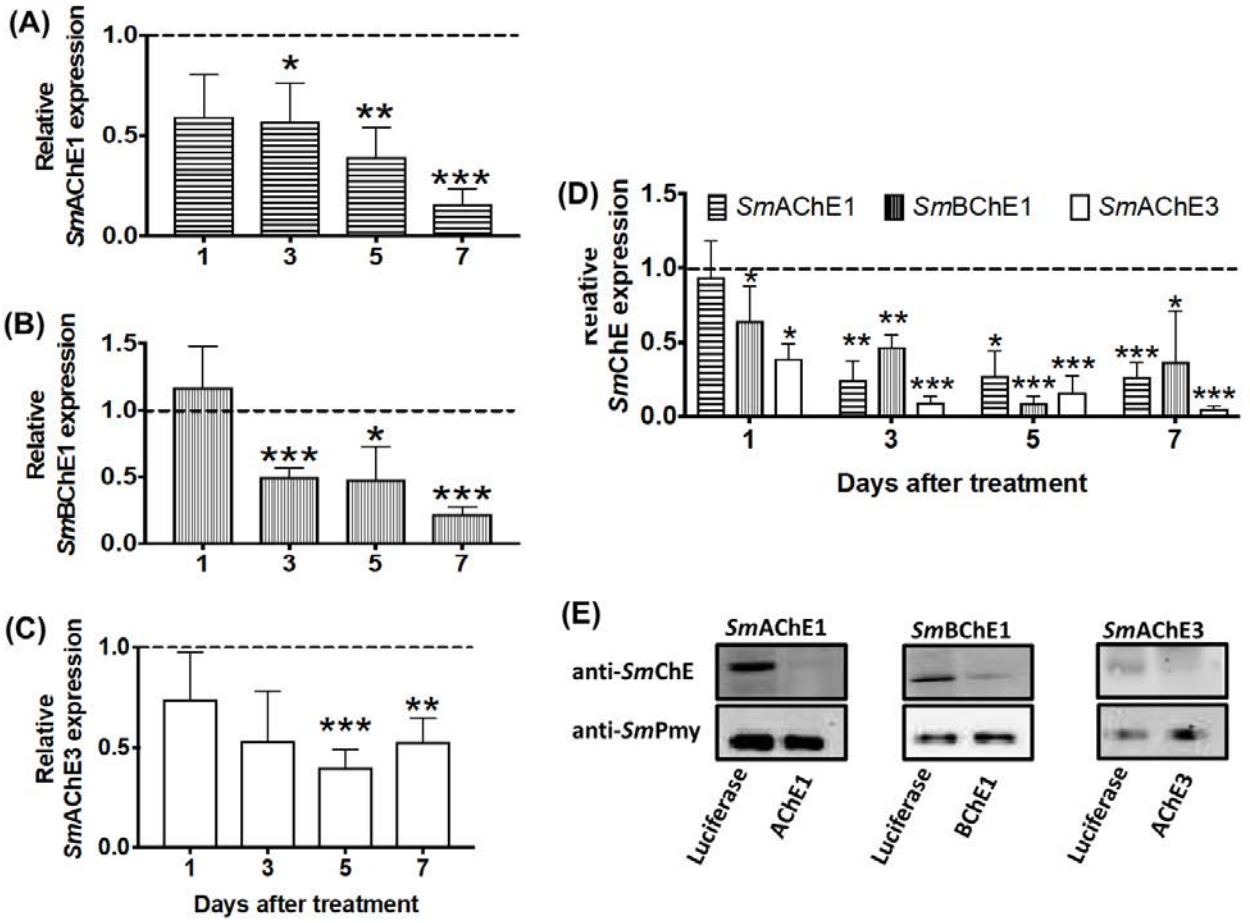


Fig 4.1. Suppression of *Sm*ChE mRNA transcript and protein expression in schistosomula by RNAi.

(A) *Sm*AChE1, (**B**) *Sm*BChE1, (**C**) *Sm*AChE3 individual siRNA treatment (**D**) treatment with cocktail of *Sm*AChE1, *Sm*BChE1, and *Sm*AChE3 siRNAs. *Sm*ChE transcript levels in parasites treated with *Sm*ChE siRNAs are shown relative to *Sm*ChE transcript expression in schistosomula treated with the luciferase control (mean ± SEM of triplicate assays from 2 biological replicates of each treatment). Transcript expression in all parasites was normalized with the housekeeping gene, *Sm*COX1. Differences in transcript levels were measured by the student's *t* test. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001. (**E**) Western blot of day 7 schistosomula extracts following treatment with *Sm*ChE or luciferase siRNAs**.** Extracts were immunoblotted with the corresponding anti-*Sm*AChE1*,* anti-

*Sm*BChE or anti-*Sm*AChE3 polyclonal antibody. An antibody against *Sm*Pmy was used as a loading control.

4.2.14 Suppression of *Sm*ChE activity

Suppression of AChE activity was seen in *Sm*AChE1 and *Sm*AChE3 siRNA-treated parasites at 5 and 3 days after electroporation, respectively (Fig 4.2A), compared to the luciferase control, while schistosomula treated with *Sm*BChE1 siRNA did not show any significant reduction in BChE activity, even 7 days after electroporation (Fig 4.2B). Parasites electroporated with a cocktail of all 3 *Sm*ChE siRNAs showed significant decreases in AChE activity at 3 days (62% reduction, $P \leq 0.001$), 5 days (67% reduction, $P \leq 0.001$) and 7 day (71% reduction, $P \leq 0.001$) after treatment (Fig 4.2C). BChE activity was not measured in the cocktail siRNA treatment group.

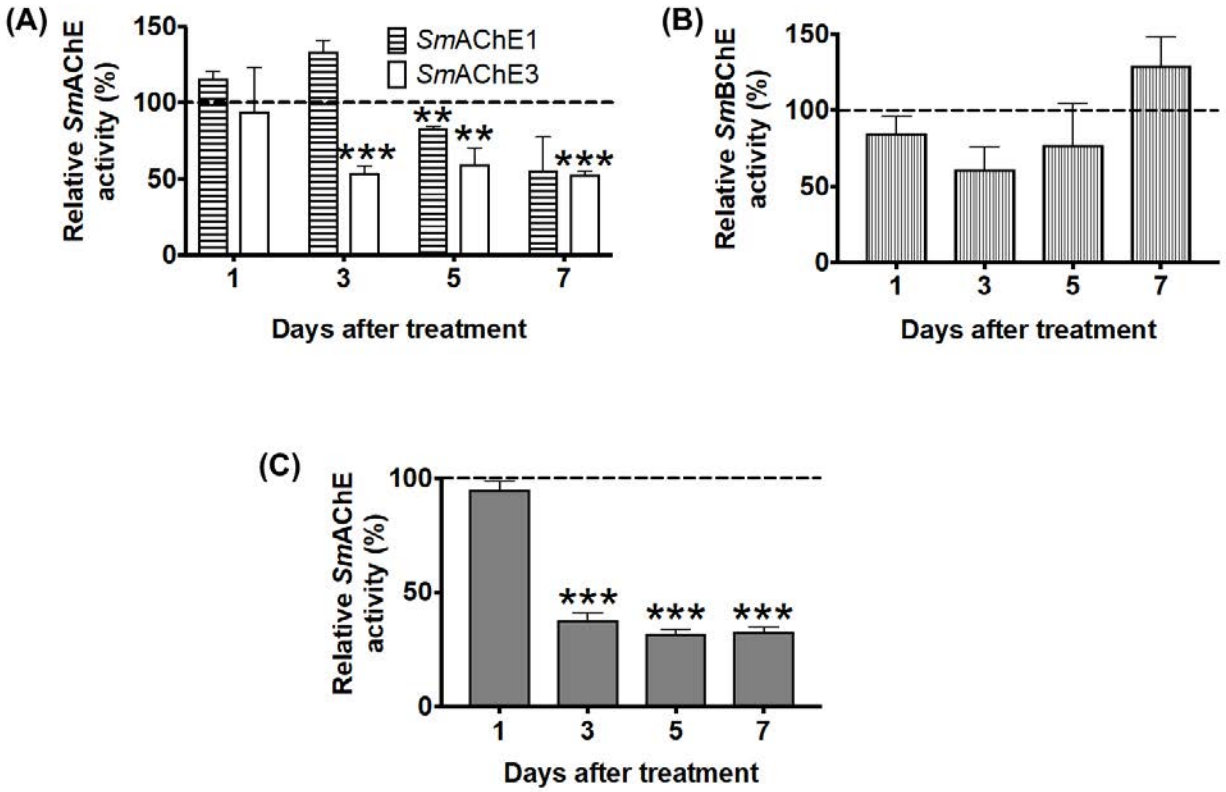


Fig 4.2. Effects of *Sm*ChE knockdown on cholinesterase activity. (**A**) AChE activity of parasites treated with *Sm*AChE1, *Sm*AChE3 or luciferase siRNA (dashed line). (**B**) The BChE activity of parasites treated with *Sm*BChE1 or luciferase siRNA (dashed line). (**C**) AChE activity of parasites treated with all 3 siRNAs or luciferase siRNA (dashed line). Data represents mean ± SEM of duplicate assays from 2 biological replicates of each treatment. Differences were measured by the student's *t* test. ***$P \leq 0.001$.

4.2.15 Suppression of parasite viability

Parasites treated with SmAChE1, SmBChE1 or SmAChE3 siRNA showed significant decreases in viability at day 3, 5 and 1 after treatment, respectively, compared to luciferase controls. At day 5

and 7 post-treatment, the most significant decreases in parasite viability was seen in the group which received the cocktail siRNA treatment, compared to luciferase controls. Further, viability in this group was also significantly lower than any individual treatment at these two time periods.

