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Identification and structural bioinformatics of druggable proteins in

Schistosoma species

By

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MASTERS IN BIOCHEMISTRY

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DECLARATION

I, Raphael Taiwo Aruleba (Student No: 201633175), declare that "Identification and structural bioinformatics of druggable proteins in *Schistosoma species*" is my own work and has not been submitted for any degree in any university. Also, I have acknowledged by references all the resources I used.

Candidate

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ABSTRACT

Schistosomiasis is a debilitating disease caused by a parasitic flatworm found in freshwater. After malaria, this disease is the second most prevalent disease in Africa and is endemic in both tropical and sub-tropical regions of the world. Morbidity and mortality attributed to this disease are very high with about 240 million people infected, 800 million persons at risk of the infection and an approximately 280,000 deaths occurring annually. With the exponential increase in morbidity and mortality resulting from Schistosomiasis, there is an urgent need for the development of new drug since studies have shown that schistosomes are becoming resistant to the widely accepted first-line drug-of-choice Praziquantel (PZQ). Therefore, the present study describes the exploration of broad-spectrum therapeutic potentials of Antimicrobial peptides (AMPs) in the design of alternative anti-schistosomal treatment regimen. AMPs are natural antibiotics produced by all living species; they have multifunctional properties and are currently explored as a vital source for the development of new drugs. The use of therapeutic peptides in various disorder treatment has been receiving significant and great attention in recent years. In this study, six putative AMPs (TAK1-TAK6) were identified to possess very strong anti-schistosomal capabilities using Hidden Markov Model. Added to this, glycosyltransferase and axonemal dynein intermediate chain schistosomal proteins were identified using *in silico* methods as vital proteins for the survival of the parasite in the host. The 3D structures of the AMPs and the proteins were modelled using the I-TASSER, while PatchDock was employed to ascertain the interaction between these schistosome proteins and the AMPs. Results obtained show that the putative AMPs have good binding affinity to the schistosomal proteins. More so, TAK3 and TAK6 showed highest binding affinities to glycosyltransferase and Axonemal dynein intermediate chain respectively. Site-directed mutagenesis studies based on the putative anti-schistosomal AMPs was carried out to increase

their biological activities; homology modelling of the mutated AMPs using I-TASSER showed that they are identical to the parental AMPs. More so, results from molecular docking using PatchDock showed that these mutated AMPs are capable of interacting with the schistosome proteins. In conclusion, results suggest that these peptides maybe potential "drug leads" in the design and development of alternative schistosomal therapy and could as well prove to be effective against PZQ-resistant schistosome strains, based on the strong interactions between both the AMPs (Parental and Mutated) and the schistosomal proteins.

DEDICATION

This dissertation is dedicated to God for being my Elohim Ozer Li. Also, to those who gave me full support and guidance; my parents (Elder and Mrs. T.T Aruleba) whom I call my small world, dear sister (Dr. G.O Aruleba) and my great computer scientists (Aruleba Kehinde Daniel and Aruleba Thomas Idowu) for sharing with me my dreams.

I LOVE YOU ALL

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CHAPTER ONE

Introduction and Literature Review

1.1 Neglected Tropical Diseases

Neglected tropical diseases (NTDs) are various group of contagious infections that affect approximately 149 countries, with over one billion persons from Africa, Asia and America (with over 500 million children), costing these developing areas billions of dollars annually. These diseases are caused by various infectious microorganisms such as helminths, viruses, protozoa and bacteria and their transmission mode are similar to tuberculosis and malaria in Sub-Saharan Africa (SSA) (Hotez and Kamath, 2009). These infections commonly affect poor populations that are close to domestic animals, infectious agents and bad sanitation. Concurrently, at least five of these diseases affect countries with low-income economies. Schistosomiasis, onchocerciasis, lymphatic filariasis (LF), soil-transmitted helminth (STH) and trachoma are NTDs that are most common among people with over 500 million people been affected (Fenwick, 2006; Molyneux *et al*., 2005; Olsen, 2007).

Table 1.1: Some of the most life-threatening tropical diseases globally, causative organism, prevalence of the disease and the therapy needs **(Adapted from Hotez *et al*., 2007).**

Disease	Organism	Scope	Therapy needs
Malaria	*Plasmodium spp*	500 million Infections yearly	New drugs and avoiding drug resistance
Lymphatic filariasis	*Wuchereria* *bancrofti*	120 million	Access to needed medicines
Schistosomiasis	*Schistosoma spp*	207 million existing infection	Backup drug is needed should praziquantel resistance arise
Leishmaniasis	*Leishmania spp*	2 million infections yearly	Safe, orally bioavailable drugs, especially for the visceral form of the disease
Ascariasis	*Ascaris lumbricoides*	807 million	Access to essential medicines
Hookworm infection	*Ancylostoma duodenale*	576 million	Highly effective and readily available medicine
Trypanosomiasis (sleeping sickness, Chagas disease)	*T. brucei* (sleeping sickness) *T. cruzi* (Chagas disease)	HAT: 300000 cases yearly Chagas: 16 million existing infections	Safe, orally bioavailable medicines specifically for the severe stages of the disease
Giardiasis/ amebiasi	*Entamoeba histolytica*	Millions of cases of diarrhoea annually	Well-tolerated medicines
Leprosy	*Mycobacterium leprae*	400000	Access to needed medicines
Trachoma	*Chlamydia trachomatis*	84 million	Access to needed medicines and need public health intervention.

1.2 Schistosomiasis

From a universal public health disposition, schistosomiasis continues to be the most significant waterborne disease (Steinmann *et al*., 2006) and recently, Adenowo and colleagues (2015) showed that schistosomiasis follows hookworm in the Sub-Saharan Africa on the list of NTDs. Schistosomiasis, also referred to as bilharzia is caused by parasitic trematode flatworms found in fresh water which belongs to the genus *Schistosoma* (Oyinloye *et al*., 2014; Merrifield *et al*., 2016). This disease mostly affects underprivileged rural communities particularly those located in areas where fishing and farming activities are a major occupation. Historically, women and children are subject to this infection through domestic activities like fetching or doing laundry in infected water, swimming and bad sanitation (World Health Organisation, 2014).

This disease leads to numerous unrecorded acute disabilities and illnesses such anaemia, caloric malnutrition, poor school performance and stunted growth, thus, leading poor life condition and continued poverty (Steinmann *et al*., 2006; King, 2010). Simultaneous infection of this disease alongside with HIV, malaria and hepatitis can expose the host to malignant hepatoma and raise the mortality rate. Tumour of the bladder can result from the urinary manifestation of this disease (Brindley *et al*., 2009).

Epidemiologic studies have reported the transmission of this acute disease from 78 countries (WHO, 2015), in which 192 million (93%) of the world total case (207 million) occurs in Sub Saharan Africa (Adenowo *et al*., 2015), with 800 million people at risk of being infected (El Ridi and Tallima, 2013). Moreover, approximately 18 million infected people have bladder wall pathology, 8.5 million suffers from hepatomegaly and 10 million have hydronephrosis (van der Werf *et al.,* 2004; Koukounari *et al*., 2006). Overall, schistosomiasis kills more than 280,000 persons annually (Perez-Saez *et al*., 2015).

Moreover, statistics have shown that over 4 million persons have been infected with this disease in South Africa (Mbabazi *et al*., 2011), with over 25.7 million persons at risk of infection (Wolmarans and De Kock, 2009). The southern and eastern parts of South Africa are the areas with high prevalence, especially, among children, adolescents and women. However, despite this, preventive measures to curb schistosomiasis by the health agencies remains of low importance in this country (Johnson and Appleton, 2005; Wolmarans and De Kock 2009).

1.3 The Biology of Schistosomes

The complicated life cycle of schistosomes involves harsh ambience, free living stages, many hosts and dimorphic adult worms living inside the host (Jolly *et al*., 2007; Gobert *et al*., 2009). They go through various developmental stages that allow genetic recombination and lay eggs immensely. The parasite can stay in the host blood vessels for many years; this parasitism ability highlights the *schistosoma* species aptitude for evasion and prolonged existence of the host immune system (Hsieh and Mentink-Kane, 2016).

According to Oyinloye *et al.* (2014), species parasitizing humans have similar life cycles, which starts when the eggs laid by the parasites are discharged from an infected person in urine or faeces; once the released eggs come in contact with fresh water, they hatch and become miracidia which swims freely in the water and reproduce asexually. These miracidia find a way to get into the snail's tissue and transform to a sporocysts which produce cercariae (larvae) with an ability of infecting mammals (Botros *et al*., 2005; Ukoroije and Abowei, 2012). The cercariae enter the human skin using oral and acetabulum and metamorphose into *schistosomula* which travel through the bloodstream causing conditions such as diarrhoea, Katayama fever with rash, lymphadenopathy, cough, intestinal pain, fever and malaise nausea. The *schistosomula* then mature into adult worms which lead to acute symptoms exhibited in chronic schistosomiasis. When

the infection gets to this level, patients suffer difficulty in blood flow, defects in reproductive organs, lungs, brain and guts, predisposing patient to genital schistosomiasis, pulmonary schistosomiasis, hepatointestinal schistosomiasis and neuro schistosomiasis. However, eggs lay by the female adult worm after mating, are voided with urine or stool to the environment. The eggs hatch to immature miracidia larvae, and the life cycle continues as shown in figure 1.1 (Oyinloye *et al*., 2014).

Adult worms have the ability to avoid the immune response of the host and the cercariae evade the defence response of the snail hosts. Children and teenagers can easily be re-infected even after undergoing curative therapy, such that residents around area of high prevalence will remain infected for approximately one-third to one-half of their lifetime (King and Dangerfield-cha, 2008). Schistosome responsible for urinary infections goes to the vesical plexus in the urinary bladder and the schistosomes affecting the intestine goes to the mesentery in the small intestine. Normally, schistosomes can stay paired for 3 to 10 years or more in some cases (up to 40 years), in the human hosts (Colley *et al*., 2014).
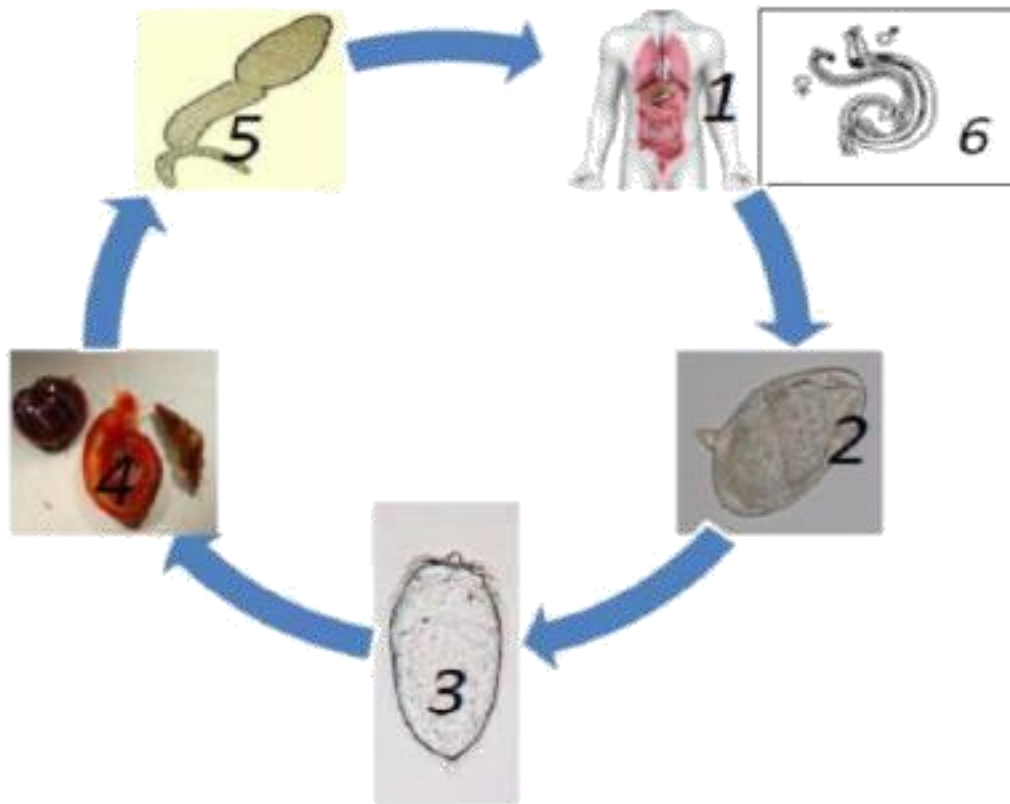
Figure 1.1. Life cycle of schistosome; 1 represents the definitive host, 2 shows the schistosome eggs discharged in stool or urine of the definitive host, 3 shows the cililated miracidia, 4 indicates the intermediate host snail, 5 Cercaria which have the ability to pierce the skin, loses its tail and turn to a *Schistosomulum,* 6 Paired adult worm (*Schistosomulum* travels to the venous system that returns blood from the liver; both the male and female worms mature together in the veins around the liver, bladder or intestines. The female lay eggs after mating, these eggs are voided with urine or stool to the environment. The eggs hatch to immature miracidia larvae, the life cycle continues. Taken from Oyinloye *et al.*, 2014.

