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Structural Characterization of the interaction between divalent cations and the novel *Schistosoma mansoni* Universal Stress Protein G4LZI3

By

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ABSTRACT

Approximately 200 000 people in sub-Saharan Africa lose their lives to schistosomiasis every year. This coupled with other severe Neglected Tropical Diseases, HIV/AIDS, malaria, tuberculosis and poverty, does not make the situation any better. Vaccines targeting this disease are still yet to be discovered and current treatment is becoming a problem as drug resistance has been reported. Thus, looking for an alternative treatment regimen is fast becoming a priority. This study took an interest in Universal Stress Proteins and focused on a novel G4LZI3 USP, which has been put forward as a possible candidate vaccine. A sufficient amount (0.518mg/ml) of recombinant G4LZI3 protein was successfully expressed using 0.5mM IPTG and subjected to a Nickel-NTA column for purification. The fractions were pooled together, concentrated down and used to investigate the binding of the protein with 3 metallic divalent cations using Isothermal Titration Calorimetry (ITC). Additionally, bioinformatics was employed to predict the secondary structure of the protein, generate a 3-dimensional model of the protein and use it for docking the divalent cations. Moreover, the interacting partners of the G4LZI3 protein were determined, thus giving a clue towards the function of the protein. Bioinformatics tools assisted in confirming Ca^{2+} , Mg^{2+} and Zn^{2+} as putative ligands and additionally identified ATP, AMP and U20 as possible molecules that interact with the G4LZI3 USP protein. However, *in vivo* studies only showed interaction between Mg^{2+} and the protein. These results provide prospects for future studies toward drug discovery for schistosomiasis.

Keywords: Docking, Isothermal Titration Calorimetry, Neglected Tropical Disease, Praziquantel, *Schistosoma mansoni*, Universal Stress Protein.

DECLARATION

I, Priscilla Masamba (Student No: 201454125), solemnly declare that the “Structural characterization of the interaction between divalent cations and the novel *Schistosoma mansoni* Universal Stress Protein G4LZI3” is my own, unaided work. It is being submitted for an MSc Masters Degree in Biochemistry at the University of Zululand and it has not been submitted before for any degree or examination in any other university.

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DECLARATION ON PLAGIARISM

I acknowledge that I have read and understood the University's policies and rules applicable to postgraduate research, and I certify that I have, to the best of my knowledge and belief, complied with their requirements.

I declare that the content of this dissertation, save for the supervisory guidance received, is the product of my own work and effort. I have, to the best of my knowledge and belief, acknowledged all sources of information in line with normal academic conventions.

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I have subjected this document to the University's text-matching and/or similarity-checking procedures and I consider it to be free of any form of plagiarism.

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DEDICATION

This thesis is dedicated to two important and irreplaceable entities in my life:

My family

&

God Almighty

This thesis is also written in loving remembrance of

Mr Romanus Kwaye

I would not have been where I am today, nor would I have done this degree if you were not the one who put it in my head in the first place. For the little time I knew you, I say thank you....for being one of my destiny helpers and a door that opened doors of blessings for me.

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Holy name for seeing me through this degree. I dedicate this degree and my life to Him forever.

LIST OF ABBREVIATIONS

3D	3-Dimensional
AIDS	Acquired Immune Deficiency Syndrome
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
Ca ²⁺	Calcium ion
CaDep	Ca ²⁺ -dependent K ⁺ channels
CNS	Central Nervous System
DALYs	Disability Adjusted Years
DNA	Deoxyribonucleic Acid
DR	Delayed Rectifier
DRC	Democratic Republic of Congo
ELISA	Enzyme-linked immunosorbent assay
EST	Expressed Sequence Tags
ExPASy	Expert Protein Analysis System
FABP	Fatty Acid-Binding Protein
FGS	Female Genital Schistosomiasis
GTP	Guanosine Triphosphate
GRAVY	Grand Average of Hydropathicity
HIV	Human Immunodeficiency Virus
HPV	Human Papillomavirus
Hsps	Heat Shock Proteins

ITC	Isothermal Titration Calorimetry
IARC	International Agency of Research on Cancer
MAFFT	Multiple Alignment using Fast Fourier Transform
MAP	Multiple Antigen Epitopes
MDA	Mass Drug Administration
Mg ²⁺	Magnesium ion
NCBI	National Centre for Biotechnology Information
NMR	Nuclear Magnetic Resonance
NTD	Neglected Tropical Disease
NS	Neuroschistosomiasis
PCR	Polymerase Chain Reaction
PCR-ELISA	Polymerase chain reaction-enzyme-linked Immunosorbent Assay
PDB	Protein Data Bank
Phyre2	Protein homology/analogy recognition engine2
PVDF	Polyvinylidene fluoride
PZQ	Praziquantel
RNA	Ribonucleic Acid

RU	Resonance Units
SAGE	Serial Analysis of Gene Expression
SCI	Schistosomiasis Control Initiative
SCC	Squamous Cell Carcinoma
SES	Socioeconomic Status
SPR	Surface Plasmon Resonance
SSA	Sub-Saharan Africa
STDs	Sexually Transmitted Diseases
TB	Tuberculosis
TBST	Tris buffered saline with tween
USPs	Universal Stress Proteins
VOCC	Voltage Operated Ca^{2+} Channels
WASH	Water, Sanitation and Hygiene
WHA	World Health Assembly
WHO	World Health Organization

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CHAPTER 1:

INTRODUCTION AND LITERATURE REVIEW

1.0 INTRODUCTION

Amongst all the Neglected Tropical Diseases (NTDs), Schistosomiasis represents one of the most extensive, virulent and debilitating parasitic diseases of poverty especially in Sub-Saharan Africa (SSA) and other economically-challenged countries around the world. Japan and other countries such as Tunisia, China, Iran and Cambodia have been successful over the past 40 years in eliminating this disease. Egypt and Saudi Arabia have successfully implemented control programmes and together with the above countries, have set themselves as exemplary models, possible through mass treatment, preventive chemotherapy and transmission control tools. In light of all this, “Neglected Tropical Diseases of Poverty” are present in over 76 countries and still burdens a great percentage of the 500 million direly poor and less privileged inhabitants of Sub-Saharan Africa (Hotez and Kamath, 2009a; Walker, 2011). In this region alone it is estimated that NTDs are responsible for about 543 000 deaths annually and liable for the loss of an additional 57 million disability adjusted years (DALYs), with schistosomiasis and geohelminths accounting for 40% of the total world tropical burden excluding malaria (Adenowo *et al.*, 2015).

Praziquantel has, since the 1970s, been used as the principle drug against all schistosome infections but the scientific community has been on edge ever since recent reports of drug resistance have come to light (Melman *et al.*, 2009). A possible venture towards unearthing alternative treatment through novel drugs or vaccines has been to look at the role Universal Stress Proteins (USPs) play in the life cycle of the *schistosoma* worm. Schistosomes experience great alterations in their morphology, cellular composition and specialization so as to survive the different environments they

pass through, as well as the different hosts they occupy during their metamorphosis (Gobert *et al.*, 2009). Two hosts are involved in their lifecycle: humans and freshwater snails; which are different hosts with different defence mechanisms to protect themselves from foreign agents that are particularly harmful. The *schistosoma* worm is able to survive these changes by up-regulating a special class of proteins known as Universal Stress Proteins (USPs), whose roles come into play during major and sudden changes in the environment. It has been hypothesized that through them schistosomes are able to withstand the many unfavourable conditions associated with their complex developmental cycle (Mbah *et al.*, 2013). An enduring ability of living organisms is their capacity to respond to their immediate environment and the changes associated therein. Environmental stress, which may appear in various forms, can result in the denaturation of proteins within the organism, eventually leading to severe harm or even death (Mbah *et al.*, 2013). Thus, it is of vital importance that the organism possesses stress response mechanisms which may be through the expression of Heat shock proteins (Hsps) or Universal Stress Proteins. The presence of this group of proteins has been confirmed in the three major *Schistosoma* species; *Schistosoma haematobium*, *Schistosoma mansoni* and *Schistosoma japonicum* as well as in a large number of other organisms including archaea, bacteria, protists, fungi as well as plants (Berriman *et al.*, 2009; Isokpehi *et al.*, 2011). USPs are responsible for enabling organisms to survive many environmental stresses such as nutrient starvation, high salinity, drought, extreme temperatures and exposure to toxic chemicals (Isokpehi *et al.*, 2011). Mbah and co-workers (2013) were able to determine the protein sequence length, subcellular location, ligand binding sites and other important characteristics of five and eight

sequences encoding the USP domain from both *S. mansoni* and *S. japonicum* respectively. The mere fact that USPs has not shown existence in the human genome singles them out as attractive drug targets (Mbah *et al.*, 2013).

The aim of this research was to investigate the putative interaction between the novel Universal Stress Protein G4LZI3, which is postulated to be a candidate vaccine, and divalent cations (Ca^{2+} , Mg^{2+} and Zn^{2+}) which have been implicated in the mechanism of action of Praziquantel (PZQ). Additionally, comparative homology modelling was used to predict the 3-dimensional structure of the G4LZI3 protein and these techniques were employed with the intent of acquiring basic data that could be useful in designing alternative treatment regimen for schistosomiasis, improving diagnostics of the disease as well as providing structural information that could contribute to treatment targeting specific developmental cycles of the worm. This putative and/or experimental interaction is crucial in the design of new drugs or vaccines in curbing the disease and in particular, focusing on the role and importance of Universal Stress Proteins in disease ecology and the lifecycle of the parasite.

2. LITERATURE REVIEW

2.1 History of Schistosomiasis

The history of schistosomiasis centres round Ancient Egypt, where this disease was and still is regarded as one of the most ancient and persistent plagues of the nation and many other countries in the sub-Saharan region. The earliest detection of schistosomiasis lies in calcified eggs found in two Egyptian mummies of the twentieth century dynasty (1250-1000BC) hence, establishing the fact that this illness has been a relentless burden to man for several years (Jordan, 2000; Oniya and Jeje, 2010). Initially, it was generally thought that Egyptian hieroglyphics for the prevalent *aaa* disease represented haematuria but it later turned out to actually represent proof that schistosomiasis was present around 2000BC. Recent evidence from new techniques which show eggs present in a mummy dating back to 5000BC as well as in a preserved corpse from China (circa 2100BC) plus signs revealing 65% prevalence of the disease among mummies, is also additional evidence of this terrible ailment.

Adamson (1976) draws a mental picture or map of how schistosomiasis could easily have spread throughout Ancient Middle East from Egypt to Punt to Thebes and throughout Upper and Lower Egypt through apes, baboons and slaves as well as the presence of the disease in biblical Jericho and Mesopotamia around the XVIII dynasty (Adamson, 1976). Other authors have also highlighted how schistosomiasis has played a significant role in some important historical events. The disease has been suggested as a possible medical explanation of Joshua's curse and why the town of Jericho remained uninhabited until the time of Elisha (Hulse, 1971). The death of Alexandra the Great was first diagnosed as "*Salmonella typhi* enteritis complicated by bowel

perforation and ascending paralysis”, then later it was suggested that exposure to *S. haematobium* may have also been a possible contributory factor to his death due to the corresponding symptoms between the disease and his deteriorating health (Berhman and Wilson, 1998). Apart from these examples, schistosomiasis has also been implicated as the cause of Great King Herod's death and a partial reason as to why Taiwan was not raided by the Chinese communist army in the late 1940s (Coon, 2005).

One of the earliest contributors toward the discovery of the trematode worm was a German anatomist, physician, helminthologist and pathologist called Theodore Maximilian Bilharz (1825-1862) (Coon, 2005; Pai-Dhungat, 2015). He studied medicine in Germany but moved to Egypt at the age of 25 where he became a Professor at Kasr El Aini Hospital in Cairo University. Bilharz discovered adult schistosome worms after conducting autopsies on infected Egyptian patients. He described the unique lateral spines found in the eggs and named the worms *Distomum haematobium* which subsequently changed to *Schistosoma haematobium*. Apart from these worms, he also discovered the tapeworm responsible for *Hymenolopsis nana* and at 37 years of age he died from typhoid fever after a trip down the Nile River. The disease was commonly named Biharziasis under the genus Bilharzia which was created by Meckel Von Hemsbach (1856) in honour of him.

The Japanese also made significant contributions as regards the discovery of schistosomiasis. Commonly known as Katayama disease, the name was attributed to Katayama district in the Hiroshima Prefecture, west of Japan (Ishii *et al.*, 2003). Yoshinao Fuji (1818-1895), an ancient Chinese medicine practitioner, (as practised in

the Tokugwa Era), first described the disease in 1847 in “Katayam-ki (Memoir)” (Tanaka and Tsuji, 1997). Fuji noticed that people who worked in flooded rice fields started experiencing pruritic papules on their legs which affected all age groups. At first these symptoms were thought to be as a result of an apparent legend where it was alleged that a ship carrying Japanese lacquer became shipwrecked in a river close to the area which caused the release of harmful toxins. He described the patients as having abdominal pains, thin arms and legs, enlarged bellies and developing swollen legs before they eventually died. Thirty years later, he again described the disease with additional information and with the suggestion that western medicine might well be a solution since it was forbidden at that time. Sasa later translated the Memoir into English in 1972 (Maki and Kuwada, 2001). Katsurada then named the parasite *Schistosoma haematobium japonicum* in 1904 (Jordan, 2000). A year later, John Catro (1905) discovered the parasite independently during an autopsy in Singapore. Fujinami and Nakamura then confirmed the route of infection in 1909 and their discovery started the first attempts in implementing disease control (Jordan, 2000). Eventually, Miyagawa (1912), as well as Miyain and Suzuki (1913) showed that the invasive form of the parasite undergoes alteration in a small fresh water snail. Local campaigns against Katayama began in 1918 and continued over the next 25 years which led to the decrease of patients and infected snails. Most of these programmes focused on environmental improvement more than chemotherapy. Since 1973, no patients or snail vectors have been seen in the Katayama area (Jordan, 2000).

2.2 Schistosomiasis: A Neglected Tropical Disease of Poverty

Neglected Tropical Diseases (NTDs) have been a constant burden to man from time immemorial and have succeeded in being a relentless cause of morbidity and mortality. Some of them were the most feared of plagues throughout history but were eventually overcome, whilst others are still considered as “global infectious disease threats” (Morse, 1995). NTDs such as leprosy, ascariasis, lymphatic filariasis, Chagas’ disease and schistosomiasis fall under the thirteen (13) tropical infectious diseases identified by the World Health Organization (WHO), which affect the mostly forgotten, destitute and poverty-stricken people predominantly concentrated in sub-Saharan Africa (see Table 1).

Table 1: List of the 13 Neglected Tropical Diseases of Poverty Ranked by Prevalence. The data presented in this table were taken from Hotez *et al.*, 2007; Hotez *et al.*, 2009; Liese *et al.*, 2010

Disease (Ranked by Prevalence)	Global Prevalence (millions)	Population at Risk	Disability-adjusted Life-years (millions)	Deaths
Ascariasis	807	4.2 billion	1.2 – 10.5	60 000
Trichuriasis	604	3.2 billion	1.6 – 6.4	10 000
Hookworm Infection	576	3.2 billion	1.8 – 22.1	65 000
Schistosomiasis	207	779 million	1.7 – 4.5	280 000
Lymphatic filariasis	120	1.3 billion	5.8	0
Trachoma	84	590 million	2.3	0
Onchocerciasis	37	90 million	0.5	0
Leishmaniasis	12	35 million	2.1	51 000
Chagas' disease	8 – 9	25 million	0.7	14 000
Leprosy	0.4	ND	0.2	6 000
Human African Trypanosomiasis	0.3	60 million	1.5	48 000
Dracunculiasis	0.01	UD	<0.1	UD
Buruli Ulcer	UD	UD	UD	UD

*UD – Undetermined

These diseases struggle to get international recognition as compared to the 'big three' (HIV/AIDS, malaria and tuberculosis). Factors such as severe poverty coupled with inadequate access to basic services like drinking water, lack of hygiene and appropriate housing, prejudice, social stigma and marginalisation, all contribute to the neglect of these diseases. Between the years 2003 to 2007, control projects for HIV/AIDS, malaria and TB stood at 42.4% in comparison to the 0.6% given for the control of NTDs (Liese *et al.*, 2010). Additionally, a huge 80% in terms of research and development has been allocated to the relief of these 'big three'. Surprisingly, it would only take 10% of the money allocated to these diseases for the elimination of most NTDs within 7 years (Fenwick, 2012). The mere fact that they result in unspeakable stigma, instigate severe disfigurement and disabilities, blindness and other serious health impairments should attract more efforts towards abolishing them.

Therefore, it is now no secret that the burden neglected tropical diseases cause needs serious reassessment. It has been suggested time and time again, by numerous authors, that the inexpensive eradication of most NTDs could in turn eliminate poverty since there is an intricate link between the two. Poverty results in the prevalence of NTDs and NTDs cause poverty. Most tropical low-income countries that are afflicted with NTDs are usually burdened with 2 or more NTDs simultaneously (polyparasitism), further complicating the situation (Feasy *et al.*, 2009). The chronic disabilities associated with schistosomiasis and other NTDs have severe and serious impacts on human performance during both adult and childhood stages (King, 2010). Infected children become malnourished and anaemic, stunted in growth and experience

reduction in physical fitness, which all have a negative impact on school performance and attendance since they are too weak to attend classes and are hardly able to concentrate even when they do make an effort to show up at school (Fenwick, 2012; King, 2010). Young women of childbearing age suffer from anaemia, which in turn results in poor birth and pregnancy outcomes. Careers are also negatively affected due to disabilities caused by this disease which lead to low agricultural productivity and limited job performance. Local economies weaken and millions of people get caught up in the “poverty trap” cycle where they struggle to survive and can not break free. Although elimination and control interventions, involving snail control and chemotherapeutics such as Praziquantel, have been put in place to try and eliminate/control schistosomiasis and other NTDS, such efforts are still not enough in most endemic countries. Thus, any alternatives/solutions will be welcomed by many and are even considered by some as long overdue.

2.3 Counting the Cost of Schistosomiasis

Disability adjusted years (DALYs) measure productive years lost due to premature mortality and disability. They take into consideration the prevalence of a disease and basically measure the overall burden caused by the disease. The 13 most important neglected tropical diseases (Table 1) are considered the fourth most important and significant group of communicable diseases in humans after lower respiratory tract infections, HIV/AIDS and diarrhoeal diseases and are responsible for 543 000 deaths and the loss of 56.6 million disability adjusted years (Engels and Savioli, 2006). Of the 200 million people assumed to be infected worldwide with schistosomiasis, 93% of

those cases occur in SSA with the total number of DALYs lost estimated at 1.532 million per year worldwide (Hotez and Kamath, 2009a). The disease also account for the deaths of more than 250 000 people in SSA alone and it has additionally been documented that between 600 and 700 million people are still in danger of encountering infection (Cioli, 2014; Gryseels *et al.*, 2006; Walker, 2011). Out of the 17.5 million people who underwent treatment for schistosomiasis in 2008, 17.1 million were from sub-Saharan Africa (Adenowo *et al.*, 2015).

DALY estimates, however, are calculated based on mortality rates and are therefore not a true reflection of the severity of chronic disease and the effects they have on the quality of life of a person, thus many DALY scores are underestimated (Conteh *et al.*, 2010). An example of this is a study that showed that the average DALY score for schistosomiasis caused in 1990 should actually have been between 2–15% in comparison to the estimated 0.5–0.6% reported by the WHO. Underestimated values have serious effects on economic growth especially on that of endemic countries both directly and indirectly. Thousands of dollars are pumped from government-funded health facilities for the treatment and control of NTDs (direct costs) while costs at the micro-level which cover economic households and businesses (productive labour) are lost due to morbidity and mortality. Expenses include media campaigns, health education and training as well as the purchase and distribution of drugs. Another flaw exhibited by the DALYs system is that it does not acknowledge the ecology of the particular disease which in turn does not focus on the degree of disease burden on the poor (King and Bertino, 2008). It also fails to represent the impact of poverty on

disability and lastly, the influence that poverty has on disease control and treatment of NTDS. Thus, the DALY framework needs to be revisited and revised so that this system can be used properly and effectively in determining health priorities.

2.4 Geographical Distribution of Schistosomiasis

Poor rural communities and urban slum populations in Africa, Latin America and Asia are mostly hit with the heavy brunt of neglected tropical diseases (Conteh *et al.*, 2010; Feasy *et al.*, 2009). Over 90% of the world NTDs burden is concentrated in Africa (Molyneux *et al.*, 2005). Schistosomiasis has been labelled second after malaria as the most significant tropical disease according to public health significance (Mbah *et al.*, 2013). Hookworm infections (192 million cases), followed by schistosomiasis, account for 93% of cases of NTDs in sub-Saharan Africa (Hotez and Kamath, 2009). Nigeria bears the largest brunt of the disease having 29 million people infected, followed by Tanzania (19 million people). Democratic Republic of Congo (DRC) and Ghana follow as the countries with the third highest prevalence with 15 million cases each (Adenowo *et al.*, 2015). Other countries outside SSA affected by this disease include Venezuela, Brazil, some West Indian islands as well as certain parts of the Middle East. Of the other countries apart from SSA affected, Brazil, with 4 to 6 million people infected out of the 25 million people living in endemic areas, is labelled as the worst affected (Lambertucci, 2010).

Schistosoma mansoni is responsible for intestinal schistosomiasis, while *S. japonicum* and *S. haematobium* are associated with hepatic and urinary schistosomiasis

respectively. Of these three species, *S. mansoni* is the one most studied. This species is endemic in 55 countries including Egypt, Libya, Venezuela, Suriname, Sudan, Arab Peninsula, Brazil, sub-Saharan Africa and some Caribbean Islands while *S. haematobium* is endemic in 53 countries, both in Africa and in the Middle East such as Iran, Iraq and Saudi Arabia (Barakat, 2013; Coon, 2005). *S. japonicum* is found in the Philippines, China, Indonesia and Southeast Asia and was successfully eradicated in Japan in 1977. *S. mekongi* is found in many areas in Cambodia and Laos People Democratic Republic while *S. intercalatum* is found in the rain forests of central Africa (Barakat, 2013). Its presence has been reported in Nigeria, Chad, Mali, Congo and Central African Republic; however, these reports still need to be confirmed (Chitsulo *et al.*, 2000).

Globally, it has been documented that 1 in every 30 people is infected with schistosomiasis and an estimated 2.5 million people are thought to be infected with the disease in South Africa alone (Appleton and Kvalsvig, 2006; Thomassen Morgas *et al.*, 2010). In this country, both *S. haematobium* and *S. mansoni* are present with the latter mostly endemic in both Northern and Southern Transvaal areas such as Venda and Kruger National Park as well as in the Rustenburg area (World Schistosomiasis Chart, 2012). In KwaZulu-Natal, high levels of infection are particularly present in the coastal regions of Natal extending to the Port St Johns area of the Eastern Cape. Other regions apart from KwaZulu-Natal include the lower Orange River in the Northern Cape Province, Ciskei region as well as Uitenhage in the Eastern Cape.

Since the 1960s, schistosomiasis has struggled to get recognised as a serious disease in South Africa despite the fact that it is endemic to the country and over 25.7 million people are at risk of infection (Magaisa *et al.*, 2015). Additionally, South Africa has the highest prevalence of HIV in the world, of which a link between female genital schistosomiasis (FGS) and HIV infection has been documented (Berge *et al.*, 2011). The first helminth control programme was set up in the KwaZulu-Natal province in 1998 with the aim of decreasing morbidity and prevalence by 2010. Three weeks following chemotherapeutic treatment with praziquantel, eggs reduced by 95.3% and a 94.1% cure rate was achieved amongst those heavily infected (Saathoff *et al.*, 2004). Sadly, the programme received inadequate support and has failed to continue or revive any treatment plans in any part of the country since then.

2.5 Taxonomic Classification of Schistosomes

Schistosomes are blood-dwelling parasitic digenean flukes that belong to the phylum Platyhelminthes (flat worms) and cause the disease schistosomiasis (Table 2). They fall under the Digenea sub-class which experience alternation of generations, where offspring resulting from asexual reproduction reproduce sexually (Coon, 2005). This reproductive behaviour continues as a cycle with each consecutive generation. Out of the more than 2700 variations of genera belonging to the Digenean class, schistosomes are characterized as being quite different from the rest in four distinct ways: they require two different hosts to complete their lifecycle in comparison to the three hosts other parasites require, the male and female reproductive organs are present in the two separate sexes (i.e. they are dioecious), infection is by skin penetration rather than

orally (being eaten) and they parasitize the intravascular niche (Pierce and McDonald, 2002).

The trematodes are 'ordered' based on the morphology of their cercariae (one of the stages in the worm lifecycle). Schistosome cercariae have forked tails and thus are placed under the order *Strigeida*. Since schistosomes are dioecious and dwell in the host circulatory system, they belong to the family *Schistosomatidae*. Under the sub-family *Schistosomatinae* are species which possess a gyneacophoric canal which is a deep groove that holds the thin female in a permanent embrace. This sub-family is then divided into many genera based on egg morphology and types of intermediate snail hosts. Historically, there are nineteen (19) species under the *Schistosoma* genus but of most clinical and veterinary importance are *S. mansoni*, *S. japonicum* and *S. haematobium*, which are known to infect and parasitize humans while *S. mekongi* and *S. intercalatum* serve as less prevalent species.

Table 2: Taxonomic Classification of Schistosomes. Table taken from Coon (2005)

Kingdom	Animalia
Phylum (flatworms)	<i>Platyhelminthes</i>
Class	<i>Trematoda</i> (Flukes)
Subclass	<i>Digenea</i>
Order	<i>Strigeida</i>
Family	<i>Schistosomatidae</i>
Subfamily	<i>Schistosomatinae</i>
Genus	<i>Schistosoma</i>
Species	<i>S. mansoni</i>
	<i>S. japonicum</i>
	<i>S. haematobium</i>
	<i>S. mekongi</i>
	<i>S. intercalatum</i>

2.6 Transmission cycle of Schistosomes

The disease is a water-borne parasitic infection whose symptoms are more of a result of eggs which become entrapped in tissues such as the heart and lungs rather than the disease being caused by the worms themselves (Walker, 2011). Many physiological and morphological changes take place in the worm as they pass through a number of different and sometimes unfavourable conditions which enable them to adapt and thrive in such situations. The lifecycle of the worm includes two hosts: a definitive vertebrate human host and an intermediate snail host (Oyinloye *et al.*, 2014; Walker, 2011).

Human excreta (either faeces or urine) harbouring schistosome eggs first contaminate fresh water sources to begin the transmission cycle (Figure 2.1). Motile ciliated miracidia hatch from the eggs and actively swim about searching for freshwater snails which they penetrate and infect (Criscione *et al.*, 2009). Each schistosome species has its own snail preference: *S. mansoni* usually infect snails belonging to the *Biomphalaria* genus while the *Bulinus* and *Oncomelania* genera usually host *S. haematobium* and *S. japonicum* respectively (Coon, 2005, Elbaz and Esmat, 2013). These intermediate hosts promote and support the development of sporocysts emerging asexually from the miracidia stage. In the case of *S. mansoni*, thirty-four (34) species have been described; eighteen (18) of which have been reported capable of sustaining full larval development, nine (9) are known to be refractory and seven (7) have not yet undergone testing (DeJong *et al.*, 2001). *Biomphalaria glabrata*, *Biomphalaria pfeifferi*, *Biomphalaria alexandria* and *Biomphalaria sudanica* fall under the most susceptible species. It has been shown that *S. mansoni* infections and the geographical distribution and relative abundance of snails are closely related.

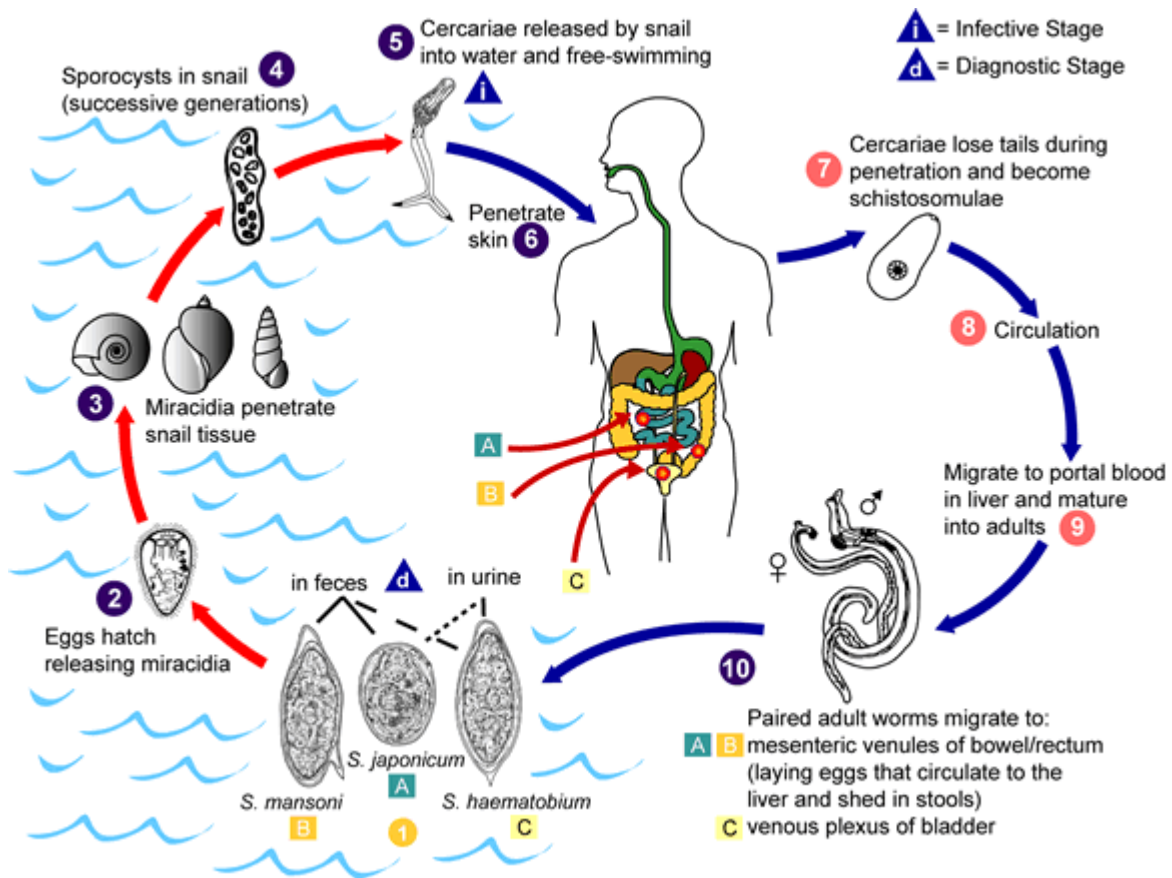


Figure 2.1: Lifecycle of Schistosomes. Eggs are eliminated from the human host via defecation, depending on the species and through urine when one is *S. haematobium* infected. Miracidia then hatch from the eggs and penetrate snails to become sporocysts which are subsequently released as cercariae. These in turn infect the human host by penetration and simultaneously shed their tails to become schistosomulae that circulate the hepatic portal system and reside in the veins to develop into adults. However, the mesenteric venules where adult worms reside are mostly species-dependent. Adults then lay eggs that relocate toward the lumen of the intestines (*S. mansoni* and *S. japonicum*) and the bladder (*S. haematobium*) to be eliminated by defecation thereby restarting the cycle again. Figure taken from the following website: <https://web.stanford.edu/group/parasites/ParaSites2003/Schistosomiasis/cycle.htm> [Accessed 12 February 2016].

Once in the snail, it takes 4–6 weeks for each miracidium to transform into countless free-swimming cercariae that possess small bifurcated or forked tails. About 1,500 cercariae are released into surrounding waters per day for 18 days aided by embryonic suckers and stimulated by light. They swim for up to 70 hours until they come into contact with human skin (Walker, 2011). These penetrate the unbroken skin of mammalian hosts, simultaneously shedding their tails to immediately transform into larval forms called schistosomulae. Generally, the lifecycle of the *schistosoma* species are all similar except for sites of schistosomula migration, which is dependent on the *schistosoma* specie. The worms first migrate to the lungs and then eventually, in the case of *S. mansoni* and *S. japonicum*, move to the veins of the intestine while *S. haematobium* schistosomulae make their way to the bladder where they mature into adults. These then mate and lay eggs which are shed in the stools of the infected individual, beginning the cycle all over again. Production of eggs occurs 4 to 6 weeks after infection and usually continues for the next 3 to 5 years throughout the lifespan of the worm (Gobert *et al.*, 2009). During circulation a large number, about half of the eggs, get wedged in the liver and form granulomatous lesions caused by a strong immune response (de Walick, 2015). These are forced out from the vessel wall and penetrate the intestinal wall to be excreted with urine and faeces. The worm can live up to 30 years producing several hundred eggs per day while feeding on the host blood. A schistosome pair is estimated to produce around 600 billion schistosomes, all capable of producing eggs most of which are excreted (Bruun and Aargaard-Hansen, 2008; Gryseels *et al.*, 2006).

2.7 Signs and Symptoms

Not all the eggs are expelled in urine and faeces; some get trapped in key organs (mostly the liver) and give rise to the signs and symptoms of the disease in three broad but distinct phases: the acute phase, the chronic phase and the advanced stage (Figure 2.2). Infection with the parasite mostly goes without notice and may only display severe disease years later. However, the intensity of infection and morbidity is mostly dependent on the pattern and amount of exposure to the parasite, the degree of egg and worm burden, which specie one is infected with, host-parasite immune response and co-infection with other biological agents.

2.7.1. Acute Manifestations: Katayama fever

There is a significant difference between travellers and tourists that are exposed to and infected with schistosomiasis as compared to people who live in endemic areas. Swimmers' itch or a temporal rash first appears 1–2 days after the skin is penetrated by cercariae and this is brought about by the release of monokines (Barsoum *et al.*, 2013; Conlon, 2005). Cercarial dermatitis, a more severe form of swimmers' itch, may also develop but mostly in those infected with species that rarely parasitize humans. It presents itself as raised, pruritic and itchy lesions that vary from 1–3cm in size caused by non-migrating schistosomulae.

Katayama syndrome then develops 4–8 weeks after infection characterized by fever, headache, diarrhoea, malaise, eosinophilia and hepatosplenomegaly (Coltart and Whitty, 2015). This stage manifests in people who travel to endemic areas for the first time thus, it is virtually non-existent in locals. Immigrants usually just manifest the

chronic symptoms of schistosomiasis. Travellers usually get infected through water sports and other recreational activities that involve large water bodies such as rafting and swimming (Clerinx and Gompel, 2011). Spontaneous recovery occurs 2–10 weeks after infection but some may develop more serious disease, exhibiting weight loss, abdominal pain, dyspnoea, widespread rash and toxæmia, caused by the body immune response to migrating immature schistosomulae (Barsoum, 2013; Coltart and Whitty, 2015). In very rare cases, Katayama fever may turn fatal (Conlon, 2005).