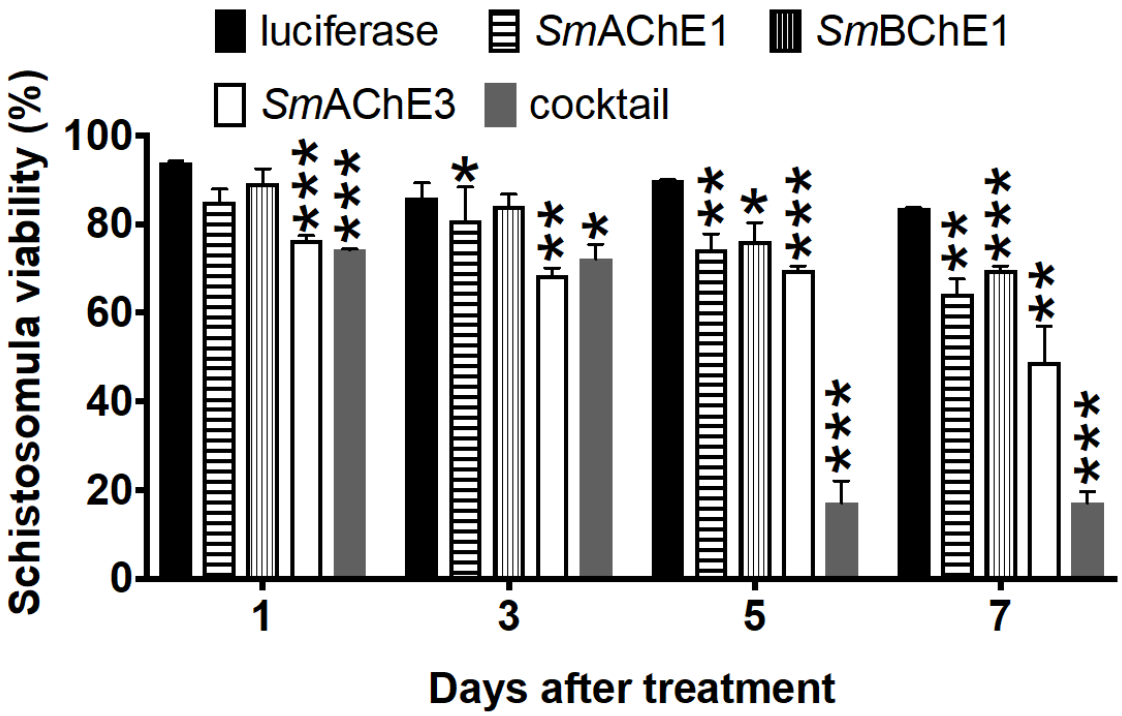


Fig 4.3. Effects of *Sm*ChE silencing on schistosomula viability. Schistosomula treated with (**A**) individual or (**B**) all 3 siRNAs were cultured for 7 days in complete Basch medium with viability determined at day 1, 3, 5 and 7 after treatment by Trypan Blue exclusion (mean ± SEM of duplicate assays from 2 biological replicates of each treatment. Differences in viability between *Sm*ChE and luciferase treated groups and individual and cocktail treated groups were measured by the student's *t* test. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001.

4.2.16 RNAi-mediated suppression of glucose uptake

Individual silencing of *Sm*AChE1 or *Sm*AChE3 and combined silencing of all three *Sm*ChEs reduced glucose uptake in schistosomula by 24.9% ($P \leq 0.001$), 32.34% ($P \leq 0.001$) and 38.61% ($P \leq 0.001$) at 48 h post-treatment, respectively, relative to the luciferase control. However, S*m*BChE1-silenced parasites showed no significant changes in glucose uptake at the same timepoint. There was no difference in the glucose consumed by the *Sm*AChE1 or *Sm*AChE3 siRNA-treated groups compared with the cocktail siRNA-treated group (Fig 4.4A). Transcript levels of the glucose transporters, SGTP1 and SGTP4, were neither decreased nor significantly increased in individual or cocktail *Sm*ChE-silenced parasites (Fig 4.4B).

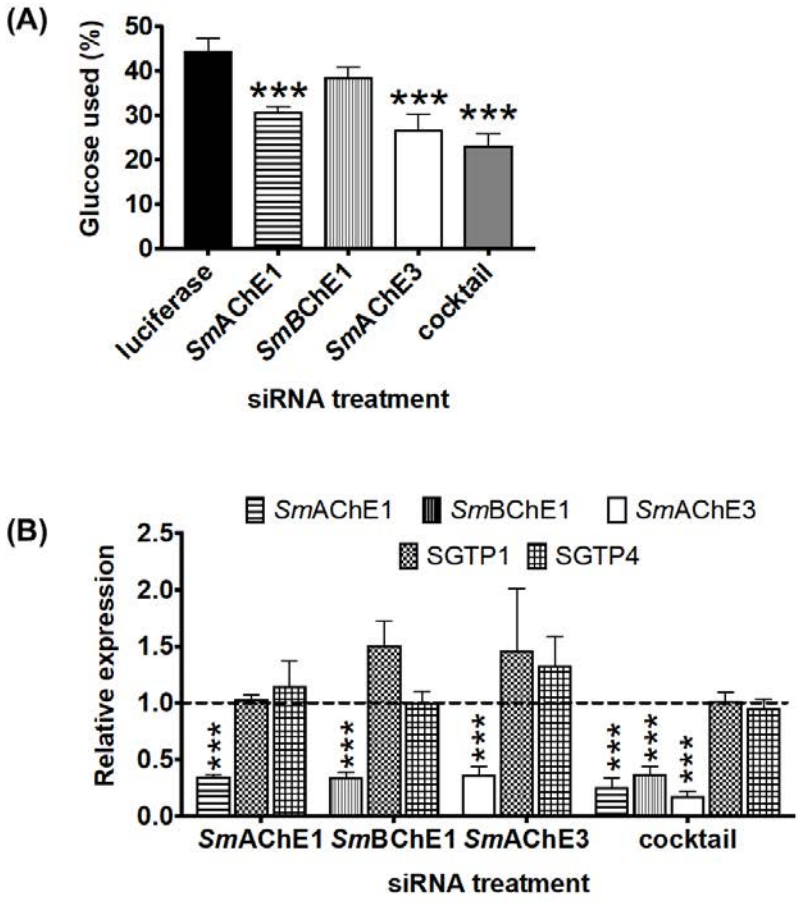


Fig 4.4. Schistosome glucose uptake is affected by *Sm*ChE suppression. (A) Glucose uptake by *S. mansoni* schistosomula 48 h after treatment with *Sm*ChE siRNAs. Schistosomula (5 days old - 5000/treatment) were electroporated with either luciferase or *Sm*ChE siRNA and glucose consumption normalized by parasite viability was measured 48 h after treatment. **(B)** Transcript levels of glucose transporters SGTP1 and SGTP4 and *Sm*ChEs in individual and cocktail *Sm*ChE-siRNA treatment parasites, relative to luciferase-treated controls (dashed line). Data are representative of the mean ± SEM of two independent experiments. *P* values were calculated using the student's *t* test. *p value ≤ 0.05, **p value ≤ 0.01, ***p value ≤ 0.001.

4.2.17 *Sm*ChEs are essential for parasite development and survival in mammalian hosts

To examine whether RNAi-mediated *Sm*ChE suppression reduced parasite viability *in vivo*, mice were infected with *Sm*ChE-silenced parasites and worm burdens measured after three weeks. From two independent experiments, there was an average 88.15%, 55.15%, 75.95% and 88.35% decrease in worm burden from mice infected with *Sm*AChE1-, *Sm*BChE1-, *Sm*AChE3- and *Sm*ChE cocktail-silenced parasites, respectively, compared to mice infected with luciferase-treated parasites. All worm burden decreases were significant and there was no significant difference between mice infected with luciferase-treated and non-electroporated control parasites. There was no longer any difference in *Sm*ChE transcript levels from recovered parasites (data not shown) and all mice had been successfully infected with parasites, as serum from necropsied mice contained parasite-specific antibodies (data not shown).

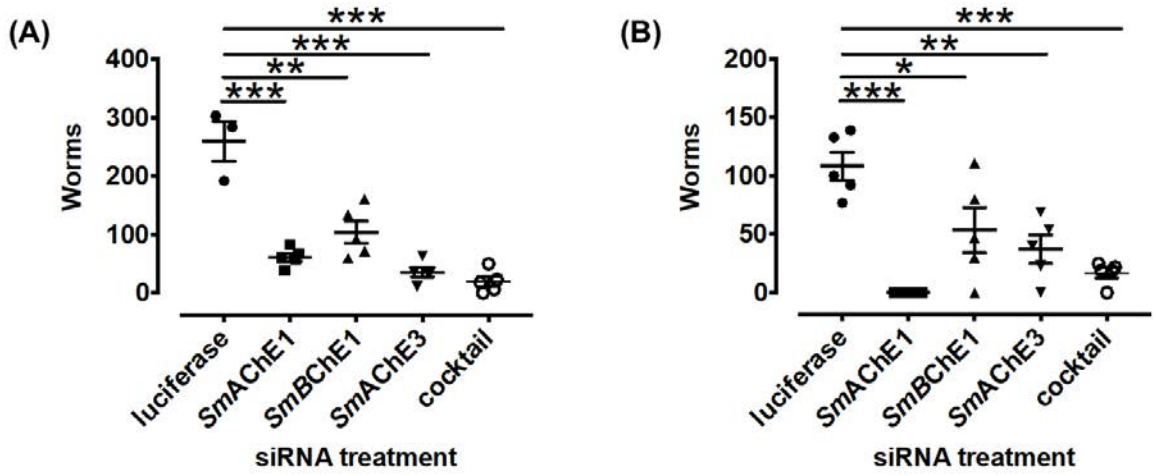


Fig 4.5. Silencing of *Sm*ChEs suppresses parasite establishment *in vivo*.

One-day-old schistosomula were treated with *Sm*AChE1, *Sm*BChE1 or *Sm*AChE3 siRNA, a cocktail of all three *Sm*ChE siRNAs, or luciferase siRNA and then intramuscularly injected (2,000 parasites) into mice. Non-electroporated parasites were similarly used as controls After 3 weeks, adult worms were recovered and counted. Data from two independent experiments (A and B) are shown. Significant differences were determined using the student's *t* test. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

4.4 Discussion

RNA interference (RNAi) is a mechanism employed by most eukaryotic cells to control coding transcript levels of targeted genes which, in turn, results in downregulation of targeted protein expression (Ndegwa et al. 2007; Da'dara et al. 2015). Schistosomes have the cellular machinery to carry out this gene-specific targeting of protein expression (Krautz-Peterson et al. 2008) and so RNAi has been used to characterize schistosome proteins through loss of function studies

(Krautz-Peterson et al. 2008; Krautz-Peterson et al. 2010; Patocka et al. 2014). Further, and relevant to this study, RNAi has been used to disrupt schistosome neurotransmitter transporters (Patocka et al. 2013) and neuronal receptors and channels (Patocka et al. 2014; MacDonald et al. 2015). Accordingly, this gene-silencing method was used to determine the functionality and essentiality of *Sm*AChE1, *Sm*BChE1 and *Sm*AChE3 through targeted knockdown, either individually or in combination, with a view to validating these proteins as therapeutic targets against schistosomiasis.