1.4 *Schistosoma Species*

Etiological studies have shown that *Schistosoma mansoni, Schistosoma haematobium, Schistosoma mekongi, Schistosoma japonicum* and *Schistosoma intercalatum* are the species parasitizing human (Gryseels *et al*., 2006; Davis, 2009). Amongst these *S. mansoni, S. japoncium* and *S. haematobium* are the ones related to the disease (Gryseels *et al*., 2006; McManus and Loukas, 2008). *Schistosoma mansoni* causes the intestinal and hepatic type of this disease and it is transmitted by *biomphalaria* in Africa, South America and Arabian countries (Adenowo *et al*., 2015). Daily, adult *S. mansoni* lays 200-300 laterally spine ovoid eggs. A study by Rambau and colleagues (2011) reported that lodging of these eggs in the human scrotum result in pain and scrotum swelling that may require surgery. Also, patients suffering from either hepatitis B or C and *S. mansoni* simultaneously have been reported to have rapid development of a renal disorder (AbouZahr, 2003). Furthermore, in Sub-Saharan Africa an estimated 700,000 to 4.4 million people have bloody diarrhoea associated to intestinal *S. mansoni* (Koukounari *et al*., 2006).

Oncomelania a fresh water snail is responsible for the transmission of *Schistosoma japonicum* which is also the cause of bone marrow, brain, spleen, liver and intestinal infections in China, Indonesia and Phillipines. *S. japonicum* lays small, round, laterally spine eggs, 500-3500 each day. If untreated, infection from this specie leads to periportal fibrosis as hepatomegaly and splenomegaly (Vennerval and Dunne, 2004).

In Africa and Arabs countries, the *Schistosoma haematobium* have been reported as the main cause of urinary and genital type of this disease as well as tumour of the bladder (Adenowo *et al*., 2015; Hsieh and Mentink-Kane, 2016). This specie lays 20-200 rounds of terminally spine eggs daily and has been linked with the acquisition of HIV/AIDS due to ulceration and mucosal inflammation caused by genital schistosomiasis in millions of females (AbouZahr, 2003; Merrifield *et al*., 2016).

In SSA, approximately 70 million people suffer from haematuria because of this *Schistosoma* specie (Lengeler *et al.,* 2002; Gryseels *et al.,* 2006). An early study by Mostafa and co-workers (1999) reported that at late stage, urinary schistosomiasis causes cancer of the bladder. In 2014, Colley and co-workers reported that the urogenital schistosomiasis affecting males comes with prostatitis, oligospermia, haematospermia, dyspareunia and orchitis; in contrast with the female urogenital schistososmiasis, the male infection resolves after schistosomicide treatment. Additionally, other reports have shown that fibrotic responses can progress to hydronephrosis and failure of the kidney in the long run (King, 2008).

1.5 The Immunology of Schistosomiasis

The mechanisms that trigger the formation of pathology has not been well-defined (Mbow *et al*., 2013). However, the morbidity and indication of this disease is determined by the quantity and position of eggs stuck in the tissues (Gryseels *et al*., 2006); but differences in immunology has been related with pathological outcome (de Jesus *et al*., 2004; Caldas *et al*., 2008). At the onset, the inflammatory reaction can easily be reversible. However, at the final phase of the disease, the pathology is associated with accumulation of collagen and fibrosis, which subsequently leads to organ impairment that can only be reversible to some degree (AbouZahr, 2003). Therefore, having knowledge of the molecular mechanisms that are associated with the immune pathogenesis of schistosome can help in combating this disease (Rutitzky *et al*., 2005).

Lambertucci (2010) reported that the *schistosomal* disease like other parasitic worm infections, is linked with a strong CD4+ T-helper (Th2) reaction. According to Colley and co-workers (2014), adult worms are not responsible for morbidity resulting from this infection, but morbidity develops as a result of responses from granulomatous tissue reaction facilitated by CD4+ T cell to eggs that are stuck in the urogenital tissues, liver or intestinal tissues (Elfaki *et al*., 2016).

The immunological response elicited in the first 3-5 weeks mostly involves the Th1 cells, when pro-inflammatory cytokines such as TNF-alpha, IL-2 and gamma interferon can be evaluated in the plasma. The IL-2 is a major cytokine involved in maintaining the regulatory T cells, and during patent infection, the activity of this cytokine may increase (Milner *et al*., 2010).

The Th2 response which is followed by egg laying produces various cytokines such as IL-4, IL-5, IL-10 and IL-13 (Hsieh and Mentink-Kane, 2016). The Th1 pro-inflammatory response is suppressed by the Th2 cells and creates eosinophil-rich granulomatous cuts around freshly laid eggs but allows growth of fibrosis. Therefore, the inability to control the early pro-inflammatory response by the Th2 response can be fatal. The IL-13 produced by the Th2 plays a key role in regulating possible severe disease during the early phases of schistosomiasis, however, its continuous induction also plays a role in the development of asthma, hepatic fibrosis (Caldas *et al*., 2008) and acute lung injury (Chiaramonte *et al*. 1999; Cheever *et al*., 2000). In human, balanced immune system during this infection is related with improvement of the regulatory cell types, rise in IL-10 levels and elevated IgG4 levels which has been predicted to regulate morbidity (Adjobimey and Hoerauf, 2010).

Conclusively, eradication of schistosome at the global level has been challenging and the elimination at areas of high prevalence have proven to be difficult (Hsieh and Mentink-Kane, 2016). This infection has shown affinity of spreading to areas that were not endemic previously due to water resources management and development (Steinmann *et al*., 2006; Fenwick 2006; Li *et al.,* 2007), a condition that may be worsened by changes in climate (Yang *et al*., 2005; 2010).

1.6 Current Drugs

The introduction of praziquantel (PQZ) has improved the treatment of schistosomiasis in the last two decades. This drug is effective against the three main species parasitizing human (Caffrey, 2007). Metrifonate and oxamniquine are the two alternative schistosomiacide but unfortunately, they are not effective against the three species. Oxamniquine (1,2,3,4-tetrahydro-2-(isopropylamino)methyl-7-nitro-6-nitro-quinoline methanol) is active *in S. mansoni* but shows no activity against *S. haematobium* or *S. japonicum*. In contrast, Metrifonate only shows activity against *S. haematobium*. In 2000, Conceic and co-workers reported resistance to oxamniquine which was attributed to the alteration in the schistosome gene that encodes the bioactivating enzyme. Since then, these two drugs have been seldomly administered as anti-*schistosoma* (Utzinger and Keiser, 2004; Danso-Appiah *et al*., 2008).

Currently, the praziquantel a pyrazinoisoquinoline derivative that was found in the 1970's, has become the singular *schistosomacide* that is widely used due to its ready accessibility, high efficacy and inexpensiveness (Fenwick *et al*., 2003). However its inability to destroy the parasite 2 to 4 weeks post-infection (Aragon *et al*., 2008), and potential of schistosome developing resistance (Greenberg, 2005) against it are limitations that still need special attention since the mode of action and molecular target of this drug still remains a mystery.

Studies have suggested that PZQ anti-*schistosomal* properties is as a result of the destruction of the parasite Ca^{2+} homeostasis; *in vitro* treatment with PZQ leads to a rise in the influx of Ca^{2+} (Kohn *et al.,* 2001; Kohn *et al.,* 2003). The α1 and β inside the voltage gate of Ca^{2+} channels are being targeted by the PZQ resulting to the death of the parasite (Kohn *et al*., 2001). More studies have also suggested that PZQ's mode of action causes impairment of the worm tegument resulting in the alterations in the host immune system response against *schistosoma* water and antigen

presentation (Ribeiro-dos-Santos *et al*., 2006). PZQ can also cause death of the parasite by inhibiting the uptake of adenosine and uridine (Angelucci *et al*., 2007).

Moreover, re-infection may occur in children after treatment due to the lack of proper immune response to the disease, a misery of preventive drug-based disease control programmes (Rollinson *et al*., 2013). Recently, a study by Sokolow and co-workers (2016) has suggested that schistosomiasis can be reduced by snail control.

1.7 Challenges

Regardless of the accessibility of PZQ, which is effective against *Schistosoma*, treatment of schistosomiasis is still with numerous drawbacks and challenges. These shortcomings include;

a. Poor diagnosis

b. Inadequate transmission data and precise statistics of death cases in areas of high prevalence

c. Unavailability of vaccines

d. Provision of drugs to patients in need

e. Combination of effective frontline treatments are too expensive for patients

f. Lack of production of drugs to combat this disease in past years

g. Poor snail control

h. Resistance development by parasites to existing drugs

1.8 Drug Discovery and Design

Annually, pharmaceutical companies and other sources spends over 100 billion dollars on drug development and health research, but health complications affecting poor Asia, Latin America and Africa gets less than 10% in this budget. Traditional techniques involved in drug development and

design waste a lot of time, require high capital and are synonymous with high clinical failure. Currently, most of the drugs available in market are manufactured by developed country whose people can afford expensive drug therapies which are too expensive for developing or under developed nations. Every year, millions of people, especially rural dwellers who do not have access to good health systems are affected by infections such as trypanosomiasis, malaria, leishmaniasis and tuberculosis (Kesselheim, 2008). A previous study by Trouiller and colleagues (2002), showed that only 16 out of the 1393 drugs developed between 1975 and 1999 were for neglected diseases which accounted for 10% of the world total health complications. Therefore, additional research must be done to amend the fatal imbalance in drug development.

Historically, phenotypic assays have been the basis for schistosomiasis drug discovery such as *in vivo* and *in vitro* screening (Caffrey and Secor, 2011). All existing anti-helminthics were discovered by employing these methods (Ferreira *et al*., 2015). Drawbacks such as inadequate understanding of the worm biology and lack of efficient genomic techniques contributed to impair, the advance of target based and molecular methods for schistosomiasis. This background is the main cause of the small number of comprehensively endorsed molecular targets, which is also applicable to other NTDs (Gilbert, 2013).

By employing the latest biotechnological hypothesis, the identification of drug targets starts the drug discovery procedure. The current technologies in molecular biology allow for identification of novel targets in fields such as proteomics, genomics and transcriptomics (D'Agostino *et al*., 2013). In recent drug discovery campaigns, target discovery is a key step because earlier documentation has linked unsuitable target selection with drug development failure (Lindsay, 2003; Butcher, 2003).