Figure 2.2: Some of the disabilities and infections associated with schistosomiasis a). Cercarial dermatitis. Source: <http://www.omicsgroup.org/articles-admin/disease-images/schistosomiasis-1471.jpg>. b). Tortuous Varicosities which manifests during Chronic Schistosomiasis. Source: <http://www.nejm.org/doi/full/10.1056/NEJMicm0803551#t=article> c). Physical disability resultant from schistosomiasis. Source: <http://diseasespictures.com/wp-content/uploads/2012/12/schistosomiasis.jpg> d). Children in endemic areas usually have a kwashiorkor-like appearance. Source: <https://userscontent2.emaze.com/images/7cf1a078-27a9-4f08-b71f-eb2b8379f7a0/74d935d0a0a4e24d9ba80bd9a97e6a0c.jpg> . [Accessed 08/10/2016]

2.7.2 Chronic Phase: Intestinal (Hepatosplenic) Schistosomiasis

Some schistosome infections may present themselves through subclinical symptoms but schistosomiasis itself is generally considered to be a chronic disease, which causes internal organ damage in people and impaired growth coupled with reduced cognitive development in children (Ukoroije and Abowei, 2012). Eggs and dead worms deposited and trapped in host tissue, mainly the intestines and the liver, give rise to the chronic forms of the disease which include inflammatory and obstructive infections in the intestine (in the case of *S. mansoni*). Proteolytic enzymes are released from the eggs provoking the aforementioned reaction, which are eventually supplanted by fibrotic lesions (liver fibrosis) (Gryseels, 2006). Almost 50% of all schistosome infected individuals develop the chronic phase of schistosomiasis. However, infection seldom results in end stage liver disease, with only 5% of the liver showing granulomatous inflammation, vascular obstruction or perivenular fibrosis. Cirrhosis rarely transpires and if it does, this is mainly due to co-infection with Hepatitis B or C and is more common in countries such as Egypt or in the Middle East as well as other endemic regions (Patel *et al.*, 2015). More advanced forms of hepatosplenic schistosomiasis has been associated with hepatitis C as well as alcoholism (Strauss, 2002). Other clinical signs include enlargement of the liver and spleen, periportal fibrosis, superficial abdominal blood vessel dilation and in severe cases death due to ruptured esophageal varices (Olveda *et al.*, 2013). Gastrointestinal schistosomiasis (which is a severe progression of disease from the chronic to the advanced stage) may cause pain in the left iliac fossa, constipation, bloody stools (haematochezia) or diarrhoea in children, accompanied by enlarged abdomen, anaemia and malnutrition, and growth impairment. Other signs also include headache, lumbar pain, seizures, muscle weakness and visual impairment.

Nonetheless, the degree to which these symptoms affect the infected individual is dependent on genetic factors, intensity of parasitic load and individual immune responses (Gryseels, 2006; Strauss, 2002).

2.7.3 The Advanced Stage: Neuroschistosomiasis

Advanced stages of this disease may result in neuroschistosomiasis, gastrointestinal schistosomiasis or liver cirrhosis (Gryseels *et al.*, 2006). Neuroschistosomiasis (NS) is a rare, but not uncommon, under-recognised disorder, more prevalent among endemic populations and tourists, which affect both the brain and spinal cord (Ferrari and Moreira, 2011). It has been stated that the central nervous system (CNS) is the second most common site of schistosome infection (Nascimento-Carvalho and Moreno-Carvalho, 2005). Early prognosis is the key to early treatment since it may actually develop at any stage. Once eggs or adult worms migrate and reach the CNS, NS occurs. This may occur in one of two ways; ova may be transported through arterial blood flow via the perivertebral plexus of Batson or they may be deposited in the spinal cord and/or small vessels of the brain via ectopic migration and embolization of worms, provoking an eosinophilic granulomatous immune response (Clerinx and Gompel, 2011; Nascimento-Carvalho and Moreno-Carvalho, 2005). Depending on the intensity and duration of infection as well as the formation of granulomas, different signs and symptoms will occur. However, in the case of Katayama syndrome, eggs are not formed. Immature worms are not yet capable of egg production; therefore the neurological manifestations which occur are not caused by the presence of eggs but rather by an eosinophil-mediated toxicity (Ross *et al.*, 2012). *S. mansoni* infections

mostly causes myelopathy *i.e.* neuroschistosomiasis that affects the spinal cord rather than the brain. Clinical manifestation usually begins with lower back pain which then eventually escalates to pain and weakness in the lower limbs (enough to even prevent or interfere with an individual ability to walk), cognitive impairment, constipation, slow reaction times, bladder dysfunction, weak short-term memory and sexual impotence (Ferrari *et al.*, 2008; Ross *et al.*, 2012).

2.7.4.1 Other Complications caused by Schistosomiasis: Female Genital Schistosomiasis (FGS) and its association with HIV/AIDS

Female genital schistosomiasis, also referred to as vaginal schistosomiasis, is a complication that arises due to egg-induced lesions that form in the lower reproductive tract (from the vulva to the ovaries) of females (Feldmeier *et al.*, 1998). It was first reported in Egypt in 1899 and was recently placed on the list of highly-deserved science research priorities by the World Health Organization. *S. mansoni*, *S. haematobium* and *S. japonicum* have all been associated with FGS where eggs are deposited in the cervix, which is labelled as the most favourable site for trapped schistosome eggs, via blood vessel anastomoses located in the pelvic organs (Kjetland *et al.*, 2012). The fallopian tubes and vagina are also common sites for egg deposition. Chronic and persistent lesions, sandy patches and contact bleeding arise as a result of the eggs. This eventually leads to abnormal discharge, substantial genital itch, decreased fertility, open wounds, spontaneous abortions and amplified exposure to co-infection with bacteria, sexually transmitted diseases (STDs) and viruses such as human papillomavirus (HPV).

Several reports have also stated and corroborated a relationship between FGS and higher chances of HIV infection in both males and females, mainly in Africa (Pearce and MacDonald, 2002). A cross-sectional study by Ndhlovu *et al.* (2007) on 544 women in Mupfure, Zimbabwe, showed higher HIV prevalence (33.3%) in those infected with urinary schistosomiasis than in uninfected women (25.6%). Additionally, a community study on 457 women in Northwest Tanzania showed 17% HIV prevalence amongst women with urinary schistosomiasis in comparison with 5.9% occurrence in uninfected women (Downs *et al.*, 2011). CD4⁺ T-cells are a class of white blood cells characterized by a T_H1 (which protects against intracellular pathogens) or T_H2 response (which promotes immunity against helminth parasites). Schistosomiasis has been shown to stimulate a T_H2 response while down-regulating the T_H1-type response thereby posing more risk for cells to be infected and destroyed by HIV infection (CDC, 2015). Inflammatory cells brought about by the presence of schistosomal eggs cause the cells to express CD4⁺ T-cell receptors, consequently attracting HIV infection (Mbamazi *et al.*, 2011).

A typical feature of FGS is the eroding, ulceration and thinning of the epithelial surface, in most cases on the cervix. The epithelium acts as a mucosal barrier against infections like HIV. Unfortunately, impairment on the surface of this barrier by FGS provides access to HIV infection (Poggensee and Feldmeier, 2001). Nearly 90% of all HIV-1 infections take place through the mucosal route. Thus, schistosomiasis, which disrupts this barrier and causes cells susceptible to HIV-1 to assemble in HIV exposure sites, in turn increases host susceptibility to HIV infection (Secor, 2012). In males, it has been hypothesized that schistosomiasis (precisely caused by *S. haematobium*), because of

inflammation caused by schistosome eggs in the seminal vesicles and prostate, intensifies loading of the virus in the semen of HIV-infected men, thus increasing the risk of HIV transmission to their female partners (Kjetland *et al.*, 2012).

2.7.4.2 Schistosomiasis and its association with bladder cancer

The World Health Organization (WHO) together with the International Agency of Research on Cancer (IARC) has labelled *S. haematobium* as a Group 1 carcinogen with recent documentation stating that the disease increases the risk of an infected individual developing bladder cancer (Oyinloye *et al.*, 2014). Evidence of this mainly relies on the fact that bladder cancer levels are much higher in areas where the parasite is endemic. Bladder cancer has been shown to be the most virulent disease in countries such as Egypt, Malawi, Zambia and Iraq, where the parasite is endemic or where prevalence of schistosomiasis is really high, caused either by mixed species or solely by *S. haematobium* alone (Mostafa *et al.*, 1999). Squamous cell carcinoma (SCC), which is a type of tumour of histological cell type, is implicated in bladder cancer where the disease is endemic, mainly caused by urinary schistosomiasis. In comparison to Western countries, which are not under the schistosome burden, such as the United States, the United Kingdom or Germany, bladder cancer is a less common cancer, mostly affecting men. Histopathological findings have indicated that in comparison to areas that are endemic to infection, SCC of the bladder is of a higher proportion than that from schistosome-free regions.

Additionally, cancers of the female genital tract, adenocarcinoma of the prostate, ovarian cystadenocarcinoma and hepatocellular carcinoma have been reported to have some type of association with *S. haematobium* infection (IARC, 2012). It has also been put forward that schistosomal lesions render bladder epithelium susceptible to mutagens from chemicals and tobacco (Barsoum *et al.*, 2013). FGS, apart from cervical lesions and HIV/AIDS, has also been associated with cervical cancer (Christinet *et al.*, 2016). However, the relationship between schistosomiasis and cervical carcinoma is still unclear. It is generally believed that irritation, inflammation and infection of the bladder by chronic forms of bacteria pave the way for the development of bladder cancer. Worms and eggs deposited in tissue also initiate chronic inflammatory reactions which contain macrophages and neutrophils, capable of producing endogenous oxygen radicals, which in turn form carcinogenic N-nitrosamines and trigger mutations (Khaled, 2013). Recently, an estrogen-DNA adduct pathway has been proposed (Botelho *et al.*, 2015). Gouveia and co-workers (2015) were able to show that carcinomas associated with urinary schistosomiasis exhibit higher levels of 8-oxodG and 8-Nitroguanine, the former of which is a major chromosomal lesion caused by oxidation of guanine and is a crucial factor in the initiation and promotion of mutations. Added to this, it also acts as an indicator for measuring the extent of DNA damage due to endogenous oxidative stress. There is a clear relationship between constant DNA damage, urinary schistosomiasis and increased oxidative stress.

Estrogens, in relation to the human host, are viewed as carcinogenic chemicals. The hydroxylation and subsequent oxidation of these estrogens form 2-, 3- and 4-catechol estrogens, quinones, semiquinones and 4-quinones. Through the identification and

characterization of novel estrogen-like metabolites that form as a result of urinary schistosomiasis and FGS infection, the pathway suggests that these estrogens (which also act as electrophilic compounds) have the ability to react with DNA forming depurinating adducts that pave the way for mutation and cancer (Botelho *et al.*, 2015).

2.8 Diagnostic Tools and Options

Diagnosis is extremely important in the treatment and control of schistosomiasis. Several factors such as individual and mass treatment coupled with mass drug administration, assessment of control efforts and measures put in place, possible routes of transmission and estimates on the severity of disease all depend on accurate diagnostic testing (Useh, 2013). However, most of the tools used for diagnosis of this disease today have their own limitations. Early symptoms and expected signs may manifest and act as a diagnostic tool but local health authorities rarely recognise the common flu-like and mild symptoms until the disease has progressed to its advanced phase and then treatment or medication is almost unreceptive. Thus, this method fails to be a specific and sensitive diagnostic tool (Bruun and Aargaard-Hansen, 2008). Additionally, those in rural and endemic settings rarely seek treatment until it is too late.

The golden standard for diagnosis is inspection of eggs in the stools and urine of an infected individual through the lens of a microscope (Adenowo *et al.*, 2015). Eggs are identified due to their unique size and shape as well as their distinguished lateral spine. The Kato-Katz thick smear or Kato techniques are widely used especially in Africa for detection of *S. mansoni*, *S. japonicum*, *S. haematobium*, *S. mekongi* and *S.*

intercalatum eggs. Although these tests are relatively cheap to perform and almost have 100% specificity, multiple samples need to be collected for increased sensitivity which may not be culturally acceptable and may actually be an impractical procedure in wide scale field diagnosis (Olveda *et al.*, 2013; Richter, 2013). Ultrasonography has also been listed as a safe and effective tool for detection since it allows for direct visualization of infected tissues. However, this method drawback is that it still remains quite expensive to implement and requires specialists. Sigmoidoscopy, colonoscopy, rectoscopy, antibody detection assays and several protein biomarkers from excretory-secretory antigens and schistosomula and adult worm teguments such as Annexin2, CAA, Sm21.7 and Sm29 (just to mention to a few) can also be used for detection (Richter, 2003; Silva-Moraes *et al.*, 2014). However, the afore-mentioned techniques (sigmoidoscopy, colonoscopy, rectoscopy) are not always acceptable or suitable for use in the field. The latter can be identified by use of molecular biology approaches such as PCR, PCR-ELISA and Western blot analysis.

The formol-ether concentration technique is also used for detecting *S. mansoni*, *S. japonicum*, *S. haematobium* and *S. intercalatum* eggs by employing ether in a microscope and a centrifuge. However, the technique is not field-applicable because of the equipment and ether is considered a health hazard due to its highly flammable nature (Useh, 2013). Flotation methods have been used, not only for schistosome detection but also for identifying soil-transmitted helminthes such as *Trichuris trichiura* and *Ascaris lumbricoides*. One such example is the FLOTAC technique developed in 2006. It is generally thought to be more sensitive than the Kato-Katz and ether-

concentration method. However, Utzinger *et al.* (2011) reported the FLOTAC technique as detecting lower schistosome faecal egg counts than other techniques.

2.9 Elimination and Control Strategies

2.9.1 Schistosomicides

Antimonial compounds were first used as schistosomicides for about 50 years where they were theorized to inhibit schistosome phosphofructokinase, an enzyme that caused the catalysis of fructose-1,6-diphosphate to fructose-6-phosphate (El Ridi and Tallima, 2013). In 1948, a metabolite identified as Hycanthone (which was later shown to be the active form of Lucanthone) was then used as a drug against *S. mansoni* infections and studies of its mode of action showed that this drug disrupted RNA and DNA synthesis in the worm (Cioli *et al.*, 1993). However, side effects were reported in animals and although it was not very clear if these effects would also manifest in humans, Hycanthone is no longer in use.

Niridazole and Metrifonate were also later introduced as anti-schistosomal drugs with Niridazole effective against *S. mansoni* strains and Metrifonate against *S. haematobium* strains exclusively (Cioli *et al.*, 1993). These drugs are currently also not in use mainly because schistosomes developed the ability to resist these drugs. In spite of this, Metrifonate is still actively used in some countries like Kenya especially due to it being relatively cheap. However, one of the other drawbacks of the drug is the need to administer it twice, instead of once in comparison to other therapies (Richter, 2003). Oxamniquine came into the picture as a drug that was administered both *in vivo* and orally and mostly targeted male *S. japonicum* worms. Reports of drug resistance,

particularly in Brazil where it has been shown to actively reduce morbidity, have also contributed to its discontinued use (Ferrari *et al.*, 2003).

Artemether, although established primarily as an anti-malarial drug, has also shown anti-schistosomal properties. Although its mechanism of action still remains unconfirmed, the drug seems to play a role in schistosomal carbohydrate metabolism by indirectly decreasing the formation of lactate via glycogen metabolism enhancement and lactate dehydrogenase inhibition in adult schistosomes (You *et al.*, 2014). Mefloquine, another anti-malarial drug, also acts as a schistosomicide by killing schistosomulae as well as adult schistosomes. However, it has been put forward as a matter of concern that the use of these anti-malarial drugs as anti-schistosomal agents could result in resistance of *Plasmodium* parasites to the drugs. Biltricide, a pyrazinoisoquinoline derivative, is the drug of choice in the United States. It causes worm detachment from the host tissue by increasing muscular contractions and reducing the worm capacity to regulate or control cation levels across the tegument at high concentrations (Coon, 2005).

Myrrh at some point, (trademark name called Mirazid at the time by an Egyptian company), was said to exhibit positive anti-schistosomal properties. Reports state that this natural extract from the stem of the *Commiphora mol* plant disrupts the tubercles and tegument of *S. mansoni* worms thereby causing a reduction in worm burden (Ali, 2011). However, it was later discovered and published that myrrh did not stand as an effective drug and actually displayed low cure rates as well as insignificant reductions in egg count. Nonetheless, the drug was still used and even made appearances outside

Egypt up until 2006. The WHO eventually stepped in to produce documentation admonishing governments and individuals to reject any Mirazid from any source (Fenwick and Webster, 2006).

Other natural products have been listed as having anti-schistosomal properties and possible leads in schistosome drug discovery and development due to the fact that most of them are well-documented and have a large coverage pertaining to chemical space. However, it is still unclear as to how most of them act as anti-schistosomal agents and therefore more research is needed in that respect and is in fact underway. Some of them include tashinones, alkaloids, arachidonic acid, *Allium sativa* (garlic), pumpkin seeds, lapachol, *Citrus reticulata*, berberine, wormwood, endod and *Acacia arabica* just to mention a few (Ali, 2011; Neves *et al.*, 2015).

2.9.2 Vaccines

The discovery and development of vaccines for schistosomiasis, hypothetically speaking, seems like the most effective way for control against this disease. Not only adults but also young children, especially in endemic and high risk settings, will be able to take them which would contribute to reduced mortality and morbidity, by inducing immune responses to decrease parasitic burden and egg production, thus providing long-term protection (Siddiqui *et al.*, 2011). However, development of a vaccine, not only against schistosomiasis but also against other parasitic helminthes and virulent diseases such as HIV/AIDS and malaria, has proven to be quite difficult due to poor and limited understanding of host-parasite interactions and mechanisms of protective

immunity. Moreover, it would be safe and proper to say that the development of vaccines against schistosomiasis will be a laborious and demanding process with the aim of having certain characteristics in mind like efficacy, easy administration, high minimal degree of protection against infective cercariae and a prominent level of safety, stability and formation (Todd and Colley, 2002). Nevertheless, new concepts regarding the generation of schistosomal vaccines have begun. A 28kDa glutathione-S-transferase (Sm28GST) from *S. mansoni* was discovered a number of years ago as a plausible vaccine candidate due to its ability to reduce the degree of female worm fertility, egg production and overall worm burden (Capron *et al.*, 2005). However, it has failed to reach Phase II in clinical trials; therefore its efficacy has not yet been determined. It falls under a group of five other *S. mansoni* antigens which include Sm14, Sm23, Ir-V5, paramyosin and triosephosphate isomerase, which were tested in the 1900s by a UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR/WHO) committee and were, and in some cases are still, regarded as the most promising targets for vaccine development. Unfortunately, none of the antigens were able to reach the 40% protection barrier, showing insufficient antigen stability (McManus and Loukas, 2008). However, research into the 14kDa Sm14 antigen as a vaccine has continued in Brazil by the Oswaldo Cruz Foundation (FIOCRUZ) which has close ties with the Ministry of Health in the country. Schistosomes lack the ability to synthesize sterols and fatty acids. Hence, they are totally reliant on their host for these and other lipid components through the action of FABPs (Fatty Acid-Binding Proteins), which are imperative in the transport and uptake of fatty acids gained from the host (Tendler and Simpson, 2008). Sm14 is an FABP homologue that has not only shown

immunity against schistosomiasis, due to its ability to mediate a T_H1 immune response, but it has also displayed protection against *Fasciola hepatica*, a trematode that parasitizes livestock, particularly cattle and sheep. The molecule displays great promise as a candidate vaccine against the disease and in 2006, a total sum of US\$3 million was awarded for Phase I clinical trials to begin in Brazil and Phase II trials to continue in both Brazil and Egypt.

Many other attempts toward schistosomiasis vaccine development have been sought through the study of tetrasanins (SmTSP-1 and SmTSP-2), MAP (multiple antigen epitopes) (Knox, 2010) and adjuvants like alum, saponins and papaine (Stephenson *et al.*, 2014) just to mention a few. However, Wilson and Coulson (2006) conclude by saying “...schistosomes are not stupid. They have had tens of millions of years to evolve mechanisms that help them survive immune attack from the mammalian host, even an attack orchestrated by our vaccine strategies. Schistosomes are only a formidable adversary that won’t be easily beaten”. This is not to say that attempts should be abandoned. Thus, this particular study considers Universal Stress Proteins (USPs) as a possible approach toward vaccine development and new therapeutic interventions against schistosomiasis. The role of this important and interesting class of proteins shall be focused on subsequently in the following chapter.

2.9.3 Molluscicides, Education, Sanitation and Safe Water Interventions

Before antischistosomal drugs came onto the scene, molluscicides were used to tackle snail intermediate hosts and have been found to be very effective and highly recommended such that the method has participated in control programmes in Egypt and Peoples Republic of China and has been used in Morocco and Japan to successfully eliminate schistosomiasis (Rollinson *et al.*, 2013). Their advantages range from having the ability to interrupt the transmission cycle, the use of unspecialised equipment and expertise and its simple methods of application (de Souza, 1995). However, the use of molluscicides has its own shortcomings. Compounds such as pentachlorophenol, Yurimin (3,5-dibromo-4 hydroxy-4-nitroazobenzene), Frescon (N-tritylmorpholine), copper and B-2 (sodium 2,5, dichloro-4-bromophenol) have been previously used as molluscicides but most have been shown to be toxic to the environment. Examples of those identified with better activity and lower levels of toxicity are Fluoroacetamide, Bromocetamide and Chlorocetamide. Other disadvantages are their costly prices, short-term effects and toxicity to biodiversity and the environment. Niclosamide, another well-known molluscicide, is very effective against all stages of the snail lifecycle but constantly requires re-application because it fails to disrupt snails from re-colonizing their primary habitats (Rollinson *et al.*, 2013). Additionally, it is very expensive, especially when huge bodies of water need treatment, causes discolouration of water bodies and is toxic to fish and other aquatic organisms (Cioli *et al.*, 2014). Alternatives in the form of biological agents have also been looked at such as leaches, birds, fish and crayfish, plant-derived molluscicides such as *Phytolacca dodencanda* (commonly known as Endod), ducks and turtles. Introduction of competitor or exotic

snail species have also been suggested to naturally displace snails susceptible to schistosomes. An example of such includes *Biomphalaria straminea* displacing *Biomphalaria glabrata* (Sturrock, 1995). Bringing snail replacements into the picture might seem like an attractive idea but such an approach may have its own dangers and limitations unless proper and severe precautionary steps have been taken.

Other factors towards schistosomiasis elimination should also be taken into consideration and used in conjunction with the already mentioned tactics as an “integrated approach”. Examples of such include health education, ecological interventions, improved hygiene, water and sanitation all combined with change in behaviour (Cioli *et al.*, 2014; Rollinson *et al.*, 2013). It has been put forward by the World Health Assembly (WHA) in May 2012 that the availability of proper water, sanitation and hygiene (WASH) shows great possibility towards the feasibility of schistosomiasis elimination (Grimes *et al.*, 2015). Those with decent sanitation and access to safe water have significantly low chances of schistosome infections and these are mostly those with higher socio-economic status (SES). However, SES may prove unimportant in certain areas where contact with infested/infected waters is inevitable. Nevertheless, most people with higher SES are more educated and have a broader knowledge of good behavioural practices, have better access to healthcare and/or treatment and are less exposed to infested waters, thus this set of people are more exempt from infection. Unfortunately, WASH and SES conditions are lacking in most schistosome-endemic countries. Change in behaviour is possible but extremely difficult especially when there are no alternatives and this mainly falls on the poor and rural population. Most blame such on governments and health policy makers and this view is

seen from two broad perspectives. Firstly, most endemic countries fall between low and middle class and are severely under-resourced. On top of that, most countries contribute to half the cost of control programmes of most NTDs while donors and non-governmental organizations take care of the rest (Conteh *et al.*, 2010). However, most countries fail to sustain such practices because of limited resources. On the other hand, poor governance may play a role where certain individuals or parties abandon welfare programmes, are less interested in the health and wellbeing of the less fortunate and are more interested in pocketing money and resources (Bruun and Aargaard-Hansen, 2008). Either way, the ultimate goal of all schistosome efforts is to deal with morbidity by 2020, interrupt schistosome transmission and finally eliminate this dreadful disease by 2025 (Colley *et al.*, 2014).

2.9.4 Praziquantel (PZQ)

For the past 30 years or so, Praziquantel (PZQ), (generic name: 2-(cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4H-pyrazinol[2,1-a]isoquinole-4-one), has been the drug of choice against all schistosome infections due to its availability, cost effectiveness and the minimal side effects it exerts on an infected individual (Figure 2.3). It is a white crystalline powder, bitter to the taste, insoluble in water and one tablet contains 600mg of the active ingredient (Doenhoff *et al.*, 2008). The drug was at first synthesized with the aim of identifying tranquilizers until its anti-schistosomal properties were discovered by Bayer AG of Darmstadt (Caffrey, 2015). Currently, it is now viewed as the backbone of schistosomiasis treatment and control through mass drug administration (MDA) (Secor, 2016). About 60 million tablets were administered to patients in Egypt between

1997–1999 (Doenhoff *et al.*, 2000). During the years 2005, 2006 and 2007 respectively, approximately 13 million, 9 million and 8 million school children underwent PZQ treatment (Hotez and Fenwick, 2009b). Despite the relative affordability of the drug (US\$0.10 per tablet), a dramatic amount of money will be needed to carry out treatment in schools and various populations; budgets that most disease-endemic countries do not have. Additionally, the cost of delivery and distribution of drugs, training and evaluation of staff should also be taken into consideration. Hotez and Fenwick (2009b) recommended US\$200 million annually to purchase 1,200 million PZQ tablets for the treatment of more than 400 million people over a period of 5 years. The Bill and Melinda Gates Foundation with pharmaceutical companies like Shin Poong (Korea), Merck (Germany) as well as the Schistosomiasis Control Initiative (SCI) have assisted in funding and implementing national schistosomiasis endeavours (Adenowo *et al.*, 2015; Utzinger *et al.*, 2009). However, this just stands as one of the many drawbacks and concerns of PZQ.

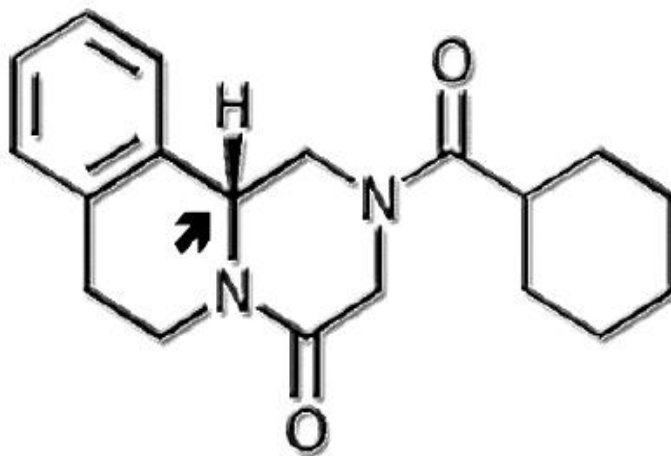


Figure 2.3: Chemical structure of Praziquantel. Praziquantel, 2-(cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a]isoquinoline-4-one. Diagram taken from Alsaqabi and Lofty, 2014.

PZQ is a drug that is a combination of two stereoisomers, one which exhibits schistosomal activity while the other is relatively useless and is actually responsible for the mild side effects that sometimes accompany treatment. It is for this reason, bulkiness and the bitter taste of the pill that make children find the tablet hard to swallow (Caffrey, 2015; Cioli *et al.*, 2014). Although the drug is well tolerated and easily absorbed by the system, it does exert side effects which include nausea, diarrhoea with/or without blood, vomiting, headache, abdominal pain, dizziness and skin rash with edema (although this is rare). However, these appearances are only seen in 30–60% of patients and are short-lived, mostly disappearing within 24 hours (Alsaqabi and Lofty, 2014). These are also mostly as a result of dying schistosomes that release their products or due to the host response to them.

Other issues regarding PZQ is its failure to kill the parasite 2–4 weeks post infection (Brandt & Kofoed, 2008). Due to the fact that the drug is stage-dependent, PZQ in turn fails to prevent morbidity. Immature parasites may survive exposure to the drug and develop into adults which lay eggs and have the opportunity of becoming lodged in host tissue which is the actual root cause of schistosomiasis itself. Thus, it is recommended that treatment should be repeated at least twice a year especially in high prevalence areas (Conteh *et al.*, 2010). This is extremely important because PZQ does not interrupt the transmission cycle, meaning that one may be re-infected with schistosomiasis again. In such cases, treatment may also fail due to immature worms migrating after re-infection particularly in high transmission areas (Alsaqabi and Lofty, 2014).

The current and greatest worry in respect to praziquantel is drug resistance. The drug has never been shown to have 100% cure rate, only developing 60–90%. Legitimate

fears concerning drug resistance are justifiable especially since the drug has been utilized for several years and evidence of this has already been reported. Melman *et al.* (2009) reported the reduced susceptibility of the KCW isolate from a patient from Kisumu, Kenya who had undergone several treatments with praziquantel but never made a full recovery. *In vitro* and *in vivo* tests carried out on this isolate, even after a space of 3 years, confirmed the fact that it had become less susceptible to PZQ and that its susceptibility had become a heritable trait that was able to maintain resistance more than 6 generations later (Melman *et al.*, 2009). Apart from the KCW isolate from Kenya, a Senegalese isolate also from *S. mansoni* showed marked susceptibility to PZQ as compared to other isolates that were obtained from Puerto Rico and Kenya (Fallon *et al.*, 1996). In addition to that, there have also been reports of low cure rates, especially in Senegalese patients, after praziquantel administration. One of the theories, although evidence is unavailable to support it, suggests that one of the possible reasons for this may be drug resistance or isolate tolerance to the drug (Fallon *et al.*, 1996). To finally top this list of concerns the mechanism of action of the drug, despite years or research, is still not well-defined.

2.9.4.1 Mechanism of Action of PZQ

So far, it has been suggested that PZQ affects inorganic ion transport, particularly having an effect on calcium levels. Present on the surface of adult schistosomes are antigens which become exposed once the tegument is disrupted by the action of PZQ (Aragon *et al.*, 2009). Shortly after the parasite comes into contact with the drug, the muscles may either rapidly contract which is also associated with the exposure of antigens on the worm's surface, or they may become paralyzed, resulting in a speedy

flood of calcium ions (Figure 2.4), a slower inflow of sodium ions and subsidiary entry of potassium ions (Aragon *et al.*, 2009). The disruption of calcium homeostasis is thought to be linked to these responses, hence the role of this inorganic ion seems too vital to ignore.

The tegument of the worm is a particularly important factor in the response of the worm to PZQ. The tegument and sarcolemma of schistosomes when treated with the drug seem to suggest that there are sites that are particularly sensitive to PZQ. For example, un-tegmented worms treated in a Ca^{2+} free medium produce no muscle contraction while worms with their tegument intact, rapidly respond to PZQ (Greenberg, 2005). This shows that the presence of the tegument is necessary for the drug to have any effect. It has been proposed that praziquantel interferes with the calcium imbalance of the schistosome worms by causing a swift entry of calcium ions into the worm leading to prompt or brisk muscular contractions (Greenberg, 2005). Calcium ion (Ca^{2+}) channels of schistosomes consist of α and β subunits (Figure 2.5). Studies have shown that the channels containing the β -type are the ones that cause calcium influx. There are two $\text{Ca}^{2+}\beta$ -subunit types: a conventional subtype which is similar and present in all other vertebrates and invertebrates, as well as a novel variant subtype with unfamiliar and essential physical and operative properties that are particularly PZQ-sensitive. These channels are very important because they are indirectly responsible for a number of functions such as muscle contraction and neurosecretion in muscles and nerves via Ca^{2+} . Moreover, Ca^{2+} together with protein kinase C plays the role of regulating the production of proteolytic enzyme that assists with the schistosome muscular activity and is also largely responsible for motor-related activities as well as schistosomal egg

hatching in fresh water. Additionally, Ca^{2+} in conjunction with cAMP assists miracidia to transform into sporocysts (Mbah *et al.*, 2013).

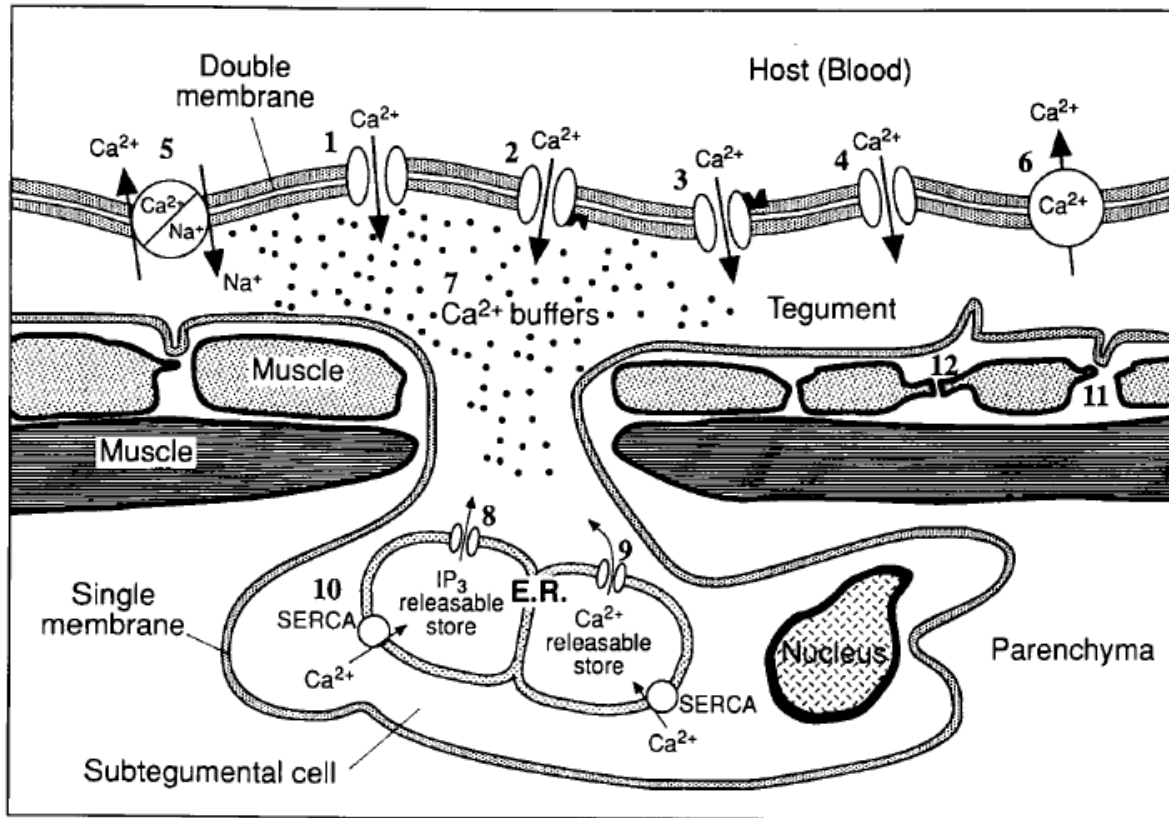


Figure 2.4: Possible mechanism by which Ca^{2+} travels in and out of the *Schistosoma mansoni* tegument and probable locations of this mechanism on the worm body wall. 1-6 indicate various channels that allow the flow of Ca^{2+} . Experiments have shown that caffeine (stood in for praziquantel) stimulates the CIRC channel (Ca^{2+} induced Ca^{2+} release channel) [9] which are in the sarcoplasmic reticulum. The channel is also activated by increase in cytosolic Ca^{2+} and adenosine triphosphate (ATP) but is inhibited by Mg^{2+} . Figure taken from Martin, 1997.

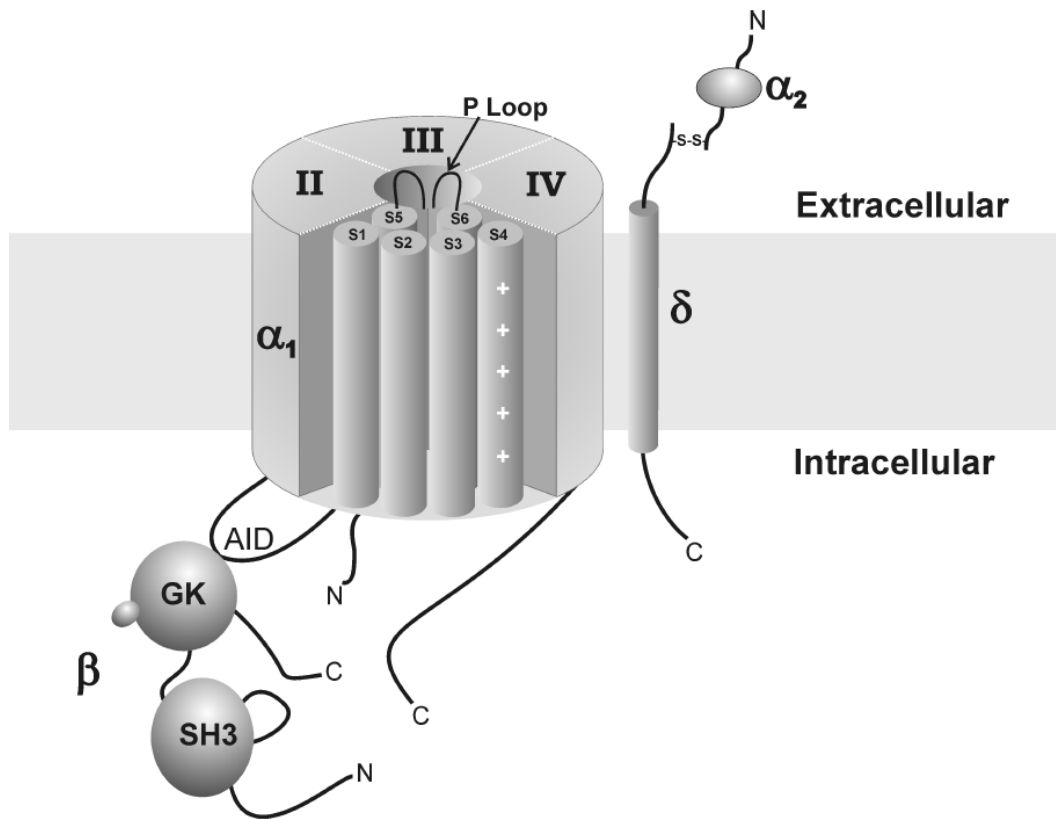


Figure 2.5: Voltage-gated Ca²⁺ channel formation. The subunit contains four homologous domains which enclose a central pore. Each domain is made up of six transmembrane regions (S₁–S₆). The P loop between the S₅ and S₆, forms the selectivity filter of the channel while the S₅ is thought to be the voltage sensor of the channel due to the presence of positively charged residues. Also shown are auxiliary β , α_2 ^{δ} and γ subunits. Figure taken from Greenberg, 2005.