Treatment of schistosomula with *Sm*ChE siRNAs, either individually or in combination, resulted in transcriptional suppression of each target by at least 50% three days after electroporation. Knockdown had increased at each timepoint until termination of the experiment at day 7 post-treatment, and there was a comparable reduction in protein expression of each paralog at this time, indicating that silencing of *Sm*ChE genes can be achieved effectively by siRNA-mediated RNAi and demonstrating the feasibility of simultaneous transcript suppression in schistosomes.

*Sm*AChE1- and *Sm*AChE3-silenced parasites showed decreases in AChE activity, consistent with reductions in transcript and protein expression levels. Moreover, inhibition of this biochemical activity was greater in schistosomula treated with the *Sm*ChE siRNA cocktail than parasites receiving any of the individual treatments, further evidence suggestive of simultaneous silencing of *Sm*AChEs. AChE activity inhibition in *Sm*AChE3-silenced parasites was more pronounced than in *Sm*AChE1-silenced parasites, which was inconsistent with protein level reductions and this may be due to the increased AChE activity reported for *Sm*AChE1 (Vmax = 5.57 nmol/min/mg, Km =

5.83 mM) compared to *Sm*AChE3 (Vmax = 5.59 nmol/min/mg, Km = 10.87 mM) (section 3.3.5). It is also possible that there may not be a direct correlation between AChE activity and protein expression, given that additional, non-cholinergic functions have been ascribed to ChEs (Camacho et al. 1995; Lee 1996; Soreq et al. 2001). This may also be the reason why no significant decrease in BChE activity was observed in *Sm*BChE1-silenced parasites, despite significant reductions in transcript and protein expression levels.

Individual *Sm*ChE silencing each resulted in significant decreases in parasite viability at various timepoints after treatment with SmAChE3-silenced parasites showing the most significant decrease in viability most quickly after treatment. *Sm*AChE3 is the only one of the *Sm*ChE paralogs studied whose expression is remarkably upregulated between *S. mansoni* cercariae and schistosomula (Parker-Manuel et al. 2011), an observation consistent with qPCR data (section 3.3.2), and so silencing this relatively highly expressed gene may have the most profound effects of all *Sm*ChE silencing on parasite viability. The viability of parasites treated with all three *Sm*ChE siRNAs was significantly decreased compared to parasites treated with an individual siRNA, suggestive of the fact that functional overlap exists between the paralogs. This redundancy has been documented in AChE-knockout mice where BChE has the ability to hydrolyze ACh in the absence of AChE (Li et al. 2000; Mesulam et al. 2002). Moreover, AChE deletion is found to be lethal in *Drosophila* only because there is no alternative BChE paralog to compensate for the lack of ACh hydrolysis (Greenspan et al. 1980; Xie et al. 2000). Similar to the observations in this study, simultaneous knockdown of multiple ChE genes have been reported to have deleterious effects

on their target organisms including the insects *Plutella xylostella* (Smith et al. 2012)*, Chilo suppressalis* (X.-M. et al. 2011) and *Tribolium castaneum* (Lu et al. 2012) and the nematodes *Nippostrongylus brasiliensis* (Hussein et al. 2002) and *Caenorhabditis elegans* (Johnson et al. 1988). Similarly, chemotherapy with "broad spectrum" ChE inhibitors has shown to be effective against a range of organisms, including pest insects (Lang et al. 2012), schistosomes (Davis et al. 1969; Bueding et al. 1972; Feldmeier et al. 1982; Sundaraneedi et al. 2017) and *Trichuris muis* parasites (Sundaraneedi et al. 2018). It is likely that the simultaneous silencing of the *Sm*ChE genes in this study has a profound effect on parasite viability due to the knockdown of cholinergic signaling, a process to which all paralogs contribute, as they have all been shown to hydrolyze ChE substrates (section 3.3.5). Also possible is that knockdown of these three genes might have also resulted in the ablation of multiple other functions that have been suggested for these molecules (Pritchard 1993; Soreq et al. 2001; Lu et al. 2012). Whatever the reason, it is evident that the targeting of all these paralogs may be necessary in the development of an intervention strategy against schistosomiasis.

Previous studies have documented the involvement of *Sm*ChEs in the uptake of exogenous glucose by schistosomes through the ablation of the glucose uptake pathway by organophosphorus (Camacho et al. 1994) and large molecule (Sundaraneedi et al. 2017) AChE inhibitors so I sought to identify the *Sm*ChE paralog(s) responsible for this mediation through the use of RNAi targeting *Sm*ChE genes. Individual *Sm*ChE gene knockdown of *Sm*AChE1 and *Sm*AChE3 suppressed glucose uptake in schistosomula, implying that both genes were involved

in regulation of this mechanism. Tegumental AChE is speculated to mediate glucose uptake by limiting the interaction of ACh with tegumental nicotinic ACh receptors which is thought to decrease the amount of glucose uptake through surface glucose transporters and so the fact that both molecules are localized to the tegument (section 3.3.4) and can hydrolyze ACh (section 3.3.5) provide evidence for their role in this pathway. *Sm*BChE1-silenced parasites did not show any difference in glucose uptake and is probably reflective of the molecule's limited role in ACh hydrolysis (section 3.3.5). Transcript levels of SGTP1 and SGTP4 were not significantly increased in *Sm*AChE1- and *Sm*AChE3-silenced parasites, suggesting that *Sm*AChEs may facilitate glucose uptake in a manner which does not directly involve glucose transporters. Indeed, at least in nematodes, AChEs have been proposed to be involved in altering the permeability of surrounding host cells, allowing nutrients (such as glucose) to leak into the parasite niche and be uptaken (Lee 1970).

Given that suppression of *Sm*ChEs affected parasite viability and inhibited biochemical activity *in vitro*, it was decided to examine whether the establishment of *Sm*ChE-silenced schistosomula would be impaired *in vivo*. Worm recovery from mice infected with all groups of *Sm*ChE-silenced parasites was significantly less than controls, indicating that suppression of *Sm*AChE1, *Sm*BChE1 or *Sm*AChE3 could inhibit schistosome establishment and/or development in the host. Viability of silenced parasites was decreased in *in vivo* compared to *in vitro* experiments. It may be that *in vitro* killing of parasites silenced for individual *Sm*ChEs could have been achieved had the experiment been conducted for longer than 7 days. Another possible explanation is that *Sm*ChE

silencing may be more "effective" in vivo as knockdown is inhibiting processes that are influenced by host biology and not evident in an *in vitro* system. For example, it has been indirectly shown that the nematode *N. brasiliensis* employs parasite-derived AChE to alter the host cytokine environment so as to be more favorable to worm survival (Vaux et al. 2016); the consequence of silencing this interaction could only be observed *in vivo* and not through the use of an *in vitro* RNAi experiment. This contrast between *in vitro* and *in vivo* RNAi experimental results has been observed previously in schistosomes; Krautz-Peterson et al (Krautz-Peterson et al. 2010) have shown that, while the mortality of schistosomula silenced for the glucose transporters SGTP1 and SGTP4 was unaffected *in vitro*, similarly silenced parasites displayed an impaired ability to establish infection when introduced into mice. Further, and consistent with *in vitro* data, schistosomula silenced for all three *Sm*ChEs exhibited the highest mortality *in vivo* when worm recovery was averaged across the two independent trials, suggesting that, not only is simultaneous knockdown of *Sm*ChEs required to overcome any functional redundancy between the molecules, this treatment has the largest impact on parasite pathogenesis due to the inhibition of multiple biological functions collectively orchestrated by these proteins.

*Sm*ChE transcript levels of silenced worms recovered from mice were no different from control parasites and it is likely that the surviving worms were parasites which received less siRNA than others in the population, resulting in less gene suppression, and/or recovered from the effects of RNAi, which is a transient treatment. CRISPR/Cas9 technology offers an advantage over RNAi in that it permanently "knocks out" the targeted gene by introducing mutations in the genome of

an organism and the recent application of this procedure to schistosomes (Ittiprasert et al. 2018) means that the functions of biologically important molecules such as *Sm*ChEs can be comprehensively deciphered using *in vivo* models of schistosomiasis due to the permanent silencing effects of this technology.

Taken together, these results confirm that *Sm*ChEs are essential for parasite survival, a claim reinforced by numerous studies in a variety of organism where suppression of AChE genes affected survivability (Kumar et al. 2009; X.-M. et al. 2011; He et al. 2012; Gong et al. 2013; Malik et al. 2016; Kishk et al. 2017; Ye et al. 2017). While ChEs have previously been validated as lethal chemotherapeutic target in *Schistosoma spp* (Davis et al. 1969; Bueding et al. 1972; Hillman et al. 1975; Feldmeier et al. 1982; Sundaraneedi et al. 2017), this study is the first report on the effect of ChE gene silencing on the *in vivo* development of any *Schistosoma spp*. Given the essentiality of *Sm*ChEs, determined by the RNAi studies in this chapter, the vaccine efficacy of recombinant *Sm*ChEs will be explored in Chapter 5.

CHAPTER 5

Vaccine efficacy of recombinant S*. mansoni* cholinesterases in a mouse model of schistosomiasis

5.1. Introduction

Despite decades of concentrated research, there is still no effective and practical vaccine against schistosomiasis (Egesa et al. 2017). Further, mass chemotherapy using praziquantel (PZQ), the only effective anti-schistosomal drug, is complicated by rapid and frequent re-infection (Mduluza et al. 2001). There is also evidence that resistance to PZQ is emerging (Crellen et al. 2016). So far, a considerable number of schistosome antigens have been identified and tested as vaccines and, although a number of these vaccine candidates (for example *Sm*TSP-2, *Sm*14, *Sm*29, *Sm*CB1 and *Sm*p80) have shown promising efficacy in animals models and are in various stages of pre-clinical or clinical development, none has been approved for licensure [reviewed in (Tebeje et al. 2016)].