A druggable target is a nucleic acid, protein or peptide that a drug can control its activity, with biological compounds such as recombinant proteins, antibodies or small molecular weight chemical compounds (Gashaw *et al*., 2011). It can also be from biological pathways, molecular entities such as genes, disease biomarkers and vital nodes located on regulatory networks.

Knowing that the treatment of schistosomiasis is still fragmentary, the need to employ modern disciplines (e.g. bioinformatics, medicinal chemistry, cell biology and target-based screening) that augments the opportunities for designing and developing a new drug is abundantly well-defined.

1.9 Bioinformatics

Bioinformatics is a multidisciplinary research field that connects biology and computer science. It uses computer to employ techniques that are effective, and useful in identifying lead compounds before synthesis in the laboratory. Blundell (1996) refers to bioinformatics as a faster and cheaper method when compared with laboratory test, this is because in synthesis of drug like molecules, computer aided techniques are able to reduce time, cost, and failure during clinical trials. Furthermore, species name, size, genetic information, habitat need, descriptions, interaction status, distributions and diseases related data can be collected from various databases such as Swiss-Prot Protein, STRING, DAVID, NCBI etc.

1.10 Computer Aided Drug Discovery Techniques

According to Lybrand (1995) these techniques are paramount in drug discovery and historically help in getting vital understanding and ideas on the synthesis of novel drugs and laboratory investigations prior to synthesis. Many computer aided design efforts have been successful in using lead optimization to improve the pharmacokinetics, activity and specificity of lead compounds. Drugs such as teveten (antihypertensive), dorzolamide (Glaucoma), NVPAUY922 (anticancer),

donepezil hydrochloride (Alzheimer's disease), indinavir sulphate (anti-HIV), LY 517717 (factor Xa inhibitor) and zolmitriptan (migraine) were developed by employing computer aided drug discovery design (Keenan *et al*., 1993; Greer *et al*., 1994; Glen *et al*., 1995; Kawakami *et al*., 1996; Talele *et al*., 2010).

Moreover, recent studies have shown that discovery of new natural products with anti-schistosomiasis activity can be accelerated using computational approaches like chemoinformatics and bioinformatics, particularly in the detection of unexploited, biologically efficient chemical scaffolds. These computational techniques have progressed, aiming at predicting, understanding and investigating the bioactivity of novel compounds (Schuster and Wolber, 2010; Geldenhuys *et al*., 2012).

1.11 Data Mining

Data mining, also known as Knowledge Discovery Data (KDD), basically means to obtain interesting, significant, accurate, previously unknown and vital information from data (Wang *et al*., 2004). With swift rise in technology know how, there is a lot of new research and new systems in data mining. This is a multidisciplinary field with diverse fields like business, biology, security, e-commerce, and medicine (Chen *et al*., 1996; Vicentini and Menossi, 2009). In bioinformatics, there are diverse applications of mining such as determination of protein functional domain, gene discovery, deducing protein functions, reconstruction of protein and gene interaction networks, discovery of functional motif, identification of disease, prognosis of disease, data cleansing, designing effective treatment for disease and predicting the location of protein sub-cellular (Raza, 2010). With rapid advances in biological data, data analysis, solving future and developing problems will be aided significantly by KDD (Wang *et al*., 2004).

Most importantly, combination of present and developing computational KDD techniques with accurate, thorough and systematic assessment have been anticipated to assist in releasing the extensive potential of proteomic profiling (Sugimoto *et al.*, 2012). Generated data will be subjected to KDD in order to identify proteins involved and a complex design, which can function as a biomarker (Patterson and Aebersold, 2003)

1.12 Biological Database

Biological databases are online repositories that contain structured information about living organisms (Helmy *et al.*, 2016). These databases are crucial in research because they are easily accessible and give computable insight which aids in proper preparation for future experiments (Helmy *et al.*, 2016). Based on the stored data, biological databases are divided into three groups namely; (a) the primary databases, made up of sequences of DNA and protein, (b) the secondary databases, which derive their information from a primary database (c) composite databases, joins different databases from the primary to derive their information (Kapushesky *et al.*, 2011).

1.13 Hidden Markov Model

An application of profile hidden Markov models (profile HMMs) for biological sequence analysis is as known HMMER (Krogh *et al.*, 1994; Eddy, 1998). This application was introduced to computational biology in the year 1994 by Krogh and colleagues. HMMER is used to query sequence repositories for homologs of gene or protein sequences and to align the sequences. This application is created to operate on POSIX-compatible platforms such as MacOS/X, UNIX and Linus. Also, this application searches protein sequence similarity using probabilistic techniques (Finn *et al.*, 2011).

When compared to FASTA, search tools built on older scoring approach, BLAST and other sequence alignment tools, HMMER is more significantly accurate and identifies remote homologs due to the strength of its fundamental probability models (Eddy, 1998). There are several programs on HMMER to build models and align sequences, as well as, search protein queries against protein database;

- hmmalign; this program aligns many sequences to a common profile HMM

- hmmbuild; this program is designed to build a new profile HMM from the multiple alignment

- hmmcaliberate; this program calibrates the HMM search statistics

- hmmsearch; search and query a profile HMM against protein sequence database.

1.14 STITCH

STITCH ('search tool for interactions of chemicals') is accessible at http://stitch.embl.de. It is a protein-chemical interaction database that combines various experimental databases, drug-target databases, drug-target predictions into an integrated network, pathway databases and manually curated data with reports derived during text-mining and interaction predictions (Kuhn *et al*., 2007; Kuhn *et al.,* 2011). It is an aggregated database of interaction network linking over 3.6 million proteins and 390,000 chemicals originating from 1133 organisms (Kuhn *et al*., 2013). Indeed, this database mines information from different sources in order to provide a detailed and comprehensive result.

STITCH allows the investigator to request for information from the database for chemical or protein names, for InChIKeys (hashed InChi) as well as SMILES strings. If a chemical is inputted into the search bar and no target family for the interacting proteins was picked, the family with the highest confident interaction will be selected robotically. More so, this database provides the

investigator a network analytical view in which edges and nodes can be clicked to know more about the protein. The elemental STITCH database is available for users in numerous means; through download files (for large scale query), via a user-friendly web interface and via an application program interface (API) (enabling automated access on a small to medium scale.

1.15 Computational Modelling of Protein Three-dimensional (3-D) Structure

According to several studies in computational biology, structural bioinformatics is one of the main research fields (Clote and Backofen, 2000; Liljas *et al*., 2001; Zhang *et al*., 2005; Altman and Dugan, 2005; Gopakumar, 2012). This field involves the prediction and analysis of biological macromolecules (e.g. Proteins, RNA and DNA) 3-D structures (Zhang *et al*., 2005; Altman and Dugan, 2005). From a computational structural biologist perspective, proteins are long sequences of building blocks called amino acid residues with unique sequences. Proteins fold into a specific 3-D shape due to these sequences and the prediction of the protein 3-D structure remains a key research hitch in structural bioinformatics. Various algorithms and techniques have been set to remedy these hitches ranging from comparative modelling (homology modelling), threading (fold recognition methods) and *ab-initio* method. These techniques outline the sets of the notorious Critical Assessment of Structure Prediction (CASP) (Al-Lazikani *et al*., 2001).

There are many databases (such as PSIPRED, HHpred, PORTER, Jpred, Phyre2, I-TASSER etc.) for predicting the function and generating 3-D models of a given query sequence. Specifically, I-TASSER an example of composite method for modelling was used in this study.

1.15.1 I-TASSER

I-TASSER 'Iterative Threading ASSEmbly Refinement' server is an accessible online tool (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) for automated prediction of protein structure and structure-based function paradigm. When evaluated with other online structure predictions

platform, I-TASSER is exceptional in the accuracy and reliability of the full-length structural prediction for targeted proteins of varying problems and broad structure to function predictions (Roy *et al*., 2010).

In the first phase of I-TASSER, queried sequence is threaded through structural templates from the PDB structure library (Berman *et al*., 2000) using LOMETS (Wu and Zhang, 2007). LOMETS is a locally installed meta-threading server that combines seven state-of-the-art threading programs which are PPA-1, FUGUE, SAM-T02, MUSTER, HHsearch, SPARKS2 and PROSPECT. In each program, the templates are graded using various scores that are based on structure and sequence, with the top template hits being selected from each program.

The excellence of the template alignment is evaluated based on a normalized Z-score, which is defined as:

$$Norm.\ Z\text{-score} = \frac{Z\text{-score}}{Z_0}$$

'where, Z-score is the energy score in stand deviation units relative to the statistical mean of all alignments, Z_0 is a program-specific Z-score cutoff established based on large-scale threading benchmark tests to differentiate good from bad templates.'

The continuous-aligned fragment structures in threading alignment are cut off from the template and are employed in constructing structural conformations of the segments that are properly aligned, where the unaligned structures are built by *ab initio* modelling (Wu *et al*., 2007) based on replica-exchange Monte Carlo simulations (Zhang *et al*., 2003). The structure curves are clustered by SPICKER. In order to evaluate the accuracy of the structure predictions, a score termed C-score is used, mathematically formulated as;

$$C - score = \ln\left[\frac{M}{M_{tot}} \times \frac{1}{\langle RMSD \rangle} \times \sum_{i=1}^{N} Norm.\, Z - score(i)\right]$$

'where M is the multiplicity of the structures in the SPICKER cluster; M_{tot} is the overall number of decoys presented to the clustering; ‹RMSD› is the average RMSD of the decoys to the cluster centroids; Norm. Z-score (i) is the normalized Z-score of the topmost templates obtained from *i*th threading program in LOMETS; N is the number of servers used in LOMETS.'

The C-score provided by this server is a confidence score, which evaluate the quality of the forecasted models. This score ranges from -5 to 2, however, scores greater than -1.5 denotes correct folding of the model. The TM-score determines the structural similarities between the predicted model and the template used in building the predicted model. TM-score greater than 0.5 denotes a 3-D model of correct topology, but TM-score lower than 0.17 denotes random similarities (Roy *et al*., 2010). Root Means Square Deviation (RMSD) evaluates the distance among atoms in superimposed proteins. An RMSD lower than 1 Å reflects perfect identical structures, while an RMSD of about 2 Å or greater indicates that there was fewer distance among atoms of the proteins and the template used in the construction of the model.

1.16 Molecular Docking

The molecular docking process predicts the ligand posing and conformation in a targeted binding site, these steps are associated with scoring schemes and sampling processes (Meng *et al*., 2011). Early research has showed that this technique can be employed in modelling the interaction between a protein and small molecules at the atomic level, allowing for the characterization of the activity of the small molecules in the binding site of the aimed proteins (McConkey *et al*., 2002). Moreover, information about the strength of the interactions between the target biological system and the compound (drug) can be obtained from this technique (Kitchen *et al*., 2004). Various steps

are involved in this technique in which each step presents one or more extra steps of complexity (Brooijmans and Kuntz, 2003). In fact, this technique does not work on its own but is embedded with various *in silico* and experimental processes (Kroemer, 2007).

Molecular docking is the most widely used tool in drug design (Ferreira *et al*., 2015). Meng and colleagues (2009) showed that the combination of docking data from the laboratory and other computational biology techniques can be used in evaluating the metabolism of drugs which can be used to get valuable report from the cytochrome P450 system.

Overall, molecular docking aims at precise structural modelling and accurate prediction of the activity (Kitchen *et al*., 2004). In this study, numerous *in silico* methods such as PatchDock (Schneidman-Duhovny *et al*., 2005), ZDOCK (Chen *et al*., 2003), HADDOCK (Dominguez *et al*., 2003), ClusPro (Comeau *et al*., 2004), HexServer (Macindoe *et al*., 2010), have been employed.