Therefore, it is obvious that calcium as a cation as well as voltage-operated calcium channels play very important roles in the mechanism of action of PZQ. However, other cations may also be involved although not much work has been attributed to their participation. It has been shown that apart from Ca^{2+} , Mg^{2+} is able to activate maximal activity of ATPase although activation is not as much as that of Ca^{2+} (Nechay *et al.*, 1980). Studies have shown that detegumented worms bathed in media with high concentrations of magnesium also react, although not as severely as when the tegument is present. A more definite and pronounced contraction is observed rather than the normal biphasic contraction that takes place due to PZQ, thereby confirming the requirement of the tegument for the full effect of contraction (Greenberg, 2005).

Increased levels of extracellular K^+ causes depolarization leading to a rise in muscle contractions in the worm, which is dependent on extracellular Ca^{2+} (Mendonça-Silva *et al.*, 2005). Removal of this extracellular Ca^{2+} leads to blockage of depolarization-induced contractions. Figure 2.6 depicts the flow of Ca^{2+} into *S. mansoni* muscle and its corresponding physiological response. This depolarization-induced contraction seems to further confirm the presence of Voltage-operated Ca^{2+} channels (VOCCs) in the muscle fibres of the worm. It is proposed that the depolarization causes the influx of Ca^{2+} through an unusual L-type VOCC and that this Ca^{2+} influx is enough to cause muscular contraction (Mendonça-Silva *et al.*, 2005). Increased intracellular Ca^{2+} levels trigger the activation of Ca^{2+} -dependent K^+ channels (CaDep). Additionally, two types of voltage gated K^+ channels, a delayed rectifier (DR) and a shaker type (Shaker) are activated. These channels facilitate K^+ to flow out thereby repolarizing the membrane as well as closing the VOCCs.

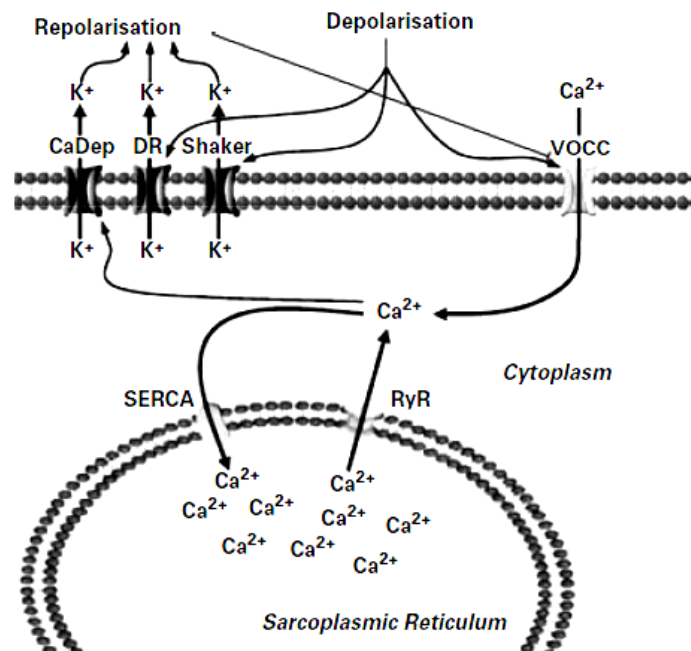


Figure 2.6: Flow of Ca^{2+} into the Schistosoma worm. Abbreviations: CaDep, Ca^{2+} -dependent K^+ channels; DR, Delayed Rectifier; Shaker, Shaker type. Figure taken from Mendonça-Silva *et al.*, 2005

Although, the mechanism of action of PZQ remains elusive, various postulations abound as to how the schistosome worm survives harsh conditions both in the human and snail host. Living organisms have evolved in certain ways to overcome environmental stresses; these include and are not limited to Heat Shock Proteins and Universal Stress Proteins (USPs). In schistosome worms, the up-regulation of the latter is hypothesized to assist the worms to survive the hostile conditions they face during their developmental cycle (Mbah *et al.*, 2013).

2.10 Universal Stress Proteins (USPs)

An enduring ability of living organisms is their capacity to respond to their immediate environment and the changes associated therein. Environmental stress, which may appear in various forms, can result in the denaturation of proteins within the organism, eventually leading to severe harm or even death (Mbah *et al.*, 2013). Thus, it is of vital importance that the organism possesses stress response mechanisms which may be through the expression of heat shock proteins (Hsps) or universal stress proteins (USPs) (Mbah *et al.*, 2013). Universal Stress Proteins (USPs) are a group of proteins that have been shown to exist in all three genomes of *S. haematobium*, *S. mansoni* and *S. japonicum*, belonging to the PF00582 superfamily, as well as in a large number of organisms including archaea, bacteria, protists, fungi as well as plants (Berriman *et al.*, 2009; Tkaczuk *et al.*, 2013). Universal stress proteins are responsible for enabling organisms to survive many environmental stresses such as nutrient starvation, high salinity, drought, severe temperatures and exposure to toxic chemicals (Isokpehi *et al.*, 2011). These proteins are up-regulated during sudden environmental changes that could harm organisms and hence, remain one of the reasons the schistosome worm is

able to withstand different and unfavourable environments, as well as different hosts they invade during their complex developmental cycle.

The Usp gene may encode different forms of the protein; either as a small protein (14–15kDa), a large protein (~30kDa), a Usp protein consisting of 2Usp domains or as a large protein with 2 peptides functioning as a single protein (Aanandhi *et al.*, 2014). Through the crystal structure of the wild-type Rv2623 from *Mycobacterium tuberculosis*, Drumm *et al.* (2009) were able to report the 2-fold symmetric dimer form of the protein. USPs also has the ability to bind cAMP, thereby acting indirectly as ATP energy regulators (Banerjee *et al.*, 2015). The mechanisms by which these proteins protect cells, as well as their biochemical pathways, is still uncertain but seemingly the importance of these proteins cannot be ignored due to their ubiquitous expression across different genre of organisms. Most are expressed in the cytoplasm of cells and may even function or act as enzymes (Bangera *et al.*, 2015). UspA (universal stress protein A) was the first to be identified from *Escherichia coli* stationery phase cells (Nyström and Neidhardt, 1993). More studies later revealed its role in cellular protection from nutrient starvation, exposure to toxic chemicals, osmotic stress and UV light damage (Kerk *et al.*, 2003). Apart from UspA, other usp genes have been reported and classified in 4 distinct classes based on similarities in their amino acid sequences: UspA, UspC and UspD belonging to Class I, UspF and G in Class II and UspE, which has the E1 and E2 domains classified in Class III and IV respectively (Kim *et al.*, 2012). So far, analysis of the eight genes encoding the USP domain of the *S. mansoni* genome has been done by Isokpehi and co-workers (2011) which include: Smp_001000, Smp_001010, Smp_031300, Smp_043120, Smp_076400, Smp_097930, Smp_136870

and Smp_136890. Figure 2.7 depicts the role of USPs in the different life stages of the *schistosoma* parasite.

In the freshwater environment, the eggs, miracidia and cercariae have to withstand the stress conditions associated with that environment. More so, certain toxic substances in the snail host are exposed to cercaria and sporocytes as they develop in the snail. Oxidative and nitrosative stress are mainly associated with these toxic substances which the parasite has to tolerate and respond to (Negrao-Corrêa *et al.*, 2012). In the human host, the immature forms of the worm have to mature into adults after which the schistosomulae migrate to organs such as the liver, bladder and intestines. Other adult worms may migrate to the heart and lungs. Even though defence mechanisms are present in these organs to help eradicate these parasitic stages via the production of nitric oxide and hydrogen peroxide, all the parasitic stages are able to survive these harsh conditions by the action of various molecular mechanisms which include the up-regulation of universal stress proteins (Loverde, 1998).

Apart from environmental factors, other cellular dynamics can also contribute to the action of USPs such as Adenosine triphosphate (ATP) binding. ATP binding is a biochemical process able to control the activity of USPs via phosphorylation (Mbah *et al.*, 2013). USPs are categorized into two main groups based on whether an ATP-binding motif G-2x-G-9x-G(S/T) is present in their primary amino acid sequence or not. ATP as a donor phosphate and guanosine triphosphate (GTP) assist in phosphorylating USPs onto serine which causes up-regulation of the protein. It is therefore vital to

understand how this important family of proteins function and how they are regulated, as this may serve as a basis for alternative treatment via drug design and/or improve diagnosis of the disease.

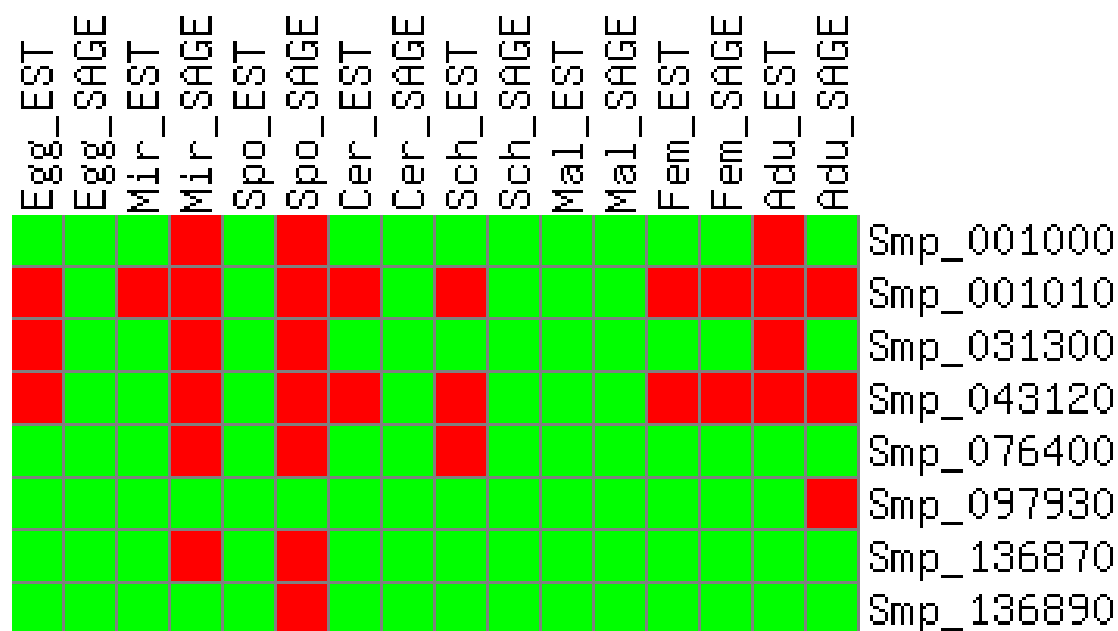


Figure 2.7: Image depicting the incorporation of evidence pertaining to gene expression from Expressed Sequence Tags (EST) and Serial Analysis of Gene Expression (SAGE) of the 8 *Schistosoma mansoni* USP genes during its developmental stages. The colour red indicates that expression has been detected whilst green shows otherwise. The Schistosome Database Identifiers (SchistoDB IDs) are shown on the right-hand side. Abbreviations: Mir, Miracidia; Spo, Sporocyst; Cer, Cercariae; Sch, Schistosomula; Mal, Male; Fem, Female; Adu, Adult. Diagram taken from Isokpehi *et al.*, 2011.

Mbah and co-workers (2013) showed the presence of conserved sites consisting of aspartate (Asp), histidine (His) and proline (Pro) in five *S. mansoni* Usp sequences, suggesting that these residues could be biologically important in the regulation of USPs. Furthermore, the authors showed two novel proteins, (G4LZI3 from *S. mansoni* and Q86DW2 from *S. japonicum*) had the same values regarding multiple annotation features, such as ligand binding sites and cellular ligands. Hence, understanding the action of these novel proteins could enable interventions to take place at certain susceptible developmental stages so as to control or eradicate the disease since they are part of the organism natural biological defence mechanism (Isokpehi *et al.*, 2011; Tkaczuk *et al.*, 2013).

The tolerance of the worm in every stage of its developmental cycle seems to an extent, largely dependent on the action of USPs. However, calcium, potassium and magnesium ions are three cations that have been postulated to play a significant role in the mode of action of PZQ. Therefore, Mbah and co-workers (2013) proposed that cations like magnesium, calcium and zinc somehow interact with the G4LZI3 novel Usp and thus further studies should be aimed at functionally characterizing these interactions, particularly that of calcium. Additionally, it would be biologically exciting to ascertain if there could be an interaction between these cations and USPs. If so, this interaction could be manipulated to assist in schistosomiasis intervention endeavours. It has been documented that tools like X-ray crystallography, Nuclear Magnetic Resonance (NMR) spectroscopy, Isothermal Titration Calorimetry (ITC) and Surface Plasmon Resonance (SPR) fall under the very few techniques that can be used to characterize the binding or interaction of diverse ligands to proteins or any other biomolecular interaction. Thus, this

study incorporated one of these powerful and versatile techniques to try and investigate this school of thought.

2.11 Nuclear Magnetic Resonance (NMR) Spectroscopy

First developed in 1946 and over the next 50 years, Nuclear Magnetic Resonance (NMR) spectroscopy has ceased from simply being a means for merely analysing chemicals but has also proven to be a potent technique in a wide range of solution state protein studies such as protein-ligand interactions, protein-nucleic acid interactions and protein-membrane interactions (Clarkson and Campbell, 2003; Edwards, 2003). NMR is fast becoming a growing field of interest especially in pharmaceutical and biotechnology industries involved in drug design and development. Biologically, the structure of a protein is paramount in understanding its functions; hence NMR is a useful tool in achieving this through the use of both hetero- and homonuclear techniques (Scheich *et al.*, 2004).

Apart from NMR spectroscopy, X-ray crystallography is also used for structure determination of proteins but has its own limitations. X-ray crystallography makes use of formed crystals which may or may not form due to disorders within one or more components in the complex, or due to weak protein-protein interactions (PPI) of which when/if they do eventually form, they might not contain the relevant biological conformation of the protein (O'Connell *et al.*, 2009). For these reasons, NMR spectroscopy is seen as the ideal approach for determining high resolution structures especially when the K_D or dissociation constant is low ($K_D > 10^{-4} M$) (Vaynberg and Qin, 2006).

One dimension proton ($1D-^1H$) NMR spectrum is first done in determining whether a protein is folded or not as this will determine if the protein can be probed with NMR. The folding state of a protein is the rate determining step in subjecting the protein to NMR spectroscopy. This "foldedness" is ascertained by the acquisition of a $1D-^1H$ NMR, $2D-^{15}N$ -HSQC and $^1H-^1H$ -NOESY spectrum. These spectra reveal certain resonances or shifts which are indicative of the protein foldedness and its amenability to NMR spectroscopy. HSQC (heteronuclear single quantum coherence) is a two dimensional NMR experiment used to further confirm the folding or unfolding of a protein especially when the $1D$ spectra is too complex for interpretation by adding more dimensional spectra. This simplifies the spectra and provides further explanations pertaining to the structure of the protein.

Two dimensional ($2D-^{15}N$) HSQC is most useful in investigating the binding of ligands or other proteins to proteins. Observation of binding is determined when the concentration of the ligand is increased gradually and an HSQC spectrum is acquired at each interval. This is done until there is no difference observed between the last HSQC spectrum and the preceding one. At this stage, the protein would have absorbed all available ligand and is "fully saturated," meaning that strong binding has taken place (Onyemata *et al.*, 2005; Pugh, 2005). The overlaying of all the HSQC spectra then reveals resonances that are perturbed, have moved or 'disappeared' from their original position in the first HSQC, appearing in another location in the last HSQC. A clear distinction in the perturbations between the 'original' resonances in the first HSQC and resonances of the last HSQC is indicative of the residues involved in binding (Figure 2.8). Once this is ascertained, the strength of binding can then be determined and this may be done by

calculating the biophysical constant called the dissociation (K_d) or binding constant (K_a) which may also be achieved by using Isothermal Titration Calorimetry (ITC).

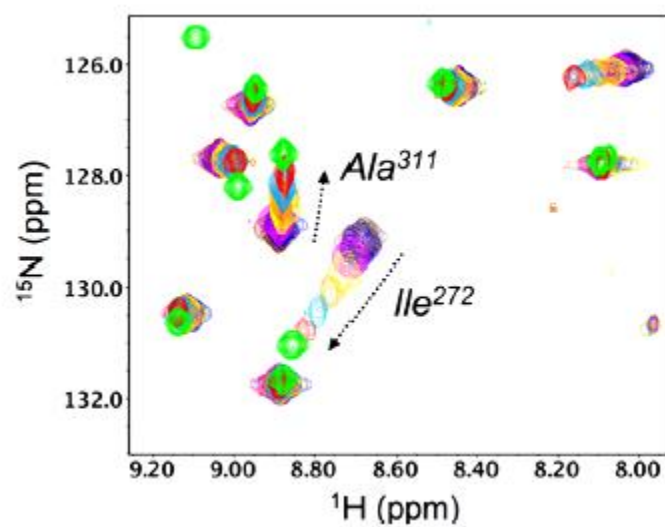


Figure 2.8: Overlay or superposition of ^1H - ^{15}N HSQC spectra recorded from 1000–25 μM concentration. Residues have clearly perturbed (shifted) due to concentration dependence. Figure taken from Kappo *et al.*, 2012.

2.12 SURFACE PLASMON RESONANCE (SPR)

This phenomenon was first discovered by Wood in 1902 and physically refers to the “excitation of surface plasmons” (Tudos and Schasfoort, 2008). In other words, during certain circumstances, photons may have the ability to excite surface plasmons present on a metallic film or surface and in turn transform a photon into a surface plasmon (Pattnaik, 2005). The Surface Plasmon Resonance (SPR) sensor technique has been shown to be very useful and suitable in detecting biomolecular interactions and a number of SPR biosensor systems have been on the market such as TI-SPR-1, Kinetic Instrument 1 and IBIS; the most common being the BIAcore SPR instrument with several models underneath it (BIAcore[®] 3000, BIAcore[®] 2000, BIAcore[®] X, BIAcore[®] 1000, BiacoreQuant[™]) (Homola, 1999). To detect any biomolecular interaction, a refractive index is measured based on the binding of the ligand to the protein under a continuous flow (Van Der Merwe, 2001). As binding takes place, changes in the refractive index occur and these are plotted on a sensorgram where the response or resonance units (RUs) are plotted against time (Figure 2.9).

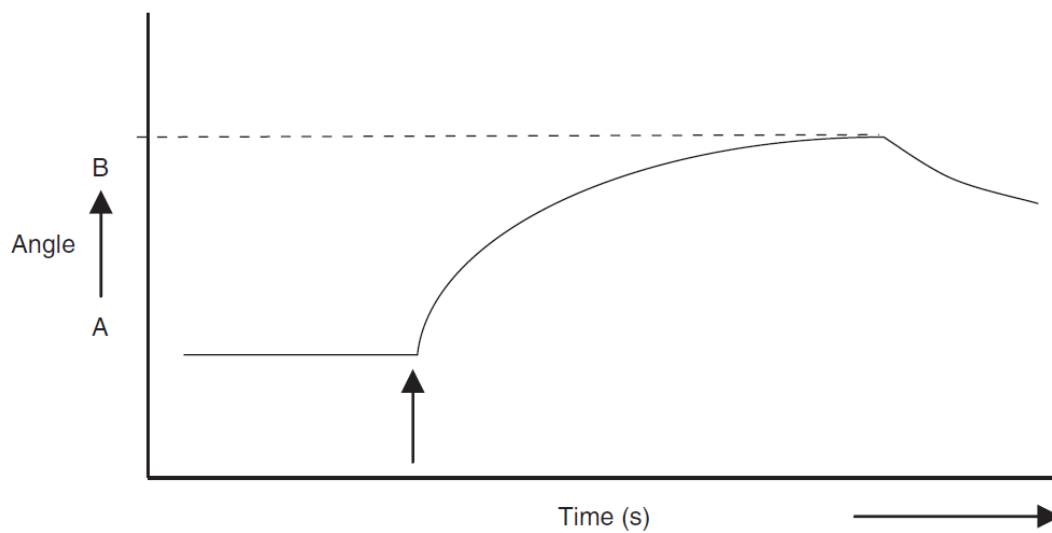


Figure 2.9: Diagram of a Sensogram. A baseline is first measured and a dip occurs at angle A. The arrow represents the sample injected and biomolecules adsorb onto the surface. This results in a shift or change in the refractive index (angle B). The amount of species adsorbed can then be determined in real time. Diagram obtained from Tudos and Schasfoort, 2008.

2.13 Isothermal Titration Calorimetry (ITC)

Very few experiments are able to study protein-protein or protein-ligand interactions. Isothermal Titration Calorimetry (ITC) stands as the best means for quantitatively measuring and characterizing the thermodynamic properties of protein-protein, protein-ligand, protein-nucleic acid and/or small molecule associations (Chilom *et al.*, 2006, Pierce *et al.*, 1999). This label-free technique is able to measure the heat absorbed or produced, (whether endothermic or exothermic), when binding takes place in a “sequential (titrating) manner” (Draskowski *et al.*, 2014). In a single ITC experiment, thermodynamic parameters like the affinity or binding constant (K_a or K_d), difference in enthalpy (ΔH) and entropy (ΔS), Gibbs free energy (ΔG) and stoichiometric index (n) are determined (Chilom *et al.*, 2006, Pierce *et al.*, 1999).

A standard isothermal titration calorimeter contains two cells; a reference and reaction/sample cell. The ligand is injected successively into a sample cell containing the macromolecule and the heat generated or absorbed after each injection is measured based on the interaction and temperature difference between the two cells (Draskowski *et al.*, 2014). The production or absorption of heat is mainly dependent on and proportional to the ability of the ligand to bind to the protein. The difference in temperature between the two cells is kept at a constant value known as the baseline. During each injection, ligand gets bound to the macromolecule and releases a signal, recorded by a computer (Chilom *et al.*, 2006), which then returns to the baseline before the next injection. With every injection, the ligand binds onto all available sites of the macromolecule, producing a decrease in heat change until it is fully saturated. At this point in time, the ligand can bind no more and only records heat of dilution. These

values are recorded as absorbed or released heat peaks and are then used to plot a thermogram using heat fluxes versus time (Figure 2.8). The sum of these peaks in turn gives rise to what is known as the titration isotherm or ITC curve which can be drawn by software like ORIGIN. Based on the shape of the curve, the thermodynamic properties (amplitude representing the enthalpy of the reaction, the inflection point symbolising the stoichiometry and width denoting binding affinity) of the reaction are generated (Figure 2.9) (Rajarithnam and Rösger, 2014). However, the shape depends on the c-value which represents the relationship between protein concentration and the dissociation constant. It is recommended that the c-value be between 1 and 1000 (and not <1 or >1000) (Chilom *et al.*, 2006, Pierce *et al.*, 1999). Values between 10 and 100 are considered very good, between 5 and 500 are regarded as good and values between 1-5 and 500-1000 are deemed acceptable. However, optimal values are recommended to be ≥ 10 and ≤ 500 (Draskowski *et al.*, 2014). Large c-values indicate strong binding and give rise to rectangular shapes while values less than 10 result in poor inflection points and loss in sigmoidal shape.

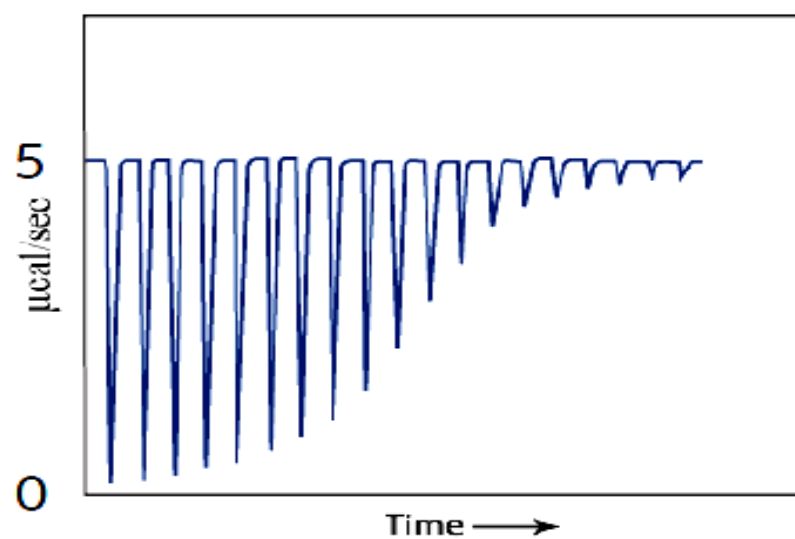


Figure 2.10: Diagram representing ITC Measurement. The ligand solution is injected into the protein solution. The heat released (enthalpy) from each injection is equivalent to the area under each peak. This is then recorded as power versus time. Diagram taken from Milev, 2013.

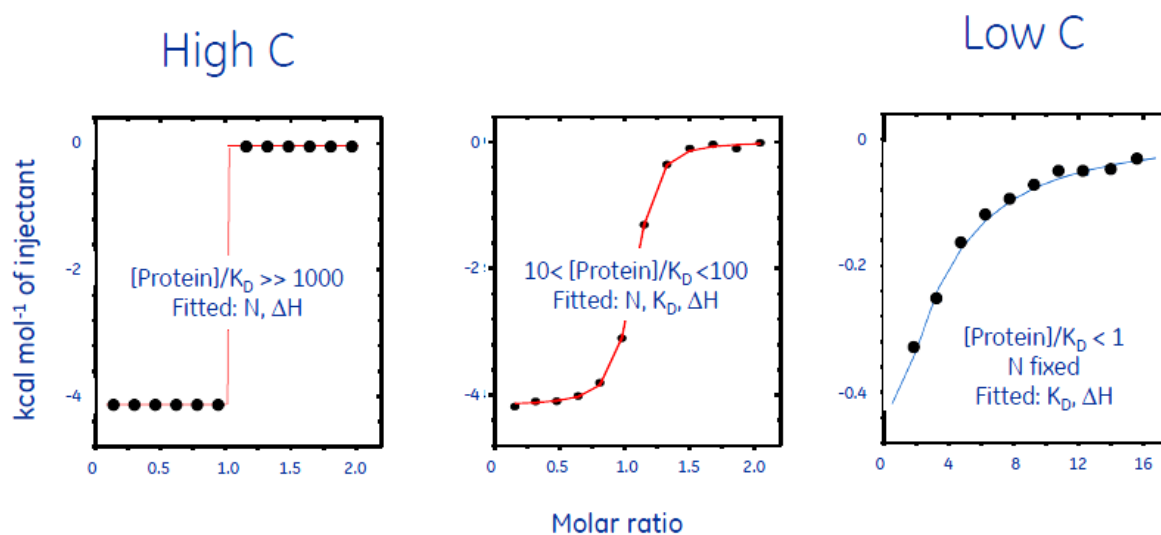


Figure 2.11: Illustration of ITC Data. The sum of all the individual peaks is calculated into a curve using best-fit parameters. This may yield different shapes of the curve indicative of binding affinity. Diagram taken from Milev, 2013.

A number of factors need to be taken into consideration when performing an ITC experiment. The 30, 30, 30 approach is considered a preliminary start especially where binding affinities have not been performed before. It is proposed that the protein concentration be 30 times higher than the anticipated K_a , the ligand concentration to be injected into the sample cell be 30 times more than the protein concentration and approximately 30 injections of 5 μ L amounts of ligand be injected into the cell (Rajaratnam and Rösger, 2014). Optimization may have to take place if signals are weak so as to avoid misinterpretation of results. This can be done by optimizing protein and/or ligand concentrations, choosing a different buffer and/or detergent in the case of membrane proteins, changing temperature and ITC instruments as well as taking a look at injection time and peak spacing.

2.14 Comparative/Homology Modelling

As much as the above experimental techniques provide critical and important information concerning any protein of interest, they each have their own technical challenges and limitations. Thus, several structures of proteins which would have been essential for drug design and several other purposes do not exist (Ramachandran and Dokholyan, 2012). Hence, comparative/homology modelling bridges the gap between sequence and structure space by providing researchers with 3-dimensional structures of proteins that have remained undiscovered for a number of different reasons or when experimental techniques fail (Mishra *et al.*, 2012; Krieger *et al.*, 2003). This process is characterized mainly by 4 steps as described by Krieger and colleagues (2003) and Sateesh and co-workers (2010).

2.14.1 Template Recognition and Alignment

Firstly, a similar sequence to the sequence of interest is obtained by sending a query to an online server called mGentreader for threading and the Protein Data Bank (PDB) will give out similar sequences, their modelling templates and alignments. Using a programme called Python, the template with a 30% minimum homology and the protein of interest is then aligned.

2.14.2 Backbone Generation

Model building commences by copying the coordinates of the template in the alignment with the model sequence. The backbone coordinates can only be copied if the aligned residues are different but if not, the side chains will be included.

2.14.3 Loop Modelling

If gaps occur during alignment, the continuous backbone from the template is cut out and the missing residues inserted in loops and turns. From the PDB, proteins with endpoints having matching residues will be searched for and the loop conformation is then selected from the one in which the loop has to be inserted. Using MODELLER software, this loop conformation is then copied into the model.

2.14.4 Model Validation

Outputs with the lowest energy function are then selected and the legitimacy of the structure is checked using another online server called RAMPAGE. The Ramachandra Plot is then obtained and lastly, the modelled protein is visualized using PYMOL Molecular Graphics (DeLano, 2003).

2.15 Molecular Docking

Molecular docking is a computational technique that is of paramount importance in drug design and discovery and is used to study molecular recognition (Mishra *et al.*, 2012). Basically, it entails finding the best fit between a ligand and a receptor (protein) of which the main aim is to predict the structure of the subsequent complex (Mukesh and Rakesh, 2011). This technique helps to solve the problem of trial and error in drug development which tends to be very expensive and time-consuming (Plewczynski *et al.*, 2010). The process mainly constitutes two parts but mostly concentrates on the ligand. Prior knowledge of the target protein (receptor) is obtained using homology modelling whereby the 3D structure helps to determine the binding site of the receptor. The first part has to do with how flexible the receptor is since most proteins generally change their conformational state once bound to a ligand. This mostly takes place in the essential binding site residues, thus there may be many possible conformations of the protein centred on flexibility (Mishra *et al.*, 2012). Based on the countless possibilities of different conformational changes inherent with ligand binding, a way of assessing changes best suited to the binding site as well as testing binding affinity is achieved using “scoring functions” (Mishra *et al.*, 2012; Warren *et al.*, 2006). The scoring function distinguishes a good from a bad docking conformation and is mathematically calculated based on van der Waal repulsions, surface complementarities and electrostatic interactions just to mention a few (Dominguez *et al.*, 2003). All in all, docking is a scientifically complex process but many of its applications together with homology modelling have been successful in assisting in the identification of important pharmacological and drug target proteins in several drug discovery projects without any bias of any hits or leads (Hillisch *et al.*, 2004; Kitchen *et al.*, 2004).

2.16 PROBLEM STATEMENT

After hookworm infections, schistosomiasis is the second most common Neglected Tropical Disease in the sub-Saharan region and clinical deformities such as hepatomegaly and periportal fibrosis have been attributed to the disease, leading to high morbidity and mortality mostly among teenagers, children and women (Adenowo *et al.*, 2015). Praziquantel has been the drug of choice for the past three decades for treating schistosomiasis but its continued use has recently raised some issues of concern. Firstly, the drug does not prevent the development of chronic schistosomiasis *i.e.* it fails to kill the parasite 2-4 weeks post-infection; this is the stage that is particularly crucial since it is around this period that eggs are produced (Brandt and Kofoed, 2008). Moreover, Praziquantel seems to be stage-dependent as it focuses largely on worms that are 1-2 weeks old and 5 weeks or older which are the larval and adult stages respectively (Fallon *et al.*, 1996). Secondly, praziquantel plays no role in interrupting transmission making re-infection a great possibility. Thirdly, since schistosomiasis is one of the greatest NTDs in the world, large scale programs have been implemented to try and eliminate the disease by making praziquantel readily accessible, cheap and easy to use, thus making it available to a large human population. However, due to mass treatment, some individuals may be left untreated or they may be treated but left uncured. Hence, resistance may be due to drug pressure. Finally, alternative treatment is unavailable and even after decades of research, the mechanism of action of the PZQ drug still remains obscure (Aragon *et al.*, 2009).

Universal Stress Proteins (USPs) have been shown to play a vital role in the lifecycle of the *Schistosoma* worm due to the fact that they are up-regulated during conditions of environmental stress. Additionally, divalent cations such as Mg^{2+} and Ca^{2+} have been implicated in the mechanism of action of PZQ and others like Zn^{2+} have been put forward by Mbah and co-workers as one of the metal ions predicted to bind to the G4LZI3 protein (Mbah *et al.*, 2013). Hence, characterizing the relationship or interaction between these cations and a novel Usp G4LZI3 protein, which has been identified as a 'lead' molecule in the design of new drugs against schistosomiasis, is being proposed as an avenue for confronting the problem related to alternative drug discovery or new vaccines against schistosomiasis.

2.17 RESEARCH QUESTION

Mbah and co-workers (2013) postulated that divalent cations such as magnesium, calcium and zinc are important regulators of the ability of the *Schistosoma mansoni* worm to be resistant to Praziquantel. Thus, this study seeks to ascertain if there is any interaction between the novel G4LZI3 Usp protein and divalent cations. If so, what is the strength of interaction between these divalent cations and the novel G4LZI3 protein?

2.18 AIM AND OBJECTIVES OF THE STUDY

The aim of this study is to investigate the putative interaction between divalent cations with the novel G4LZI3 protein using biophysical methods such as isothermal titration calorimetry. This aim will be achieved through the following objectives:

- ❖ Recombinant expression of sufficient quantities of the G4LZI3 protein and the development of a purification strategy in purifying the protein to homogeneity
- ❖ Building of the G4LZI3 protein model using the software PHYRE2
- ❖ Mapping of experimentally-identified amino acid residues onto the surface of the modelled G4LZI3 protein
- ❖ Determination of the strength of interaction (dissociation constant; K_d) between divalent cations and the G4LZI3 protein

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CHAPTER 2: REVIEW ARTICLE

Priscilla Masamba, Abiola Fatimah Adenowo, Babatunji Emmanuel Oyinloye and Abidemi Paul Kappo (2016). Universal Stress Proteins as New Targets for Environmental and Therapeutic Interventions of Schistosomiasis. *International Journal of Environmental Research and Public Health* **13**(972): 1–13.