Due to the fundamental roles they play in parasite biology (reviewed in (Arnon et al. 1999), schistosome cholinesterases (*Sm*ChEs) have been posited as intervention targets against schistosomiasis and there are several indications to support the feasibility of their use as vaccines. Firstly, *Sm*ChEs have been localized to the tegument of schistosomula and adult worms (Espinoza et al. 1991) (Chapter 3) and anti-*Sm*ChE antibodies have been shown to bind to and kill schistosomula (Arnon et al. 1987), suggesting that the enzymes are accessible to immune attack. Anti-*Sm*ChE antibodies also showed no cross-reactivity against human AChE (Espinoza et al. 1991), indicating that a vaccine safe for human use could be designed. Thirdly, protein array studies have detected significantly high levels of antibodies to *Sm*ChEs in humans exhibiting resistance and low pathology to schistosomiasis, suggesting an involvement of these antibodies in a protective anti-schistosomal response (Pearson et al. 2015; Driguez et al. 2016).

Lastly, RNAi-mediated suppression of ChEs in both *S. mansoni* (Chapter 4) and *S. japonicum* (You et al. 2018) revealed that the enzymes are essential for parasite survival. However, it remains to be determined which *Sm*ChE paralogs are effective vaccine targets in *S. mansoni*, particularly as there appears to be some functional redundancy between the enzymes (Chapter 3).

Herein, I have demonstrated that purified IgG against each of the three *Sm*ChE paralogs identified in chapter 3 inhibit ChE activity in both larval and adult worms *in vitro*, which results in eventual parasite death. Further, I document the efficacy of these *Sm*ChEs, when administered as recombinant vaccines in isolation or as a triple combination, in reducing parasite burden, stunting worm growth and decreasing egg viability.

5.2. Material and Methods

5.2.1. Ethics statement

All experimental procedures reported in the study was approved by the James Cook University (JCU) animal ethics (Ethics approval numbers A2391). Mice were maintained in cages in the university's quarantine facility (Q2152) for the duration of the experiments. The study protocols were in accordance with the 2007 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the 2001 Queensland Animal Care and Protection Act.

5.2.2. Parasites

Biomphalaria glabrata snails infected with *S. mansoni* (NMRI strain) were obtained from the

Biomedical Research Institute (BRI) and cercariae were shed by exposure to light at 28°C for 1.5 h (Ramalho-Pinto et al. 1974). Cercariae were mechanically transformed to obtain schistosomula (Ramalho-Pinto et al. 1974). To obtain adult worms, 6-8 week old male BALB/c mice (Animal Resource Centre, WA) were infected with 180 cercariae via tail penetration and parasites harvested by vascular perfusion at 7-8 weeks post-infection (Lewis et al. 1986).

5.2.3. Recombinant protein expression and purification

Recombinant *Sm*ChE subunit proteins (p*Sm*ChEs) were expressed in *E. coli* BL21 (DE3) and purified by IMAC, as described in Section 3.2.6-3.2.7.

5.2.4. Effect of polyclonal anti-*Sm*ChE IgG on larval worms

Newly transformed schistosomula (1000/ml) were cultured in DMEM (supplemented with 4x AA) at 37°C and 5% CO_2 in the presence of 50 µg of either anti-*Sm*AChE1, *Sm*BChE1 or *Sm*AChE3 polyclonal IgG (section 3.2.8) or a combination of all three antibodies (equal amounts - 50 µg total). Separate sets of parasites were similarly incubated with 50 µg of naïve mouse IgG, which served as a control. After 2 and 14 h (separate experiments were conducted for each timepoint), surface and secreted ChE activity was measured by Ellmann assay (section 3.2.11) by incubating the parasites in assay buffer for 1h and schistosomula viability determined by Trypan Blue exclusion (section 2.x). Enzyme activities of IgG-treated parasites were expressed relative to parasites cultured with naïve IgG (negative control). Data are presented as the average of two biological and three technical replicates ± SEM.

5.2.5. Effect of polyclonal anti-*Sm*ChE IgG on adult worms

Enzyme inhibitory effects 24 h after addition of IgG were measured as for schistosomula using 5 pairs of freshly perfused adult worms in 1 ml of media. Data are presented as the average of two biological and two technical (four total) replicates ± SEM. To investigate the effects of polyclonal anti-*Sm*ChE IgG on worm viability, ten pairs of worms were similarly incubated with antibodies for 10 days, monitored every 24 h for motility by microscopic examination and considered dead if no movement was seen. Glucose uptake was also measured calorimetrically using a glucose assay kit (Sigma) as described earlier (section 2.17), 24 h after antibody addition. Glucose levels were expressed relative to media collected from worms which received naïve IgG (negative control). Data are presented as the average of two biological replicates ± SEM.

5.2.6. Anti-*Sm*ChE IgG responses in *S. mansoni*-infected mice during infection and before and after PZQ treatment

Sera from *S. mansoni* infected male BALB/c mice (6-8 weeks) (n=5) was collected at day 3, 14, 28, 42 and 56 post infection (p.i.) to assess anti-*Sm*ChE responses during the course of parasite infection. In a separate experiment, sera from *S. mansoni* infected male BALB/c mice (6-8 weeks) (n=11) was collected at 5 weeks p.i. and then mice were treated orally with PZQ (100 mg/kg) at 35, 37 and 39 days p.i. Sera was again collected at day 49 p.i. (2 weeks post-PZQ treatment). Anti-*Sm*ChE responses during infection and before and after PZQ treatment were screened by ELISA with plated p*Sm*ChEs (100 ng/well) using standard methods. The cutoff value for each dilution

was established as three times the mean OD of the naïve sera for that dilution and the endpoint was defined as the highest dilution above the cutoff value.

5.2.7. Vaccine trials

Five groups of 10 male BALB/c mice (6-8 weeks) were immunized intraperitoneally on day 1 (50 µg/mouse) with either p*Sm*AChE1, p*Sm*BChE1, p*Sm*AChE3, a combination of all three p*Sm*ChEs (17 µg each - 50 µg total) or PBS, each formulated with an equal volume of Imject alum adjuvant (Thermofisher) and 5 µg of CpG ODN1826 (InvivoGen). Immunizations were repeated on day 15 and 29 and each mouse was challenged (abdominal penetration) with 120 *S. mansoni* cercariae on day 43. Two independent trials were performed to ensure reproducibility. Blood was sampled at day 28 and 42 and on the day of a necropsy to determine pre- and post-challenge antibody titers.

5.2.7.1 Mouse necropsy and estimation of worm and egg burden:

Mice were necropsied at day 91 (7 weeks post-infection) and worms harvested by vascular perfusion and counted. Livers were removed and halved, with one half weighed and digested for 5 h with 5% KOH at 37^0C with shaking. Schistosome eggs from digested livers were concentrated by centrifugation at 1,000 g for 10 minutes and re-suspended in 1 ml of 10% formalin. The number of eggs in a 5 µl aliquot was counted in triplicate and the number of eggs per gram (EPG) of the liver was calculated. Small intestines were removed and cleaned of debris before

being weighed and digested as per the liver halves. Eggs were also similarly concentrated and counted to calculate intestinal EPG.

5.2.7.2 Egg viability assays

The other half of each liver was pooled according to the group, homogenized in H_2O and placed in identical foil-covered volumetric flasks under bright light to hatch eggs released from the livers. After 1 h, the number of miracidia in 10 x 50 µl aliquots of H_2O (sampled from the extreme top of each flask) were counted. The number of eggs in each flask at the start of the hatching experiment was determined by liver EPG calculations, allowing the egg hatching index of each group to be calculated by expressing the hatched eggs (miracidia) as a percentage of the total eggs.

5.2.7.3 Glucose consumption and glycogen storage assays

Five pairs of freshly perfused worms from each vaccinated group were cultured in DMEM (1000 mg/l glucose). Media (50 µl) from each experiment was collected after 24 h, and the amount of glucose was quantified using a colorimetric glucose assay kit (Sigma) according to the manufacturer's instructions. Glucose levels were expressed relative to media collected from worms, recovered from PBS treated mice (negative control). To measure the glycogen content of the same worms used for glucose uptake assay were used for Triton X-100-soluble extraction and assayed for glycogen in a modified procedure described by Gomez- Lechon et al. (Gómez-Lechón et al. 1996). Briefly, 0.2 M sodium acetate, pH 4.8, was added to 30 µg parasite extract

and 50 µl glucoamylase (10 U/ml) to make a reaction volume of 150 µl. The mixture was incubated at 40°C for 2 h with shaking at 100 rpm, 40 µl added to a new microplate with 10 µl 0.25 M NaOH and the amount of glucose quantified using the colorimetric glucose assay kit. Extracts were made from triplicate sets of parasites (five pairs of worms) and assays were performed three times. Data are presented as the average of each triplicate biological and technical experiment ± SEM.

5.2.7.4 Immune responses in vaccinated mice

Sera were collected from all mice in each group before cercarial challenge and at necropsy. Serum anti-*Sm*ChE IgG antibodies were measured by ELISA using standard methods.

5.2.8. Statistical analyses

Statistical differences for all experiments in this chapter were calculated by the Student's *t* test using GraphPad Prism 7 software. Results are expressed as the mean ± standard error of the mean (SEM).

5.3. Results

5.3.1. Anti-*Sm*ChE polyclonal antibodies block enzyme activity and decrease viability of larval *S. mansoni in vitro*

To determine the ability of anti-*Sm*ChE specific polyclonal antibodies to inhibit ChE activity in *S. mansoni*, and the effect this had on parasite viability, I studied the effects of paralog-specific antibodies on at two different timepoints. Treating schistosomula with anti-*Sm*AChE1 IgG, anti-*Sm*AChE3 IgG, or a cocktail of all three anti-*Sm*ChE IgGs caused significant inhibition ($P \leq 0.01$) of AChE activity by 56.2%, 57.1% and 59.74%, respectively, 2 h after treatment, in comparison with the naïve IgG control (Fig 5.1A). When schistosomula were incubated with anti-*Sm*BChE1 IgG or a cocktail of all three anti-*Sm*ChE IgGs for 2 h, BChE activity was inhibited by 37.4% ($P \leq 0.01$) and 49.3% ($P \leq 0.001$), respectively, compared to the control (Fig 1B). Schistosomula viability was not significantly affected at this timepoint (Fig 1C). Extending the treatment with anti-*Sm*AChE1 IgG, anti-*Sm*AChE3 IgG, or a cocktail of all three anti-*Sm*ChE IgGs for 14 h significantly decreased ($P \leq 0.001$) AChE activity by 66.9%, 70.5% and 72.6%, respectively, compared to the control (Fig 5.1D). Similarly, when schistosomula were incubated with anti-*Sm*BChE1 IgG or a cocktail of all three anti-*Sm*ChE IgGs for 14 h, BChE activity decreased significantly ($P \leq 0.01$) by 26.5% and 35.6%, respectively, compared to the control (Fig 5.1E). Schistosomula viability was significantly decreased ($P \leq 0.01$) by all treatments at this timepoint, with the biggest decrease seen in the anti-*Sm*ChE cocktail IgG-treated group (Fig 5.1F).