1.16.1 PatchDock

The PatchDock server accessible on https://bioinfo3-D.cs.tau.ac.il/PatchDock/ was utilized in this study. This is an online server with a geometry-based molecular docking algorithm designed to ascertain docking transformations that will yield good molecular shape complementarity (Schneidman-Duhovny *et al*., 2005). This online algorithm is based on a rigid-body server with a scoring system that gives the best 3-D coordinates for two proteins or protein-peptides involved in the complex formation. It is a server with high efficiency because of its swift transformational search, powered by local features complementing rather than using six-dimensional transformation space search by brute force (Schneidman-Duhovny *et al*., 2005).

1.17 Problem Statement

Today, schistosomiasis is on a rapid and swift rise globally, claiming thousands of lives every year with 800 million persons at risk of infection. With the high prevalence of this disease and steady increase in infections, praziquantel remains the only effective drug against this acute disease and no significant approaches have been made in recent years in discovering a new drug. Sadly, resistance to this drug has been reported in some regions and this drug is not effective against the juvenile schistosome parasite. Thus, it is of paramount importance to urgently develop novel targets and lead compounds using *in silico* approaches for this disease to substitute for PZQ.

1.18 Aim

The aim of this study was to identify and perform structural bioinformatics on 'druggable' protein targets in *Schistosoma* species.

1.19 Objectives

The specific objectives of this research work are as follows;

- Identification of putative AMPs for schistosomiasis therapy *in silico*

- Identification of novel 'druggable' targets in *Schistosoma* spp

- Structural and functional characterization of the identified AMPs and protein using *In silico* techniques

- Ascertaining the interaction of identified proteins with the AMPs (receptor and ligand interaction).

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CHAPTER TWO

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PZQ therapy: how close are we in the development of effective alternative anti-schistosomal drugs?

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ABSTRACT

Today schistosomiasis, caused mainly by the three major schistosome species (*S. mansoni*, *S. haematobium* and *S. japonicum*), has for many decades and still continues to be on a rapid and swift rise globally, claiming thousands of lives every year and leaving 800 million people at the risk of infection. Due to the high prevalence of this disease and the steady increase in infection rates, praziquantel (PZQ) remains the only effective drug against this acute disease although it has no effect on the juvenile schistosome parasite. However, no significant approaches have been made in recent years in the discovery of new or alternative drugs and unfortunately, resistance to this drug has been reported in some parts of the world. Therefore, it is imperative to develop a new drug for this debilitating disease. In this review, a brief history of past, present, and new promising anti-schistosomal drugs is presented.

Keywords: Drugs, praziquantel, schistosomiasis, *Schistosoma mansoni*, *Schistosoma haematobium.*

INTRODUCTION

Schistosomiasis continues to be one of the most significant water and vector-borne diseases from a universal public health disposition [1]. This disease is said to follow hookworm infections as the second most prevalent Neglected Tropical Disease (NTD) in sub-Saharan Africa [2]. Currently, schistosomiasis is a debilitating disease that is transmitted in 78 developing countries and affects over 200 million people globally [3, 4]. In the sub-Saharan African region alone, 193 million cases occur mostly due to bad sanitation, poor treatment, and very few control programs. The disease is responsible for the loss of 10.4 million disability adjusted life years (DALYs) and accounts for over 280,000 deaths annually [5]. Additionally, over 800 million people are still vulnerable to this disease [6].

Schistosomiasis, also referred to as bilharziasis [7], is caused by parasitic trematode flatworms found in fresh water belonging to the genus *Schistosoma* [8]. The lifecycle starts asexually once schistosome eggs are voided into freshwater with the urine or faeces of the definitive host (human) and miracidia are produced from the eggs. These swim freely in water and find their way into the tissue of the intermediate host (snail). In the snail, the miracidia transform into sporocysts that produce many cercariae through asexual reproduction. The infection starts when humans are exposed to waters infested with cercariae, which have the ability to penetrate human skin. Once this takes place, the cercariae lose their bifurcated tails and metamorphose into schistosomulae, which migrate to various tissues such as the heart, liver and lungs. In the liver, the schistosomulae undergo rapid growth and mature into adult worms. Eggs are then produced from mating adults, which are either shed in human excreta, or lodged in various tissues that ultimately lead to various complications of the disease such as genital, pulmonary, hepato-intestinal or neuro-schistosomiasis and other clinical outcomes such as gastrointestinal and hepatic pathologies, anaemia, caloric

malnutrition as well as a heightened risk to HIV/AIDS and cancer of the bladder. The worms are able to produce several hundred eggs daily which are all capable of developing into schistosomes. *S. mansoni* adults lay about 200-300 laterally-spined ovoid eggs daily while *S. haematobium* and *S. japonicum* parasites lay 20-200 round, terminally-spined eggs and 500-3500 small and round laterally-spined eggs respectively. As discussed by Hsieh and Mentink-Kane [9], the parasite can stay in host blood vessels for many years and this parasitic ability highlights the *Schistosoma* species' aptitude for evasion and prolonged existence in the host immune system.

Not until 1984 when the World Health Organisation Expert Committee proposed chemotherapy, elimination of snails was often used in tackling this chronic debilitating disease [10]. Chemotherapy remains the only means for schistosomiasis control but this has relied solely on one single effective treatment, praziquantel (PZQ), which is now deemed unsatisfactory [11, 12, 13]. In recent times, not much approach has been made to develop new drugs for this disease because pharmaceutical companies snub diseases affecting poor nations hence, schistosomiasis was referred to as one neglected tropical diseases. Over the last few decades, many drugs have been used to remedy this disease. In this article, past, recent and currently-used schistosomicides are reviewed and the question of how close we are in the development of effective treatment against this debilitating disease is addressed.

PRAZIQUANTEL (PZQ)

Praziquantel-(2-cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4H-pyrazino-(2,1-α), Figure 2.1, marketed under the brand name Biltricide, is a bitter-tasting white crystalline powder. Under normal storage conditions, this drug is stable and practically insoluble in water but soluble in some organic solvents. PZQ was first selected for its action against helminths in the mid-1970s, and was initially used in treating veterinary cestode and trematode infections; subsequently, it was and

continues to be used in treating various trematode infections in humans [14]. Over the years, PZQ has remained the best mono-therapeutic agent and drug of choice for all forms of schistosomiasis due to its ready accessibility, inexpensiveness, safety, and high efficacy [11, 15, 16]. However, its cure rate of only 60% to 95%, inability to hinder re-infection [17, 18], ineffectiveness against the juvenile stage of the parasite [19], and resistance have raised concerns.

To date, the mechanism of action of PZQ still remains a mystery, thus many researchers have suggested ways in which the drug may be responsible for the parasite's death. In 2001, Kohn and colleagues [20] hypothesized the drug has a negative effect on the Ca^{2+} homeostasis of the worm. They speculated that PZQ allows the opening of several channels that lead to the disruption of the interface between the α_1/β inside Ca^{2+} voltage gated channel. Other reports have suggested that PZQ induces muscle contraction and disruption of the tegument system resulting in antigen presentation [21, 22]. PZQ is lipophilic and its action on worm-antigen exposure may be as a result of interaction with hydrophobic areas of the tegumental outer membranes [23].

The cure rate for *S. mansoni* ranges between 60% and 99%. For instance, 25mg/kg bodyweight in two oral doses every 4 hours achieves a cure rate of between 63% and 97%. A single oral dose of 40mg/kg bodyweight attains a 72% to 100% cure rate, while 20mg/kg of three divided oral doses every 4 hours kills 71%-99% of the parasites. Yet, a 78.6% to a 90% cure rate may be attained with a single dose of 40 mg/kg, while an 84.6% to 98% decrease in egg output among non-cured individuals may be achieved [24]. Intramuscular, oral and intradermal administration of this drug is said to be effective. It is generally a well-tolerated and non-toxic drug. However, nausea, vomiting, hepatomegaly and headache are some of the known negative side effects [25].
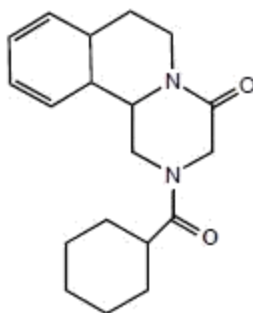
Figure 2.1: Chemical structure of Praziquantel.

PAST AND PRESENT DRUGS USED IN THE TREATMENT OF SCHISTOMIASIS

METRIFONATE

Historically, metrifonate (Figure 2.2) was used in treating urinary schistosomiasis but the administration of multiple doses in the course of treatment made the drug to lose public approval and acceptance [26]. Metrifonate is an organophosphorous, 0,0-dimethyl-2,2,2-trichloro-hydroxyethyl-phosphonate, previously known as trichlorphone and trichlorfone. This compound has variable and selective anti-*Schistosoma haematobium* activity, due to its incomplete metabolism as an effective acetylcholinesterase inhibitor and organophosphate (dichlorvos). In 1991, Shekhar [27] reported that this drug was the best in treating urinary schistosomiasis caused by *S. haematobium* but sadly, it showed no activity against other species parasitizing humans [28]. The mechanism of action behind this drug exhibiting anti-*S. haematobium* action is still a mystery. However, an early study by James and colleagues [29] suggested that the inhibition of acetylcholinesterase by metrifonate leads to sweeping of the worm to the lungs where they cannot develop. The results showed that 40% of *S. haematobium* worms were found in the lungs and 60% in the liver but there was an increase in the quantity of the *S. haematobium* in the lungs after treating with metrifonate for 2 or 3 days. It was concluded that after 5 days of metrifonate treatment, there was a significant decrease in the quantity of *S. haematobium*. But, the death of the

pharmacologically damaged parasite could also be assumed to be as a result of physical factors, inadequate supply of nutrients or by possible cell-mediated mechanisms of cytotoxicity due to the abundance of immunocompetent cells like alveolar macrophages and eosinophils presence in the lungs [30]. One study suggested that metrifonate can cause a stunning effect on the adult parasite. According to Shekhar [27], the *S. haematobium* worms are stuck, enclosed and killed in the arterioles of the lungs after being stunned.

Moreover, the prescribed oral dosage of metrifonate is 7.5 mg/kg to 10 mg/kg thrice and it must be administered two weeks apart [30]. In the course of treatment, metrifonate roughly decreases 90-95% of the parasite eggs and a 44% to 93% cure rate is attainable in treated individuals [31]. Metrifonate use can results in minor side effects such as diarrhoea, vomiting, colic, and nausea. Other effects include tiredness, vertigo, syncope, headache, myasthenia, bronchial spasms, sweating and muscular tremor. To date, this drug has been withdrawn from the market as a result of economic, operational and medical standards [32, 33]. However, with optimization, it can be reassessed as the medication or alternative drug for urinary schistosomiasis [34].
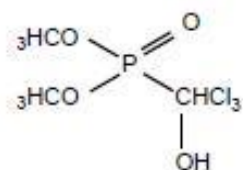
Figure 2.2: Chemical structure of Metrifonate.

OLTIPRAZ

Oltipraz (OPZ), Figure 2.3, is a synthetic dithiolthione ($C_8H_6N_2S_3$; 5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione) which is similar in structure to the dithiolthiones found in cruciferous vegetables. In humans, OPZ can be used against infections caused by *S. intercalatum*, *S. mansoni*, and *S.*

haematobium. Nare and colleagues [35] reported that the action of this drug against schistosomiasis is very slow, taking approximately 2 months to cure. Although the mode of action of this drug is poorly understood, it is assumed that after nine days of use it causes a hepatic shift that moves the parasite to the liver from the mesenteric veins [36]. Exposure of the worm to OPZ causes a reduction in glutathione synthetase (GSS) levels [36, 37], which is assumed to interfere with the metabolism and possible elimination of the worm by the host immune system, probably by decreasing protection against the reactive oxygen intermediates [38]. Other studies have documented that OPZ assists the host to increase its detoxification ability [36, 39]. According to Shekhar [27], an oral dose of OPZ, 3.0 g to 4.5 g three times a day for curative treatment and a dose of 35 mg/kg twice a day only produces 90% cure in *S. mansoni* infected individuals. Gentilini and colleagues [40] stated that a dose of 1.25 to 4.50 g should be administered for *S. intercalatum* infection for 3 days to achieve a cure rate of 76.5% to 92%. While on the other hand, a dose of 25mg/kg should be administered for 1 or 2 days to achieve a cure rate of 86% to 94% in *S. haematobium* infected persons [40]. Nausea, vomiting, weakness, stomach pain and insomnia are the frequent side effects of OPZ. At present, OPZ is currently not available in the market for schistosomiasis treatment again due to its induced photosensitivity [39].
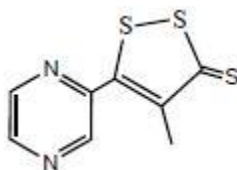
Figure 2.3: Chemical structure of Oltipraz.