Review

Universal Stress Proteins as New Targets for Environmental and Therapeutic Interventions of Schistosomiasis

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Abstract: In spite of various control measures and eradication methods that have been in progress, schistosomiasis still prevails as one of the most prevalent debilitating parasitic diseases, typically affecting the poor and the underprivileged that are predominantly concentrated in sub-Saharan Africa. The parasitic schistosome blood fluke responsible for causing the disease completes its complex developmental cycle in two hosts: humans and freshwater snails, where they physically undergo gross modifications to endure the different conditions associated with each host. Just like any other organism, the worm possesses mechanisms that help them respond to environmental insults. It has been hypothesized that a special class of proteins known as Universal Stress Proteins (USPs) are up-regulated during sudden environmental changes, thus assisting the worm to tolerate the unfavourable conditions associated with its developmental cycle. The position of praziquantel as the drug of choice against all schistosome infections has been deemed vulnerable due to mounting concerns over drug pressure and so the need for alternative treatment is now a matter of urgency. Therefore, this review seeks to explore the associations and possible roles of USPs in schistosomiasis as well as the functioning of these proteins in the schistosomulae stage in order to develop new therapeutic interventions against this disease.

Keywords: cercariae; *Schistosoma mansoni*; schistosomiasis; schistosomulae; praziquantel; universal stress proteins

1. Introduction

In comparison to equally virulent diseases such as tuberculosis, malaria and HIV/AIDS, schistosomiasis still persists as one of the most neglected, prevalent and parasitic diseases of poverty [1]. Its high degree of morbidity and consequential disabling effects are still severely underestimated though millions of lives are affected [2]. The disease is endemic in more than 76 countries, affecting more than 200 million people with a mortality rate estimated to be more than 250,000 deaths per annum in sub-Saharan Africa alone [3,4]. Additionally, it has been documented that more than 700 million people are still at risk of infection [3]. Countries such as Venezuela, Brazil, Nigeria and the Democratic Republic of Congo fall under those most hit with the disease burden [5,6].

The disease mostly affects poor rural dwellers who are in constant and regular contact with open water sources and largely dependent on agricultural and irrigation activities. Poverty is one of the key underlying factors that form a vicious “poverty-trap” cycle in developing countries. There is a clear link between schistosomiasis and poverty due to the fact that the disease mostly affects individuals during

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The disease mostly affects poor rural dwellers who are in constant and regular contact with open water sources and largely dependent on agricultural and irrigation activities. Poverty is one of the key underlying factors that form a vicious “poverty-trap” cycle in developing countries. There is a clear link

between schistosomiasis and poverty due to the fact that the disease mostly affects individuals during their most productive years. The effect of schistosomiasis on agricultural productivity, pregnancy outcome, child development and most importantly, the economy has been documented as stimulating poverty, which in turn increases risk of infection [6–8]. Poverty and underdevelopment in areas with limited healthcare resources and personnel compound the problem. With insufficient education, unchanged habits and inadequate water supply from proper sources, re-infection rates still remain high. About 6–13.5 million disability-adjusted life-years (DALYs) of healthy lives are lost globally and therefore reducing transmission, infection and re-infection is quickly becoming a control priority [9].

Schistosoma mansoni, *Schistosoma haematobium* and *Schistosoma japonicum* are the three main species mostly responsible for parasitizing humans while *Schistosoma mekongi* and *Schistosoma intercalatum* are more rare and confined to only a few countries [4,10]. Each of these species has their own geographic pattern: *S. mansoni* infection mostly occurring in sub-Saharan Africa, the Middle East, South America; *S. intercalatum* in Congo, Nigeria, Chad and Central African Republic; *S. haematobium* is mostly prominent in sub-Saharan Africa, North Africa, Middle East, India and the islands of Madagascar and Mauritius; *S. japonicum* is endemic to Asian countries only such as China, Philippines and Indonesia and finally *S. mekongi* is found exclusively in Laos and Cambodia (Figure 1) [11,12]. Japan, Algeria, Morocco and Tunisia have successfully eliminated the disease, while China and Egypt have effectively implemented control programmes, all showing that elimination is a target that can be achieved with the use of preventive chemotherapy and transmission control tools [13]. However, the disease has now spread to regions that were non-endemic before and it is feared the building of dams, development of irrigation systems and global warming could further increase schistosomiasis transmission [1]. The development of water resources have the advantages of increasing crop yields, dealing with water scarcity, generating hydropower and accumulating export earnings, but schistosomiasis easily detects ecological make-overs due to its wide distribution and changing infection rates [14]. Other factors have also complemented the problem such as the presence of intermediate snail species. Urinary schistosomiasis was last detected in Europe in the 1950s. However, the presence of *S. haematobium* freshwater snails such as *Bulinus contortus*, *Bulinus truncatus* and *Planorbis metidjensis* in Portugal, Spain and especially in Corsica France (particularly by the Caviu River) could very well cause the re-emergence of the disease in Europe, [15]. This also substantiates the suggestion that schistosomiasis not only has the ability to persist locally but can also spread from Africa to countries of higher latitudes [16].

Throughout their complex lifecycle (Figure 2), schistosomes face a number of hostile and unfriendly environments, transitioning between intra-mammalian, aquatic and snail stages to develop into full maturity [17]. Despite the harsh conditions they are challenged with, the worms have the ability to withstand all these conditions and evade different defense systems they encounter. Various complications, which have been associated with the disease, then result from eggs produced by females, which become trapped in host tissues. It is with this background that this review suggests that Universal Stress Proteins (USPs) have possible roles in *Schistosoma* ecology and therefore, seeks to explore the associations and possible roles of USPs in schistosomiasis in order to develop new environmental and therapeutic interventions against this prevalent debilitating parasitic disease.

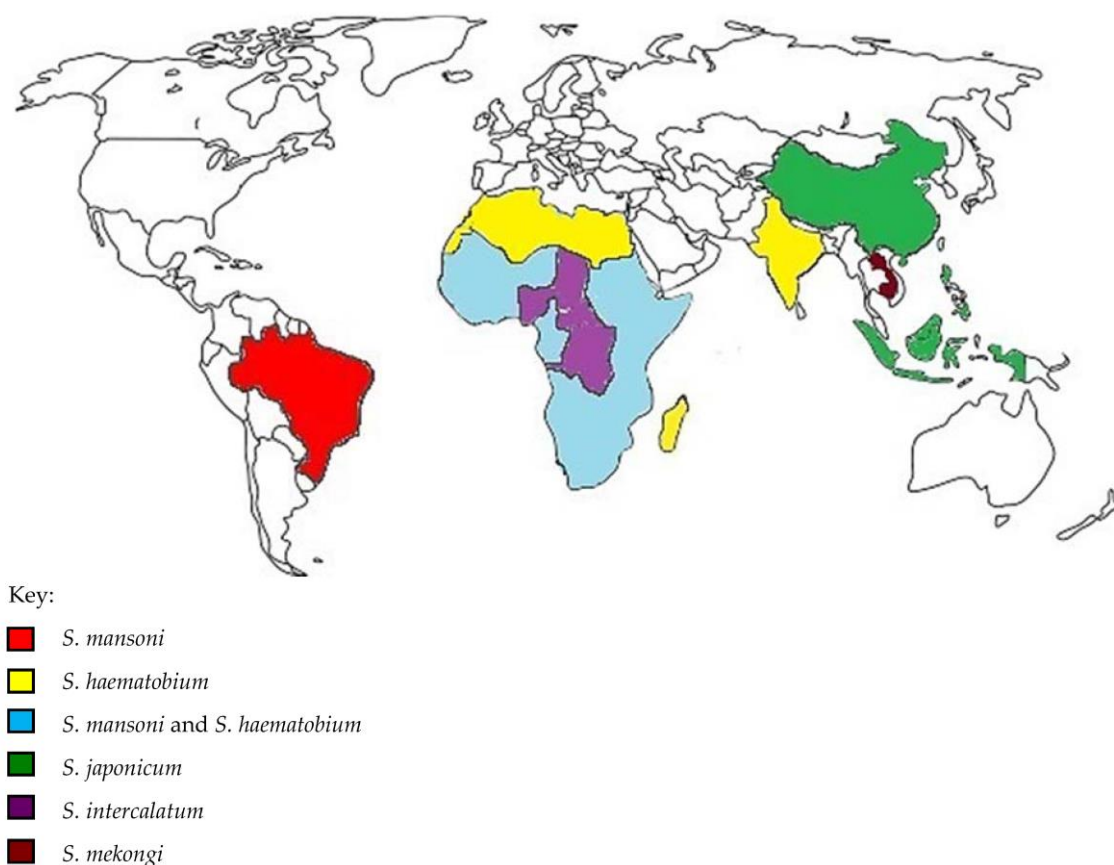


Figure 1. Geographical distribution of the different schistosome species. Each of these species has their own geographic pattern: *Schistosoma mansoni* infection mostly occurring in sub-Saharan Africa, the Middle East, South America; *Schistosoma intercalatum* in Congo, Nigeria, Chad and Central African Republic; *Schistosoma haematobium* is mostly prominent in sub-Saharan Africa, North Africa, Middle East, India and the islands of Madagascar and Mauritius; *Schistosoma japonicum* is endemic to Asian countries only such as China, Philippines and Indonesia and finally *Schistosoma mekongi* is found exclusively in Laos and Cambodia.

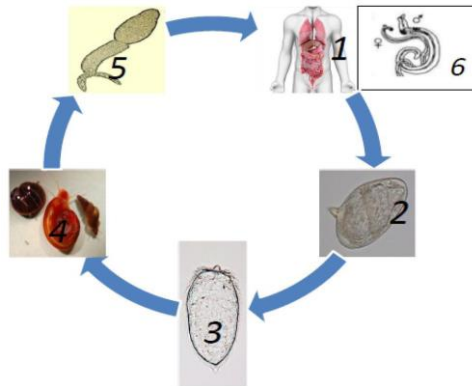


Figure 2. Life cycle of Schistosomes. 1 & 2: Eggs excreted, either in the urine (in the case of *S. haematobium*) and/or faeces (depending on the species) of infected individuals, begin the devastating cycle and eggs may stay viable for up to 7 days [4,10]. Only then do the eggs hatch in fresh water to become 3: miracidia under the most favourable environmental conditions [3]. With the help of light and chemical stimuli, the miracidia burrow the tissue of a 4: freshwater snail. Production of sporocysts and 5: cercariae occur asexually over a period of 4–6 weeks and are released from the snail. The cercariae have forked or bifurcated tails and embryonic suckers and swim around until they come into contact with the human skin [4,18]. Thereafter, they shed their tails whilst simultaneously penetrating the epidermis to develop into larval forms called schistosomulae which remain in the skin for 48 h [3]. They then circulate the hepatic portal system via the bloodstream to mature into 6: adults [19]. The worms then mate 28–35 days after infection, producing about 100–300 eggs per day (female *S. mansoni* and *S. haematobium* female worms) and 500–3500 eggs produced daily by *S. japonicum* worms which are released from the human host via defecation, thereby continuing the destructive cycle [20,21]. At this point, the disease seems species-dependent in that adults from *S. mansoni* and *S. japonicum* dwell in the mesenteric venous plexus causing hepatic and intestinal schistosomiasis; while adults belonging to *S. haematobium* cause urinary schistosomiasis and reside in the perivesical venous plexus [22]. The worms are characterised by having a blind digestive tract, advanced neural, excretory and reproductive organs, two ending suckers and a greyish-white tegument that covers the 7 mm–20 mm body of the worm [3,22]. Females reside in the gynaecophoric canal of the male and together form a nematode-like appearance [5,23].

2. Anti-Schistosomal Drugs

Originally, schistosomiasis control was tackled by elimination of snails until 1984 when chemotherapy was suggested by the WHO Expert Committee [18]. Presently, the likes of praziquantel, oxamniquine and artemisinin are considered as defence systems against the disease. However, oxamniquine does not treat all schistosome infections, its target is only limited to *S. mansoni* adult males, with less action against female and juvenile worms [23]. Use of the drug is mainly restricted to Brazil and South American countries but has been replaced by praziquantel as the principal drug and main line of treatment against schistosomiasis since the mid-2000s [24,25]. Artemisinin is an antimalarial drug which when used with artesunate and artemether combined has been shown to exhibit anti-schistosomal activity against juvenile worms [22]. However, the development of drug resistance in endemic areas against the *Plasmodium* strain has caused a bit of concern.

Discovered in the 1970s, praziquantel (PZQ) is a pyrazinoisoquinole antihelminthic that stands as one of the very few anti-schistosomal drugs available commercially with few known side effects [26]. The drug has been in use for more than 30 years and stands as the most efficient chemotherapeutic drug effective against all forms of schistosome infection. The drug is generally well-tolerated with mild common side effects such as abdominal pains, dizziness, weakness and headache. However, there have been mounting worries over the line of treatment by this drug [27].

As reported by Aragon et al. and several other authors, PZQ is not effective against all stages involved in the life cycle of the worm and fails to kill immature worms 3–4 weeks post-infection [24,28–30]. The drug seems stage-dependent, only effective on 1–2 week old larvae and 5 weeks and older adult worms [23]. Additionally, the mode of action still remains obscure despite decades of research [31]. So far, it has been suggested that antigens on the surface of the parasite become exposed due to disruption of the tegument caused by the drug. Rapid contraction of the muscle occurs (which is also associated with antigen exposure) as well as muscle paralysis accompanied with a rapid influx of calcium ions, a slower influx of sodium ions and a decreased influx of potassium ions [26,31]. All these responses are thought to be linked to the disruption of calcium homeostasis. An unusual variant β subtype Ca^{2+} channel with unusual structural and functional properties that is especially F-sensitive is thought to cause this calcium influx [31].

Most importantly, reports of drug resistance to praziquantel due to drug pressure have now put the health and scientific community on edge. Despite countless treatments made with the drug, the “KCW” isolate found in a patient from Kisumu in Kenya had grown remarkably resistant to praziquantel and even developed a heritable trait that maintained resistance to the drug more than six generations later [32]. A Senegalese isolate also showed less susceptibility to praziquantel as compared to other *S. mansoni* worms from Puerto Rico and Kenya [27]. In Egypt, by the Nile Delta region, reports of *S. mansoni* strains growing resistance to praziquantel were reported after villagers were treated with the drug three times successively 6–8 weeks apart but still continued to shed viable eggs in their stool [33]. These villagers were then revisited and retreated 7–8 years later with the same drug but 4% were still left uncured after as single dose [34]. Apart from field isolates, experimentally induced isolates have also been reported. Coeli et al. observed and reported a 3-fold increase during seven parasite generations amongst mouse models treated with increasing doses of PZQ [35]. Fallon and co-workers were also able to mention 2 isolates in two separate laboratories, where both exhibited PZQ-resistant behavior [27]. More recent experimentally-induced PZQ resistance has been reported by Couto et al. where infected *Biomphalaria glabrata* snails underwent PZQ treatment and subsequent cercariae produced (LE-PZQ isolates) were used to infect mice which also underwent PZQ treatment. Results showed accumulated resistance and reduced susceptibility to PZQ [36].

With this disease still prevalent in many countries and even exhibiting signs of resistance on PZQ, which at the current moment is the most reliable drug, depending on this one line of treatment and the

very few other available therapies is quite unreasonable. Targeting the parasitic lifecycle and its different developmental stages could well provide a lasting solution. However, one needs to also consider the abiotic and biotic stresses associated with them.

3. Parasitic Lifecycle and Stress

The environment undergoes constant change spontaneously and this may have severe effects on the lifecycle of the *Schistosoma* worm. The intermediate hosts are also equipped to defend themselves internally against infective parasitic stages. Such conditions may manifest or be grouped in either of two forms: biotic or abiotic stress.

3.1. Abiotic Stress

The eggs, miracidia and cercariae are exposed to the freshwater environment and have to endure the stress conditions associated with it [17]. The range in water temperature has important bearing on the hatching of eggs and shedding of cercariae. Warming of water may decrease or increase the number of snails, especially in endemic areas, which in turn has an effect on snails being infected with cercariae [37]. Eggs hatch between 10 °C and 30 °C even though temperatures vary between 30 °C and 38 °C in a period of 17 days based on the time miracidia first penetrate snail tissue and snails release cercariae [38]. Hatching of the eggs is also dependent on osmotic pressure and light. The speed and movement of water, either rapidly moving or standing water can also affect the ability of miracidia to effectively locate and infect snails and it has been reported that free-flowing water increases the chances of miracidia to come into contact with their intermediate snail hosts [39].

3.2. Biotic Stress

Miracidia penetrate the freshwater *Biomphalaria* intermediate host, usually at the base of its antennae and cephalopodal mass to mature into primary sporocysts, later spawning into secondary sporocysts that migrate from the muscles of the cephalopodal region to the digestive glands or hepatopancreas of the snail [40]. From there, the structure changes to form cercariae. The internal defence system (IDS) of the snail consists of hemocytes, which are responsible for killing these larval stages [41]. Additionally, in the hemolymph are soluble elements that produce toxic substances that recognize the parasite as well as activate the hemocytes [41]. Resistance or susceptibility of the parasite to the snail host is mostly controlled by genetic factors [42]. However, new evidence has suggested that snail susceptibility or resistance for schistosome infection may also depend on epigenetic factors. It has already been previously established that indicators of stress such as Hsp70 and Hsp90 proteins indicate stress levels in susceptible (NMRI) rather than in resistant (BS-90) snails. However, four generations of BS-90 resistant snails grown and maintained at a mild heat shock temperature of 32 °C lost their resistance when returned to room temperature (25 °C), shedding cercariae 4 weeks after exposure to the environment [16]. More so, the snails exhibited greater expression of Hsp70 protein. Additionally, the parasites also need to be compatible with the snails they encounter. Compatibility is heritable, either for the resistant or susceptible snail or in the parasite's ability to infect an intermediate snail host. It may also be strain specific in that snails susceptible to a particular schistosome strain might not be so susceptible to another strain [43]. Theron and co-workers were able to show that among five *B. glabrata* and five *S. mansoni* strains, some snails had greater ability in recognizing parasites and producing ROS thus having a more efficient and effective defence system [43]. Other biotic factors directly or indirectly having an impact on these schistosomal stages include natural predators like invertebrates and fish which may reduce miracidia population by feeding on them [38].

The human host also has defence systems set in place to protect it from foreign harmful agents. Cercariae penetrate the skin of a human host, simultaneously shedding their tails to transform into

schistosomulae. An antibody-dependent cell-mediated cytotoxic (ADCC) response is responsible for eliminating this parasitic stage by the production of reactive oxygen species such as superoxide radical anion and hydroxyl radicals [44]. Additionally, the release of cytotoxic proteins to produce nitric oxide may also contribute to the removal of schistosomulae [44]. Infection with schistosomiasis stimulates a T_H1 (which is the dominant response) and T_H2 response which provides immunity against the parasite and this has been shown based on experimental mouse models. The T_H1 response is up-regulated during the migration of immature worms during the first few weeks of infection and later down-regulates to allow for the expression of the T_H2 response during mating and egg production [45]. Cytokines with immunoregulatory functions like IL-10 are generated after cercariae exposure along with other pro-inflammatory cytokines like IL-12, IL-1 β , MIP1 α , IL-6 and TNF α [46]. Responses are more T_H2 -associated in the event of re-infection. IgG4 has been shown to act as an antibody by blocking IgE action thereby decreasing susceptibility to re-infection while in contrast; IgE receptors have also been associated with protecting the host against re-infection [47].

In short, major morphological transitions have to take place between the three different and distinct environments. The miracidium larval stage has to tolerate freshwater aquatic environments. The sporocysts, parasites of the intermediate snail host, reside in a freshwater environment while the cercariae habituate an aquatic environment before finding a human host. Finally, the parasite develops in a different setting of a well-defended warm-blooded mammalian host to develop into two different sexual stages to produce eggs and continue the cycle.

Therefore, the question to be asked is this: how then does the schistosome parasite have the ability to withstand the inimical conditions associated with each hostile environment? Mbah and co-workers suggested that amid other cellular and molecular mechanisms that may also have an input in their survival, the presence of Universal Stress Proteins (USPs) could be an important factor or an interesting drug target that could be developed for therapeutic interventions to take place in the developmental cycle of the parasite [17].

4. Universal Stress Proteins, Mode of Action and Factors that Induce and Regulate Their Activity

An environment undergoes constant change, which may either be good, bad, sudden or seasonal. Organisms should therefore be equipped with tactics to survive and respond to these environmental changes. Environmental stress may appear in various forms, which can result in the denaturation of proteins of an organism eventually leading to severe harm or even death [17]. Various molecular and response mechanisms may be responsible for this quality in many organisms such as the expression of Heat shock proteins (HSPs). It has been suggested that in schistosomes, the up-regulation of a special class of proteins could enable them to withstand the different and mostly unfavourable environments and different hosts the parasite occupies during their complex developmental cycle called Universal Stress Proteins (USPs) [17].

USPs have been shown to be present among a diverse group of organisms such as bacteria, protists, fungi and plants (Table 1) [48-59], assisting them to tolerate different environmental and adverse conditions such as high salinity, high temperatures, drought and toxic chemicals [49]. Originally, universal stress protein A was first discovered in *Escherichia coli* but lately, genes encoding proteins with the USP domain (Pfam Identifier: PF00582) have recently been shown to be present in the *S. mansoni*, *S. japonicum* and *S. haematobium* species [17,49,60]. This protein is a member of what is known as the “universal stress protein (USP) domain” family, which is a conserved domain, constitutes about 140–160 residues [50]. Foret and co-workers also identified and reported the presence of USPs in metazoans based on EST data encoding sets (Figure 3) [49]. However, many of the gene families have been lost during animal evolution suggesting that either their roles or functions are no longer needed or

they have evolved to suit different and specific animals [49]. Majority of the genes are small single domain proteins with some considered ATP-binding.

Table 1. Universal stress proteins (USPs) found in different living organisms.

Organism/Kingdom	Specie	USP Name	ATP-Binding Potential	Reference
Bacteria	<i>Escherichia coli</i>	UspA	Non ATP-binding	[48,49]
		UspC, UspD	ATP-binding?	
		UspE, UspF, UspG	ATP-binding	
	<i>Heamophilus influenza</i>	H10815	Non ATP-binding	[50]
	<i>Mycobacterium tuberculosis</i>	RV2623	ATP-binding	[51]
	<i>Nitrosomonas europea</i>	NE1028	Non ATP-binding	[52]
	<i>Salmonella typhimurium</i>	YdaA	Assumed to bind ATP	[53]
		YnaF	ATP-binding	
	<i>Thermos thermophiles</i>	TTHA0350	ATP-binding	[54]
	<i>Halomonas elongate</i>	HELO1754	ATP-binding	[55]
Archaea	<i>Methanococcus jannaschii</i>	MJ0577	ATP-binding	[56]
	<i>Archeaoglobus fulgidus</i>	AF0836	Non ATP-binding	[52]
Plants	<i>Solanum pennellii</i>	SpUSP	ATP-binding region	[57]
	<i>Grosspyiumarbo-retum</i>	GUSP ₁ GUSP ₂	Non ATP-binding	[58]
	<i>Arabidopsis thaliana</i>	At3g01520	Suggested to be ATP-binding	[59]
Schistosomes	<i>S. japonicum</i>	Q86DX1, Q5DDH7 Q5DED2, Q5DHK1, Q86DW2, Q5DG19, Q5DH64, Q5D136	ATP-binding	[17]
	<i>S. mansoni</i>	G4V552, G4VPM6, G4LZI3, C1MOQ2, G4VIW9	ATP-binding	[17]

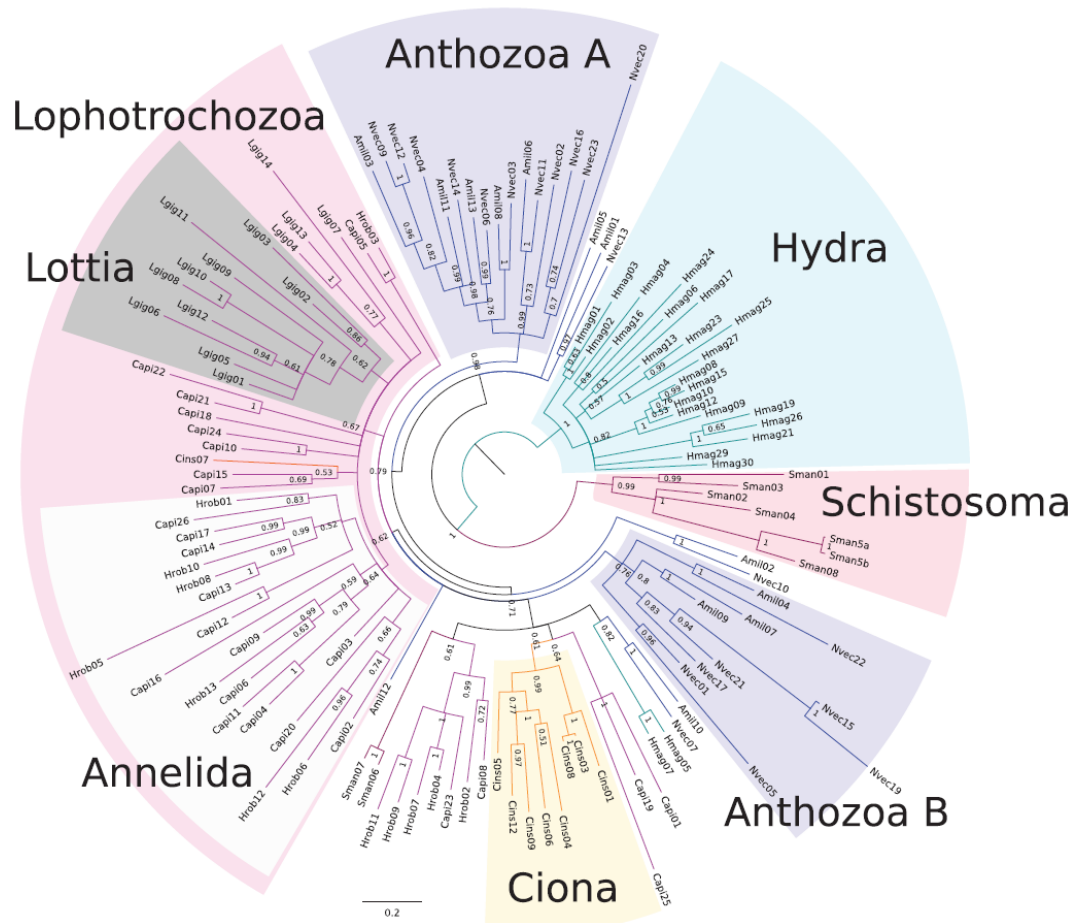


Figure 3. Phylogenetic analysis of USP sequences from animals using maximum likelihood and Bayesian methods. The figure was taken from Foret et al. [49].

USPs may exist as small proteins (~14–15 kDa) with a single domain, as larger proteins (~30 kDa) also with a single domain or in a combination with one or two USP domains having functional domains that include, voltage-gated channels, protein kinase domains, antiporters and amino acid permeases [60]. Several duplicates of USPs may exist in certain organisms such as the six Usps present in *Escherichia coli* (UspA, UspC (*yecG*), UspD (*yjiT*), UspE (*ydaA*), UspF (*ynaF*) and UspG (*ybdQ*)) and they may also be encoded by different gene families [52,58]. There are 8 known *S. mansoni* USP genes: (Smp_001000, Smp_001010, Smp_031300, Smp_043120, Smp_076400, Smp_097930, Smp_136870 and Smp_136890) and all have shown conserved sites for Aspartate, Leucine, Glycine, Histidine and Proline which seem to be common functional sites for regulating these USPs [60]. USP proteins are generally categorized into two main groups based on the presence or absence of an ATP binding motif G-2x-G-9x-C(S/T) (which is a molecular mechanism responsible for regulating their functions) [17]. UspAs and those categorized as UspA-like proteins do not bind ATP while UspFGs bind ATP [48]. All 8 *S. mansoni* USP genes contain this motif [60]. ATP binding up-regulates the protein by acting as a donor phosphate and together with guanosine triphosphate (GTP), phosphorylates USPs onto serine, activating the protein and showing that other cellular factors such as ATP contribute to the activity of the protein through phosphorylation. It has been suggested that Usp proteins that bind ATP are part of the ancient HUP (HIGH-signature proteins, USP-like domains and PP-ATPases) superfamily whose domains contain an α/β core and characterized by 5-stranded parallel β sheets and 4 α helices [53,54].

Another contributing factor is the predicted presence of metallic cations such as magnesium, calcium and zinc. It has been suggested that by binding to the Mg-ATP-binding groove during the phosphorylation process or any response to stress that is dependent on ATP, Mg^{2+} could play a vital role in protein functional efficiency [17]. The role of calcium in certain developmental stages and its association with the mechanism of action of PZQ has been suggested while work is ongoing to establish the transcriptional response of *Schistosoma* USP genes to PZQ which could eventually lead to new schistosome control interventions [17].

5. Role of USPs in Disease Progression

Jolly and co-workers reported the up-regulation of 1154 genes with gene expression transitions observed between the sporocysts, cercariae and adult stages of *S. mansoni* [61]. Out of these genes, 406 of them are said to currently have unknown functions. Factors like genes involved in protein synthesis (such as heat shock genes), apoptosis, ubiquitination, and proteolysis in the sporocyst stage, ATP-producing and utilizing genes in the cercariae as well as stress-response genes in adults have been observed in these stages [61].

As reported by Mbah et al. USPs has actually been observed in different stages of the parasite as it transitions through different environments. The *S. mansoni* G4LZI3 USP has been shown to be expressed in the sporocyst, miracidium and schistosomula stages while the C1M0Q2 USP from the same species is expressed in adult schistosomes [17].

However, we propose that the schistosomula stage is most beneficial for therapeutic interventions using universal stress proteins. This is because up-regulation of these proteins has been observed in this stage, both in *S. mansoni* and *S. japonicum* worms. More so, at this stage, there is a transition from an aquatic environment to a mammalian environment, accompanied with a change in temperature. The parasite has to have the ability to interact with an external environment and also have the skill to adapt to an internal environment. Interestingly, these proteins have not been observed in humans thus making them an interesting and attractive drug target [17]. Secondly, once the cercariae penetrate the human skin to transform into schistosomulae, there should be an adaptation from aerobic surroundings to an anaerobic location. Furthermore, praziquantel has no effect on young schistosomes aged 3–4 weeks post

infection but is active against sexually mature adults (6–7 weeks post infection), miracidia and cercariae [31].

Lastly, the UspA family with UspD has been shown in *E. coli* to act as defence mechanisms against superoxide-generating agents [62]. Reactive oxygen species are usually produced under anaerobic conditions and have the potential to oxidize proteins, lipids and nucleic acids, which may eventually lead to enzyme inactivation and genome damage [63]. Nitric oxide and hydrogen peroxide are produced in the mammalian host with the intent of defending against these larval forms by killing them [17]. Under normal circumstances, schistosomulae are more susceptible to oxidative stress and are killed once exposed. However, we suggest that by the action of USPs and through evidence of expression patterns, this parasitic stage escapes or avoids this defence mechanism and goes on to develop into adults, which are able to produce SOD, and GPx antioxidant enzymes that are not found in the miracidia, sporocysts or cercariae [63].

6. Recent Advances in Novel Promising Schistosomicides

Because of the risks involved in the continued dependence on praziquantel as the sole chemotherapeutic drug for schistosomiasis, suggestions have been given on what can be used or developed into alternative and promising anti-schistosomal drugs. Thioredoxin reductase and glutathione reductase (which are mammalian detoxifying enzymes), phosphinic amides and oxadiazoles are all enzymes that are proposed to have an effect on schistosomes as inhibitors of the thioredoxin-glutathione reductase schistosome enzyme [24]. Although they are expensive and their mechanism of action is unknown, cyclosporins could also be considered as a new drug alternative due to their ability to kill juvenile schistosome worms as well as the ability to prevent diseases or infections [24]. Cysteine proteases have been considered as chemotherapeutic targets after their activity was shown to be closely linked with hemoglobin digestion and their inhibition shown to disrupt schistosomulae and also reduce egg and worm burden [64]. To avoid toxicity that could result from the use of anti-helminthics, medicinal plants such as garlic, tobacco, pineapple, wormwood and walnut have been suggested as natural anti-helminthic sources [65]. Ali gave an account of different examples of natural products that have promising anti-schistosomal properties [66]. Recently, antimicrobial peptides (AMPs) have come onto the scene as promising alternative anti-schistosomal tools due to their multifunctional properties. It has been suggested that AMPs may play a role against schistosomiasis by acting as immunostimulators, ROS scavengers or through elimination of schistosomulae by selective cytotoxicity [23].

Taken together, since up-regulation of USPs has been detected at the schistosomula stage, and via a variety of mechanisms; the up-regulation in USPs assists the schistosomula to tolerate different environmental and adverse conditions, we propose that selective identification of small molecule inhibitors or peptides as an inhibitor of USPs at this stage could provide a means of targeting schistosomiasis and preventing re-infection of the disease. More research however, has to take place due to the minimal literature on universal stress proteins in schistosomes. However, unveiling of the *S. mansoni*, *S. haematobium* and *S. japonicum* genomes as well as the recent work on the discovery of universal stress proteins in schistosomes could well unfold new opportunities in vaccine development and alternative anti-schistosomal drugs as well as the development of new diagnostic tools.

7. Conclusions

In most developing countries, the vicious cycle of poverty and daunting existence of detrimental parasitic infections such as schistosomiasis are some of their biggest challenges. Therefore, addressing poverty should be at the centre of elimination strategies. Intensifying already-in-place interventions such as improving sanitation and health facilities, providing clean and safe water sources, use of molluscicides,

as well as continuing with mass treatments using chemotherapeutic drugs are necessary and critical steps to attaining this goal. Praziquantel has been successful so far in decreasing morbidity in endemic areas but mortality rates still soar high and the disease claim thousands of lives every year. Because of its shortcomings, other anti-schistosomal drugs and discovery of vaccines have to be developed as soon as possible. The presence of universal stress proteins in schistosomes presents a very interesting avenue for tackling the problem. Thus, it is believed that future research integrated with understanding social behavior and targeting poverty could abolish this dreadful disease and provide more permanent solutions.

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CHAPTER 3:

MATERIALS AND METHODS

3.1 Introduction

Parasitic trematodes, belonging to the *Schistosoma* genus, are responsible for schistosomiasis which is a poverty-related infection of public human health significance. Infective cercariae in open freshwater bodies released from infected snail hosts actively penetrate exposed human skin where eggs are produced by mature worms (Trainor-Moss and Mutapi, 2016). These are excreted along with faeces and urine while others are distributed throughout the body causing clinical and pathological disease (Christinet *et al.*, 2016). However the primary cause and majority of pathology results from granulomatous reactions responsible for killing eggs but which simultaneously result in widespread fibrosis of host tissue (Driguez *et al.*, 2016; Gobert *et al.*, 2015). Praziquantel (PZQ), which is a broad spectrum anthelmintic drug, has dominated most schistosome control endeavours because of its effectiveness, affordability and safety. Nevertheless it is unsuccessful in correcting or reversing tissue fibrosis complications, ineffective against immature schistosome worms and its mode of action is still not yet fully understood (Driguez *et al.*, 2016; Weerakoon *et al.*, 2016). Additionally, extensive use of the drug for over three decades could result in drug resistance which has already started to emerge. Other efforts in combination with PZQ have also failed to control, prevent and/or eliminate the schistosomiasis global disease burden. Thus, alternative treatment is currently on the lookout. It has been suggested that the discovery of a vaccine could be a means towards a more permanent solution to this problem as it would control the disease, reduce morbidity and provide long-term immunity especially for young children who live in endemic areas (Siddiqui *et al.*, 2011). This project focused on characterizing the novel G4LZI3 Universal Stress Protein (USPs) which has been

proposed as a candidate vaccine and also looked at its interaction with divalent cations as they have been shown to play a role in PZQ mechanism of action. It has been documented that the up-regulation of this protein in schistosomes during its lifecycle renders it 'immune' to environmental and host stress. Hence, the aim of this project was achieved by expressing, purifying and determining the dissociation constant using ITC. However, bioinformatics studies were first done with the use of both on and offline tools for the analysis of DNA and protein sequences for model building as well as for docking. This chapter focuses on the dry- and wet-lab strategies used to achieve the aim of this study.