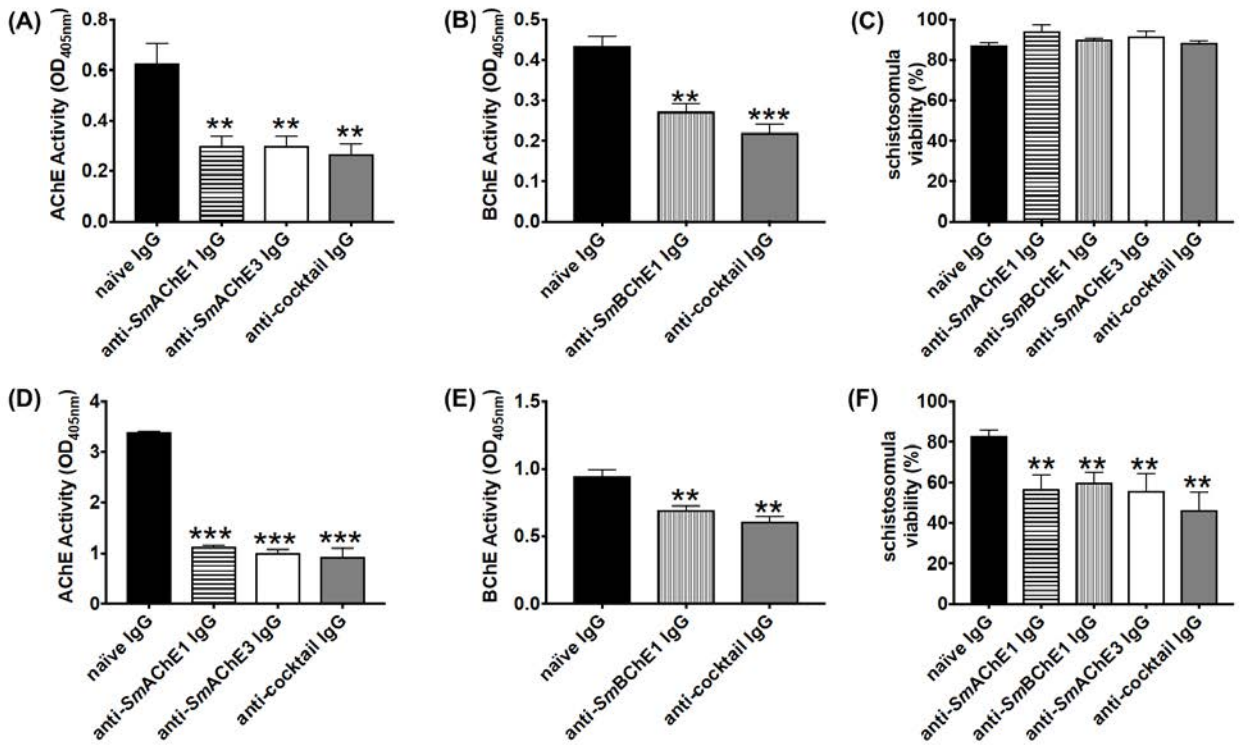


Fig 5.1. Anti-*Sm*ChE antibodies inhibit ChE activity in schistosomula which leads to decreased parasite viability. Newly transformed schistosomula (1000/treatment) were incubated in DMEM in the presence of anti-*Sm*ChE IgG and incubated at 37 °C in 5% CO_2. Naïve IgG served as a negative control. **(A)** AChE activity 2 h after treatment, **(B)** BChE activity 2 h after treatment, **(C)** schistosomula viability 2 h after treatment, **(D)** AChE activity 14 h after treatment. Data represents the mean ± SEM of two biological and three technical replicates. Significance (relative to the naïve IgG control) determined by the student's *t* test ** $P \leq 0.01$, *** $P \leq 0.001$.

5.3.2. Effects of anti-*Sm*ChE antibodies on adult worms

The effects of anti-*Sm*ChE antibodies on *S. mansoni* adult worms was also tested *in vitro*. Freshly perfused worms cultured in the presence of anti-*Sm*AChE1, anti-*Sm*AChE3, or a cocktail of all

three anti-*Sm*ChEs, showed no significant inhibition of AChE or BChE (data not shown) activity at 2 h post-treatment, compared to controls. After 24 h treatment, however, all anti-*Sm*ChE IgG-treated groups showed significant inhibition of AChE and BChE activity with the anti-*Sm*ChE cocktail IgG-treated group displaying the greatest inhibition of AChE activity (Fig 5.2A and 5.2B). The rate of glucose uptake over 24 h was also measured at this timepiont and all antibody treatments significantly reduced glucose uptake in adult worms, compared with naïve IgG-treated controls, again with the anti-*Sm*ChE cocktail IgG-treated group displaying the greatest inhibition (Fig 5.2C). To determine if anti-*Sm*ChE antibodies can play a role in killing adult worms, the antibody experiment was repeated with 5 pairs of adult worms per treatment and worm viability post-treatment was assessed. Consistent with inhibition of AChE activity and glucose uptake, the cocktail of anti-*Sm*ChE antibodies were the most effective in killing (all worms dead at day 7 post-treatment), compared to controls (Fig 5.2D).

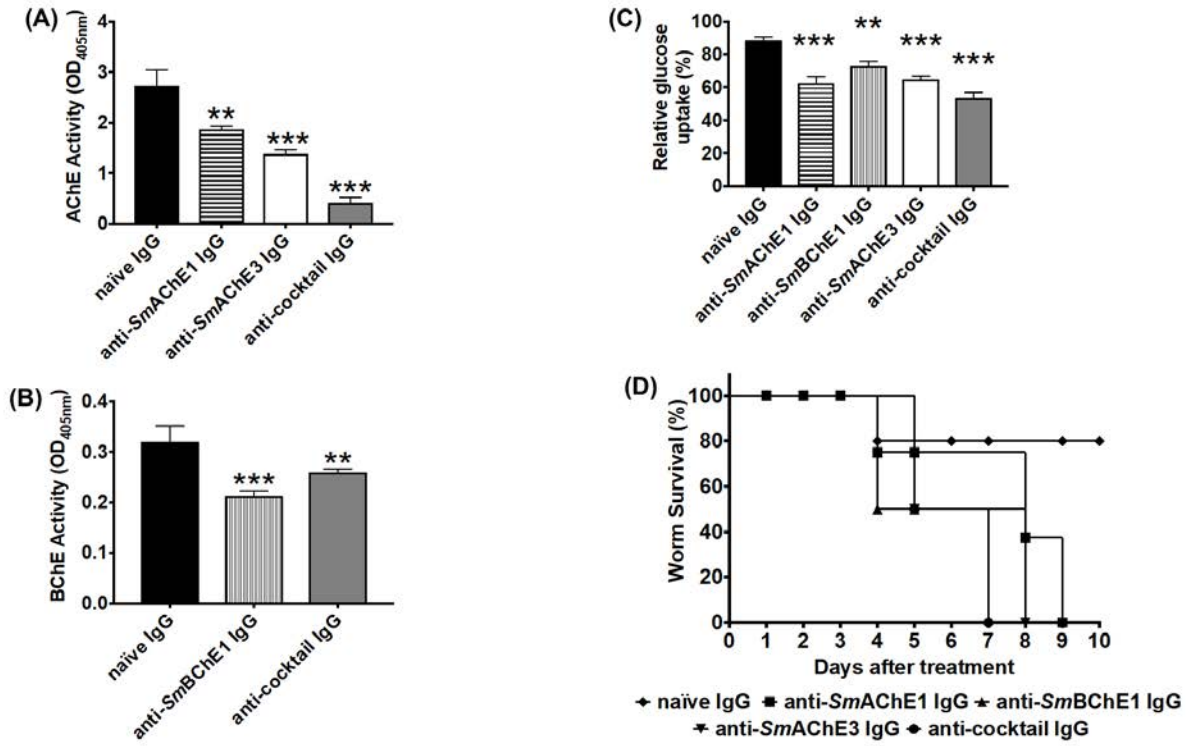


Fig 5.2. Effects of anti-*Sm*ChE antibodies on adult *S. mansoni* worms.

Five pairs of freshly perfused worms were incubated in the presence of anti-*Sm*ChE purified IgG in DMEM at 37 °C in 5% CO_2. Naïve IgG served as a negative control. **(A)** AChE activity 24 h after treatment, **(B)** BChE activity 24 h after treatment, **(C)** glucose uptake over 24 h, one day after treatment **(D)** survivability up to 10 days after treatment. The results are the mean ± SEM of two biological and three technical replicates (A-C) or two biological replicates (D). Significance (relative to the naïve IgG control) determined by the student's *t* test *$P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

5.3.3. Antibody responses to *Sm*ChEs during the course of infection and following PZQ treatment in mice

Antibody responses to all *Sm*ChEs were significantly higher in infected mice than before infection, even at just 3 days post-challenge ($P \leq 0.05$), and increased as infection progressed (Fig 5.3A). In a separate experiment, all anti-*Sm*ChE IgG responses were shown to significantly increase ($P \leq 0.001$) after PZQ treatment (Fig 5.3B).