NIRIDAZOLE

Niridazole (1-(5-nitro-1,3-thiazol-2-yl)imidazolidin-2-one (Figure 2.4), is an orally administered anti-schistosomal agent, which shows activity against all the three major schistosome species [41]. This drug is shown to be an effective long-lasting suppressor of delayed intolerance [42]. In addition to its anti-schistosome effects, Moczon and Swiderski [43] documented that this drug destroys schistosomes by decreasing glycogen levels of the parasite, thereby inhibiting glucose and lactate uptake and is also responsible for the degeneration of the female reproductive system. This drug acts by taking up [14C]-niridazole which binds covalently and leads to nitro-reduction and subsequent bond formation with the parasite macromolecules [44, 45, 46].

The prescribed dose is 25 mg/kg each day for a week or 35 mg/kg daily for 5 days [45]. It shows more activity against *S. haematobium* infections with 80%-100% cure rate mostly in children and approximately 50% and 30%-70% cure rate in *S. japonicum* and *S. mansoni* infections respectively. Niridazole side effects include vertigo, skin rashes, non-specific destruction of the T waves in the electrocardiogram (ECG), nausea, diarrhoea, brown urine and vomiting. Other unpleasant effects include the central nervous system (CNS) and renal toxicity besides alteration of digestive and cardiac functions [47]. An *in vivo* study by Urman and co-workers [48] showed that this drug is carcinogen. Therefore, due to the many adverse effects attributed to this drug, the populace and medical practitioners have abandoned it in the treatment of schistosomiasis.
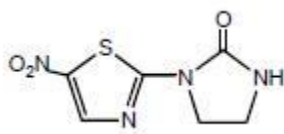
Figure 2.4: Chemical structure of Niridazole.

OXAMNIQUINE

Oxamniquine (6-hydroxymethyl-2-isopropyl-aminomethyl-7-nitro-1,2,3,4 tetrahydroquinoline), Figure 2.5, is the only drug active against *S. mansoni* worms, particularly the male parasite, but has no effect on *S. haematobium* or *S. japonicum* worms [49]. This is due to the fact that the conversion of this drug to its active form require the activity of sulfotransferase [50], which is only available in the *S. mansoni* parasite. When converted to its active state (sulfate ester), it dissociates non-enzymatically and alkylates schistosome DNA leading to the inhibition of nucleic acid production, disruption of protein synthesis, delayed destruction and death of the parasites [46, 51]. Over the last two decades, this drug has been the main drug for treating *S. mansoni* infection in South America [52]. However, resistance to oxaminiquine has been reported in Brazil [53], which may be as a result of alteration in the schistosome gene that encodes the esterifying enzyme [54]. Currently, praziquantel has replaced oxaminiquine as a schistosomicide, not only because of its effectiveness but also largely due to its cost effectiveness [52]. For three consecutive days, oral doses of 20 mg/kg bodyweight of oxaminiquine can be administered and depending on the geographic area, this dose provides a cure rate of between 80-90%. Some of the negative side effects of this drug include fever, headache, drowsiness, dizziness, convulsions and occasional orange-red urine discoloration.
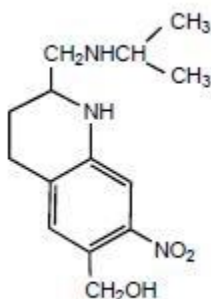
Figure 2.5: Chemical structure of Oxamniquine.

MEFLOQUINE

Mefloquine (Figure 2.6), an aryl-amino-quinoline used in the treatment of malaria, has been reported to show effective activity against various stages of the schistosome parasite *in vivo* [55]. In 2008, this antimalarial was used for the first time to treat schistosomiasis by Van Nassauw and colleagues [56] who documented that a dose of 150 mg/kg caused a significant reduction in *S. mansoni* eggs in infected mice [56]. *In vivo* investigations further revealed that mefloquine shows good activity against *S. mansoni* at a single dose of 200 mg/kg resulting in 72.3% total worm burden reduction [57]. On the contrary, oral administration of a higher dose at 400 mg/kg achieved 86.7% and 95.1% worm burden reduction of both immature and mature female worms in infected mice [58]. Although the mechanism of its anti-schistosomal activity has not been investigated, interference with the digestion of hemoglobin and raising intravacuolar pH, have both been suggested to play a role in its mechanism of action against *Plasmodium* [59]. In addition, mefloquine possesses a wide range of antimicrobial activity as it shows activity against larval and adult stages of *Brugia malayi* and *Brugai patei* [58]. It has a high tolerability rate in children and adults with dose-dependent negative effects like gastrointestinal disorders and neuropsychiatric side effects [60]. Therefore, the efficacy of mefloquine in the treatment of schistosomiasis does deserve further and extensive study.
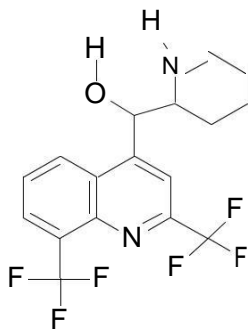
Figure 2.6: Chemical structure of Mefloquine.

ARTEMISININ

Artemisinin (qinghaosu), Figure 2.7, is a key ingredient extracted from *Artemisia annua* plant leaves, a plant endemic to China, USA, Argentina and Central Europe [61]. It is a sesquiterpene lactone possessing a peroxide bridge that is considered to be the active pharmacophore. This compound constitutes a class of potent drugs used in the treatment of malaria which is known for their good safety profile and tolerance [62, 63]. Thus far, artemisinin-based combination therapies (ACTs) have been documented as the most potent antimalarial drugs [64]. Interestingly, artemisinin derivatives such as artemether, artesunate, dihydroartemisinin and arteether have been reported in several studies to possess anti-schistosomal activity, both in human and animal experiments [65, 66,67]. In areas of highly endemicity such as in China and Cote d'Ivoire, *S. japonicum* and *S. mansoni* infections have been effectively controlled with the administration of artemether in human trials [68].

In contrast to PZQ, which shows the highest activity against adult worms [69, 70], artemether shows the highest activity against the juvenile worms of the three main species affecting humans, while leaving the invasive and adult stages of the worm less vulnerable [71, 72]. Thus, joint treatment with PZQ and artemether would cover the entire lifetime of the parasite in its definitive host [73] because once PZQ kills the adult worms, artemether will then subsequently kill the surviving schistosomula, which would have repopulated the host resulting in abolishment of reinfection. Therefore, it was concluded that ACTs stimulate the destruction of both the juvenile parasite tegument and adult parasite [74].

Moreover, artemisinin derivatives can affect the gut heme in the parasite resulting in heme alteration to an unstable specie which can generate reactive oxygen species (ROS) with subsequent worm death [75]. A dose of 6mg/kg is given once in every 2-3 weeks [76].
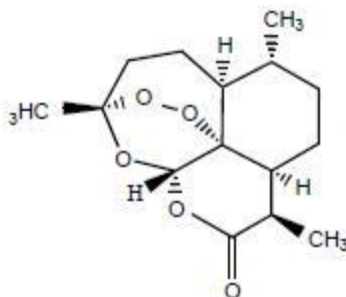
Figure 2.7: Chemical structure of Artemisinin.

HYCANTHONE

Hycanthone, 1-(2-(diethylamino) ethylamino)-4(hydroxymethyl)-thioxanthen-9-one (Figure 2.8), is a hydroxylated version of lucanthone. Although this drug is no longer in clinical use, studies have documented that when administered in a single intramuscular dose of 3 mg/kg, hycanthone is effective against *S. mansoni* and *S. haematobium* parasites [77] but shows no activity against *S. japonicum* worms [78]. According to Cioli and Knopf [79], male parasites are more susceptible to this treatment than females. In mice, the hepatic shift hits the highest point at approximately 6 days post-treatment [80]. It is believed that this drug irreversibly binds to acetylcholine receptors, therefore paralyzing the digestive system of the parasite, leading to starvation, followed by the death of *S. mansoni* worms [81]. During hycanthone therapy, Senft and co-workers [82] observed loss of hemoglobin pigment from the gastrointestinal tract, weakening of the tegument, and a decrease in blood volume, as well as body size in *S. mansoni* worms. However, it was also observed that this drug can be carcinogenic and can induce liver damage [83, 45].
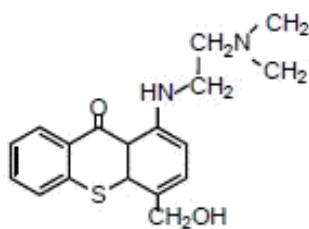
Figure 2.8: Chemical structure of Hycanthone.

Table 2.1: Different anti-schistosomal drugs and their mechanisms of action

Drug Tested	Parasite	Study Type (*in vitro/in vivo*)	Dose	Route	Mechanism	Reference
Metrifonate	Schistosoma haematobium	Hamster (*in vivo*)	7.5 mg/kg to 10 mg/kg × 3 for 2 wks	Oral	Inhibition of acetylcholinesterase	[29, 30]
Oltipraz	*S. intercalatum*, *S. mansoni* and *S. haematobium*	Mice (*in vivo*)	3.0 - 4.5 g/day × 3. Curative treatment: 35 mg/kg/day × 2 = 90% cure in S. mansoni. 1.25 - 4.50 g for 3 days in S. intercalatum. S. haematobium, 25 mg/kg × 1 or 2 days	Oral	Causes hepatic shift	[40, 27]
Niridazole	*S. japonicum*, *S. mansoni* and *S. haematobium*	Mice (*in vivo*)	25 mg/kg each day × 1week or 35 mg/kg daily × 5 days.	Oral	Takes up [14C]-niridazole, binds covalently leading to nitroreduction and subsequent bond formation with the parasite macromolecules	[44, 45, 46]

Oxamniquine	*S. mansoni*	Mice (*in vivo*)	20 mg/kg × 3days	Oral	Converts to its active type sulfate ester which alkylate the schistosome DNA	[46, 51]
Artemisnin	*S. japonicum*, *S. mansoni* and *S. haematobium*	Human (*in vivo*)	6 mg/kg once every 2–3 weeks	Oral	Affect the gut heme in the parasite resulting in heme alteration to an unstable species which can generate ROS with subsequent worm death	[75].
Praziquantel	*S. japonicum*, *S. mansoni* and *S. haematobium*	Human (*in vivo*)	Single dose of 40 mg/kg	Oral	Disruption worm Ca^{2+} homeostasis Impairment of the worm tegument	[84, 24, 20]

OTHER DRUGS USED AGAINST SCHISTOSOMIASIS IN THE PAST AND POTENTIAL FUTURE DRUGS

Mirazid is a commercial product extracted from myrrh, an aromatic gum resin. This extract was proposed as an alternative to PZQ as it elicits anti-schistosomal activity by extravasation and uncoupling of the parasite [85]. Earlier studies have shown it to be a promising emerging drug with low toxicity relative to PZQ. For example, Sheir and colleagues [86] showed an initial 91.7% cure rate in *S. mansoni* at a dose of 10mg/kg three times daily and a 98.1% cure rate after 2 months of initial treatment (6 mg/kg × 6 daily). However, throughout the years, results from various sstudies on this drug have been inconsistent, which has led to its use being stopped by the World Health Organisation (WHO) [87].