3.2 *In silico* Studies/Bioinformatics Analyses of the G4LZI3 Protein

3.2.1 *Acquisition of the Physicochemical Properties of the G4LZI3 Protein*

The nucleotide database of the National Centre for Biotechnology Information (NCBI) was consulted and the mRNA sequence comprising exons and excluding introns was selected. The gene sequence was then translated into an amino acid sequence online using the ExPASy (**Ex**pert **P**rotein **A**nalysis **S**ystem) translate tool at www.expasy.org. The biochemical and biophysical properties of the protein were then first and foremost obtained so as to have insight into the primary structure and function of the protein. The ProtParam tool was used to obtain these results via the ExPASy server using the following url: <http://web.expasy.org/cgi-bin/protparam/protparam>. These properties include the amino acid composition, estimated half-life, theoretical pI, instability index as well as the grand average of hydropathicity (GRAVY). These results are shown in Appendix II.

3.2.2 Multiple Sequence Alignment of G4LZI3 Homologues

Multiple sequence alignment was done between G4LZI3 and its homologues to identify similar regions caused by any evolutionary, structural or functional relationship that could exist between the different sequences. The online BLAST (**B**asic **L**ocal **A**lignment **S**earch **T**ool) tool (<http://web.expasy.org/blast/>) via the ExPASy server was consulted and a BLAST search was run to acquire similar sequences in the NCBI database by using the G4LZI3 sequence as the query. Sequences apart from schistosome sequences were then selected, copied in FASTA format and aligned using a high speed multiple sequence alignment programme called MAFFT (**M**ultiple **A**lignment using **F**ast **F**ourier **T**ransform) which can be accessed using the following url: <http://www.ebi.ac.uk/Tools/msa/mafft/> (Kato and Standley, 2013). The selected sequences and their corresponding accession numbers are tabulated in Appendix III. To produce a phylogenetic tree for phylogenetic analysis, an online server called ClustalW was used (<http://www.genome.jp/tools/clustalw/>; Larkin *et al.*, 2007). Using a sequence analysis program Bioedit Version 7.2.5 (Hall, 1999), the sequences were aligned and used to construct a conservation table highlighting the percentage of identities and similarities between organisms by pairwise alignment. This table is available in Appendix III.

3.2.3 Secondary Structure Prediction and Homology Modelling

The amino acid sequence of the G4LZI3 protein was used to predict its secondary structure by using Phyre2 (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>; Kelly *et al.*, 2015) and Psipred (<http://bioinf.cs.ucl.ac.uk/psipred/>; Buchan *et al.*, 2013). Thereafter, Phyre2 was used to build a 3D structural model and this was visualized

using PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC and Chimera, Version 1.11.2 (Pettersen *et al.*, 2004). A Ramachandra Plot (used to evaluate violation of stereochemical parameters) was then generated by logging onto the RAMPAGE server (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>), which classifies and views residues in their respective energy levels based on phi-psi torsion angles, thus evaluating the quality of predicted models (Mbah, 2013.) Thereafter, an online search tool called MOTIF Search (<http://www.genome.jp/tools/motif/>) was explored to quickly ascertain which motifs are present in the protein. The protein sequence was simply copied onto the server in FASTA Format, submitted and results were generated a few seconds later.

3.2.4 Protein-Ligand Molecular Docking

To perform docking, the 2D-structures of Ca^{2+} , Mg^{2+} and Zn^{2+} were first retrieved from Protein Data bank and converted into pdb files using a programme called OpenBabel. Thereafter, these pdb files together with the 3D model in pdb format from Phyre were used as input files in an online docking software called Patchdock. The output files were then sent to the user email and were then downloaded, viewed and edited using both Chimera and PYMOL.

3.2.5 Mapping of Predicted Interactors of the G4LZI3 Protein

Search Tool for the Retrieval of Interacting Genes/Proteins (STRING 10) database (<http://string-db.org/>) was used to predict the interacting partners of the G4LZI3 protein. The amino acid sequence was loaded onto the online server and the organism selected. Nodes and edges were then displayed once the network was constructed and set at a

medium confidence of 0.4 with a maximum of 10 interactors. The colour, thickness and line shape indicate evidence of interaction, strength of data support and the predicted mode of action respectively. Lastly, an open web-based tool called Galaxy was used to predict other different interacting partners with the G4LZI3 protein which include ATP, AMP and U20. These models were viewed with Pymol and Chimera and revealed the possible binding pockets as well as the ligands they bind to.

3.3 General Stock Solutions, Buffers and Media

2X Sample Buffer: 1ml 1M Tris-HCL, pH6.8, 4ml 10% SDS, 2ml glycerol, 2.5ml β -mercaptoethanol/DTT, 500 μ l 1% Bromophenol blue. This was made up to 10ml with water.

5X Running Buffer: 15g/l Tris, 72g/l Glycine and 5g/l SDS.

Acrylamide/Bis: 29.2g acrylamide, 0.8g N,N-methylene-bis-acrylamide in 100ml of deionised water. The solution was filter sterilized and stored in a dark bottle at 4°C.

Agarose: 12g/l agarose in 1L TAE buffer.

Ammonium persulfate: 1g ammonium persulphate powder was dissolved in 10ml distilled water for a 10% stock solution. This was aliquoted into 2ml eppendorf tubes and stored in -20°C.

Ampicillin: 100mg/ml stock solution was made by dissolving 1g in 10ml water.

Cell Lysis Buffer: 50mM Tris-HCL, 300mM NaCl and 10mM Imidazole (pH 7-8).

DTT: 1.5g DTT was dissolved in 8ml deionised water and the volume was adjusted to 10ml to obtain a 1M stock solution. This was then aliquoted into 2ml eppendorf tubes and stored in -80°C.

Elution Buffer: 50mM Tris (pH 8) and 300mM Imidazole.

Equilibration/Wash Buffer: 50mM Tris (pH 8) and 40mM Imidazole.

IPTG: 1M stock solution was made by dissolving 2.38g IPTG in 8ml of distilled water and topped up to a final volume of 10ml. The solution was filter sterilized, aliquoted and stored in -80°C.

Luria Agar: 16g/l Tryptone powder, 10g/l Yeast extract, 5g/l NaCl and 17g/l Bacterial agar.

Luria Broth (2x YT): 16g/l Tryptone powder, 10g/l Yeast extract, 5g/l NaCl and 4g/l Glucose.

Lysozyme: 0.01g lysozyme was dissolved in 10ml distilled water to make a 1mg/ml stock solution.

Lysis Buffer: 50mM Tris, 100mM NaCl and 10mM Imidazole.

PBS: 8g NaCl, 0.2g KCl, 1.44g Na₂HPO₄, 0.24g KH₂PO₄. These were all diluted in 800ml of deionised water. The pH was then checked and adjusted to 7.4 then topped up with distilled water to 1L.

PMSF: 100mM stock solution made by dissolving 17.4 mg of PMSF to 1ml of isopropanol.

Staining Solution: 1g Coomassie Blue, 50% Methanol, 10% Glacial acetic acid, 40%distilled water.

Sodium Azide: 10% stock solution was made by dissolving 10g NaN₃ in 100ml distilled water.

Tris Buffered Saline (TBST): 50mM Tris, 150mM NaCl and adjusted to pH7.5. 1ml Tween 20 was added in 1L TBS to make TBST.

Tris for SDS PaGE: 1.5M Tris (pH8.8) [running/separating gel], 0.5M Tris (pH6.8) [stacking gel].

Western Blot Transfer Buffer: 25mM Tris, 192mM Glycine and 20% Methanol. This buffer was always made fresh and cool.

3.4 Bacterial Strains Used

1. *E. coli* BL21 (DE3) (Promega) pLysS strain: B F⁻ *ompT gal dcm lon hsdS_B(r_B⁻m_B⁻)* λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*⁺]_{K-12}(λ^S) pLysS[T7p20 *ori_{p15A}*](Cm^R).
2. *E. coli* JM109 (Sigma) strain: *endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB*⁺ Δ(*lac-proAB*) e14- [F' *traD36 proAB*⁺ *lacI*^q *lacZ*ΔM15] *hsdR17*(r_K⁻m_K⁺).
3. *E. coli* XL1-Blue (Stratagene) strain: *endA1 gyrA96*(nal^R) *thi-1 recA1 relA1 lac glnV44* F' [::Tn10 *proAB*⁺ *lacI*^q Δ(*lacZ*)M15] *hsdR17*(r_K⁻ m_K⁺).

3.5 Preparation of Competent Cells

Agar plates without antibiotics were streaked with glycerol stocks of competent cells and allowed to grow overnight at 37°C. The next morning, colonies were picked and inoculated in 5ml 2xYT broth with ampicillin and were left to incubate overnight with

shaking at 37°C. The overnight culture was then diluted the next morning with 50ml YT broth with ampicillin and left to grow with shaking at the same temperature till the early log phase (OD_{600} 0.4–0.6). The cells were then harvested by centrifugation at 5000rpm for 10mins at 4°C and the supernatant discarded. The cells were then kept on ice from this point forward and re-suspended in 10ml sterile ice cold 0.1M $MgCl_2$. This was left on ice for 30mins and then centrifuged for 10mins at 4000rpm and supernatant discarded. The pellets were again re-suspended in 10ml sterile ice cold 0.1M $CaCl_2$ followed by incubation on ice for 4–5hrs. The mixture was then centrifuged at 4000rpm for 10mins and the pellet re-suspended in equal volumes of 3ml sterile ice cold 30% glycerol and 3ml 0.1M $CaCl_2$. The cells were then aliquoted into pre-chilled microcentrifuge tubes and stored at -80°C for later use.

3.6 Transformation of pQE30-G4LZI3 constructs

Competent cells were allowed to thaw on ice and 2 μ l of the pQE30-G4LZI3 construct (purchased from GenScript) [Figure 3.1] was added to 100 μ l of competent cells in sterile microcentrifuge tubes. The tubes were placed on ice for 15mins then heat shocked for 45s at 42°C. Thereafter, the tubes were placed back on ice for 5mins. A 900 μ l of warm 2XYT broth was added to the cells and mixed by gently inverting the tubes a few times. The tubes were then incubated at 37°C with shaking for 1h30–2hrs and 100 μ l of the mixture was spread out on ampicillin-agar plates. The remaining 900 μ l was centrifuged for 2mins at high speed and 800 μ l of the supernatant discarded. The remaining 100 μ l was mixed gently with the pellet, then spread out on agar plates containing ampicillin. The plates were then allowed to grow overnight at 37°C.

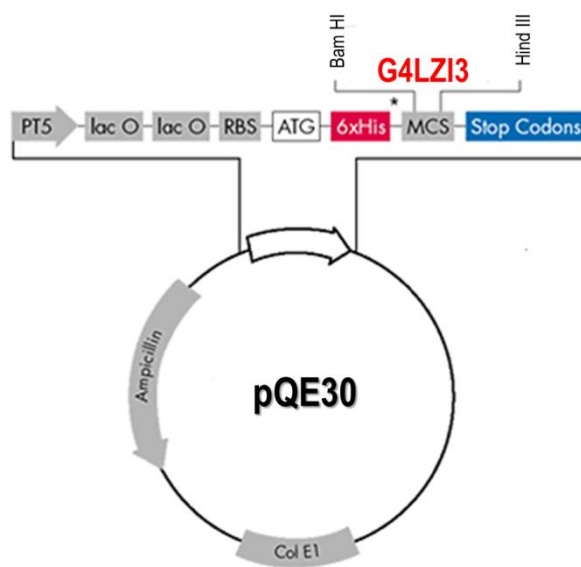


Figure 3.1: pQE30 map. The G4LZI3 construct was cloned into a pQE30 vector.

3.7 Mini-preparation of Plasmid DNA

Mini-preparation (miniprep) of the construct was done so as to extract, purify and create larger amounts of plasmid DNA for subsequent use. This was done using the Zippy Plasmid Miniprep kit (Zymo Research), following the manufacturer instructions. Colonies from transformed *E. coli* XL1Blue competent cells were picked and inoculated in 5ml 2X YT broth with ampicillin and left to grow overnight with shaking at 37°C. Out of the bacterial culture 600µl was taken out and placed in a microcentrifuge tube which was centrifuged for 30s at maximum speed. The supernatant was then discarded and the pellet was completely re-suspended in 600µl TE buffer/water. A 100µl volume of 7X Lysis buffer was then added and mixed by inverting the tube 4–6 times. The colour then changed from opaque to clear blue indicating complete lysis. Thereafter, 350µl of cold Neutralising Buffer was mixed thoroughly with the sample which then turned yellow and formed a yellow precipitate. This was centrifuged for 2–4 mins at 11 000–16 000xg. The supernatant (~900µl) was then transferred into the provided Zymo Spin™ IIN column, taking great care not to disturb the cell debris pellet. The column was then placed into a collection tube and centrifuged for 15s. The flow-through was discarded and the column placed back into the same collection tube. This was followed by the addition of 200µl of Endo Wash Buffer to the column and this was centrifuged again for 30s. After that, 400µl Zippy™ Wash Buffer was added to the column and centrifuged for 1min. The column was then transferred to a clean and sterile microcentrifuge tube and 30µl Zippy™ elution buffer was added directly to the column matrix and allowed to stand for 1min at room temperature. This was then centrifuged for 30s to elute the plasmid DNA.

3.8 Expression Screening

Four colonies were picked out randomly from transformed JM109 plates. Each colony was incubated in 5ml YT broth with antibiotics (ampicillin) and left to grow at 37°C with shaking for 4hrs. Once the OD was between 0.4–0.6, 1ml of the bacterial culture was taken out and induced with 0.5mM IPTG. An additional 1ml of the bacterial culture was also taken out and served as the un-induced sample. The remaining 3ml was made into glycerol stock and stored at -80°C for future use. The two samples were incubated at the same temperature with shaking for 2hrs and cells harvested by centrifugation for 5mins at the highest speed. The pellets were then re-suspended in 50µl 2X sample buffer and the mixture heated for 10-15mins at 95°C. Thereafter, the sample was centrifuged again at top speed for 20-30mins and 20µl of the sample was immediately analysed on a 15% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis. The SDS PAGE gel system was set up is shown in Table 3.1 below.

Table 3.1: Depicting the composition of a 15% SDS PAGE gel.

	Running/Separating Gel	Stacking Gel
Deionised Water	2ml	3ml
1.5M Tris pH8.8	2ml	-
0.5M Tris pH6.8	-	630µl
30% Acrylamide/Bis	4.2ml	830µl
20% SDS	342µl	50µl
10% APS	150µl	50µl
Temed	16µl	5µl

3.9 Large Scale Protein Expression

The best expressing clone from the expression screen, based on the results from the SDS-PAGE gel, was used to induce large scale protein expression. A 100µl volume of the best expressing clone was inoculated in 100ml 2X YT broth with ampicillin overnight with shaking at 37°C. The next morning, this was scaled up to 2L with 2X YT broth and left to incubate at the same temperature till the early log phase (OD₆₀₀ 0.4–0.6). Once the OD was reached, the culture was then induced with 0.5mM IPTG, the temperature was reduced to 25°C and left to grow overnight. The next morning, the cells were harvested by centrifugation and the pellets re-suspended in lysis buffer. These were then left at -80°C until needed.

3.10 Western Blot Analysis

This was done to confirm or detect the presence of the protein of interest using antibodies. Western transfer buffer was poured into the bath and nitrocellulose membrane and filter paper were chilled in the buffer. Nitrocellulose paper was then placed on a piece of filter paper using a pair of forceps. Thereafter, a freshly-ran SDS-PAGE gel was placed on the membrane and another filter paper placed on top. This was then set to run at 100V at 1.5Amp for 10mins. The membrane was then removed from the filter paper and placed in a container. Ponceau staining solution was added and allowed to sit for a few minutes. The membrane was then washed 2–3 times with TBS-T (Tris buffered saline with Tween20) for about 5mins each time until the stain disappeared. Milk solution (5g milk + 100ml TBS-T) was then added to the container until the membrane was covered and was left to incubate on a shaker at 4°C overnight.

The next morning, the milk was discarded and anti-His antibody (5 μ l of anti-His in 10ml of milk) was poured onto the membrane. This was then left to shake in 4°C for an additional 1–2hrs. The membrane was then washed 3 times with TBS-T for 5mins at a time and then viewed under a CHEMI-DOC visualization system.

3.11 Extraction and Purification of the G4LZI3 Protein

Total bacterial cell lysates from the large scale expression stored at -80°C were taken out and subjected to 3–5 cycles of freeze-thawing and sonication (4mins, 30% Amp, 15–20 pulse). Thereafter, the lysates were centrifuged at 5000rpm for 20mins at 4°C until a clear supernatant was obtained. A Nickel-NTA column was used for purification. HisSelect Nickel-linked agarose beads were first packed onto a plastic column and the storage ethanol was allowed to flow by gravity. The beads were then washed and re-suspended in deionized water and left at 4°C overnight to swell and pack properly. The next morning, the beads were equilibrated using equilibration buffer for a number of times. The supernatant was then poured down the column and allowed to flow by gravity. The flow-through was collected and 20 μ L taken for analysis. The protein was then eluted with 7 column volumes of elution buffer and the fractions collected separately. A 20 μ L sample was taken at each stage of the purification for SDS-PAGE analysis. Thereafter, 30% ethanol was added to the beads which were stored in 20°C. All the 20 μ L samples underwent SDS-PAGE analysis while 1M DTT and 0.02% of 10% sodium azide was added to the flow-through and elution fractions.

3.12 Isothermal Titration Calorimetry (ITC)

Prior to ITC, all the purified fractions were pooled together, poured into a piece of dialysis tubing and clipped down on both sides. The protein was then dialysed overnight in 50mM Tris buffer at 4°C so as to dialyse Imidazole from the protein. The protein was then concentrated down to 5ml using Zymospin concentrators at a speed of 4000Xg for 45mins apiece. The concentration of the protein was then checked using a NanoDrop ND2000 and thoroughly degassed using a dessicator chamber with an ordinary tap vacuum (Figure 3.2) before subjecting it to ITC. The G4LZI3 protein sample was poured into an eppendorf tube whose cap was left open and placed into a septum vial. The vial was closed with a tight rubber lid and placed in the dessicator. The vacuum hose was placed at the top of the stopcock and turned on. The dessicator tap was then slowly opened to form a vacuum seal after which the tap was then closed to turn off the vacuum. The samples were then left in the dessicator for about 3 hours to ensure complete degassing.

The ITC experiments were performed using a modular titration nanocalorimeter TAMIII (Figure 3.3) which was always cleaned prior to any experiment and calibrated until a baseline was reached. Thereafter, 750µl protein sample was poured into the sample cell. Standard 1M PBS buffer (pH 7.4) was used to dilute the ligands and was also placed in the reference cell. The ligands (CaCl_2 , MgCl_2 and ZnSO_4) were diluted with PBS to a concentration 10 times the concentration of the target protein and 250µl of the ligand was placed in a syringe. This 'titrant' was then set to inject approximately 10µl (9.975µl) for a maximum of 20 injections for each experiment at a 5mins interval so that

each titration peak could return back to the baseline before the next injection. The stirrer was kept at a constant speed of 40rpm and a calorimetric titration isotherm was recorded for each experiment.



Figure 3.2: A glass vacuum dessicator. This was done to produce an ‘anaerobic’ protein solution to prevent any spurious heat interference caused by air coming out of the sample.

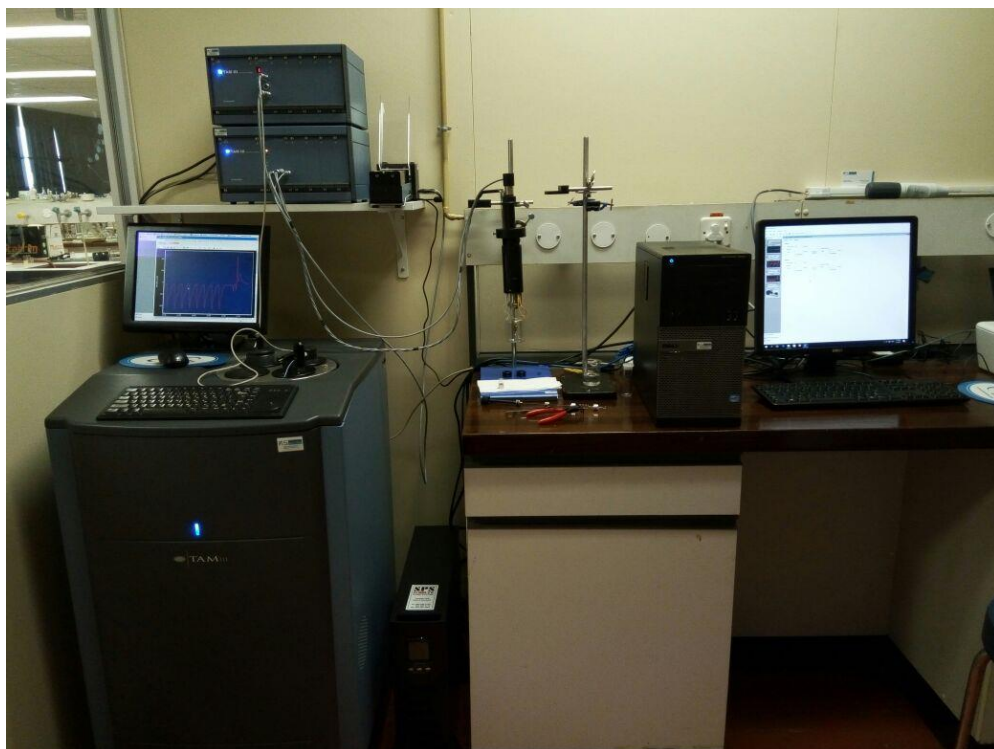


Figure 3.3: Isothermal Titration Calorimeter. All ITC experiments were performed using this modular titration nanocalorimeter TAMIII

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CHAPTER 4:

RESULTS AND DISCUSSION

4.1 Introduction

This project took into account one of the two novel proteins that Mbah and co-workers (2013) drew attention to, which could be used to target schistosomiasis since they form part of the worm natural biological defence system (Mbah *et al.*, 2013; Tkaczuk *et al.*, 2013). Both computational and experimental studies were conducted on the G4LZI3 Universal stress protein. Bioinformatics was first used to gain preliminary or predictive insight into the protein function, structure, evolutionary history and possible active sites. This was achieved by conducting multiple sequence alignment, prediction of the protein secondary structure, determination of the 3-dimensional structure of the protein, as well as molecular docking of important residues and ligands onto the G4LZI3 protein.

Thereafter, wet-lab experiments were undertaken to confirm these predicted *in silico* parameters. Bacterial JM109 *E. coli* cells were first made competent and used for transforming G4LZI3-pQE30 construct. The transformed colonies were then used for expression screening and large scale expression. The protein was then purified, dialysed, quantified and concentrated down for interaction studies with divalent cations using Isothermal Titration Calorimetry (ITC). This was done with the prospects that in future, crystallization trials can be set-up to provide structural information that could be useful in designing alternative treatment regimen for schistosomiasis as well as to contribute to treatment targeting specific developmental cycles of the worm.

4.2 Bioinformatics Analyses

The term bioinformatics was first brought about by Paulien Hogewag and Ben Hesper in 1970 who described it as the “study of informatics processes in biotic systems.” In the true sense of the word, bioinformatics can be defined as “conceptualising biology in terms of molecules (in the sense of physical chemistry? Applying “informatics techniques” (derived from disciplines such as applied maths, computer science and statistics) to understand and organise information associated with these methods, on a large scale” (Luscombe *et al.*, 2001). Though an interdisciplinary field, bioinformatics is slowly moving from being an applied to a basic science. Today this tool is used in a broad spectrum of biological fields such as biotechnology, gene therapy, molecular medicine, waste clean-up, and crop improvement. Bioinformatics was employed in this study using various online and offline tools to acquire, compare and group, analyse and interpret data in a biologically meaningful manner.

4.2.1 Sequence similarity analysis between G4LZI3 and various homologues

Multiple sequence alignment is a very important and useful method in predicting protein structure and function. This sequence analysis tool groups protein sequences into a rectangular formation with a view to discover homologous residues in a given column which may most likely play a common functional and significant role or represent a similar structure amongst related proteins (Edgar and Batzoglou, 2006). Thus, a multiple sequence alignment in other words is a “hypothesis of evolutionary history” and “statement of positional homology” (Thompson, 2004). CLUSTALW, MUSCLE, T-COFFEE, MAFFT and PROBCONS fall under some of the most well-known and widely used global alignment tools today. Before any alignment was done, a BLAST search was conducted via the NCBI server. Table B (Appendix III) highlights

the accession number, name of the organism and source, BLAST value and E-score of the chosen sequences. These were then entered and aligned using CLUSTALW. Figure 4.1 is a visual depiction of the comparison between the G4LZI3 protein sequence against 14 other protein sequences from distantly related organisms, which were chosen so as to have an idea on the conservation of important amino acids. The results shaded in black represent the most conserved residues or regions indicating they are probably of critical structural and functional importance since they have been more resistant to evolutionary change. Mutations amongst the sequences also took place such as point mutation where single amino acids were replaced, whilst the hyphens indicate insertion or deletion mutation. Using an offline tool called BioEdit, a table was constructed (Table C, Appendix III) which highlighted the similarities and identities between these sequences by pairwise alignment. Although the level of similarity or identity between 2 sequences was not necessarily meaningful, pairwise alignment estimated the possibility or likelihood that a residue had been replaced by another due to natural selection.

10 20 30 40 50 60

XP_018646651.1 1 MSETEQPSTSDGLDIGET--KGTIS-----MTDATERVLMFPVVGSEHSEAEFNWYMD
EUB59302.1 1 MSSPPTPLASDPPTQRAH--QSTRNSSESEDYLANVRRVILLSIDANENSSRAFEWYTN
CDI96922.1 1 MSSPPTPLASDPPTQRAH--QCSTRNSSESEDYLANVRRVILLSIDANENSSRAFEWYTN
GAA41511.1 1 -----VNEKPRITVLVPGSEHSEAEFNWYLN
CDS35071.1 1 -----MFSQHRSLQNLNVPT-----MFNDPRVLLPVDKSESSKKAFTWYMA
XP_001637863.1 1 -----MASEEPKKVRPILLPIDSSKHSEDAFEWYVN
KXJ07042.1 1 -----MAEQKTGRIVIPVVGSVNSEAEADWY-K
XP_015754675.1 1 -----MMACVRVVALPVDGSEHSEAEADWYLK
XP_005097850.2 1 -----MA-----ASHARHCIIAVDGEHSEAEAEWYCK
XP_009060489.1 1 -----MTLDKPDMSNFRVIVLLAVDDTEYSEAEFNWYRD
XP_012553698.1 1 -----MSTANRTILMAVDDTETLLFAFEWYIE
XP_011412635.1 1 -----MATESKPSSKPSRVVAVAIQNSEYAEAEADWYLE
XP_002130444.1 1 -----MKVLIADDESIDAEKTEAEWYLN
XP_014775911.1 1 -----MS-----SSEGRTVVGMDGSEYSEAEFNWYME
XP_009167172.1 1 MVTPYVSDDEDGLTQPFDA--MASAG-----VNEKPRITVLVPGSEHSEAEFNWYMN

70 80 90 100 110 120

XP_018646651.1 51 NVMKI-TDGLYIVHIV-EPVSTSYNYGLASKPAFITDDFSRHIQELVDEGRELGKRYLYK
EUB59302.1 59 NFARE-DDGLYIVHIV-EPVSTSYNYGLASKPAFLTDDFSRHIQELVDEGRELGKRYLYK
CDI96922.1 59 NFARE-DDGLYIVHIV-EPVSTSYNYGLASKPAFLTDDFSRHIQELVDEGRELGKRYLYK
GAA41511.1 33 NVMQP-NDNVKEVINII-EPVYTPSGFGAAIELPSLP-DVSRVMAETVEAGKRCQEKMHQ
CDS35071.1 43 NIERP-TDVIHTLHIC-EPSYAANNFSLTTIGQSKTNEINAMLOEHIELSNTTSHDELRSR
XP_001637863.1 32 NMHHE-EDELIVHIVL-DSAAIQT-RVSSHGLVD--DEFKNEMNKGLEVKAEFEERYKTK
KXJ07042.1 29 DGHLLKHNDLLVHAY-DLYTAPPTPYFYG--FAPPEDWEKHMKNKNTEDARKMQYKEKK
XP_015754675.1 28 QMHKKGEDKVVIIEVN-EMTRSIET-----ITKEDWEQHVKDHTQKVQDVEEYKTK
XP_005097850.2 29 FIYKD-DDEVTLNIP-EVNPTVHKSPPNV-ALVAPEIMVAMARQEHDKINAEMQKFEADK
XP_009060489.1 35 FVHRP-ENFLVILHCT-EHDNQELR-----DASPGRTKTMIVELTERMRFIEDRFQQL
XP_012553698.1 28 NFHRS-EDVLVILHVRHMPPELPTMGLMAGTIAMSESYEL--VIRASIEKSKQILASVYENR
XP_011412635.1 35 KIRRN-DDVIVILHLP-ESYDFSL-----ASPGVIRQLLDELEKNVKFTEDEYAEK
XP_002130444.1 23 QIHKP-DNDIVVSEAG-EPHPLPTLFMSEGAVFSPDEIKNIMTQSNKKLEEFKNRYSLK
XP_014775911.1 29 QVYRQ-NDHIIIVYCP-EFQSITHS--P-Y-VMADVITLVDMREEEKQVKAQLEKFGEM
XP_009167172.1 51 NVKRP-NDNVKEFINII-EPVYTPSGFGAAELPLLP-DVSRVMTETVETGKKRCQEKMHQ

130 140 150 160 170 180

XP_018646651.1 109 CEDSGLSARFTIHVGTK-PGENIVRIAHEHGVDLVILGNRGIGTVRRFELGSVSDYVLIIE
EUB59302.1 117 CKGTGIRVRFMLHIGAK-PGEHICRLAKEQQVDVIVMGRGMRIRRRFLGSVSDYVLIIE
CDI96922.1 117 CKGTGIRVRFMLHIGAK-PGEHICRLAKEQQVDVIVMGRGMRIRRRFLGSVSDYVLIIE
GAA41511.1 90 AKAYNINSQAFHLVDSR-PGPAIVKAVQDYNADLVIMGNRGIGTVRRFELGSVSDYVLIIE
CDS35071.1 101 LEDKGVSGDFTLVGTK-PGEMIVAKARDLNIDLIVMGNRGVSTLRRFELGSVSDYVLIIE
XP_001637863.1 87 AETASLKAKIEVRGG--KPGETIQCSKDEHCDLIMGSRGLGSIIRRRFLGSVSDYVLIIE
KXJ07042.1 86 CNEKLLKCKLFNESC--EPGETIQNLAKDQKADQIVMGRGMRIRRRFLGSVSDYVLIIE
XP_015754675.1 79 MCDEEAEEFEVKLISG--KPGEMITEAMKECGADVIVMGRGIGAIIRRRFVGSVSDYVLIIE
XP_005097850.2 86 LKQHKIGGKVKTIAS-KPGEGITKAAETHADLIVIGSRGKGTVRRFLMGSVSDYVLIIE
XP_009060489.1 86 MORSGLSGRIQRGGG--KPGEMILKAAVEEGASMIVIGIRGLGKIIRRRFLGSVSDYVLIIE
XP_012553698.1 85 CKDHQVHSRIILADDDHSPGHVICKLAKSNEADVILTGRCIGKLERFELGSVSDYVLIIE
XP_011412635.1 84 VKAYGIDGKFRITGGG--KPGEMILKIAREENATLIVIGIRGLGKIIRRRFLGSVSDYVLIIE
XP_002130444.1 81 CAEKKIKCKLVFQLSDKSPGEMIVKIANEEACDVIVMGRGIGAVRRFLGSVSDYVLIIE
XP_014775911.1 83 LKNAGVGGKVKRSING--RPGEMIVNVAKEONASLIVIGIRGMRGTIRRRFLVGSVSDYVLIIE
XP_009167172.1 108 AKACNINSQAFHLVDSR-PGPAIVKAVQDYNADLVIMGNRGIGTVRRFELGSVSDYVLIIE

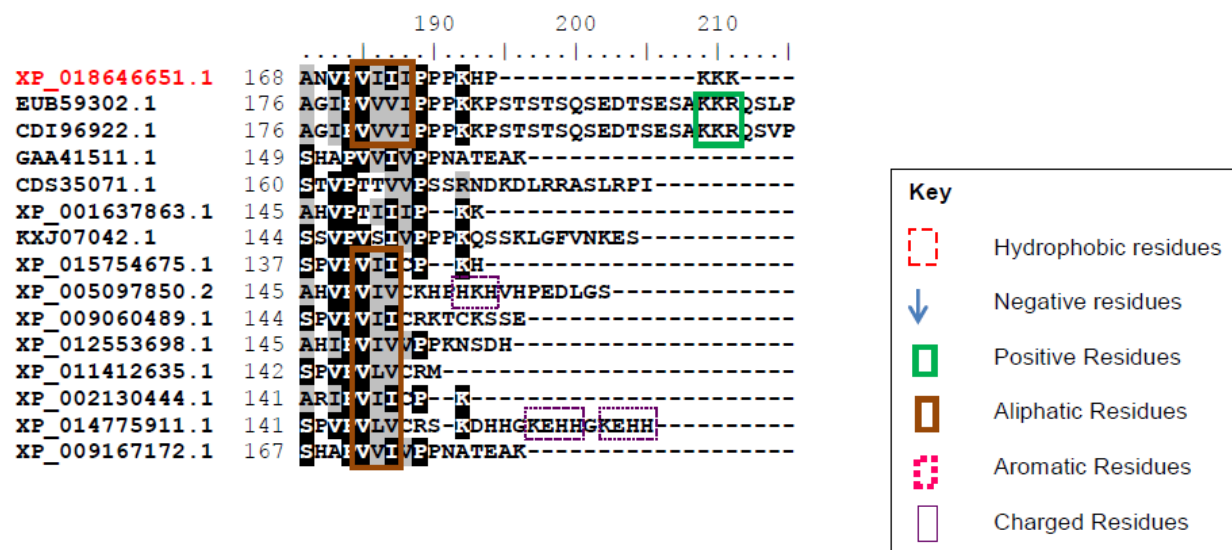


Figure 13.1: Multiple Sequence Alignment of G4LZ13 with its homologues. Protein sequences and their corresponding accession numbers were derived from NCBI. XP_018646651.1: Universal stress protein G, putative [*Schistosoma mansoni*]; EUB59302.1: Universal stress protein A-like protein [*Echinococcus granulosus*]; CDI96922.1: Universal stress protein [*Echinococcus multilocularis*]; GAA41511.1: Universal stress protein A-like protein [*Chlonorchis sinensis*]; CDS35071.1: Universal stress protein in QAH:OAS [*Hymenolepis microstoma*]; XP_001637863.1: Predicted protein [*Nematostella vectensis*]; KXJ07042.1: Universal stress protein A-like protein [*Exaoptasia pallida*]; XP_015754675.1: PREDICTED: universal stress protein Slr1101-like [*Acropora digitifera*]; XP_005097850.2: PREDICTED: universal stress protein A-like protein [*Aplysia californica*]; XP_009060489.1: hypothetical protein LOTGIDRAFT_165237 [*Lottia gigantea*]; XP_012553698.1: PREDICTED: universal stress protein A-like protein isoform X2 [*Hydra vulgaris*]; XP_011412635.1: PREDICTED: uncharacterized protein LOC105317627 isoform X4 [*Crassostrea gigas*]; XP_002130444.1: PREDICTED: uncharacterized protein LOC100180023 isoform X1 [*Ciona intestinalis*]; XP_014775911.1: PREDICTED: putative universal stress protein SAS1637 [*Octopus bimaculoides*]; XP_009167172.1: hypothetical protein T265_04215 [*Opisthorchis viverrini*]. The Accession Number and source along with their corresponding protein length and derived E-value are tabulated in Table C (Appendix III). Multiple sequence alignment was done by ClustalW online tool. Identical/conserved residues are shaded in black while semi-conserved/similar residues are represented by a gray shade.