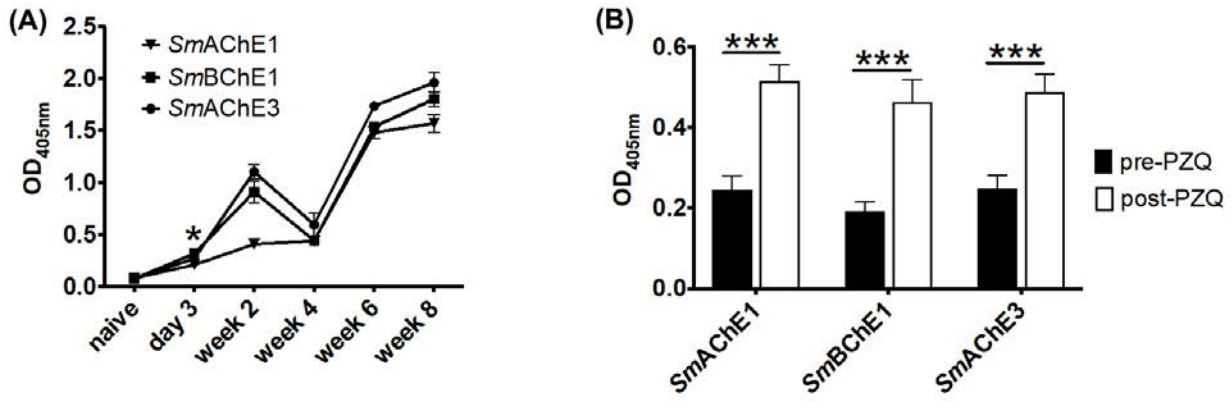


Fig 5.3. Antibody responses to *Sm*ChEs during the course of infection and following PZQ treatment in mice. Anti-*Sm*ChE IgG responses in mice (A) from 3 days to 8 weeks post-infection (n=5) and (B) before (5 weeks post-infection) and 2 weeks after PZQ treatment. Data represents the mean of 2 technical replicates and significance determined by the student's *t* test *$P \leq 0.05$.

5.3.4. Vaccine efficacy of recombinant *Sm*ChEs in a mouse model of schistosomiasis

In both vaccine trials, all four groups of mice immunized with *Sm*ChEs, either in isolation or as a combination, showed a significant decrease in worm burden (28% - 38%), compared to controls, with the *Sm*ChE cocktail-vaccinated group displaying the highest reduction in trial 1

and 2 of 38% ($P \leq 0.001$) (Fig 5.4A and 5.4B). Compared to controls, significant decreases in liver egg burdens (expressed as eggs per gram – EPG) were observed for all groups across both trials (13% - 46%), except for the *Sm*AChE1-vaccinated group in trial 1. When averaged over both trials, liver egg burdens in the cocktail-vaccinated group showed the greatest reduction (Fig 5.4C and D). Intestinal egg burdens (expressed as EPG - only determined for trial 2) were significantly reduced for all vaccinated groups, compared to controls, with the greatest reduction seen in the group vaccinated with the *Sm*ChE cocktail (33%, $P \leq 0.001$). (Fig 5.4E). Egg viability (only assessed for trial 2), as determined by egg hatching from liver homogenates, was significantly reduced in the groups vaccinated with the *Sm*ChE cocktail (40%, $P \leq 0.01$) and *Sm*AChE3 (46%, $P \leq 0.01$) (Fig 5.4F). While there was no significant reduction in glucose uptake for worms from any of the vaccinated groups, compared to controls (data not shown), the glycogen content of worms from all vaccinated groups in trial 1 was significantly lower (24% - 52%, $P \leq 0.001$) than worms from the control group (Fig 5.5A). A significant reduction in worm length (30%-50%) was also observed between worms from all vaccinated groups compared to worms from the control group (Fig 5.5B). As with the parasitology burden data, worms from the cocktail-vaccinated group showed the greatest decrease in glycogen content and body length. Glucose uptake, glycogen content and worm size was not significantly different between control and vaccinated worms in trial 2 (data not shown).

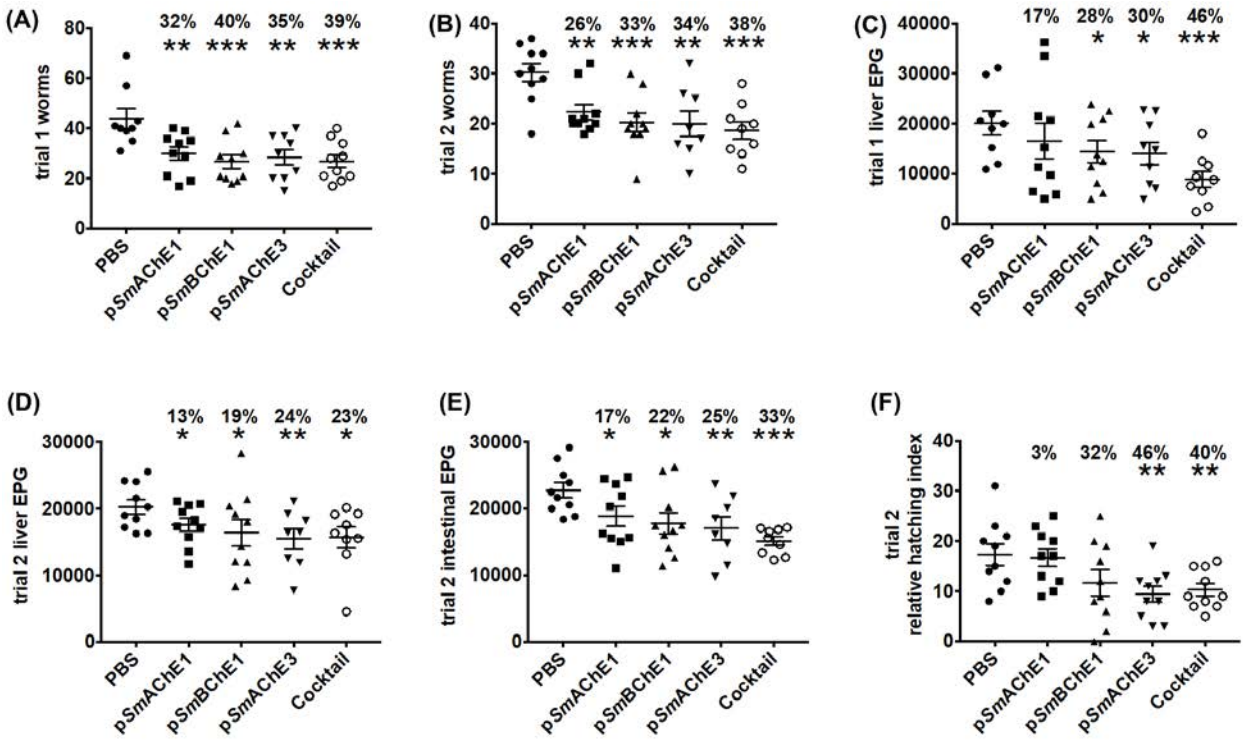


Fig 5.4. Vaccine efficacy of recombinant *Sm*ChEs in a mouse model of schistosomiasis.

Graphs show parasitology burdens from vaccinated and control mice **(A)** trial 1 adult worms, **(B)** trial 2 adult worms, **(C)** trial 1 liver EPG, **(D)** trial 2 liver EPG, **(E)** trial 2 intestinal EPG. **(F)** Hatching viability of eggs obtained from the pooled livers of control and vaccinated mice from trial 2. Data are the average of ten replicate counts ± SEM of hatched miracidia. Significance and percent reductions (if any) for all parameters are measured relative to the control group. Significance determined by the student's *t* test *$P \leq 0.05$, **$P \leq 0.01$, **$P \leq 0.001$.

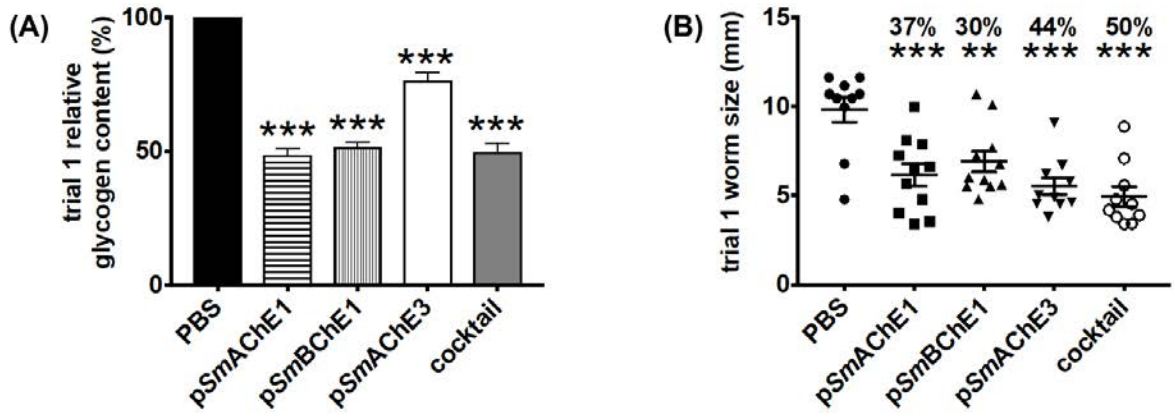


Fig 5.5. Effect of p*Sm*ChEs vaccination on glycogen storage in, and size of, *S. mansoni* adults.

(**A**) Triton-X-100 extracts were made from 5 pairs of worms freshly perfused from each vaccinated or control group and the glycogen content in these extracts was measured. Plotted data are the average of triplicate biological and technical experiments ± SEM. (**B**) Worm sizes (mm) were assessed by randomly selecting and measuring (ImageJ) at least 20 worms from each group. Significance and percent reductions (if any) for both parameters are measured relative to the control group. Differences for both experiments were measured by the student's *t* test $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

5.4. Discussion

Surface-exposed proteins, such as tegumental proteins, are effective targets for vaccine development in schistosomes (Mulvenna et al. 2010; Sotillo et al. 2015). In this regard, *Sm*ChEs are promising candidates as immunolocalization studies in *S. mansoni* has shown *Sm*AChE1, *Sm*BChE1 and *Sm*AChE3 to be expressed in the tegument of adult worms and schistosomula

(chapter 3), and proteomic analysis of ES products has confirmed the presence of *Sm*AChE1, *Sm*BChE1 (chapter 3), suggesting that these molecules are accessible to immune attack. Further, RNAi-mediated silencing of all 3 *Sm*ChE genes, both individually and in combination, significantly decrease schistosomula viability *in vitro* and parasite survival *in vivo* (Chapter 4), implying that these genes are essential for proper worm development and function. Moreover, recent protein array studies have demonstrated high levels of circulating antibodies to *Sm*BChE1 in individuals exhibiting drug-induced resistance and low pathology to schistosomiasis, implicating these antibodies in a protective anti-schistosomal response (Pearson et al. 2015; Driguez et al. 2016).