Meclonazepam (3-methylclonazepam), a derivative of benzodiazepine developed and patented in 1977 by Hoffman-La Roche, has also been demonstrated by experimental investigations to possess anti-schistosomal activity and relatively long plasma half-life of approximately 40 hours [88]. Studies have established that this drug is very effective against *S. mansoni* strains and its potency is similar to that of oxamniquine, hycanthone and niridazole [89]. A single dose of 0.3 mg/kg will successfully cure parasitic infections with harsh side-effects like drowsiness, dizziness, slurred speech, ataxia, muscle weakness, reduced mental alertness, and lateral nystagmus [90].

THE USE OF NATURAL PRODUCTS AGAINST SCHISTOSOMIASIS

For centuries, plants have not only been used to cater for the basic needs and livelihood of man such as food, shelter, clothing, biologically active products, fertilizers, flavours, fragrances, but also for curative purposes when used as medicines [91, 92]. Steadily old medicines are now becoming the modern and state-of-the-art drugs of today. It has been documented that more than 80% of Africa, Asia, and Latin America's medicinal needs have depended heavily on traditional and herbal medicines [93]. However, modern technology and orthodox medicine are currently raising interest and getting involved in these sources of alternative health care. However, in the absence of vaccine treatment for schistosomiasis, investigators are now considering natural products as new, inexpensive and effective alternatives to PZQ.

Several plants have been used in traditional African medicine as molluscicides and for curing schistosomiasis [94]. Most of the plants that have been documented for their anti-schistosomal activities and potentials have mostly been in the form of indigenous knowledge passed down through numerous generations, mostly by traditional healers. A study by Ndamba and colleagues [95] successfully interviewed 286 traditional healers from five provinces in Zimbabwe, with 85% of them registered with the largest association of traditional healers in the country– the Zimbabwe

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National Traditional Healers' Association (ZINATHA) [95]. The traditional healers reported 47 anti-schistosomal plants, of which 8 were identified as those most commonly used for treatment based on the healers' knowledge of the urinary form of schistosomiasis. These include *Ximenia caffra* (Olacaceae)*, Dicoma anomala* (Compositae)*, Phaseolas vulgaris* (Leguminosae)*, Lannea edulis* (Anacardiaceae)*, Elephantorrhiza goetzei* (Leguminosae)*, Abrus precatorious* (Leguminosae)*, Ozoroa insignis* (Anacardiaceae)*,* and *Pterocarpus angolensis* (Leguminosae).

Furthermore, animal studies using extracts from these eight plants, showed the latter three plants presenting positive lethal activity against adult schistosomes. Further studies by Molgaard and co-workers [96] investigated twenty-three of these plants by testing their leaf, stem, root, fruit and bark extracts *in vitro*. *Arbus precatorius* and *Elephantorrhiza goetzei* exhibited the best results against schistosomulae and this was attributed to the presence of natural compounds such as tannins, steroids, terpenes, flavonoids and indole alkaloids in the *Arbus* species.

Terpenoids e.g. (+)-limonene epoxide (extract from *Citrus sinensis*), Tashinones (cryptotashinone, tanshinone I and IIA), alkaloids (e.g. imidazole alkaloid epiisopiloturine from *Pilocarpus microphyllus*), quinoline methanols (e.g. quinidine), flavanoids (alpinum isoflavone from *Millettia thonningii*), arachidonic acid (e.g. oils from *Mortierella alpine*) Quinones (e.g. plumbagin from *Plumbago scandens*) and other natural products such as Aspidin, Desaspidin, Flavaspidic acid and Anisomycin are a few examples of plant products that have shown activity against schistosomes [97].

The schistosomicidal activity of some of these compounds has been shown to disrupt the mating process, diminish egg production, and increases the chances of the worms dying as well as affect the parasite's motor activity and tegument. Other potential anti-schistosomal natural products not only take into account the ability of the worm to migrate to different parts of the body and resist

host immune responses, but also consider the ability of the compound itself to kill the worms, the duration taken to do so and any reversible effects once the drug has ceased from being used [98]. These natural products include Epiisopilotulorine (from *Pilocarpus microphylus*), Piplartine (*Piper tubercalatum*), Phytol (found in chlorophyll), Phloroglucinols (Dryopteris species), Cinchona Alkaloids, Vernonia amygdalina (Asteraceae), Emetine (from *Cephaelis ipecacuanha*), Mevinolin (Lovastatin), Plumbagin and Sangunarine.

Familiar plants that have been used for several years as spices or general ingredients in food have also been tested and shown to exhibit anti-schistosomal properties. These include *Allium sativum* (garlic), pumpkin seeds, peppermint, olive leaves, wormwood, thyme, black walnut, berberine and endod, among others [99]. Garlic has shown activity against schistosomes by causing wrinkling and detrimental effects to the tegument of the worm and severe damage to the parasite tubercles by causing shortness and loss of the spines and thorns [98, 100]. Ginger (*Zingiber officinal*) on the other hand has been shown to not only kill adult worms, but to also interfere with the production of eggs and worm recovery [98]. Recently, it has been demonstrated that the plant can also ameliorate oxidative stress by increasing chloramphenicol acetyltransferase (CAT) activity in the liver of infected mice as well as cause an increase in glutathione (GSH) and superoxide dismutase (SOD) antioxidant levels just like PZQ [101]. Curcumin, the principal curcuminoid of turmeric from the Zingiberaceae, has been documented with other compounds such as vernodali, piplartina, artesunate, artemether, artemisina and avocado and soybean oils, to induce the separation of the male from the female and to disrupt the release of eggs [98, 100]. Other effects include the death of adult worms and a decrease in motor activity [97].

Other compounds such as propolis (a glue-like substance that bees collect from plants and tree barks) have also been suggested to be effective in treating schistosomiasis when in synergy with

each other natural products or with PZQ. Studies have shown administration of this substance to infected mice significantly reduced schistosomula and the number of eggs in the liver and intestines [102]. However, incomplete eradication was also observed and hence, it has been suggested that propolis in conjunction with PZQ, could result in an effective anti-schistosomal agent. Added to this, a mefloquine-artesunate combination has shown effective anti-schistosomal properties against *S. haematobium* infected children, especially since both compounds each exhibit anti-schistosomal properties [103]. Additionally, this drug combination can clear malaria infection and reduce schistosomiasis-related illnesses.

EMERGING DRUGS IN FIGHTING SCHISTOSOMISIS

Knowing that chemotherapy for schistosomiasis is still fragmentary; drug repurposing can be an alternative strategy to finding a cure for this acute disease. Drug repositioning involves investigating existing drugs and its application in treating non-related diseases for which they were not originally designed [104]. Although several researchers have studied various classes of drugs for anti-schistosomal effects, drugs used in treating cancers have also showed promising effects. For instance, miltefosine, an alkylphosphocholine with anticancer activities, showed significant activity against the larval and adult stages of the *S. mansoni* worm [105]. An *in vivo* study by Eissa and co-workers [106], showed miltefosine interfered with the *S. mansoni* lifecycle. A dose of 20 mg/kg orally administered daily for five days in invasive, immature or mature *S. mansoni* infected mice, showed a significant decrease in hepatic granulomata size and a reduction in worm burden [106]. Additionally, in a related study showed that using miltefosine and lipid nanocapsules (LNCs) as oral nanovectors resulted in a potent anti-schistosomal effect and a significant worm burden decrease in invasive and immature *S. mansoni* infected mice [107].

More so, imatinib (Gleevec®) is a kinase inhibitor employed in treating chronic myeloid leukemia; a disease caused by constitutive expression of gastrointestinal stromal tumour and active c-Kit kinases or deregulated Abi kinase activity [108]. Lately, imatinib has attracted attention in schistosomicide drug discovery as a result of its *in vitro* time and dose-dependent effect in *S. mansoni* morphology and physiology [109, 110]. Beckmann and Grevelding [109] showed this drug causes pathological changes in the gonad and gastrodermis in both male and female worms, which lead to the death of the parasite. Additionally, it has been shown to have a remarkable effect on both the ovary and testes. Thus, further research on kinases is indispensable because their inhibitors have the ability to interfere with schistosome biology, thereby making them an attractive compound in new schistosomicide discovery.

Chlorambucil is another anticancer drug that has exhibited anti-schistosomal activity. It is an alkylating agent used in the treatment of chronic lymphocytic leukemia, low-grade non-Hodgkins lymphoma and Hodgkin's disease [111]. According to Eissa and co-workers [112], this drug has negative and favourable *in vivo* and *in vitro* activity on schistosomes. In an *in vivo* experiment, this drug expressed a significant decrease in all worm burdens, achieving the best results against the juvenile worm where it attained a decrease in intestinal egg count, hepatic egg count and total worm load of 89.2%, 86.7% and 75.8% respectively [112]. Furthermore, a progressive decrease was also displayed in the parasite's viability in a dose-dependent manner [112].

UNIVERSAL STRESS PROTEINS (USPs)

USPs are a group of proteins present in various organisms that include fungi, archaea, bacteria, yeast, protists, and plants [113, 114]. Their up-regulation enables schistosomes to tolerate diverse and mainly harsh ambiences such as high salinity, toxic chemicals and high temperatures during

its developmental cycle. However, in the human genome, the genes encoding USPs have not been characterized, therefore making USPs a potential drug target against schistosomiasis [115, 116]. Additionally, their absence from the human host makes them an interesting vaccine target for the disease. It has been suggested that the schistosomulum parasitic stage, which is more prone to oxidative stress than other parasitic stages due to the production of nitric oxide and hydrogen peroxide by the human host, voids the human immune response through the action of USPs and goes through a transition in morphology and adaptation to a different environment, which are both needed for the survival of the parasite [117].

ANTIMICROBIAL PEPTIDES (AMPs)

AMPs are a subset of proteins that forms part of the innate immune system [118, 119]. They serve as the primary defence line in many organisms [120]. One of the mechanisms schistosomes use is to reduce the efficacy of the host immune system. However, AMPs have the ability of stimulating the immune system, thereby causing resistant to the disease. In addition, AMPs can scavenge the ROS produced by the schistosomes. In respect to their various mechanisms of actions and characteristics, AMPs was proposed as novel drug targets in drug design and discovery [7].

CONCLUSION

Schistosomiasis is a disease that affects underprivileged rural communities particularly those located in areas where fishing and farming activities are the major occupation. Sadly, pharmaceutical companies have ignored developing drugs for this disease because it usually affects poor people from rural areas who cannot afford highly-priced drugs and good health systems. Therefore, it is important to develop new and affordable anti-schistosomal drugs that are structurally and functionally different from PZQ so as to alleviate the pressure of existing drug

resistance by the worm. Moreover, combined chemotherapy based on merging compounds such as artemether and PZQ would serve as an advantage as both would cover all developmental stages of the schistosome worm and the rapid development of PZQ resistance would be tackled [121]. Overall, more detailed knowledge and understanding of the schistosome biology is needed in order to speed up the design and development of new drugs.