4.2.2 Phylogenetic analysis of G4LZI3 and its homologues

The amino acid sequences were analyzed using the “One Click” method on the Phylogeny fr server (Fig 4.2). Most phylogenetic analyses revolve around cladogram construction (Phillips *et al.*, 2000). The evolutionary tree basically summarizes the divergence, descent and ancestry of the selected biological entities diagrammatically (Gregory, 2008). Fig 4.2a shows that universal stress protein XP_018646651.1 (from *Schistosoma mansoni*) and CDS35071.1 (from *Hymenolepsis microstoma*) are more evolutionarily close to each other, as compared to GAA41511.1 and XP_009167172.1, since they fall within the same clade. The length of the horizontal lines or branches, depicted in red, was indicative of the phylogenetic distance or evolutionary change and/or time between the species. The longer the branch, the more evolutionary change had taken place (Dutilh and Keşmir, 2016).

As much as a phylogenetic tree is a pictorial representation of the protein historical relationships, it is a mere estimation. Thus, it is important to determine how reliable this estimate is and this was done by the bootstrap method (Hall, 2013; Rhee *et al.*, 2006). Bootstrap replicates were set to 100 and used to estimate the reliability of each node. However, the phylogenetic tree in Fig 4.2a was somehow misleading because the node at the extreme left seemed to represent the root. Fig 4.2b shows an un-rooted tree in radiation format, which clearly showed that the root or common ancestor is actually unknown (Hall, 2013; Rhee *et al.*, 2006).

4.2.3 Prediction of secondary structures present in *S. mansoni* G4LZI3 protein

Till today, correct and accurate predictions of three-dimensional protein structures directly from protein sequences have still not been achieved. This in itself, as well as the need for secondary structure predicting tools, is a challenge due to the abundance of genomic data that several sequencing projects produce (McGuffin *et al.*, 2000). Based on the residues constituting each sequence, it is possible to solve the afore-mentioned problem by first classifying these residues as coils, helices or sheets (Hua and Sun, 2001). Predicting secondary structures assist in detecting possible domains and functions of the protein, apart from determining protein flexibility and helping molecular biologists sub-clone protein fragments and add affinity tags for expression and purification, respectively (Montgomerie *et al.*, 2006). Two of the most abundant and common fold classes are alpha helices and beta sheets (also known as pleated sheets) which consist of hydrogen bonds that occur over a number of three or more successive residues (Koswatta *et al.*, 2011). GOR IV, HNN, YASPIN and PROF fall under some of the well-known amino acid sequence evaluators. For this study, an online tool called PSIPRED was used which is a “prediction method that employs two feed-forward neural networks, which perform an analysis on the output obtained from PSI-BLAST” (Koswatta *et al.*, 2011). PSIPRED predicted the G4LZI3 protein to consist of four (4) α -helices represented by the pink cylinder and six (6) β -sheets (yellow arrows) [Figure 4.3]. These secondary structures are composed of hydrogen bonds which provide strength to polar backbone groups in the hydrophobic core, thus playing an imperative role in the stability of folded proteins.

4.2.4 Generation and construction of G4LZI3 homology model

For more than 3 decades, researchers have succeeded in developing computational tools for predicting the structures of different proteins. Most of these methods involve comparing the sequence of the target protein against other sequences of known structure within a large database so as to gain an “evolutionary or statistical profile” of the sequence in question (Kelley *et al.*, 2015). The RSCB Protein Data Bank is one such database, housing over 100 000 3D protein structures and other various biological macromolecule structures, thus making it one of the best resources for biomedicine and biology globally (Rose *et al.*, 2015). Alignment between the unknown and known structures result and a model is then constructed and generated using the known structure as reference. Software such as Swiss-Model, Homer, Modweb, (PS)², (PS)²-V2, CPHmodels and Phyre2 fall under some of the most widely used tools for model generation; amongst them, Phyre2 and Swiss-model are arguably two of the best and freely available tools for this purpose (Nema and Pal, 2013). Protein Homolgy/analogy Recognition Engine 2 (Phyre2), which is a template-based modeling server, was used in this study. This tool operated by comparing and acquiring homologous sequences with HHblits. This resulted in multiple sequence alignment and prediction of the secondary structure of the protein. Fig 4.4 depicts the secondary structure of the G4LZI3 protein, which was predicted to have five (5) α -helices and five (5) β -sheets.

Thereafter, both the alignment and secondary structure prediction were then combined to form a query using hidden Markov model which was scanned against proteins of known structure. A score was then given to each alignment and those with a top-scoring value were used to construct models with crude backbones. Ultimately, loops were incorporated into the model and amino acid side chains were added to generate the final Phyre model (Fig 4.5). The model revealed a structure consisting of the C and N terminal loops, 4 α -helices and 5 parallel β sheets. These results were slightly in contrast with the Phyre secondary structure prediction and also, with that obtained from PSIPRED, which predicted 4 helices and 6 β -strands while the former predicted 5 β -sheets and 5 α helices respectively. Though somehow accurate, predictions cannot in the end be trusted, since they are merely predictions; hence it was imperative to conduct benchtop experiments to confirm these results. However, a Ramachandran plot was generated via the RAMPAGE server which employed PROCHECK principles to evaluate predicted model accuracy as well as to generate plots for Proline and Glycine residues. The Ramachandran map is a true and extremely reliable determinant of the intrinsic quality of a protein structure based on the three backbone torsion angles (ϕ , ψ and ω) which actually determine protein fold, mainly because the distribution of these angles are quite difficult to improve even when using refinement software (Hooft *et al.*, 1997). The plot showed that ~98% of the residues were found in the favoured region while a meagre ~2% were located in the allowed region (Figure 4.6a &b). This means that the model was in fact good and convincing because no stereochemical parameters were violated during model building.

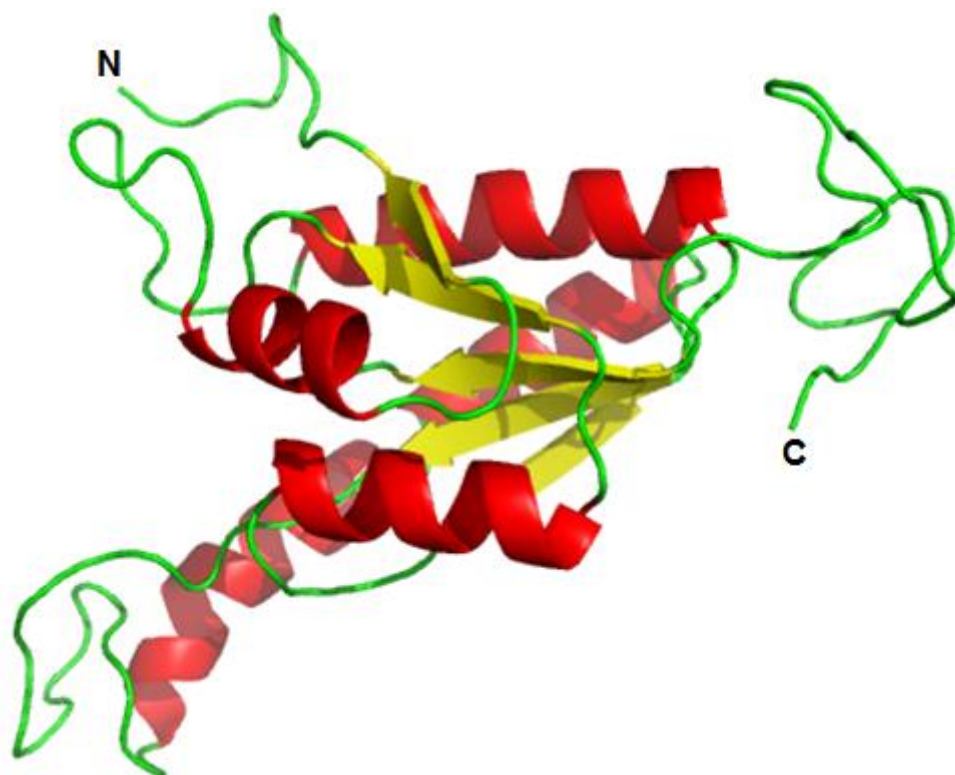


Figure 4.5: Model structure of G4LZI3. The model was generated by Phyre2 (Kelley *et al.*, 2015); viewed and edited by Pymol (DeLano Scientific) and Chimera. This cartoon representation shows the model to contain 4 α helices and 5 β sheets.

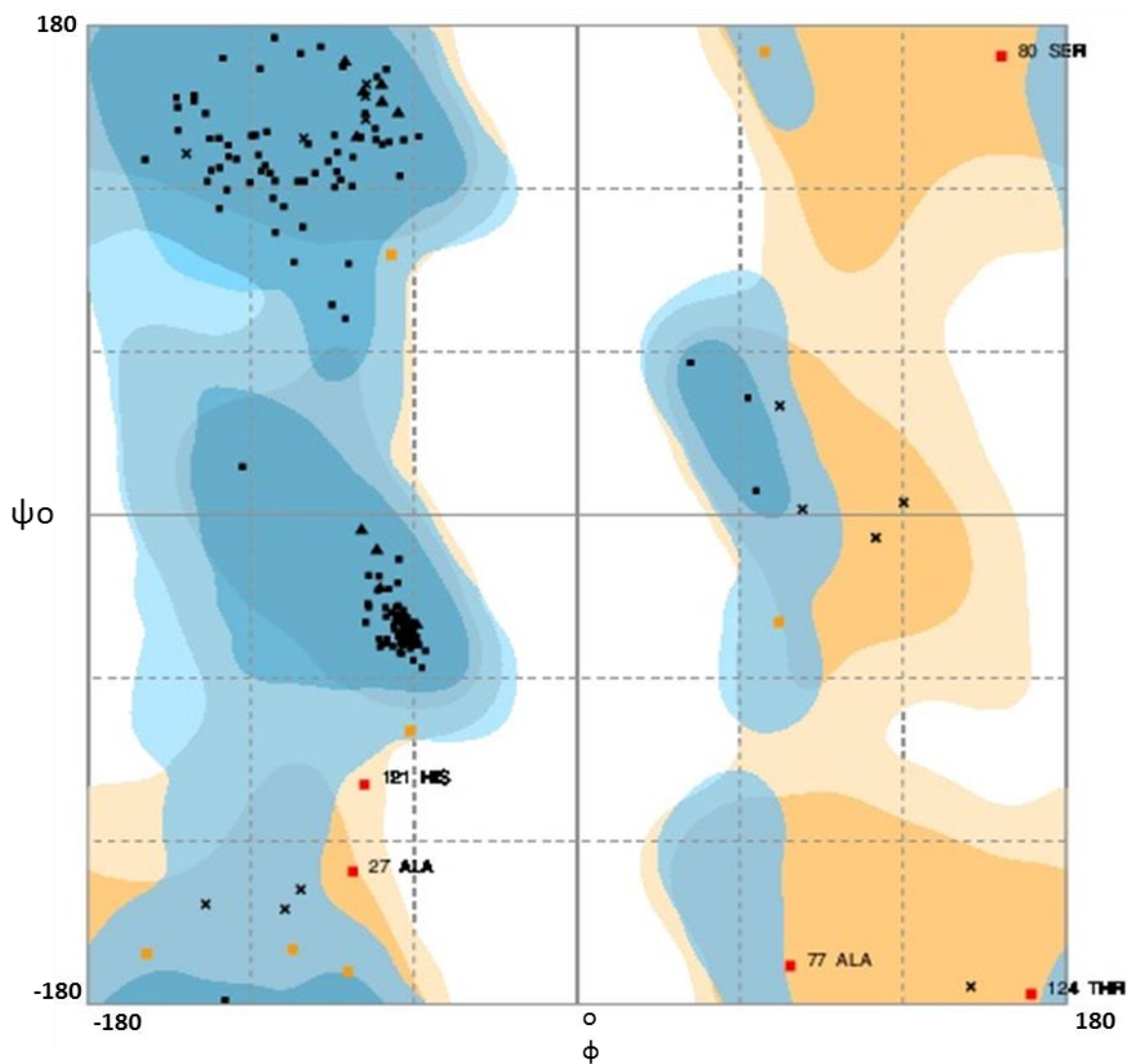
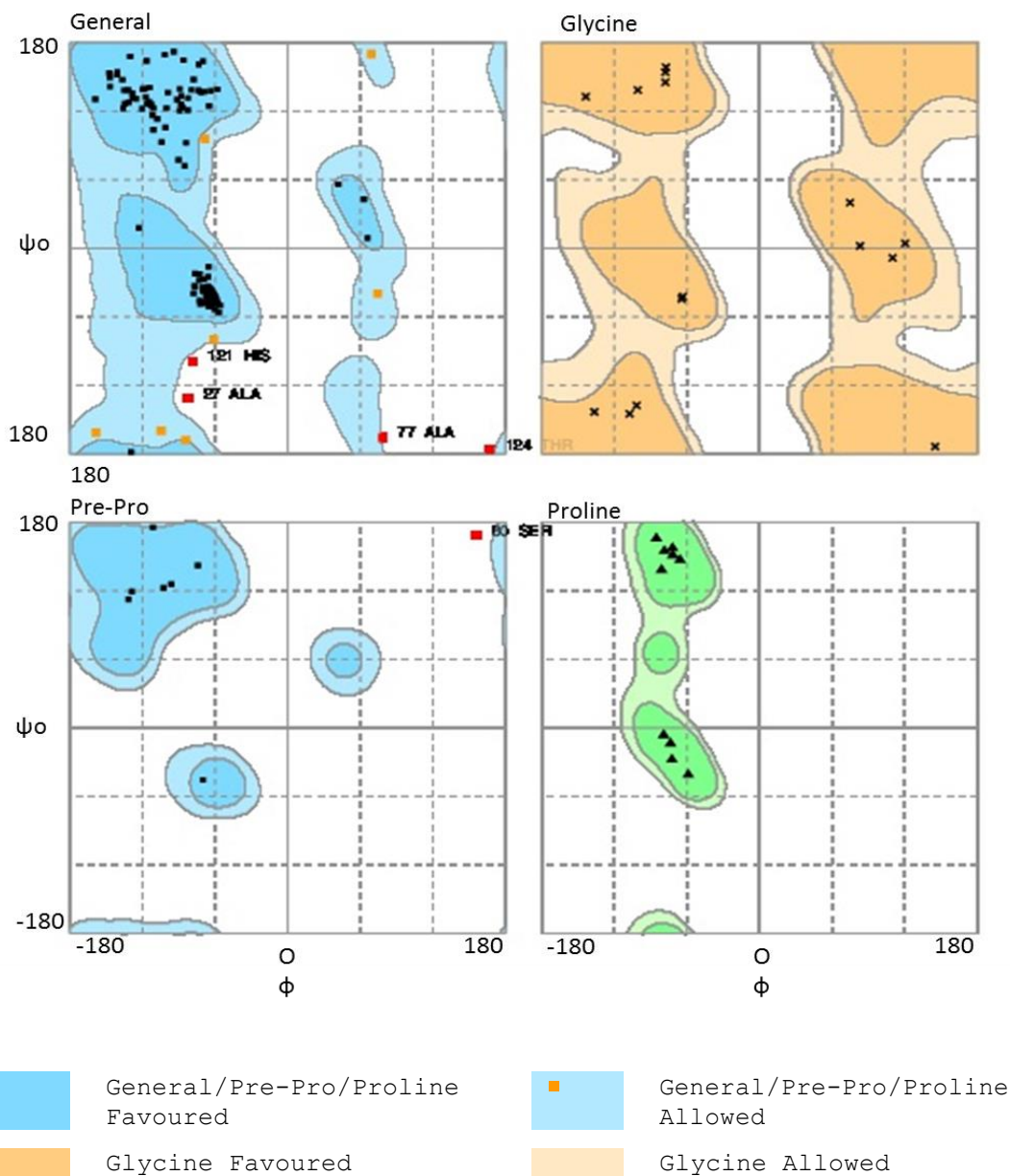


Figure 4.6a: Ramachandra Plot of modelled Universal Stress Protein G4LZ13. The figure was generated and visualized using RAMACHANDRA (Lovell *et al.*, 2003) via the RAMPAGE online server.



Number of residues in favoured region (~98.0% expected): 170 (93.4%)
 Number of residues in allowed region (~2.0% expected): 7 (3.8%)
 Number of residues in outlier region: 5 (2.7%)

Figure 4.6b: Ramachandra Plot of modelled Universal Stress Protein G4LZ13 (Glycine and Proline Plots). The figure was generated and visualized using RAMACHANDRA (Lovell *et al.*, 2003) via the RAMPAGE online server.

The detection of motifs present in a protein is beneficial and biologically important since they are in the sense of the word 'regions' or 'portions' of the protein that are biologically and functionally significant, such that they may actually be used to classify the protein (Kunik *et al.*, 2005). They may be used to understand a series of features such as drug design, gene function, transcriptional regulatory elements and binding sites. Figure 4.7 illustrates that the G4LZI3 protein is made up of only one motif which is the functional part or the backbone of the protein. The first residues were simply additional residues to keep the protein from floating in water. These results were generated using an online analysis tool called MOTIF Search (<http://www.genome.jp/tools/motif/>).

Pfam ID: Usp
Description: PF00582, Universal stress protein family
Appearance:

Position	29..176
Alignment Query Database	RKVLMPVDGSEHSERAFNWYMDNVMKITDGLYLVHIVEPLSQGLNYNLASKSPSIKDDFSKHLNSLV EsgralrakffTRCEDSGLSARFTIHVGTKPGENIVRIAHEHGVDLVIIGNRGIGTVKRTFLGSVSD YVLHHANVPVIIIPkrIlVavDgseeskkAlewAlelakargaelillhvidprasgaasdadeeae eelelaeaeleada.....akaaaeaggvkvvevvveegdpaeielevaeeedadliVvGsg rsglsrlllGSvseyvlrhapcpVlvVr
i-Evalue	9e-25

Sequence: MSETEQPSTSDGLDIGETKGTISMTDATRKVLMPVDGSEHSERAFNWYMDNVMKITDGLYLVHIVEPLSQGLNYNLASKSPSIKDDFSKHLNSLVESGRALRAKFFTRCEDSGLSARFTIHVGTKPGENIVRIAHEHGVDLVIIGNRGIGTVKRTFLGSVSDYVLHHANVPVIIIPPKHKPKK

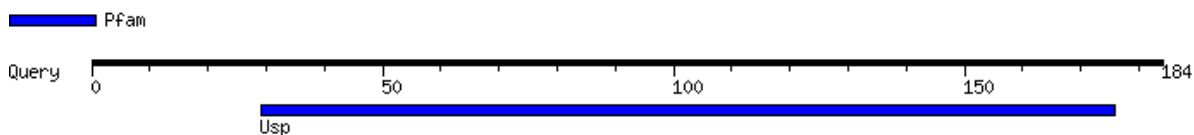


Figure 4.7: Motif found in the G4LZ13 protein by MOTIF Search. The protein is made out of one functional motif which begins from residue 29–176. This region is highlighted in red.

Additionally, ConSurf was used to calculate the degree at which evolution had taken place in the nucleic acid sequence at each position as well as regions in the protein that are of functional and structural importance (Ashkerazy *et al.*, 2010). Compared to other methods, this widely used tool has the ability to predict slowly evolving positions at the core which are assumed to be important for the protein structural stability and folding, whilst those evolving at the surface are usually critical for function (Ashkerazy *et al.*, 2016). ConSurf also pinpoints buried and/or exposed positions within the protein and highlights them onto the sequence or modelled structure of the macromolecule. Figure 4.8 depicts the degree of evolutionary preservation of the amino acids in the protein and predicted 14 functional, highly conserved and exposed residues (Arg19, Asp36, Ser38, His40, Arg43, Asp57, Glu66, Pro126, Gly127, Arg147, Arg154, Gly158, Pro171 and Lys182) while 11 residues, which were highly conserved and buried (Ala44, Tyr48, Val142, Gly145, Gly148, Thr155, Ser159, Val160, Ser161, His166, His167) are considered as structurally important.

4.2.5. Determination of different G4LZI3 interacting partners

Knowledge of protein interacting partners narrows down and helps to describe the target protein function; thus it is key in understanding this factor and other important cellular processes (Szkłarczyk *et al.*, 2015). Both direct and indirect interactions bring about a functional web of links that can give rise to different organisms and behaviours by catalysing consequent reactions in a particular metabolic process or regulating one another transcriptionally or post-transcriptionally amongst other activities (Franceschini *et al.*, 2013). By studying a protein with unknown function and its interaction with other proteins of known function, this could give clues and eventually lead to the discovery of the target protein function (Qi and Noble, 2011). However, information pertaining to such interactions and known functions is scattered across several databases and some of these are incomplete or of questionable quality. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) provides wide-spread and comprehensive direct and indirect protein networks of over 1100 organisms, and scores and integrates them. It was for this purpose that this tool was employed to search for any protein interactions and/or associations between G4LZI3 and any other proteins. These results are mapped out in the figure 4.9.

Of all the interactors, only one of the protein 3D structures was known or predicted. Smp_056970.1_mRNA is actually Glyceraldehyde-3-phosphatase dehydrogenase which surprisingly, and quite interestingly, is an antigen associated with human resistance to schistosomiasis. It has been shown that rsG3PDH (recombinant schistosome glyceraldehyde-3-phosphatase dehydrogenase) provides protective immunity to humans because it develops resistance to reinfection after chemotherapy

(Ridi *et al.*, 2001). Additionally, studies have shown that this antigen is actually expressed on the surface membrane of the lung in stage 5-day-old *in vitro* grown schistosome larvae and is therefore currently being considered as a possible human schistosomiasis vaccine (Tallima and Ridi, 2008).

Heat shock proteins (HSPs) or stress proteins fall under a group of highly conserved proteins whose expression is induced by various environmental stressors such as extreme pH and heat levels, oxygen deprivation and nutrient scarcity (Abaza, 2014). These proteins are grouped based on their molecular weight and it has been shown by Tielens *et al.* (1993) that Hsp60 is constitutively expressed throughout the complex lifecycle of the schistosome worm, aiding its ability to adjust to different temperatures between the environment and different hosts especially from cercarial to schistosomula transformation (Abaza, 2014; Tielens *et al.*, 1993).

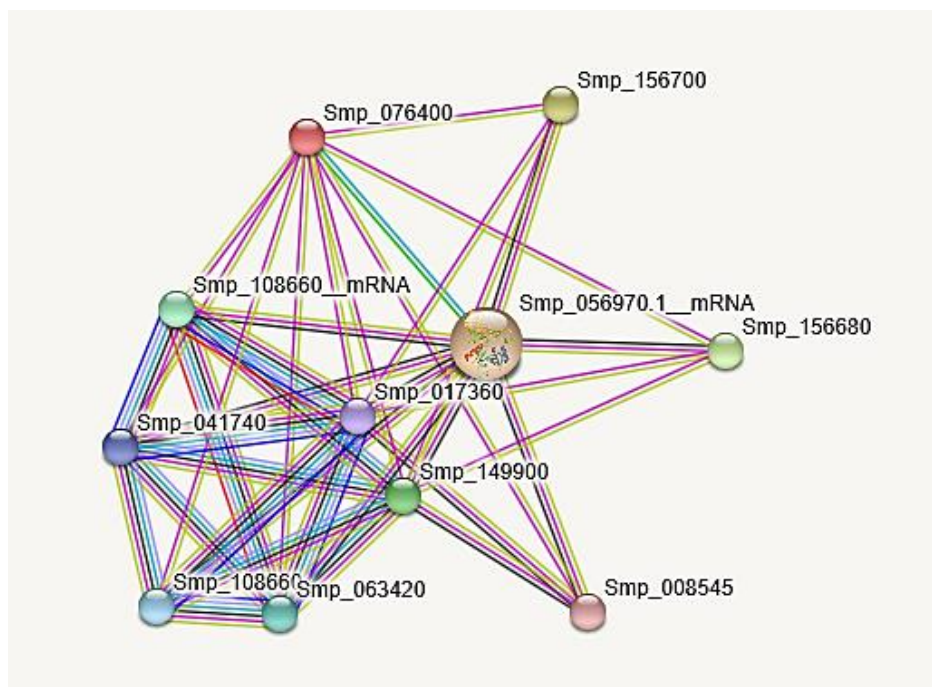


Figure 4.9: Predicted direct interacting partners of the G4LZI3 protein. Smp_076400: Universal stress protein G, putative (184aa); Smp_056970.1_mRNA: Gyceraldehyde-3-phosphate dehydrogenase; Smp_156700: Putative uncharacterized protein (894aa); Smp_156680: Fyve finger-containing phosphoinositide kinase, fyv1 putative (2289aa); Smp_149900: Chaperonin containing t-complex protein 1, eta subunit, tcph, putative (551aa); Smp_108660_mRNA: beta subunit, tcbp, putative (114aa); Smp_063420: Chaperonin containing t-complex protein 1, epsilon subunit, tcpe, putative (545aa); Smp_108660: Chaperonin containing t-complex protein 1, beta subunit, tcpb, putative (530aa); Smp_041740: Chaperonin containing t-complex protein 1, gamma subunit, tcpg, putative (545aa); Smp_017360: T-complex protein 1 subunit alpha,; Molecular chaperone; Smp_008545: Heat shock protein HSP60, putative (567aa).

4.2.6 Molecular docking of divalent cations and ligands to the G4LZI3 protein

Molecular docking has proven to be a powerful tool for studying protein-ligand and protein-protein relationships, structure-based virtual screening and has also been an important contributor in the design of new drugs (Guedes *et al.*, 2014; Kitchen *et al.*, 2004). The computer based tool is a cost-effective, cheap and time-saving technique which together with Nuclear Magnetic Resonance (NMR) and X-ray crystallography, work hand in hand to resolve 3-dimensional protein structures and have up to date solved more than 100 000 macromolecules, thus providing vital structural information pertaining to potential drug targets (Ferreira *et al.*, 2015; Rizvi *et al.*, 2013). Most structure-based drug design (SBDD) methods make use of molecular docking, molecular dynamics (MD) and structure-based virtual screening (SBVS) strategies for molecular analysis applications. Autodock, GOLD, DockIt, FlexX, GRAMM, DOCK and LIGPLOT are a few examples of some well-known docking softwares. Patchdock was used for the purpose of this study and revealed binding sites for all three docked ligands. Patchdock predicted that Ca^{2+} binds to Leucine at position 94 and Isoleucine at position 149, while Mg^{2+} and Zn^{2+} both bind to Glutamate at position 42.

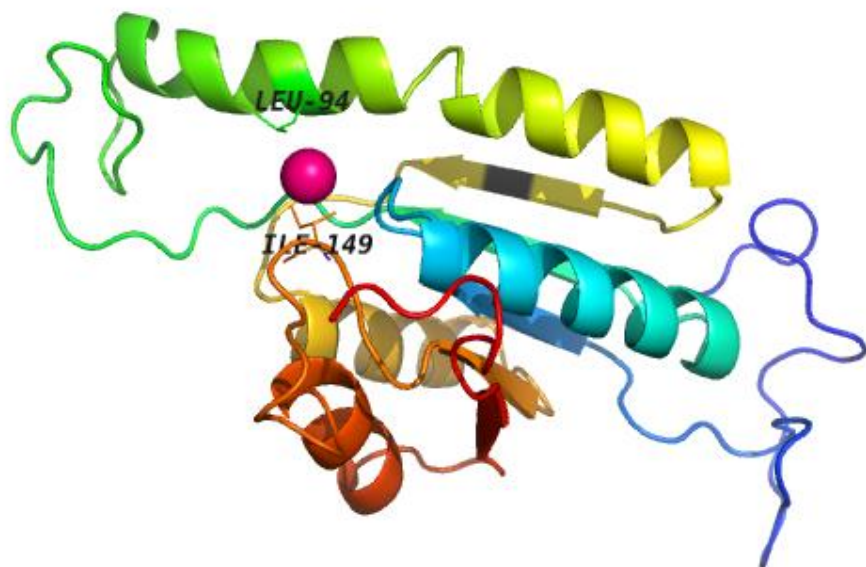


Figure 4.10: G4LZI3 protein docked with Ca²⁺. The Ca²⁺ ligand is docked to Leu94 and Ile149 respectively.

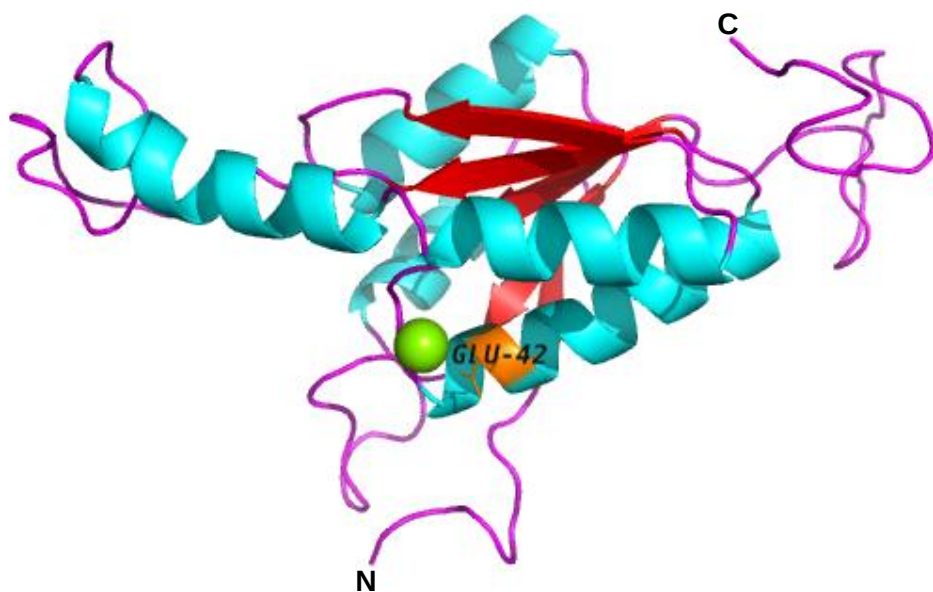


Figure 4.11: G4LZI3 protein docked with Mg^{2+} . The Mg^{2+} ligand is docked to a Glutamate on position 42.

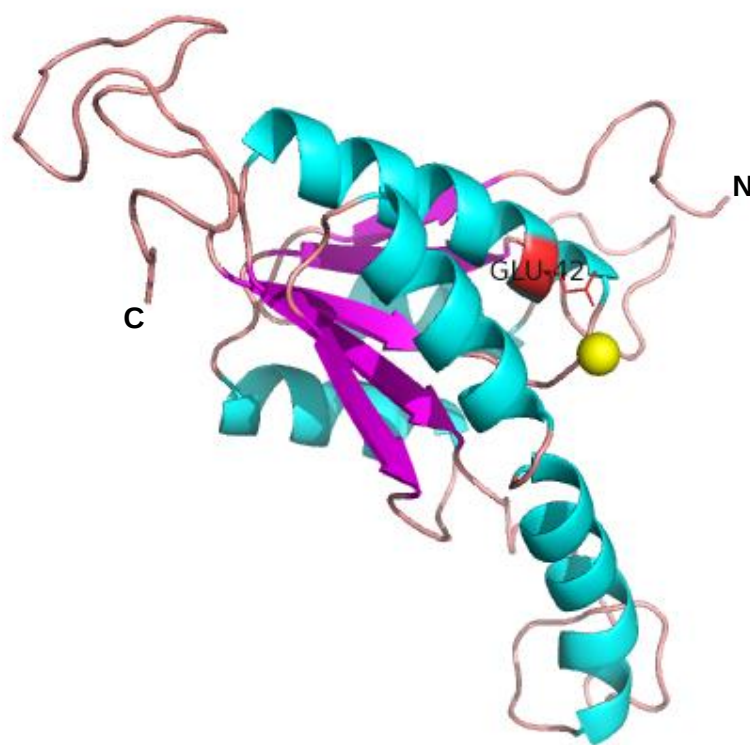


Figure 4.12: G4LZI3 protein docked with Zn²⁺. The Zn²⁺ ligand is also attached to Glutamate on position 42.

Additionally, an online server called GalaxyWeb was used to predict any ligand binding sites on the novel G4LZI3 protein. The server is available at the following url: <http://galaxy.seoklab.org/index.html> and provides a number of tools related to protein structure prediction such as GalaxyGemini, GalaxyLoop, GalaxyRefine, GalaxyRefineComplex amongst a number of other tools. By using GalaxyWeb, one was able to work on data acquired from different sources or databases and perform diverse actions such as calculations, combining this data or refining them (Giardine *et al.*, 2005). Once the server was accessed, the GalaxySite tool was selected for ligand binding site prediction and the results gave three (3) output model predictions. These were viewed in PyMOL; their surface representations are depicted in Figure 4.13, Figure 4.14 and Figure 4.15.

Adenosine triphosphate (ATP) was predicted to bind the G4LZI3 protein at the following positions: Pro34, Val35, Asp36, Ser38, Ser41, Val62, Ile64, Pro126, Ile130, Ile144, Gly145, Arg147, Gly148, Ile149, Gly150, Thr151, Gly158, Ser159, Val160, Ser161 (Figure 4.13a). USPs can be divided into two classes, either they bind ATP or they do not and thus, the G4LZI3 protein should be regarded as UspFG-type since it has been predicted to do so (Tkaczuk *et al.*, 2013). According to Mbah and co-workers (2013), ATP regulates USPs by phosphorylating onto serine and threonine residues (Mbah *et al.*, 2013). This tallies with what the model predicted and this is more evident in Figure 4.13b, where serine and threonine are two of the residues involved in ATP binding to the G4LZI3 protein.