Given *Sm*ChEs are accessible to antibody attack, and are enzymatically functional (chapter 3), catalytic activity can be used to measure the effectiveness of antibody binding as the interaction between enzymes and their corresponding antibodies generally leads to a complete or partial reduction in their enzymatic activity (Cinader et al. 1971; Arnon 1973; Arnon 1975). The data presented here show that antibodies against recombinant *Sm*ChEs are capable of inhibiting surface (and, in the case of *Sm*AChE1 and *Sm*BChE1, secreted) enzymatic activities in both schistosomula and adult worms, which is similar to previous studies that have used antibodies raised against parasite-derived AChE to inhibit AChE activity on intact *S. mansoni* (Espinoza et al. 1995), *Necator americanus* (Pritchard et al. 1991), *Dictyocaulus viviparus* (McKeand et al. 1994) and *Electrophorus electricus* (Williams 1969). The inhibitory effect of antibodies is likely to be due to steric hindrance, potentially blocking substrate access to the peripheral anionic sites or catalytic gorge of AChE. Indeed, previous studies on rabbit (Brimijoin et al. 1985), human

(Sorensen et al. 1987) and bovine (Wolfe 1989) AChE have documented the AChE-inhibitory ability of antibodies raised against epitopes other than AChE-active sites.

Consistent with the effects of RNAi-mediated *Sm*ChE gene silencing, antibody-mediated *Sm*ChE inhibition resulted in a significant decrease in parasite viability. I posit that this mode of enzyme inhibition was the cause of eventual parasite death as schistosomula were still viable, compared to controls, at 2 hours after antibody treatment, despite a significant decrease in ChE activity; it was not until after a much longer exposure (14 h) to anti-*Sm*ChE antibodies that parasite viability was significantly lower. It should be noted that, while more effective *in vitro* killing experiments have been performed (Arnon et al. 1987), these studies were conducted with the addition of guinea pig complement to facilitate complement-dependent killing and so may not truly represent the amount of parasite death due to enzyme inactivation appeared to have a similar effect on adult worms in that a significant reduction in ChE activity was evident in treated parasites before death started to occur. Glucose uptake in adult worms (not tested in schistosomula) was also significantly reduced by anti-*Sm*ChE antibody treatment. The cholinergic action of surface AChE has been implicated in mediation of the glucose scavenging mechanism in schistosomes (Camacho et al. 1995) and I have shown that AChE-inhibitory, anti-schistosomal drugs reduce glucose import in parasites (Sundaraneedi et al. 2017) so it is likely that antibody-mediated impairment of glucose uptake contributed to a decrease in parasite viability. As seen in our RNAi studies, simultaneous inhibition of multiple *Sm*ChEs by the treatment with a cocktail of *Sm*ChE antibodies resulted in the greatest reduction in parasite viability, both adult and

juvenile. It may be that there is some redundancy in the cholinergic functioning of these molecules (even BChEs, like *Sm*BChE1, can perform a cholinergic role in situations of AChE deficiency (Boudinot et al. 2005) and so collective inhibition of the molecules is required to produce a functional deficit. Also, given the multiple proposed functions for parasite ChEs (Day et al. 1996; Arnon et al. 1999), it is possible is that the neutralization of multiple enzymatic targets more profoundly interrupts varied processes of parasite biology than just cholinergic transmission.

Given the relative cytotoxic potential of antibodies against all three *Sm*ChEs as opposed to anyone *Sm*ChE, I decided to test the efficacy of this antigen cocktail as a vaccine in a mouse model of schistosomiasis. The vaccine efficacy of each individual *Sm*ChE was also tested to investigate the relative anti-parasitic effects of anyone *Sm*ChE over the cocktail or one another.

Mice vaccinated with the cocktail of *Sm*ChE antigens displayed the highest level of protection against experimental schistosomiasis, showing the greatest reductions in every parameter tested. An additive protective effect was not readily apparent, however, as protection levels were not significantly different from groups vaccinated with single antigens. Of the groups vaccinated with individual *Sm*AChEs, the *Sm*AChE3 vaccinated group engendered the highest levels of protection. Similar results were reported in a test of the vaccine efficacy of a recombinant AChE from S. japonicum (You et al. 2018). Further, vaccine trials using purified secretory AChE from the nematodes *Trichostrongylus colubriformis* and *Dictyocaulus viviparous* have resulted in

significant protection in animal models (Griffiths et al. 1994; McKeand et al. 1995).

Egg burdens did not concomitantly decrease with worm burdens, but there were significant reductions in egg viability in all but the *Sm*AChE1-vaccinated group, an observation I have previously reported when testing the *in vivo* anti-schistosomal efficacy of AChE-inhibitory drugs (Sundaraneedi et al. 2017). Studies in rats and honey bees have observed abdominal spasms and involuntary muscle contractions when AChE inhibitors have been administered to these organisms (Jarvie et al. 2008; Williamson et al. 2013) so a possible explanation for this less than expected decrease in egg number but significant reduction in viability could be that ova are being prematurely released as a result of AChE inhibition affecting reproductive tract motility. It could also be that the significant stunting of worms recovered from vaccinated groups affected fecundity and egg maturity. Indeed, previous studies on insects demonstrated that suppression of AChE considerably reduced the weight and length of surviving organisms (Kumar et al. 2009; Hui et al. 2011; Ye et al. 2017) and severely affected the hatching ability of the eggs laid (Kumar et al. 2009; Xiao et al. 2015). These researches have suggested that dysregulated cell proliferation and apoptosis during larval growth may be reasons for such phenotypic effects attributed to the absence of AChE, although such a link in trematodes remains to be established. Finally, parasites recovered from vaccinated mice had significantly depleted glycogen stores. Reduced glycogen content and glucose uptake in worms treated with AChE-inhibitory drugs have been previously observed *in vitro* (Sundaraneedi et al. 2017) and be attributed to interference with the tegumental AChE-mediated glucose scavenging pathway (Camacho et al. 1995) through

the inhibition of this enzyme. It could be that the same effect is being orchestrated by antibody-mediated inhibition of AChE (which would be consistent with the results of *in vitro* antibody-based experiments and RNAi studies - chapter 4, forcing the parasite to rely on its glycogen stores, rather than the scavenging of exogenous glucose, for nutrition.

Even though immunization with *Sm*ChEs induced high titers, these antibody levels were not sustained during the course of infection, with titers at necropsy dropping between four- and ten-fold from pre-challenge levels. This would seem to indicate that the specific antibody response induced by immunization was not augmented by natural infection, a hypothesis corroborated by the generation of modest anti-*Sm*ChE titers during the course of parasite infection in a separate experiment. That being said, the *Sm*ChEs used in this study were still capable of inducing moderate levels of protection in the face of modest antibody titers. Treatment with PZQ has been shown to induce antibody-mediated resistance to schistosomiasis in humans through the exposure of parasite antigens to the immune system (and subsequent generation of an antibody response) as a result of tegument damage (Harder et al. 1987) and protein array studies by us have shown that antibodies to *Sm*BChE1 are significantly upregulated in resistant individuals (Pearson et al. 2015). The upregulation of *Sm*ChE immune responses following PZQ treatment has been verified in this study. Given that an effective anti-schistosomal vaccine strategy would ideally be linked with chemotherapy (Pearson et al. 2015), it is possible that the vaccine efficacy of antigens such as the ones described here could be increased by vaccination after PZQ treatment due to the augmentation of an already upregulated immune response.

CHAPTER 6

General discussion and future directions

Schistosomiasis affects over 200 million people in the developing tropics. There is no approved vaccine for the infection and current treatment and disease control relies on chemotherapy with a single drug, praziquantel (PZQ) (Trainor-Moss et al. 2016), which is a concern due to the inevitable development of drug resistance (Cioli 2000). There is a strong imperative, therefore, to identify novel vaccine and drug targets which can form the basis of anti-schistosomal intervention strategies. In this respect, we need to increase our understanding of the biology of this parasite to identify essential facets of schistosome physiology that could be disrupted through immuno- or chemotherapy.

The vital biological processes of schistosomes are solely regulated and controlled by the parasite's nervous system (Halton et al. 1996), emphasizing the importance of targeting its neuromusculature and associated proteins for therapeutic development. In the present work, I show that acetylcholinesterases (AChE)s - a class of neuronal and tegumental enzymes responsible for the regulation of neurotransmission through hydrolysis of the neurotransmitter acetylcholine (ACh) – can be inhibited by inorganic metal complexes, causing death of the parasite. I then provide a comprehensive molecular characterization of three cholinesterase enzymes from *Schistosoma mansoni* (*Sm*ChEs) and give evidence that they are vital for the normal functioning of the parasite through the use of RNAi- and vaccine-based approaches that target these molecules.

The work presented in chapter two of this thesis documents the use of polypyridylruthenium (II) (Ru) complexes in the successful treatment of schistosomiasis through the inhibition of parasite AChE. Various classes of synthetic Ru complexes, including the panel screened in this study, have been shown to possess antimicrobial properties (Gorle et al. 2016), but this current work is the first documentation of the anthelmintic activity of these compounds. The AChE activity in schistosome extracts was inhibited significantly (low micromolar concentration) by a range of panel members. Further studies on live *S. mansoni* parasites also revealed that the complexes displayed efficacy against eggs and juvenile worms *in vitro*. They were also active against adult worms, which became immobile and coiled upon treatment, possibly due to the paralyzing effects of AChE inhibition (Bueding et al. 1972; Hillman et al. 1975; Pax et al. 1981). Treated parasites showed significant decreases in surface and somatic AChE activity, implying the complexes exerted their effects, at least partially, through the inhibition of the activity of these enzymes. Worms treated with Ru complexes had a significantly reduced ability to uptake glucose, an observation likely explained by the inhibition of tegumental AChE, which is a mediator of exogenous glucose scavenging by the parasite (Camacho et al. 1995). Two of the compounds were tested for their efficacy in a mouse model of schistosomiasis and were shown to have a significant impact on egg viability and development. This effect may be due to compounds stimulating reproductive tract motility (De Giorgio et al. 2004; McNamara et al. 2008), resulting in the premature release of under-developed eggs, a hypothesis supported by AChE inhibitor studies in ticks (Perez-Gonzalez et al. 2014; Prado-Ochoa et al. 2014).