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CHAPTER THREE

Manuscript already submitted for publication in the journal: Advances in Bioinformatics

In silico **studies of druggable** *Schistosoma* **proteins and interactions with putative Antimicrobial peptides towards alternative anti-schistosomal therapeutics.**

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3.1 Abstract

Schistosomiasis is a debilitating disease caused by a parasitic flatworm found in freshwater. With the exponential increase in prevalence, Praziquantel (PZQ) remains the only effective drug in the anti-schistosomal arsenal. More so, resistance to PZQ has been widely reported recently. Therefore, it is imperative to develop effective alternative anti-schistosomal compounds using bioinformatics-based tools utilizing the broad-spectrum therapeutic capabilities of antimicrobial peptides (AMPs). These AMPs are essential components of the innate immune system and are responsible for the complete destruction and immunomodulatory effects in the host defence against pathogenic organisms. In this study, Hidden Markov model was used in the identification of anti-microbial peptides with potential anti-schistosomal activities. Also, glycosyltransferase and axonemal intermediate chain were identified as Schistosome proteins using *in silico* methods. The 3D-structures of the AMPs and proteins were modelled using I-TASSER. Finally, PatchDock was employed to ascertain the interaction between these schistosome proteins and the putative AMPs. Furthermore, six putative AMPs (TAK1-TAK6) were identified with potent anti-schistosomal capability. Moreover, predicted structures of the six putative AMPs and the two proteins had low C-score, which may be due to lack of available templates for their modelling. As revealed by molecular docking, all the predicted putative AMPs have good binding affinity to the schistosomal proteins. However, TAK3 (15206) and TAK6 (15888) showed the highest binding affinities to glycosyltransferase and Axonemal intermediate chain respectively. Overall, all the generated AMPs are potential target in schistosomiasis therapeutic remedy and could prove effective against drug-resistant schistosome strains.

Keywords; Schistosomiasis, Putative anti-schistosomal, Antimicrobial Peptides, Hidden Markov Model (HMMER), Praziquantel

3.2. Introduction

Despite the mass drug administration for schistosomiasis [1, 2], it continues to be a major health threat to humans. Schistosomiasis is a neglected tropical disease, which is endemic in the tropics. This disease has been reported in 78 countries to have infected an estimated 240 million people, leaving over 700 million people at risk of the parasites [3]. *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma japonicum* are the three major schistosome species responsible for morbidity and mortality in humans [4]. The life cycle of the parasite is characterized by two hosts: an intermediate host (freshwater snails) and a definitive host (mammals). The eggs voided by the mammalian definitive host hatches in water to form miracidia that enter the tissue of the snail intermediate host. Cercariae, the *Schistosoma* larvae with the ability to penetrate the skin of the mammalian host are then released by the snails. The free-living cercaria transforms into schistosomula which travel through the bloodstream to the liver where they develop and mature into adult worms that lay eggs into the bloodstream of the host with these eggs lodging within the host tissue. At this stage, the host suffers difficulty in blood flow, defects in reproductive organs, lungs, brain, and guts, predisposing the patients to genital schistosomiasis [5], neuro schistosomiasis [6], hepatointestinal schistosomiasis [7] and pulmonary schistosomiasis [8]. Several acute and chronic clinical outcomes have been attributed to this disease such as cancer [9], pulmonary [8] and portal hypertension [10], malaise [11], liver cirrhosis [12] and skin dermatitis

[13] amongst others. However, the introduction of praziquantel (PZQ) several years ago has improved the treatment of this disease [14]. PZQ is easily accessible, less expensive and more effective against the three major species parasitizing mammals, but it has been faced with the challenge of not showing activity against the juvenile worm [15]. Also, PZQ does not prevent re-

infection and parasite resistance has been reported from some regions of the world. Moreover, various compounds have been reportedly been used in the treatment of Schistosomiasis; metrifonate [16], oxamniquine [17], niridazole [18], oltipraz [19, 20], hycanthone [21]. Additionally, some compounds have been proposed to be targeted in treating schistosomiasis; these are anti-microbial peptides (AMPs), miltefosine [22] and imatinib [23].

The ability to protect oneself from pathogens and parasites is indispensable for good health. The innate immune system, which is a conserved mechanism of defence in animals, has been responsible for this protective mechanism [24]. Several studies have documented AMPs as part of the innate immune system [24-26]. AMPs are natural antibiotics with multifunctional properties produced by all living species and are currently explored as a vital source for the development of new drugs [27]. Oyinloye and co-workers proposed AMPs as excellent candidates in the treatment and control of schistosomal infections [28], and further suggested that the mechanism by which the schistosome worm evades the innate immune system is by reducing the efficacy of the host immune system by mimicking or manipulating it. This further leads to the development of favorable ambiences for the schistosomes promoting their survival and co-habitation within the host in an adaptive host-parasite complex mechanism. This co-habitation leads to immunosuppression, resulting in severe complications and the predisposition of the host to other secondary infections. However, AMPs are known to have immunostimulatory potentials. In addition, AMPs can scavenge disease-causing reactive oxygen species (ROS) produced by the schistosomes [28]. With respect to their various mechanisms of action and characteristics, AMPs have been proposed as emerging drug targets in drug design and discovery [29-31]. With all the above-mentioned characteristics of AMPs, it is imperative to identify a class of these biomolecules that can act as therapeutic agents to tackle schistosomiasis or alternatively can act as an adjuvant

to PZQ. Therefore, *in silico* discovery of new druggable compounds using AMPs as the foundation, modelling of AMPs and schistosome proteins using various bioinformatics techniques will aid and enhance the discovery of new schistosomicides since experimental investigations are time and capital consuming.

3.3. Materials and Methods

3.3.1. Data mining and data retrieval

For the purpose of stochastic model construction, various AMP databases such as Anti-microbial Peptide Database (APD) [32, 33], Dragon Anti-microbial Peptide Database (DAMPD) [34], Collection of Anti-microbial Peptide (CAMP) [35], and PepBank [36] were queried to extract and collect experimentally validated AMPs with anti-parasitic activity. The retrieved AMPs were documented in FASTA format and three-quarters of these were utilized as the training set for the model construction, whilst the remaining one-quarter was used as the testing set. Consequently, Hidden Markov models (HMMER) [37] was employed in the construction of the stochastic models.

3.3.2. Construction of AMPs profiles using HMMER

The HMMER algorithm version 2.3.2 was used in constructing the predictive profile. The algorithm was operated on POSIX-compatible platforms such as MacOS/X, UNIX and LINUX, to create the desired profiles. With the help of this algorithm, the constructed profiles enabled the search of protein sequence similarity using probabilistic techniques [38]. However, Ubuntu 16.04.2 LTS (XenialXerus) operating system, which is built on a LINUX kernel, was used to construct all the HMMER profiles. The training set of the experimentally proven AMPs was utilized in the construction of the HMMER profile with the robustness of the created profile validated using the testing dataset.

3.3.3. Profile creation

To create the predictive profile, the training dataset was aligned with the hmmalign command of HMMER. This was carried out using the ClustalW alignment tool to perform multiple alignments for the training set using the command line:

```
Clustalw –align –output=gcg –case=upper –seqnos=off –outorder=aligned –
infile= family.fasta
```

The result from the predictive profile creation process was saved as msf(gcg) format (family.msf).

3.3.4. Building of the profile

The resulting file was used in the second step of profile HMMER building, known as build profile.

hmmbuild- builds a new HMM profile from the multiple sequence alignment file using the command line:

```
hmmbuildfile.hmmfamily.msf
```

The 'build profile' is saved in hmm format (family.hmm).

3.3.5 Calibration of the profile

hmmcalibrate- this step calibrates the HMM search statistics. This command line helps to improve the profile sensitivity.

```
hmmcalibratefamily.hmm
```

3.3.6 Testing of the profile

Following profile calibration, the hmmsearch step was carried out to search and query the testing dataset. The created HMM profile 'family.hmm' was thereafter used to evaluate the performance of the created profile by testing against a list of independent AMPs. The testing set (represented

by one-quarter of the experimentally validated retrieved AMPs) was utilized as the positive dataset hence; the testing set was queried against the constructed profile. This was achieved using the command line:

```
hmmsearch -E 1e-2 family.hmm family(test set).fasta>
```

The cut-off E-value to query the constructed model was then set to 0.01 as indicated in the command line, since lower E-values indicate a more significant hit.

3.3.7 Performance evaluation of created profile based on the prediction of both positive and negative testing sets

Using sensitivity, specificity, accuracy and Mathews Correlation Coefficient as parameters to measure the statistical performance evaluation for the created profile, the performance of the created profile was calculated. Furthermore, the constructed profile was queried against a negative dataset of 250 neuropeptide sequences with known non-anti-parasitic activity. Statistical measures were then calculated to evaluate the strength and performance of the created profile using the formula:

Sensitivity: This is the percentage of anti-schistosomal AMPs (testing set) correctly predicted as anti-schistosomal AMPs (Positive)

$$= [\quad \overline{\quad\quad\quad} \quad] \times 100 \tag{1}$$

Specificity: This is the percentage of non-anti-schistosomal AMPs (negative set) correctly predicted as non-anti-schistosomal AMPs (negative)

$$= [\quad \overline{\quad\quad\quad} \quad] \times 100 \tag{2}$$

Accuracy: This is the percentage of correctly predicted anti-schistosomal and non-anti-schistosomal AMPs.

$$= [\frac{+}{+ + +}] \times 100 \qquad (3)$$

Mathews Correlation Coefficient (MCC): This is a measure of both sensitivity and specificity. It is worthy to note that MCC=0 indicate complete random prediction, while MCC=1 indicates perfect prediction.

$$= [\frac{(\times)-(\times)}{\sqrt{(+) \times (+) \times (+) \times (+)}}] \qquad (4)$$

where: TP = True Positive, TN = True Negative, FP = False Positive, FN = False Negative and MCC = Mathews Correlation Coefficient

3.3.8 Identification of novel putative anti-schistosomal AMPs from proteome sequence databases

Approximately one thousand proteome sequences in FASTA format were retrieved from the ENSEMBL server (http://www.ensembl.org/index.html) as well as the UniProt database (http://www.uniprot.org/) respectively. More so, by employing the hmmsearch module of HMMER, all the retrieved proteome sequences were scanned against the calibrated constructed profile and the cut-off E-values for the search of anti-schistosomal AMPs was set at 0.01 using the command line:

```
hmmsearch –E 1e-2 family.hmm family(test set).fasta>
```

Based on the set E-value, all identified peptides were considered to be putative anti-schistosomal AMPs.

3.3.9 Identification of novel druggable schistosomes proteins

The STITCH (**S**earch **T**ool for **I**nteractions of **Ch**emicals) database, which was accessed at http://stitch.embl.de was used to identify schistosome target proteins using the known effective

drug against the worms, Praziquantel as the query. *Schistosoma* was selected as the organism and all families of the interacting proteins were displayed; the family with the highest interaction confidence was then selected. The database provided a network of an analytical view whereby information about a particular protein was provided.

3.3.10 Physico-chemical characterization of the putative anti-schistosomal AMPs and identified schistosome proteins

Physicochemical properties such as net charge, Boman index, instability index, extinction coefficient, hydrophobic residues and isoelectric point of the putative AMPs were calculated using

the Bactibase (http://bactibase.hammamilab.org/physicochem) and the APD (http://aps.unmc.edu/) interfaces. Additionally, the ProtParam tool of the proteomic server, ExPASy (http://web.expasy.org/protparam/) was used to compute the physicochemical parameters of the target proteins.

3.3.11 Ab initio structure prediction of putative anti-schistosomal AMPs and schistosome proteins

The I-TASSER (**I**terative **T**hreading **ASSE**mbly **R**efinement) server for predicting protein structure and function (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) was used to predict the structures of the schistosome proteins and the top 6 putative AMPs selected based on their predictive E-values. The respective amino acid sequences of the putative AMPs and the novel proteins were submitted to the online server I-TASSER. The 3D structural elements of the output data were examined and created using PyMol Molecular Graphics System (2003). DeLano Scientific, LLC, USA. http://www.pymol.org

3.3.12 Molecular docking of interaction between schistosome proteins and putative anti-schistosomal AMPs

In silico molecular docking of the two (2) identified schistosome proteins with the six (6) putative anti-schistosomal AMPs was carried out using PatchDock (https://bioinfo3d.cs.tau.ac.il/PatchDock) an online server with a geometry-based molecular docking algorithm designed to ascertain docking transformations that yields good molecular shape complementarity [39]. Briefly, the PDB files of the 3-D coordinates of the schistosome proteins (receptor) and that of the putative AMPs (ligand) were uploaded. The recommended default RMSD clustering of 4Å was used, with the complex type was set to protein-small ligand before the submission was made. The results in the form of observed interactions between the two biomolecules in the form of docked conformations were examined and visualized using PyMol Molecular Graphics System (2003). DeLano Scientific, LLC, USA. http://www.pymol.org.