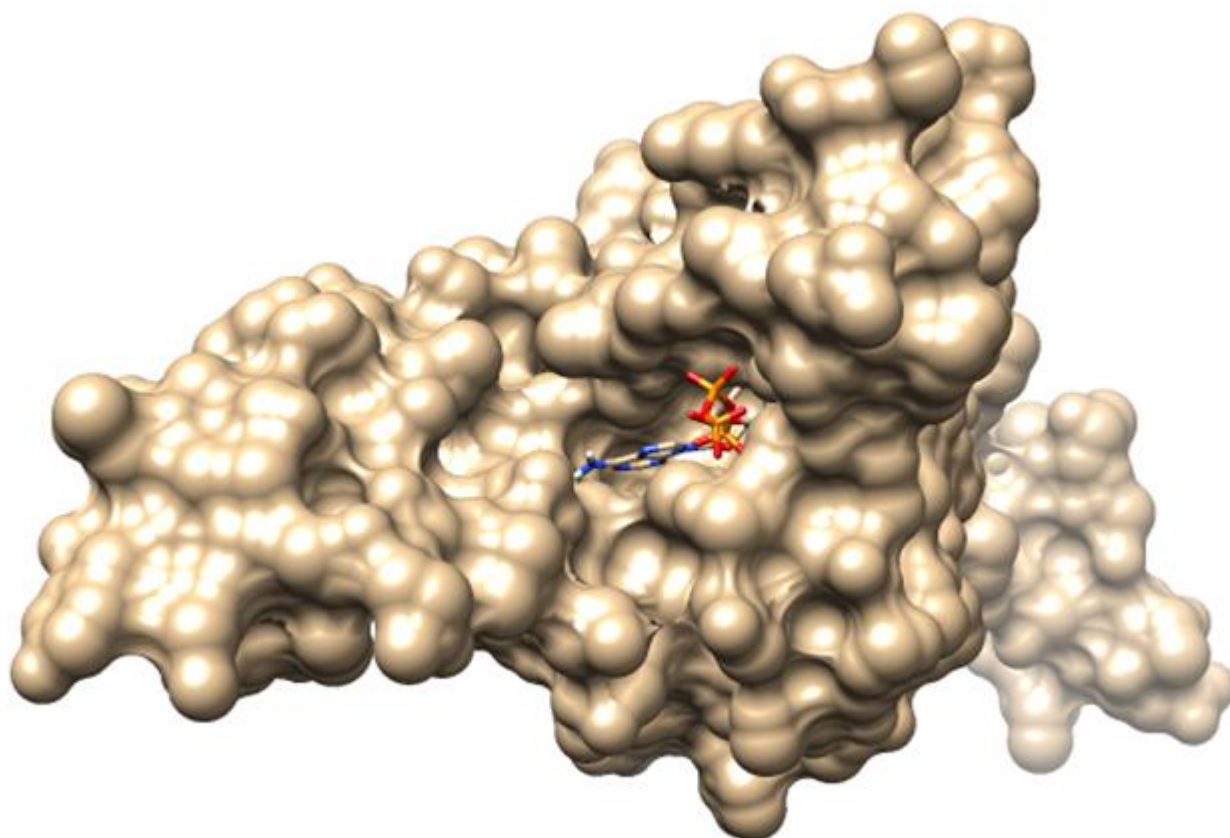
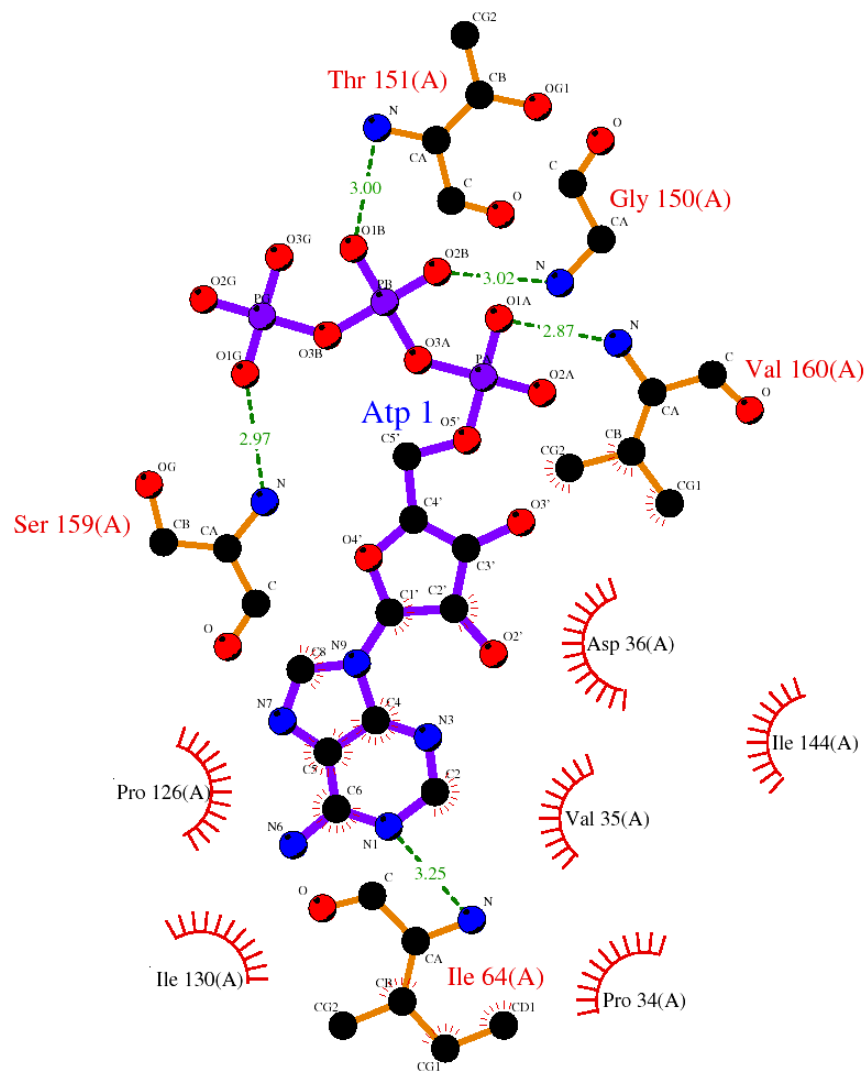


Figure 4.13a: Hydrophobic Representation of G4LZI3 protein docked with ATP. It is assumed that the protein is of FG-type since it binds ATP.



Key

- | | | | |
|--|------------------------------|--|---|
| | Ligand bond | | His 53 Non-ligand residues involved in hydrophobic contact(s) |
| | Non-ligand bond | | Corresponding atoms involved in hydrophobic contact(s) |
| | Hydrogen bond and its length | | |

Figure 4.13b: Residues involved in predicted binding of ATP to the G4LZ13 protein. Serine and threonine amongst others are two of the residues involved in the binding and are assumed to be involved in phosphorylating and regulating the protein.

Adenosine monophosphate (AMP) was also predicted to bind onto the G4LZI3 protein at positions Pro34, Val35, Asp36, Ser38, Ser41, Val62, Ile64, Pro126, Ile130, Ile144, Gly145, Asn146, Arg147, Gly148, Gly158, Ser159, Val160 and Ser161. Mbah *et al.* (2013) have also predicted the binding of AMP to the G4LZI3 protein and even went further to acquire integration and visualization of data on the sequence features, evolutionary relatedness and developmental expression of *Schistosoma* universal stress proteins. The docked model with AMP once again tallies with their observations and could almost be a confirmation of binding between the molecule and the protein (Figure 4.14a & Figure 4.14b). Additionally, Banerjee *et al.*, 2015 have shown that USPs actually bind AMP (cAMP in this case) with higher affinity than ATP.

Uridine-5'-diphosphate-3-O-(R-3-hydroxymyristoyl)-N-acetyl-D-glucosamine (U20) is one other ligand predicted by GalaxyWeb to bind to the G4LZI3 protein (Figure 4.15a & Figure 4.15b). Galaxy predicted Gly16, Ser23, Met24, Thr25, Asp26 and Thr28 as the ligand binding residues.

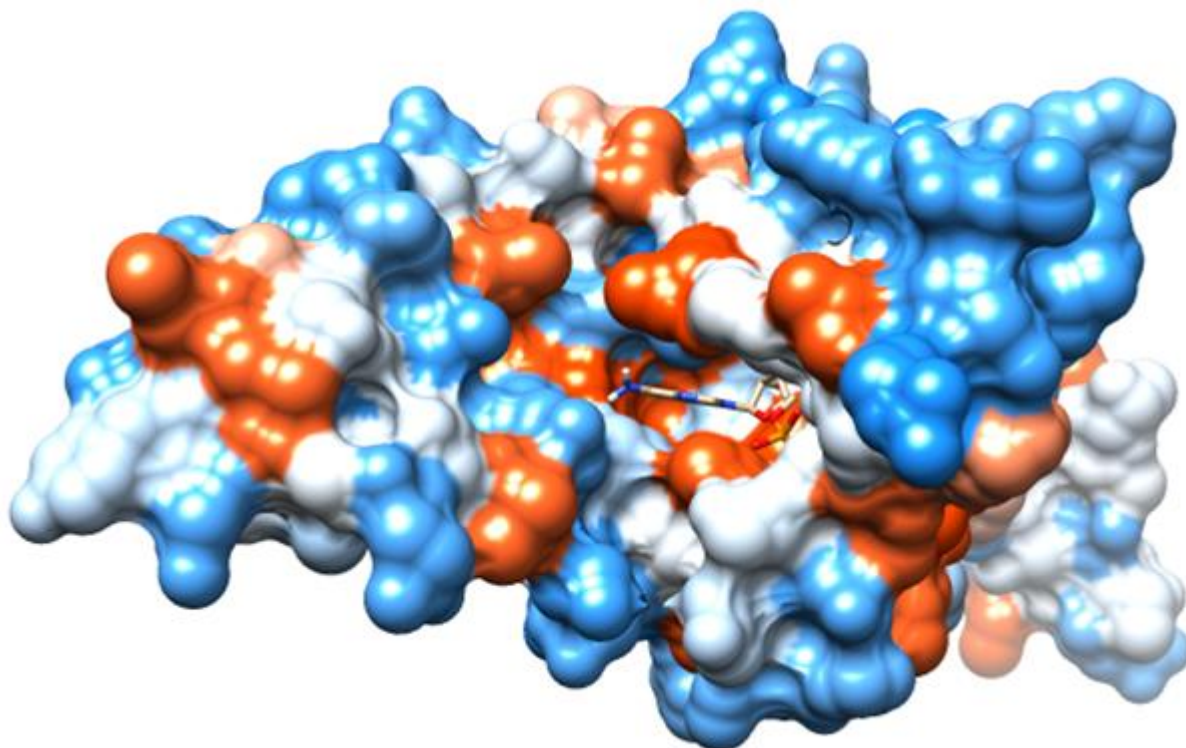
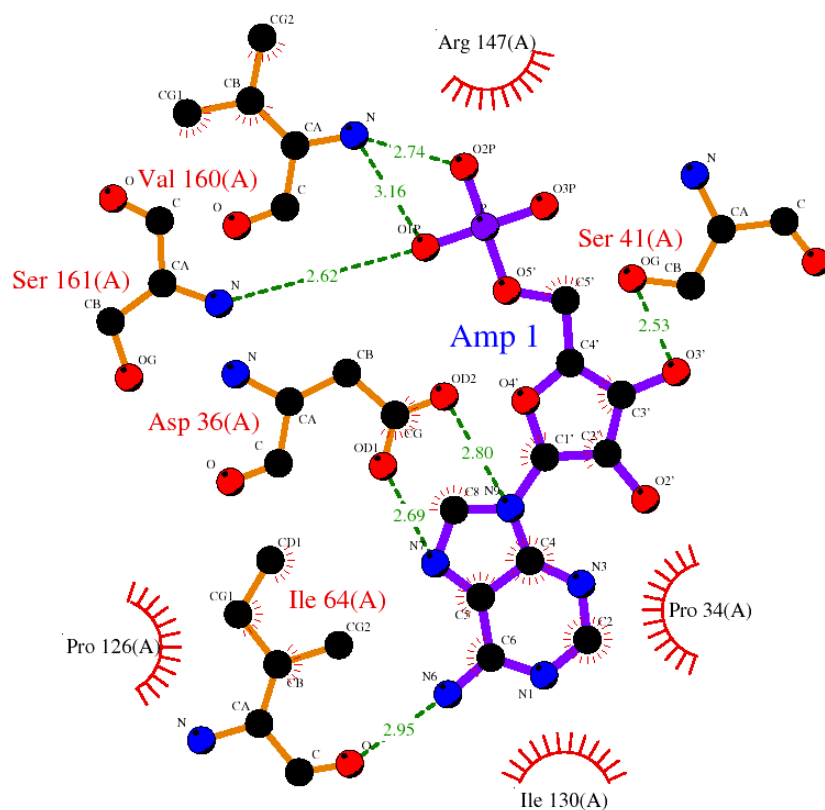


Figure 4.14a: Hydrophobic Representation of G4LZI3 protein docked with AMP. It has been documented that AMP binds to Usps with higher affinity than ATP (Banerjee *et al.*, 2015).



Key

- | | | | |
|---|------------------------------|---|--|
|  | Ligand bond |  | Non-ligand residues involved in hydrophobic contact(s) |
|  | Non-ligand bond |  | Corresponding atoms involved in hydrophobic contact(s) |
|  | Hydrogen bond and its length | | |

Figure 4.14b: Residues involved in predicted binding of ATP to the G4LZI3 protein. Valine, serine and aspartate include some of the residues involved in binding.

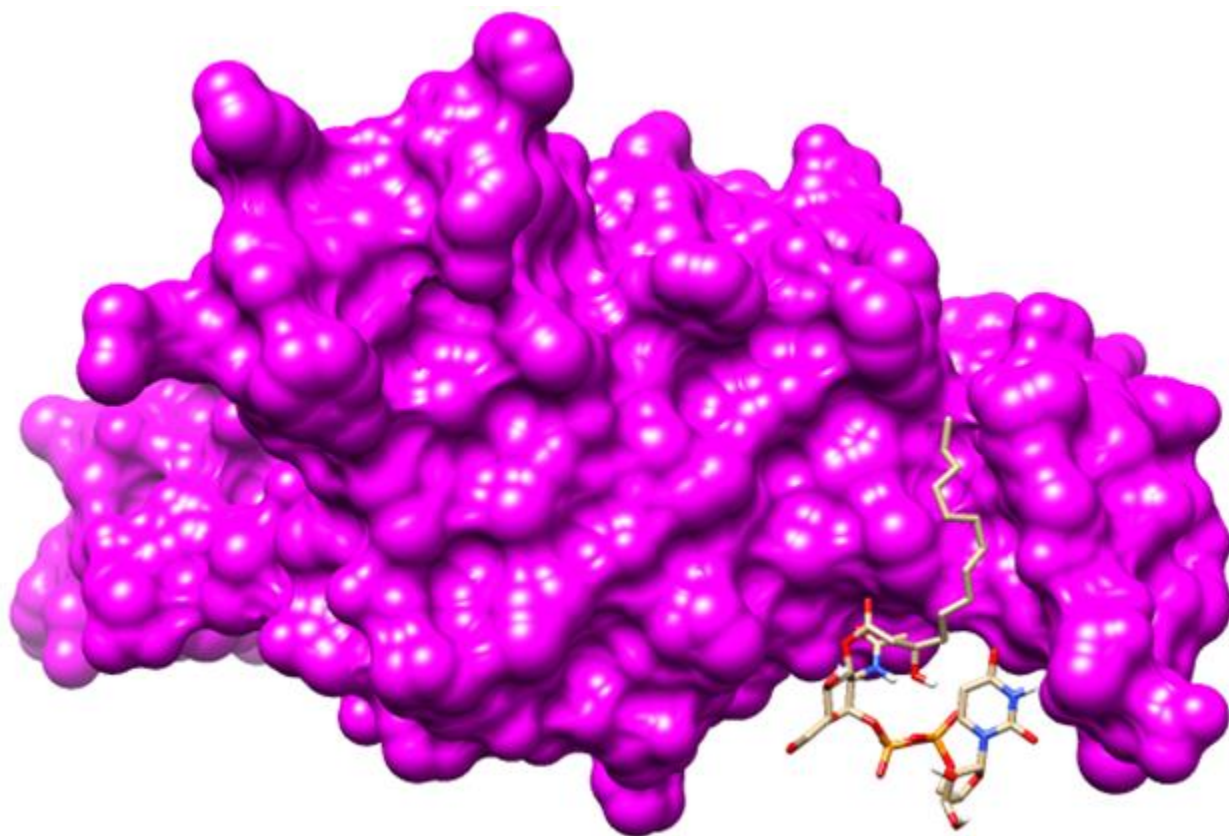
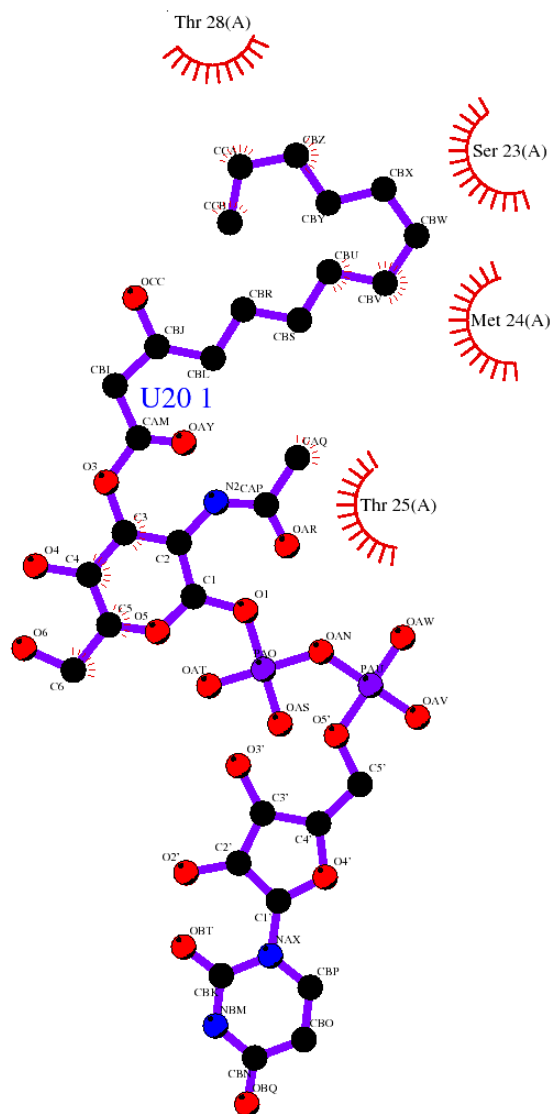


Figure 4.15a: Hydrophobic Representation of G4LZI3 protein docked with UD20. Another ligand predicted to bind to the G4LZI3 protein.



Key

- | | | | |
|--|------------------------------|--|---|
| | Ligand bond | | His 53 Non-ligand residues involved in hydrophobic contact(s) |
| | Non-ligand bond | | Corresponding atoms involved in hydrophobic contact(s) |
| | Hydrogen bond and its length | | |

Figure 4.15b: Residues involved in predicted binding of U20 to the G4LZ13 protein. Residues Threonine, methionine and serine predicted as residues involved in its binding to the protein.

4.3 *In vitro* studies

In vitro studies are those that take place in test tubes, petri dishes, and flasks, mostly with the use of biological material such as cells and microorganisms. Protein expression, purification and subsequent studies based on production of the recombinant protein are a good example of such experiments. Recombinant protein production is particularly important in the world of science today simply because it is the driving force of several biological processes in every living organism (Ismaya, 2011). Drug discovery efforts therefore look at proteins associated with the disease of interest and investigate their 3D structure, as well as other proteins they interact with at the atomic level, so as to assist in drug development efforts (Sugiki *et al.*, 2014). Since the G4LZI3 protein has been identified as a 'lead' molecule in the design of new drugs or vaccines against schistosomiasis, it was therefore necessary to first express, purify and characterize the protein which can subsequently be studied for the aforementioned purpose.

4.3.1 Transformation of *E. coli* JM109 competent cells with pQE30-G4LZI3 construct

The initial step before transformation was the preparation of competent cells which are bacterial cells with altered cell walls that allow easy uptake of foreign DNA. Most cells need special treatment (artificial competence) by suspending them in high concentrations of calcium chloride. DNA and cell membranes of bacterial cells are negatively charged, therefore under normal circumstances these two elements would repel each other. The positively charged ions of calcium chloride solution solve this problem by neutralizing the charges on both the cell membrane and DNA molecules, thus creating pores in the cell walls. A sudden increase in heat (heat shock) treatment

also assists in the competency process by causing the plasmid to enter the bacterial cell. Transformation is the process whereby bacterial cells are genetically modified due to the uptake/ingestion of foreign DNA from their immediate surroundings and this happens with the use of competent cells. *E. coli* JM109 bacterial cells were used for this experiment and were made competent with the use of ice cold sterile $MgCl_2$ and $CaCl_2$. Amongst all other expression systems, *E. coli* stands as the most appealing for heterologous protein production due to its availability, cost efficacy, well-characterized genetics and its ability to rapidly grow under favourable conditions (Sivashanmugam *et al.*, 2008). The cells were then transformed using the pQE30-G4LZI3 construct and colonies grew overnight on Luria-Agar test plates containing Ampicillin, while no growth appeared in the control plate (Fig 4.16). This was a positive indicator that the cells were competent, transformation was successful and that there was no contamination during the transformation process.

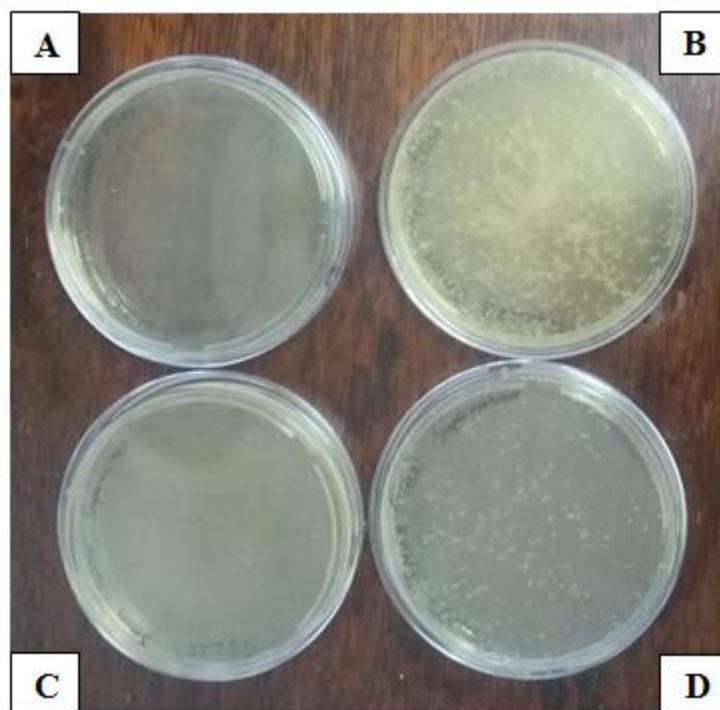


Figure 4.16: Transformation of pQE-30-G4LZ13 constructs. JM109 competent cells were used which conveyed positive transformants. A-Control (Pellet); B-Test (Pellet); C-Control (Supernatant); D-Test (Pellet).

4.3.2 Expression screening and small scale expression of G4LZI3

Expression screening was done as a predictive tool to establish which of the four colonies picked expressed soluble protein that could eventually be used for large scale protein expression. It was imperative that soluble expression be screened on a small scale first, so as to identify which constructs could be used for structural analysis as well as to provide a reliable indication (both quantitatively and qualitatively) of an estimated protein yield if a subsequent decision to invest in large scale expression and purification was to be made (Berrow *et al.*, 2006). Four of the colonies from the overnight plates were randomly picked for expression screening to check for the presence of the protein of interest and to check its expression profile which was analyzed on a 15% SDS PAGE gel. Figure 4.17 indicates that the protein expressed at the correct estimated size, at approximately 20.3kDa when induced with 0.5mM IPTG. Using this gel as a reference, the best expressing clone was used for large scale protein expression.

Thereafter, 100μl of the best protein expressing clone was inoculated overnight in 100ml YT broth and scaled up to a small culture of 300ml the next morning. Figure 4.18 shows the gel of this expression where different temperatures of 25°C and 30°C were used to optimize growth. Additionally, samples were taken periodically after 2hrs, 5hrs and 24hrs and ran on the SDS-PAGE gel. This result showed that the protein was able to express a sufficient amount at 300ml. This then gave a go-ahead for large scale expression to ensue.

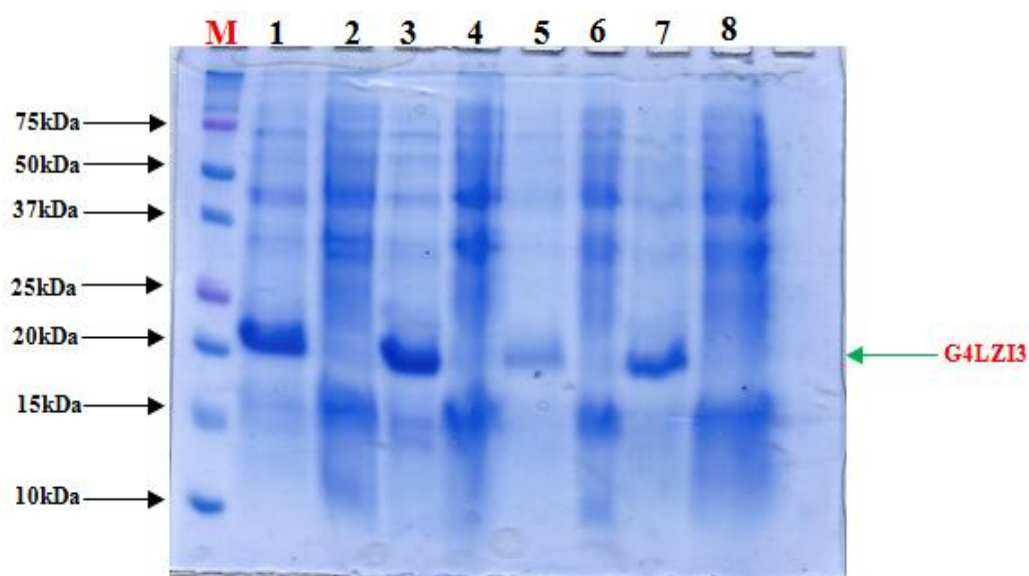


Figure 4.17: Expression Screening of G4LZI3 construct in JM109 cells. M representing the Molecular Marker; Lanes 1, 3, 5, 7 representing induced samples; Lanes 2, 4, 6, 8 representing un-induced samples.

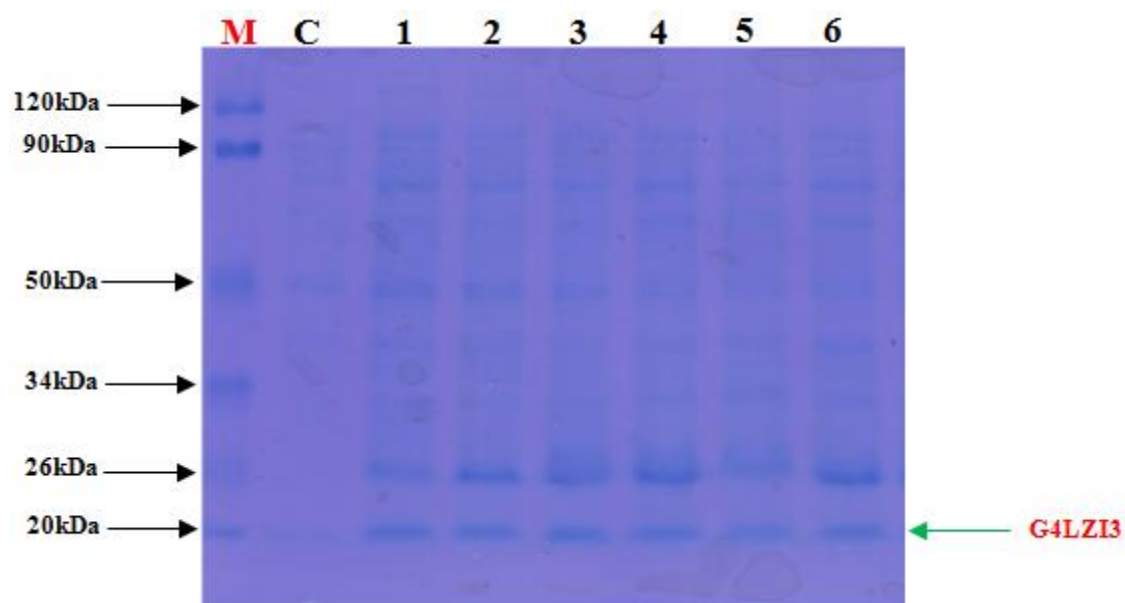


Figure 4.18: Small scale expression of G4LZI3 protein. M representing molecular weight marker; C expression with pQE30 which acted as a control; Lanes 1, 3, 5 fractions incubated at 25°C; Lanes 2, 4, 6 fractions incubated at 30°C.

4.3.3 Western Blot Analysis

Western blot (apart from enzyme-linked immunosorbent assay/ELISA), is one powerful technique for identifying, quantifying and determining the size of specific proteins (Jensen, 2012). Gel electrophoresis was used to separate the desired protein from a pool of other extracted macromolecules based on their molecular weight, then transferred onto a nitrocellulose or polyvinylidene fluoride (PVDF) membrane which is known as electric transfer (Yang and Ma, 2009). Antibodies specific to the protein of interest were then applied and revealed a thick band corresponding to the amount of protein it bound once the film was developed (Mahmood and Yang, 2012). Blocking, which is an important step in western blotting, with non-fat dry milk diluted in TBST, was done to prevent nonspecific binding of the antibody to the membrane. Fig 4.19 shows the western blot experiment done with the SDS-PAGE gel used to analyse the small scale expression of the G4LZI3 protein. Chemiluminiscent detection was used which is a reliable and sensitive method that can be used to quantify the target protein (Jensen, 2012). The figure shows that the anti-his antibody used was able to bind and detect the G4LZI3 protein. Additionally no other band was detected which confirms the size of the protein (~20.3kDa) and verifies that no nonspecific binding took place.

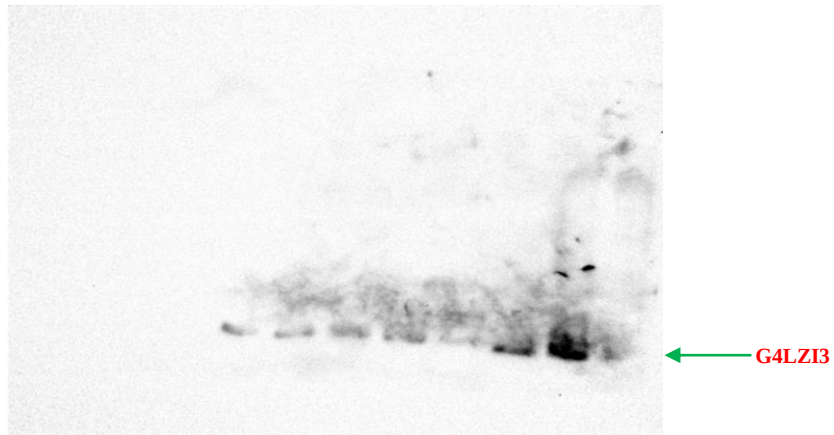


Figure 4.19: Western Blot Analysis. This was done using an anti-His antibody to bind and detect the G4LZI3 protein.

4.3.4 Large scale expression and affinity purification of the G4LZI3 Protein

A wide variety of biotechnology applications require large quantities of soluble and bioactive protein for numerous structural and biochemical analyses. Therefore, the field of recombinant protein production has highly expanded and improved over the years, yielding hundreds of therapeutic proteins (Chen, 2012). Apart from pharmaceutical purposes, recombinant proteins are also needed to study activities *in vitro* such as ligand binding, enzyme activity and protein interactions (Papanephytou and Kantopidis, 2014). The G4LZI3 protein was scaled up to 2L with 2X YT after overnight incubation of 100µl in 100ml of YT broth. Figure 4.20 is a 15% SDS-PAGE gel which shows a clear progression of protein expression at 5µl, 10µl, 15µl and 20µl. Thereafter, the protein was subjected to Nickel-NTA column for purification by affinity chromatography. Figure 4.21 shows that purification was successful with most of the protein eluting at Elution 2 and 3. These fractions were then pooled together and the concentration estimated using a Nanodrop ND2000 spectrophotometer (Figure 4.22), which gave a concentration of 0.518mg/ml.

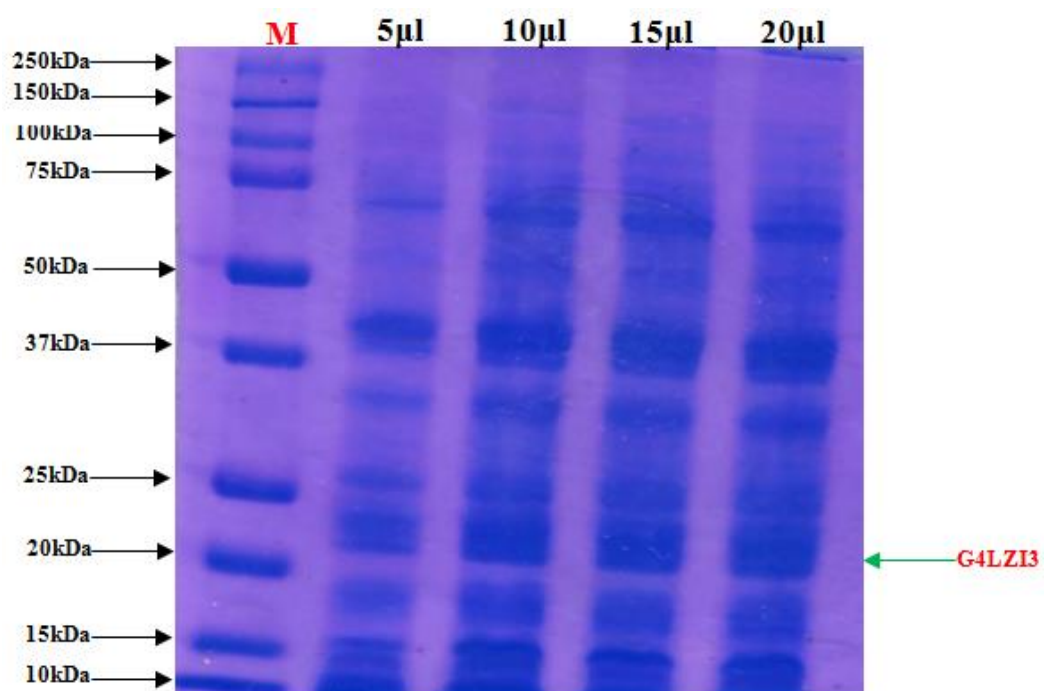


Figure 4.20: Large scale expression of G4LZI3 protein. M represents molecular weight marker and were samples loaded at different volumes of 5µl, 10µl, 15µl and 20µl.

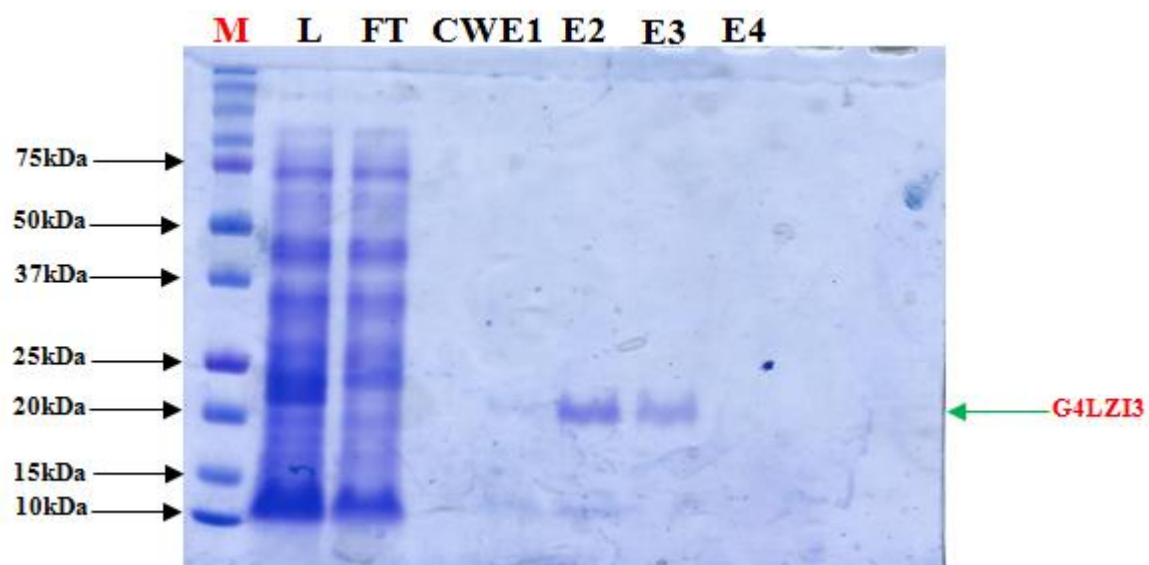


Figure 4.21: Affinity purification of G4LZI3 protein on a Nickel-NTA column. M represents molecular weight marker; L-bacterial lysate; FT-flow through; CW-clean wash; E1- elution 1; E2 - elution 2; E3 - elution 3.

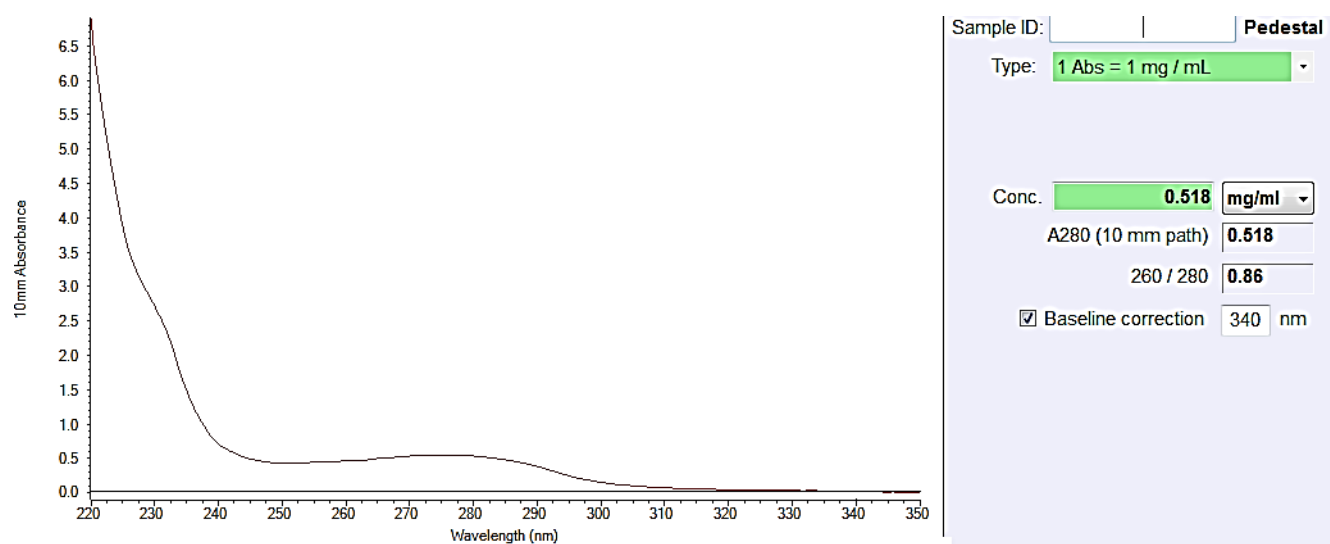


Figure 4.22: Determination of G4LZI3 protein concentration using a Nanodrop ND2000. The protein concentration was measured to be 0.518mg/ml; approximately 0.2mM concentration.