In addition to highlighting the potential of Ru compounds as effective drug leads for the treatment of schistosomiasis, the experiments in chapter two stimulated research efforts into determining the specific molecules responsible for ChE functioning in *S. mansoni* (chapter three). In contrast to previous studies, interrogation of the now comprehensively annotated *S. mansoni* genome, revealed the presence of three ChE-encoding genes: two acetylcholinesterases (AChE)s - *Sm*AChE1 (Smp_154600) and *Sm*AChE3 (Smp_136690) – and one butyrylcholinesterase (BChE) – *Sm*BChE1 (Smp_125350). Functional expression of the three paralogs verified their ChE class based on hydrolysis of AcSCh or BcSCh. Antibodies to recombinant molecules localized the proteins to the tegument and neuromusculature of adults and schistosomula and developmental expression profiling differed among the molecules, suggestive of functions extending beyond traditional cholinergic signaling for each of them. *Sm*AChE1 and *Sm*BChE1 were also found in parasite ES products. Evidence was provided for the role of *Sm*BChE1 as a bio-scavenger of carboxylic esters, using the anti-schistosomal AChE inhibitor, DDVP, as an example. It was postulated that *Sm*BChE1 might protect parasite AChE against the effects of naturally occurring AChE inhibitors by performing this function, given the role of human BChE in the detoxification of similar molecules (Lockridge 2015).

RNA interference was used to further investigate the functions of *Sm*ChEs (chapter four). Silencing of *Sm*ChEs, either individually or in a combination of all three paralogs, reduced transcript and protein expression levels significantly. For *Sm*AChE1 and *Sm*AChE3, knockdown resulted in decreased AChE activity in parasite extracts and suppressed uptake of glucose by live

schistosomula, reflective of the neuronal and non-neuronal cholinergic functions suggested from immunolocalization results. Silencing of *Sm*BChE1 did not suppress BChE activity or impair glucose uptake, implying a different function for this molecule. All *Sm*ChEs were regarded as essential for parasite development and function, however as *Sm*ChE-silenced schistosomula failed to survive both *in vitro* and *in vivo*. Further, simultaneous silencing of all three paralogs had a more profound impact on parasite survivability than each individual treatment, implying some degree of functional redundancy between the molecules but also highlighting the effectiveness in targeting numerous molecular functions in the development of a parasite intervention strategy

The tegumental localization (chapter three) and essentiality (chapter four) of *Sm*ChEs attested to the feasibility of targeting these molecules with antibodies to develop an effective vaccine against schistosomiasis, the objective of the experiments in chapter five of this thesis. Indeed, both adult worms and schistosomula treated in *vitro* with anti-*Sm*ChE IgG (either individually or in combination) showed significant decreases in ChE activity and glucose uptake ability, reflective of RNAi results. Further, when administered as vaccines, either individually or in combination, in a mouse model of schistosomiasis, recombinant *Sm*ChEs protected against parasite challenge as adult worm burdens were significantly reduced and surviving worms were stunted and nutritionally depleted. Egg loads were not as decreased as worm numbers but egg viability was affected by *Sm*ChE vaccination, reflective of results observed in the drug *in vivo* efficacy trials (chapter two).

In conclusion, the data generated in this thesis has resulted in a detailed characterization of a family of ChE molecules from *S. mansoni* and has highlighted the anti-schistosomal efficacy of a novel class of AChE-inhibitory Ru complexes. Further, it offers new approaches for targeting aspects of schistosome neurobiology as a method of controlling the debilitating disease caused by these parasites.

Future directions

In chapter two of this thesis, I have described the anti-schistosomal activity of a family of Ru complexes and, while some members showed promising efficacy *in vivo*, the maximum tolerated dose was relatively low. The modular nature of Ru complexes makes it possible to synthesize these compounds to target specific enzymes and even regions within molecules, so future work will involve tailoring Ru complexes to increase their selectivity and potency. Further, given their potency against PZQ-refractory schistosomula, these complexes could be administered in combination with PZQ, overcoming the limitations of current monotherapy and augmenting existing schistosomiasis control initiatives.

In a first for schistosomiasis research, I have documented the presence of catalytically active *Sm*ChEs (*Sm*AChE1 and *Sm*BChE1) in parasite ES products (chapter three). Secretory AChEs have extensively characterized in *N. brasiliensis* (Selkirk et al. 2005; Selkirk et al. 2005) and recent studies have suggested an immunomodulatory function for these molecules through the hydrolysis of host ACh (Vaux et al. 2016). It is tempting to speculate that secreted (and even

tegumental) *Sm*AChE1 may perform a similar function in schistosomes, given its accessibility to the host environment. Co-culture experiments with RNAi-silenced schistosomula and immune effector cells could be designed to explore this hypothesis.

The isolation of a BChE (*Sm*BChE1) from schistosomes (chapter three) is a novel finding, and one with potential translational application, given its perceived role in the detoxification of AChE-inhibitory compounds. Human BChE is currently being developed as a treatment for cocaine addicts due to its ability to rapidly inactivate the drug and there is considerable research effort focused on producing structural mutants of BChE, from humans and other organisms, to increase the catalytic turnover (and, therefore, detoxification potential) of the enzyme (Lockridge 2015). *Sm*BChE1 could be studied for this, and similar, purposes and be added to the growing arsenal of helminth molecules being exploited for therapeutic use.

The *Sm*ChEs characterized in this study have been shown to provide modest protection against schistosomiasis when administered as vaccines (chapter five). I have documented the ability of anti-*Sm*ChEs to impact parasite viability *in vitro*, thus providing evidence for the role of humoral immunity in protection, so it may be possible that the generation of only modest antibody titers in vaccinated animals may be the reason for the protection levels observed, although the role. Future studies to maximize the vaccine potential of these molecules will include the use of full-length proteins as vaccine antigens (to potentially increase immunogenicity. The role of T-cell

mediated immunity in protection cannot be ignored so additional experiments will involve the use of different adjuvant formulations to stimulate a range of immune responses.

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Appendices

Supplementary Table 3.1.

Oligonucleotides used in this project

Gene	Direction	Sequence
f*Sm*AChE1	Fwd	GAATTCGCGGCCGCGAATTC
	Rev	TCTAGAGGTCTAGAGCTCGAG
f*Sm*BChE1	Fwd	GAATTCGCGGCCGCGAATTC
	Rev	TCTAGAGGTCTAGAGCTCGAG
f*Sm*AChE3	Fwd	GAATTCGCGGCCGCGAATTC
	Rev	TCTAGAGGTCTAGAGCTCGAG
p*Sm*AChE1	Fwd	GACAGAAACCACATGATGTTGGAA
	Rev	TTCCAACATCATGTGGTTTCTGTC
p*Sm*BChE1	Fwd	TCCAGGAAGCACATGGTCTTCACT
	Rev	AGTGAAGACCATGTGCTTCCTGGA
p*Sm*AChE3	Fwd	CGCCATATGCTCTCCAAAGCGTGGTTACT
	Rev	CGCCTCGAGCGGATCCCAACTTAGTCTCATC
***Sm*AChE1 qPCR**	Fwd	ATGGATATGAGATTGAGTATG
	Rev	CTGGAAGGATGTTAGGAT

***Sm*BChE1 qPCR**	Fwd	CTACTCGTAATGATGACT
	Rev	GGCTGAATTATACAAGATT
***Sm*AChE3 qPCR**	Fwd	ATGCGACCACACTATCACCA
	Rev	CCTGATGTAAATCCACCACCA
SGTP1 qPCR	Fwd	CTGCAGCTTATTCACTGAGTCAATC
	Rev	CCACCGATGTTTTTCTGTATAACAGGAT
SGTP4 qPCR	Fwd	AGCCAAGGAGTTAACTTATTATGCAATTTATTG
	Rev	TCCAACAGATAATAACGATAACTAAAAATGGTAAGAA
***Sm*COX1 qPCR**	Fwd	TAGGGTTGGTGGTGTCACAG
	Rev	ACGGCCATCACCATACTAGC

Gene	**Target sequence for siRNA duplex**
*Sm*AChE1	CAGGAGCTTTAATGTTTGGCA
*Sm*BChE1	GTATCATCTTGTACAAAGTTTAAGA
*Sm*AChE3	CATCAAAACCAATTGGTAAATTACGT
Luciferase	ACTGAGACTACATCAGCTATTCTGAT

Supplementary Table 3.2

Identification of secreted form of *Sm*ChE by MALDI-TOF/TOF mass spectrometry purified by Edrophonium chloride

Accession number[a]	Protein name	Molecular function	Score	Seq(Sig)[b]	emPAI[c]	Peptide sequences
Smp_154600.1	Carboxylic ester hydrolase	carboxylic ester hydrolase activity	15457	19	4.64	SFKCPTINMATAVTNDYR CPTINMATAVTNDYR RAHTLPVYFYEFQHR AHTLPVYFYEFQHR TVSLPMPK QLSDIMMTYWANFAR TGDPNILPDGR HVTDNLNPDDPDEITEDQLK NPFIGWPEFR NPFIGWPEFRNSTK SAPANLLVSTRPR RWYPALLQQVER
Smp_125350.1	protein (S09 family)	acetylcholinesterase activity	89	2	0.13	ALGTGSWTSLEVVK YETYSPHSVATR
Smp_034840.1	14-3-3 epsilon	protein domain specific binding	53	2	0.38	VFSAVEQTEGNR DILELIDK

[a] Protein identification is from Uniport database
[b] number of significant distinct sequences
[c] Exponentially Modified Protein Abundance Index

Supplementary Table 4.1.

siRNAs used for gene silencing

Gene	**Target sequence for siRNA duplex**
*Sm*AChE1	CAGGAGCTTTAATGTTTGGCA
*Sm*BChE1	GTATCATCTTGTACAAAGTTTAAGA
*Sm*AChE3	CATCAAAACCAATTGGTAAATTACGT
Luciferase	ACTGAGACTACATCAGCTATTCTGAT