4.3.5 Interaction studies using Isothermal Titration Calorimetry (ITC)

Apart from NMR and SPR, ITC is now being extensively used to investigate protein-ligand and protein-protein interactions (Velazquez-Campoy *et al.*, 2004). This method quantitatively measures the thermodynamic properties of a protein-protein or protein-ligand interaction, and gives values for the stoichiometry, enthalpy of binding and binding constant in a single experiment by measuring the heat absorbed or evolved during a successive number of titrations (Pierce *et al.*, 1999).

The experiments carried out in this study were done with the G4LZI3 protein and the three ligands (Ca^{2+} , Mg^{2+} and Zn^{2+}) that have been hypothesized to interact with the protein. The ligands (reactants) each underwent stepwise addition to the protein (a total amount of 250 μl , 10 μl per titration) in a reaction cell. The energy triggered by the chemical reaction, whether released or absorbed, was then recorded. The amount of heat generated was therefore, proportional to the amount of ligand that bound to the protein at every titration (Leavitt and Freire, 2001). Figures 2.23, 2.24 and 2.25 show the isotherms for titrations done with MgCl_2 , CaCl_2 and ZnSO_4 respectively. With each experiment done, a ligand binding report was generated (see Appendix III).

Most ITC experiments, as is this one, are heterodimeric interactions i.e. the binding partners are completely different from each other (heterodimeric complex). With every titration, there was a change in heat, thus ΔH referred to the enthalpy of binding. Once the protein within the cell was fully saturated, there was no significant change in heat, meaning the concentration of the complex had reached its maximum (Velazquez-Campoy *et al.*, 2004). This heat was then used to calculate the area under each peak (peak area). With every subsequent titration, the amount of uncomplexed protein

available decreased, which in turn caused the peaks to become smaller until the protein was fully saturated such that any other injection/s after that would produce similar peaks due to dilution or mechanical effects (Leavitt and Freire, 2001).

The dissociation constant (K_d) tells the strength of the interaction between a ligand and macromolecule. For high dissociation constants, the ligand would bind to the macromolecule until fully saturated thereby producing peaks that showed the same heat flow towards the end such as in Figure 4.23. This figure shows clear interaction between $MgCl_2$ and the G4LZI3 protein. Fifteen titrations were made for this particular experiment but it may actually be possible that the protein still had available sites for binding. It is therefore recommended that an increased number of injections take place. Unfortunately, satisfactory binding cannot be said to be found in the case of Figure 4.24, which shows unfavourable binding. Ca^{2+} however, has been predicted as one of G4LZI3 binding partners, so this data may be inconclusive. Therefore, it is recommended that this experiment be carried out again using SPR to confirm or disprove this result. Figure 4.25 shows the ITC experiment between G4LZI3 and Zn^{2+} . This shows no interaction whatsoever between the ligand and macromolecule. However, this may be due to a number of factors; it may be that there is indeed no interaction between the two reactants or possibly the first injection of ligand was enough to super-saturate the protein. Thus, it will be ideal in future to do several trial and error experiments to determine which actual concentration would give interaction (if any) between the ligand and the G4LZI3 protein.

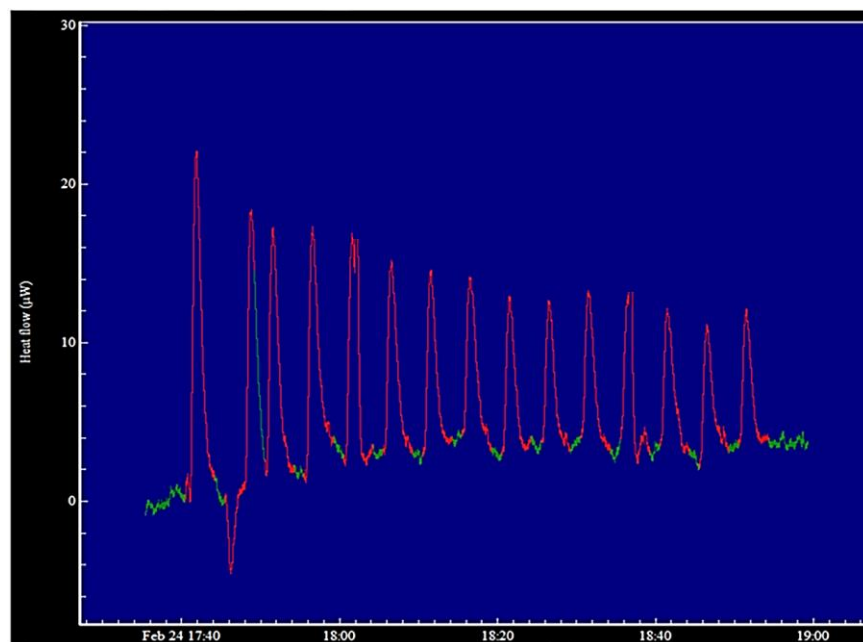


Figure 4.23: ITC experiment with G4LZI3 protein and MgCl₂ as the ligand. Thermogram shows binding between the ligand and the protein.

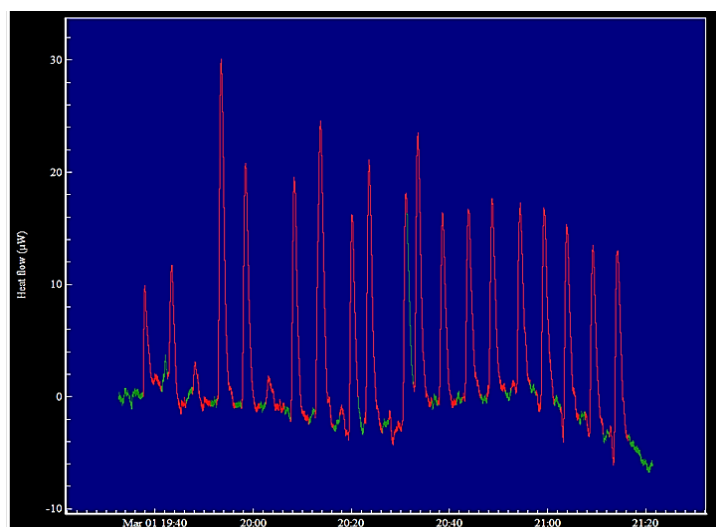


Figure 4.24: ITC experiment with G4LZI3 protein and CaCl₂ as the ligand. Thermogram shows no binding between Ca²⁺ and the protein but this result remain inconclusive.

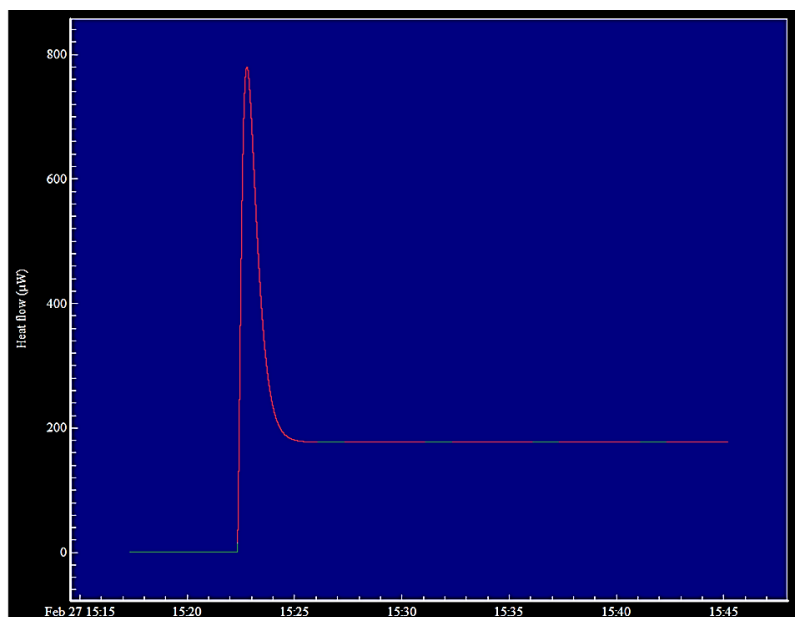


Figure 4.25: ITC experiment with G4LZI3 protein and ZnSO₄ as the ligand. Thermogram reveals that there is no binding between Zn²⁺ and the G4LZI3 protein.

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CHAPTER 5:

CONCLUSION AND FUTURE PERSPECTIVES

5.1 CONCLUSION

This study was able to detect, with the use of various bioinformatics tools, binding sites or interaction pockets for three putative ligands (Ca^{2+} , Mg^{2+} and Zn^{2+}) in the G4LZI3 protein as well as for AMP, ATP and U20. It has been proposed by Mbah and co-workers (2013) that these ligands all play important roles in regulating many biochemical and cellular processes. PZQ disrupts Ca^{2+} homeostasis in schistosome worms, causing a swift influx of Ca^{2+} ions into the worm and rapid muscular contractions. Calcium's role in the G4LZI3 protein, for instance, may be to modulate the survival mechanism of the worm throughout the adverse environmental conditions the worm faces during its developmental cycle. The results for the predicted binding of AMP and ATP to the protein confirmed previous studies which also predicted the binding of these ligands to the protein. However, *in vivo* experiments suggested otherwise; the docking done with the metallic cations only showing interaction between Mg^{2+} and the G4LZI3 protein. The Mg^{2+} ion has been suggested as an ATP metallic chemical ligand, which could play a role in binding to the Mg-ATP-binding groove during phosphorylation or in the process of stress response mechanisms that mostly depend on ATP; mechanisms which may include those that involve the G4LZI3 protein since it is a protein that responds to stress. Banerjee *et al.* (2015) have reported AMP, mostly in the form of cAMP, to bind to USPs; thus functioning as an ATP energy regulator in the G4LZI3 protein as well as in sensory transduction pathways.

Nevertheless, this study was a milestone, since for the first time, expression and purification of this novel protein was successfully done in our laboratory. Additionally, other vital information was gathered such as the different interacting partners

associated with the protein, the determination of secondary structure and the generation of a 3D model. This will contribute significantly in getting to know the exact function of the protein and in future design of a new treatment regimen for the debilitating disease schistosomiasis.

5.2 FUTURE PROSPECTS

Future interaction studies would include multi parametric Surface Plasmon Resonance (SPR) experiments using the various ligands and the G4LZI3 protein in order to confirm the results obtained by ITC. It would also be ideal to take note of certain factors that were overlooked during this study and attempt to modify them for better results. These include degassing both the ligand and protein prior to any ITC experiment, the use of different buffers like Tris-HCl, as well as increasing or decreasing the concentration of the ligand; to determine if there really is no interaction or if a certain concentration is required for the interaction. Thus, more trial and error experiments would be ideal. Future prospects would also involve subjecting the G4LZI3 protein to Nuclear Magnetic Resonance (NMR) spectroscopy, especially 2D ^{15}N -HSQC experiments. Although this will require labelling the protein with isotopes such as ^{15}N -ammonium chloride and ^{13}C -glucose which are costly, this tool will further confirm binding and subsequent triple resonance transfer experiments, such as CBCA(CO)NH and HNCACB will actually help to identify residues involved in any interaction. Finally, the ultimate goal would be subjecting the G4LZI3 protein to crystallization trials for X-ray crystallography in order to solve the structure of the protein. This aim may also be achieved using NMR spectroscopy or Electron Microscopy Tomography. Once this goal is achieved, the

structure can then be used as a 'lead' molecule in rationale drug discovery programme targeted towards effective and efficient treatment regimen and even elimination of schistosomiasis infection.

APPENDIX I: GENERAL CHEMICALS AND ENZYMES

PRODUCT	COMPANY
Acetic Acid	Merck
Ammonium Persulphate (APS)	Biorad
Ampicillin sodium salt	Inqaba Biotech
Calcium Chloride	Merck
Coomasie Brilliant Blue	Sigma
Dithiothreitol (DTT)	Merck
Ethanol	Merck
Fastcast	Inqaba Biotech
Glucose	Merck
Glycerol	Merck
Glycine	Merck
His-Select Nickel Affinity Gel	Sigma
Imidazole	Sigma
Isopropyl- β -D-Thiogalactopyranoside (IPTG)	Inqaba
Lysozyme	Merck
Magnesium Chloride	Merck

Nutrient Agar	Merck
Phenylmethylsulfonyl Fluoride (PMSF)	Sigma
Precision Plus Protein Marker	Biorad
Sodium Azide	Merck
Sodium Chloride	Merck
Sodium Dodecyl Sulphate (SDS)	Merck
N,N,N',N'-Tetra methyl ethylene diamine (TEMED)	Merck
Tris (hydroxymethyl) Aminoethane	Merck
Tryptone Powder	Merck
Yeast Extract Powder	Merck
Zymospin Miniprep Kit	Inqaba biotech

APPENDIX II: PREDICTION OF PROTEIN PARAMETERS

NCBI Reference Sequence: Smp_076400

>gi|350645018|emb|CCD60301.1| Universal stress protein G, putative [*Schistosoma mansoni*]

ATGTCAGAAACAGAACCAACCTTCGACTTCCGACGGATTAGATATTGGAGAAACGAA
AGGAACTATATCAATGACTGATGCAACACGTAAAGTATTAATGCCGGTCGATGGAA
GTGAACATAGTGAAAGAGCATTCAATTGGTATATGGATAATGTTATGAAAATAACT
GATGGTTTATATCTAGTTCATATTGTAGAACCATTATCACAAGGATTGAATTATAAT
CTTGCAAGTAAATCTCCCTCTATAAAAGATGATTTTTTCAAACATCTAAATTCCTTG
TTGAATCTGGTCGTGCATTACGTGCTAAATTTTTTACACGTTGTGAAGATAGTGGTTT
ATCAGCAAGATTTACCATACACGTTGGTACTAAACCAGGTGAAAATATTGTACGTAT
CGCCCATGAACATGGAGTTGATTTAGTTATTATTGGAAATCGTGGTATTGGAACAGT
AAAGAGAACATTTTTAGGTAGTGTAAGTGATTATGTTTTACATCATGCCAATGTTCC
TGTTATTATAATACCACCACCTAAACATCCAAAGAAAAAAATAA

Figure A: DNA sequence of Universal Stress Protein G4LZI3

10	20	30	40	50	60
MSETEQPST	DGLDIGETK	TISMTDATR	KLMPVDGSE	SERAFNWYMD	NVMKITDGLY
70	80	90	100	110	120
LVHIVEPLSQ	GLNYNLASKS	PSIKDDFSKH	LNSLVESGRA	LRAKFFTRCE	DSGLSARFTI
130	140	150	160	170	180
HVGTKPGENI	VRIAHEHGVD	LVIIGNRGIG	TVKRTFLGSV	SDYVLHGANV	PVIIIPPKH

PKKK

Figure B: Full-length amino acid sequence of G4LZI3 excluding the mRNA sequence

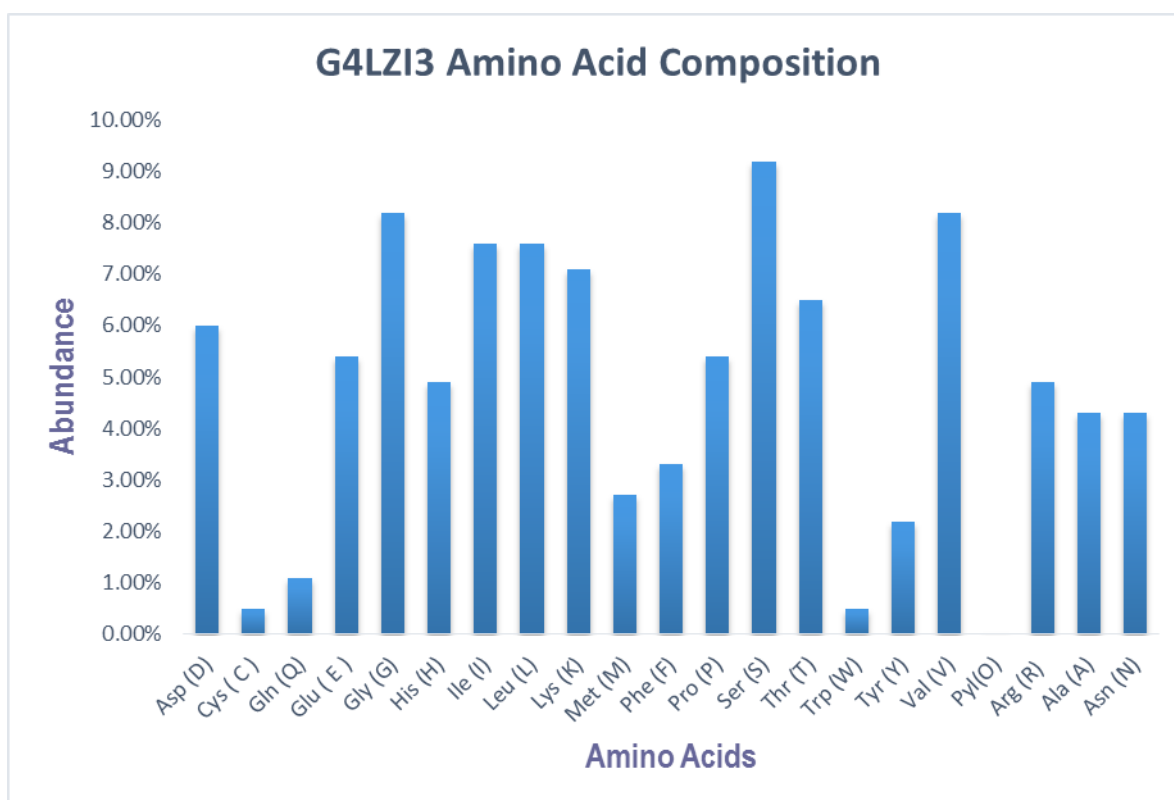


Figure C: Amino Acid Composition of *S. mansoni* Universal Stress Protein G4LZI3. Serine, Valine and Glycine are the amino acids with the highest abundance, while Cysteine and Trptophan have the lowest abundance.

Table A: Physicochemical properties of G4LZ13 calculated from the amino acid sequence

ProtParam Parameters	Values
Number of amino acids	184
Molecular weight	20276.1 Da
Atomic composition	Carbon 897 Hydrogen 1435 Nitrogen 253 Oxygen 270 Sulphur 6
Theoretical pI	7.92
Number of negatively charged residues	21
Number of positively charged residues	22
Formula	$C_{897}H_{1435}N_{253}O_{270}S_6$
Extinction coefficient	11460 Abs 0.1% (=1 g/l) 0.565, assuming all pairs of Cys residues form cystines Abs 0.1% (=1 g/l) 0.565, assuming all Cys residues are reduced
Estimated Half-life	30hrs (Mammalian reticulocytes, <i>in vitro</i>) >20hrs (yeast, <i>in vivo</i>) >10hrs, (<i>Escherichia coli</i>, <i>in vivo</i>)
Instability index	34.97
Aliphatic index	87.34
Grand average of hydropathicity (GRAVY)	-0.305
Total number of atoms	2861

APPENDIX III: SUPPLEMENTARY DATA

Table B: Accession number and corresponding names and sources of G4LZ13 homologues

Accession Number	Source & Name	Protein Length	Blast E-value
XP_018646651.1	Universal stress protein G, putative [Schistosoma mansonii]	184	2e-133
EUB59302.1	Universal Stress protein A-like protein [Echinococcus granulosus]	210	1e-59
CDI96922.1	Universal Stress protein [Echinococcus multicularis]	210	2e-59
GAA41511.1	Universal Stress protein A-like protein [Chlonorchis sinensis]	164	6e-43
CDS35071.1	Universal Stress protein in QAH:OAS [Hymenolepis microstoma]	184	1e-42
XP_001637863.1	predicted protein [Nematostella vectensis]	155	3e-35
KXJ07042.1	Universal Stress protein A-like protein [Exaiptasia pallida]	167	2e-32
XP_015754675.1	PREDICTED: universal stress protein Slr1101-like [Acropora digitifera]	147	2e-29
XP_005097850.2	PREDICTED: universal stress protein A-like protein [Aplysia californica]	166	4e-27
XP_009060489.1	hypothetical protein LOTGIDRAFT_165237 [Lottia gigantean]	159	2e-26
XP_012553698.1	PREDICTED: universal stress protein A-like protein isoform X2 [Hydra vulgaris]	159	6e-26
XP_011412635.1	PREDICTED: uncharacterized protein LOC105317627 isoform X4 [Crassostrea gigas]	151	1e-25
XP_002130444.1	PREDICTED: uncharacterized protein LOC100180023 isoform X1 [Ciona intestinalis]	150	1e-25
XP_009167172.1	hypothetical protein T265 04125 [Opisthorchis viverrini]	182	6e-42

Table C: Conservation level of G4LZI3 homologues

-	XP_018 646651. 1	EUB5 9302. 1	CDI9 6922. 1	GAA 41511 .1	CDS3 5071. 1	XP_001 637863. 1	KXJ0 7042. 1	XP_015 754675. 1	XP_005 097850. 2	XP_009 060489. 1	XP_012 553698. 1	XP_011 412635. 1	XP_002 130444. 1	XP_014 775911. 1	XP_009 167172. 1
XP_018 646651. 1	-	41.9 (60.5)	41.0 (60.5)	37.1 (58.1)	35.5 (52.8)	31.7 (50.3)	28.3 (44.4)	32.3 (47.3)	27.2 (41.0)	31.4 (42.8)	28.9 (46.0)	25.5 (44.8)	27.6 (45.4)	26.3 (41.9)	36.0 (58.1)
EUB59 302.1	41.9 (60.5)	-	97.1 (99.5)	29.0 (49.0)	26.9 (46.2)	29.4 (46.0)	29.2 (47.2)	24.5 (40.6)	24.2 (40.6)	24.6 (40.0)	26.4 (42.0)	26.1 (40.8)	25.1 (42.2)	23.7 (40.0)	30.0 (50.0)
CDI969 22.1	41.0 (60.5)	97.1 (99.5)	-	29.5 (48.6)	26.9 (45.8)	29.4 (46.0)	29.2 (47.2)	24.5 (40.6)	24.2 (40.8)	24.6 (39.8)	26.4 (41.5)	26.1 (40.8)	25.1 (42.2)	23.7 (39.8)	30.5 (49.5)
GAA41 511.1	37.1 (58.1)	29.0 (49.0)	29.5 (48.6)	-	29.9 (49.2)	30.6 (52.9)	33.3 (45.8)	32.3 (49.7)	31.0 (47.1)	26.0 (44.5)	31.7 (52.1)	28.9 (48.0)	25.9 (47.6)	27.1 (44.6)	84.1 (86.8)
CDS35 071.1	35.5 (52.8)	26.9 (46.2)	26.9 (45.8)	29.9 (49.2)	-	23.8 (43.4)	23.4 (42.6)	24.7 (44.1)	26.2 (41.7)	26.6 (37.0)	28.5 (42.5)	27.6 (39.1)	23.2 (42.7)	27.3 (43.3)	29.9 (47.4)
XP_001 637863. 1	31.7 (50.3)	29.4 (46.0)	29.4 (46.0)	30.6 (52.9)	23.8 (43.4)	-	35.5 (54.1)	38.4 (59.7)	29.1 (51.2)	30.1 (50.6)	30.3 (47.9)	32.9 (55.3)	38.1 (59.4)	28.9 (53.2)	27.1 (47.9)
KXJ07 042.1	28.3 (44.4)	29.2 (47.2)	29.2 (47.2)	33.3 (45.8)	23.4 (42.6)	35.5 (54.1)	-	38.1 (56.5)	31.4 (49.4)	26.0 (43.9)	25.4 (46.2)	26.6 (45.7)	30.2 (49.4)	32.6 (51.7)	30.3 (41.0)
XP_015 754675. 1	32.3 (47.3)	24.5 (40.6)	24.5 (40.6)	32.3 (49.7)	24.7 (44.1)	38.4 (59.7)	38.1 (56.5)	-	33.9 (53.6)	36.9 (55.0)	28.4 (45.7)	36.8 (59.4)	33.8 (55.4)	33.1 (55.4)	29.7 (44.9)
XP_005 097850. 2	27.2 (41.0)	24.2 (40.6)	24.2 (40.8)	31.0 (47.1)	26.2 (41.7)	29.1 (51.2)	31.4 (49.4)	33.9 (53.6)	-	32.6 (51.7)	25.9 (45.9)	34.9 (55.2)	32.7 (51.2)	43.2 (63.9)	28.6 (42.2)
XP_009 060489. 1	31.4 (42.8)	24.6 (40.0)	24.6 (39.8)	26.0 (44.5)	26.6 (37.0)	30.1 (50.6)	26.0 (43.9)	36.9 (55.0)	32.6 (51.7)	-	30.8 (43.8)	46.5 (68.6)	30.4 (48.2)	36.3 (59.1)	24.6 (39.8)
XP_012 553698. 1	28.9 (46.0)	26.4 (42.0)	26.4 (41.5)	31.7 (52.1)	28.5 (42.5)	30.3 (47.9)	25.4 (46.2)	28.4 (45.7)	25.9 (45.9)	30.8 (43.8)	-	28.0 (47.2)	30.4 (49.7)	26.6 (48.0)	28.1 (45.9)
XP_011 412635. 1	25.5 (44.8)	26.1 (40.8)	26.1 (40.8)	28.9 (48.0)	27.6 (39.1)	32.9 (55.3)	26.6 (45.7)	36.8 (59.4)	34.9 (55.2)	46.5 (68.6)	28.0 (47.2)	-	35.0 (50.9)	41.8 (61.8)	26.7 (42.9)
XP_002 130444. 1	27.6 (45.4)	25.1 (42.2)	25.1 (42.2)	25.9 (47.6)	23.2 (42.7)	38.1 (59.4)	30.2 (49.4)	33.8 (55.4)	32.7 (51.2)	30.4 (48.2)	30.4 (49.7)	35.0 (50.9)	-	26.5 (48.2)	23.9 (43.5)
XP_014 775911. 1	26.3 (41.9)	23.7 (40.0)	23.7 (39.8)	27.1 (44.6)	27.3 (43.3)	28.9 (53.2)	32.6 (51.7)	33.1 (55.4)	43.2 (63.9)	36.3 (59.1)	26.6 (48.0)	41.8 (61.8)	26.5 (48.2)	-	25.6 (40.0)
XP_009 167172. 1	36.0 (58.1)	30.0 (50.0)	30.5 (49.5)	84.1 (86.8)	29.9 (47.4)	27.1 (47.9)	30.3 (41.0)	29.7 (44.9)	28.6 (42.2)	24.6 (39.8)	28.1 (45.9)	26.7 (42.9)	23.9 (43.5)	25.6 (40.0)	-

Table Legend: Sequence identities and similarities are presented as percentages. Similarities are written in parenthesis. Ascension numbers are described in Table B above.

Ligand Binding Report

Mar 02, 2017 7:43

Model

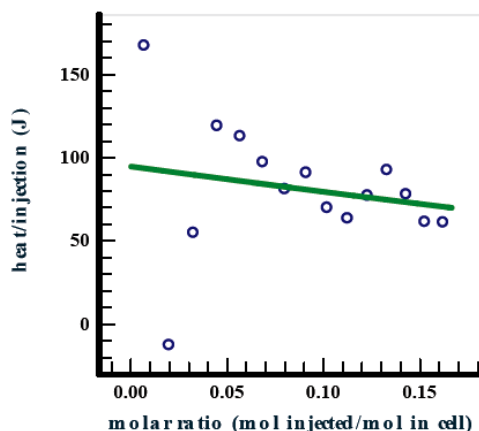


Thermodynamics Parameters

k_1	$9.7 \cdot 10^2 \pm$	$8.2 \cdot 10^3$
ΔH_1	$-13 \text{ MJ/mol} \pm$	89 MJ/mol
ΔG_1	-17.04 kJ/mol	
ΔS_1	$-42.732 \text{ kJ/(K} \cdot \text{mol)}$	

Statistics

Degrees of freedom	13
χ^2	$2.02 \cdot 10^{-6}$
Reduced χ^2	$1.55 \cdot 10^{-7}$
Standard error of point	$3.94 \cdot 10^{-4}$



Input Data

Trial Titration (2-24-17)-unizulu

Volume in vessel	750 μl
Number of datapoints	15
Concentration of [M] in vessel	265 μM
Concentration of [L] in syringe	36.4 μM

No	Volume (μl)	Q_{exp} (mJ)	Q_{calc} (mJ)	Residual (mJ)
1	10.0	1.67	0.93	0.74
2	10.0	-0.12	0.91	-1.04
3	10.0	0.55	0.90	-0.34
4	10.0	1.19	0.88	0.32
5	10.0	1.13	0.86	0.27
6	10.0	0.97	0.84	0.13
7	10.0	0.81	0.82	-0.01
8	10.0	0.91	0.81	0.10
9	10.0	0.70	0.79	-0.09
10	10.0	0.64	0.78	-0.14
11	10.0	0.77	0.76	0.01
12	10.0	0.93	0.75	0.18
13	10.0	0.78	0.73	0.05
14	10.0	0.62	0.72	-0.10
15	10.0	0.61	0.70	-0.09

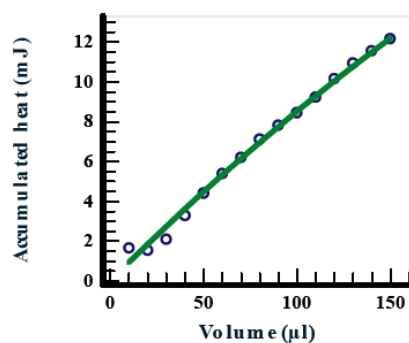
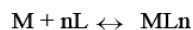


Figure D: Ligand Binding Report (MgCl_2)

Ligand Binding Report

Mar 02, 2017 7:33

Model



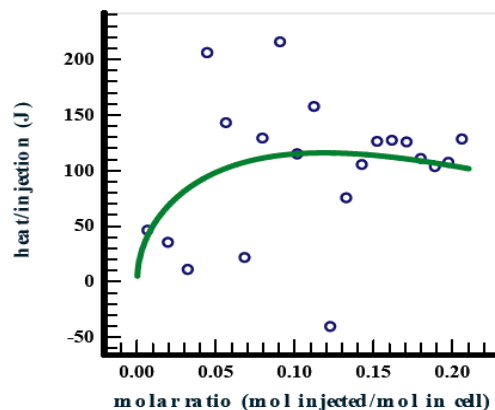
$\beta_1, \Delta H_1$

Thermodynamics Parameters

k_1	$1.7 \cdot 10^2 \pm$	$9.7 \cdot 10^2$
ΔH_1	-4.6 MJ/mol	$\pm 14 \text{ MJ/mol}$
n	$1.5 \cdot 10^0 \pm$	$5.0 \cdot 10^{-1}$
ΔG_1	-12.784 kJ/mol	
ΔS_1	$-15.473 \text{ kJ/(K} \cdot \text{mol)}$	

Statistics

Degrees of freedom	17
χ^2	$6.73 \cdot 10^{-6}$
Reduced χ^2	$3.96 \cdot 10^{-7}$
Standard error of point	$6.29 \cdot 10^{-4}$



Input Data

CaCl2 Titration final (3-1-17)

Volume in vessel	750 μ l
Number of datapoints	20
Concentration of [M] in vessel	36.5 μ M
Concentration of [L] in syringe	50.333 mM

No	Volume (μ l)	Q_{exp} (mJ)	Q_{calc} (mJ)	Residual (mJ)
1	10.0	0.46	0.39	0.08
2	10.0	0.35	0.67	-0.32
3	10.0	0.11	0.83	-0.72
4	10.0	2.06	0.94	1.11
5	10.0	1.43	1.02	0.41
6	10.0	0.22	1.07	-0.85
7	10.0	1.29	1.11	0.18
8	10.0	2.15	1.13	1.02
9	10.0	1.15	1.15	-0.00
10	10.0	1.57	1.15	0.42
11	10.0	-0.40	1.16	-1.56
12	10.0	0.75	1.15	-0.40
13	10.0	1.05	1.14	-0.09
14	10.0	1.26	1.13	0.13
15	10.0	1.27	1.12	0.15
16	10.0	1.25	1.10	0.15
17	10.0	1.11	1.08	0.02
18	10.0	1.03	1.06	-0.03
19	10.0	1.07	1.04	0.03
20	10.0	1.28	1.02	0.26

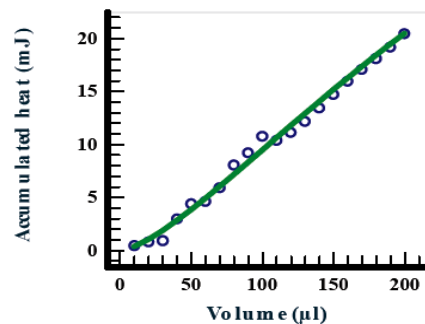


Figure E: Ligand Binding Report (CaCl₂)

Ligand Binding Report

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Model

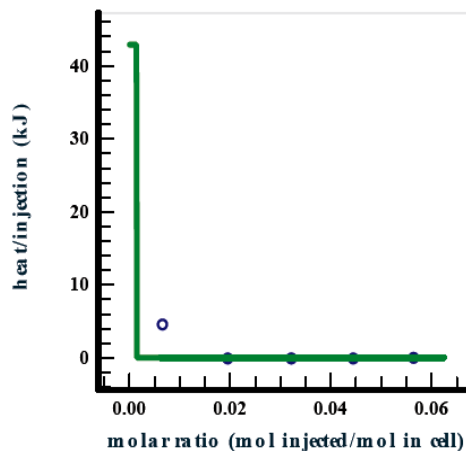


Thermodynamics Parameters

k_1	$2 \cdot 10^{17} \pm 4.6 \cdot 10^{21}$
ΔH_1	$-1.67 \text{ MJ/mol} \pm 28 \text{ kJ/mol}$
n	$1.00 \cdot 10^0$
ΔG_1	-98.635 kJ/mol
ΔS_1	$-5.269 \text{ kJ/(K} \cdot \text{mol)}$

Statistics

Degrees of freedom	3
χ^2	$1.74 \cdot 10^{-6}$
Reduced χ^2	$5.79 \cdot 10^{-7}$
Standard error of point	$7.61 \cdot 10^{-4}$



Input Data

ZnSO4 Titration (2-27-17)

Volume in vessel	750 μ l
Number of datapoints	5
Concentration of [M] in vessel	36.5 μ M
Concentration of [L] in syringe	25.7 mM

No	Volume (μ l)	Q_{exp} (mJ)	Q_{calc} (mJ)	Residual (mJ)
1	10.0	45.71	45.71	0.00
2	10.0	-0.75	0.00	-0.75
3	10.0	-0.76	0.00	-0.76
4	10.0	-0.77	0.00	-0.77
5	10.0	0.00	0.00	-0.00

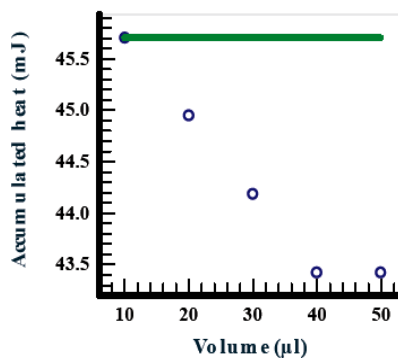


Figure F: Ligand Binding Report (ZnSO₄)