3.4. Results

Experimentally validated anti-parasitic AMPs were retrieved from various databases as well as the accompanying publication for each AMP. After retrieving the experimentally validated AMPs from the various databases, a final list of 13 AMPs was compiled using Cluster Database at High Identity with Tolerance (CD-HIT) to remove duplicated sequences. Following the creation of the anti-parasitic profile, the scanning of the created profile against the testing set confirmed that the profile would recognize AMPs of this class as it identified AMPs within the testing set which has known anti-parasitic activity. Furthermore, scanning the 250 neuropeptides (negative set) against the profile showed no hits since the negative set does not possess anti-parasitic activity (see Table 3.1a). After scanning has been done against the negative and the testing set, statistical performance

measures such as sensitivity, specificity, accuracy and MCC were calculated to ascertain the robustness of the profile as shown in Table 3.1b.

Table 3.1a: Results obtaining by querying HMMER profiles against the testing dataset

AMPs profile	True Positive	False Negative	True Negative	False Positive
AMPs	2	2	250	0

Table 3.1b: Performance measurements generated for the model created by HMMER profile

Super-family	Sensitivity (%)	Specificity (%)	Accuracy (%)	MCC
AMPs	50	100	99.3	0.5

The performance of the profile is only rated on its specificity and accuracy values. From this study, the created model had a specificity of 100% and an accuracy of 99.3% however, specificity and accuracy values around 95% implies that the constructed profile had more than 95% confidence to predict a peptide to be a putative anti-schistosomal AMP.

3.4.1 Discovery of putative anti-schistosomal AMPs

As mentioned in section 2.8, the constructed profile was scanned against the proteome sequence to identify peptides with the same motifs and activity as the profile. All the peptides recognised were termed as putative anti-schistosomal AMPs and were ranked according to their respective E-values. The significance of a hit is measured by its E-value, which is computed from the bits score, revealing the number of true positives (TP) picked by the training dataset. An E-value of 0.01 signifies that the chance of a hit being false or has come up by chance is only 1%. A final list of 20 AMPs was identified and the top 6 with the lowest E-values were selected for further study. E-values of the putative anti-schistosomal AMPs were 3.2E-55 for TAK1, 4.8e-08 for TAK2, 5e-07

for TAK3, 4.6e-06 for TAK4, 2.7e-05 for TAK5 and 0.00017 for TAK6. From the results, it can be inferred that these peptides have E-values that are well below the cut-off value (E-value: 0.01).

3.4.2 Identification of novel schistosome proteins based on interaction study

In order to identify potent latent interacting proteins with PZQ, the STITCH 5.0 database was queried and the result shown in Figure 3.1. Two new proteins, Axonemal dynein intermediate chain, putative (Smp_103920) and Glycosyltransferase (Smp_052330) were identified as potential interacting partners of PZQ with a confidence score of 0.430 and 0.425 respectively. The amino acid sequences of the proteins were retrieved for further *in silico* analysis and literature mining.

Figure 3.1 Network diagram generated from STITCH indicating interacting protein partners of PZQ.

3.4.3 Analysis of the physicochemical parameters of Schistosome proteins and putative anti-schistosomal AMPs

The physicochemical properties of the six putative AMPs were computed using Bactibase [40, 41] and APD [32, 33] to ascertain if the putative AMPs possessed similar properties to all known AMPs (Table 3.2a). The putative AMPs are shown to be novel since there was no match with any existing AMPs in various database libraries.

Table 3.2a: Physico-chemical properties for the 6 putative anti-schistosomal AMPs

	Mass(Da)	Most common amino acid	Isoelectric point	Net charge	Total hydrophobic ratio(%)	Boman index(kcal/mol)	Half-life(hrs)	Most similar sequence with active domain(%)
TAK 1	9484.17	Arg: 15.66	10.83	11	34	3.02	30	AP01161: 89.15
TAK 2	9594.76	Lys: 11.76	10.09	9	35	2.31	1.1	AP01373: 76.47
TAK 3	9321.14	Ile/Lys: 9.52	11.2	13	39	1.82	30	AP02076: 81.17
TAK 4	9446.34	Leu: 10.59	10.41	8	45	1.41	4.4	AP02185: 61.79
TAK 5	9266.44	Lys: 10.71	10.88	12	40	1.45	30	AP02077: 82.35
TAK 6	9601.04	Lys: 9.41	10.02	6	40	2.08	1	AP02185: 74.15

Additionally, the physicochemical parameters of the two proteins and their amino acids abundance were generated using the ProtParam tool of the ExPASy proteomics server as shown in Table 3.2b and Figure 3.2. Leucine is the most abundant amino acid in Glycosyltransferase, while glutamic acid is the most abundant amino acid in the axonemal dynein intermediate chain protein. The abundance of these two amino acids is an excellent indicator of the stability of both proteins as depicted by their instability index (Table 3.2b).

Table 3.2b: Physico-chemical parameters of the two schistosome proteins.

Protein	Molecular weight (Da)	Theoretical pI	Instability Index	Aliphatic Index	Half-life in mammals
Glycosyltransferase	56833.82	8.95	30.21	93.93	30 hours
Axonemal dynein intermediate chain	71781.50	5.15	55.15	73.34	30 hours
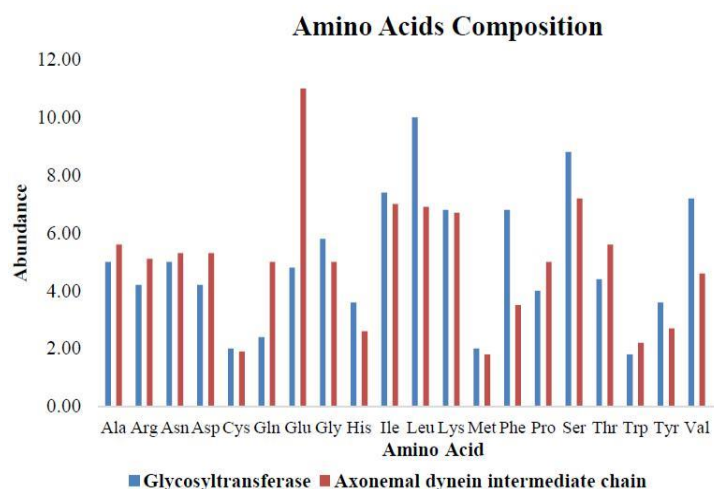
Figure 3.2: Amino acid composition of Glycosyltransferase and Axonemal dynein intermediate chain generated.

3.4.4 3D homology modelling of the Schistosome proteins and putative antischistosomal AMPs

Homology modelling of both the schistosome proteins and the putative AMPs was achieved via the I-TASSER server. The outputs generated from this process were saved and visualized using PyMol Molecular Graphics System (2003), DeLano Scientific, LLC, USA as depicted in Figures 3.3 and 3.4. The modelled AMP structures revealed the presence of secondary structure elements such as α-helices, β-sheets, β-hairpins, γ-turns and β-bulges, which are consistent with the structures of known AMPs. The C-score, TM-score and RMSD shown in Table 3.3 were used to evaluate the quality of the predicted structures of the putative anti-schistosomal AMPs and the

Schistosome proteins. The C-score data shown in Table 3.3 revealed the modelled structures were of high quality.
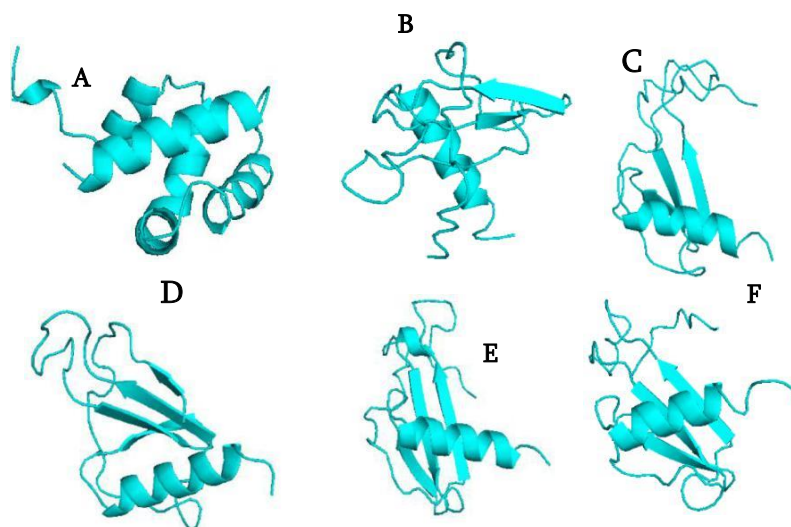
Figure 3.3: 3D structures of putative anti-schistosomal AMPs. A: **TAK1**, B: **TAK2**, C: **TAK 3**, D: **TAK4**, E: **TAK5**, F: **TAK6**. The structures were predicted using an online server I-TASSER and visualized using PyMol Molecular Graphics System (2003). DeLano Scientific, LLC, USA.
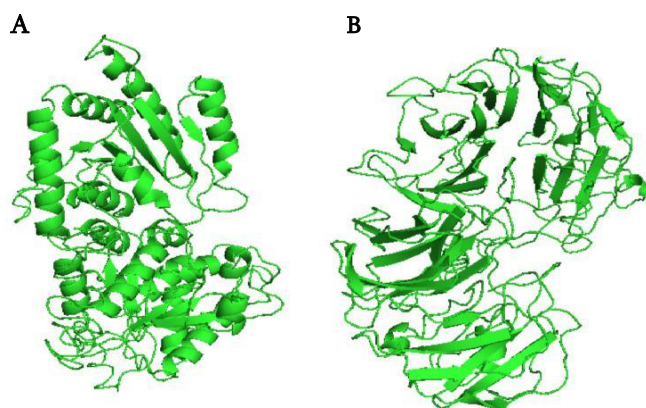
Figure 3.4: 3-D structures of schistosome proteins. A: **Glycosyltransferase,** B: **Axonemal dynein intermediate chain**. The protein structures were predicted using an online server I-TASSER and visualized using PyMol Molecular Graphics System (2003). DeLano Scientific, LLC, USA.

Table 3.3: Quality assessment scores of the predicted 3D structures of putative anti-schistosomal AMPs and schistosome proteins generated by I-TASSER

Putative anti-schistosomal AMPs	C-score	Exp. TM score	Exp. RMSD (Å)
TAK 1	0.15	0.73 ± 0.11	3.3 ± 2.3
TAK 2	-0.83	0.61 ± 0.14	5.3 ± 3.4
TAK 3	-0.55	0.64 ± 0.13	4.7 ± 3.1
TAK 4	-0.43	0.66 ± 0.13	4.5 ± 3.0
TAK 5	-0.60	0.64 ± 0.13	4.8 ± 3.1
TAK 6	-0.74	0.62 ± 0.14	5.1 ± 3.3
Glycosyltransferase	-2.28	0.45 ± 0.14	12.8 ± 4.2
Axonemal dynein intermediate chain	-1.53	0.53 ± 0.15	11.5 ± 4.5

3.4.5 Protein-peptide docking

The binding orientation, as well as the binding strength of each putative AMP (ligand) when bounded to the schistosomal proteins (receptor) was determined using online software PatchDock. This software works by removing all irregular interactions between atoms of the ligand and that of the receptor. Finally, the top 20 docking conformations for the most probable interactions based on binding affinity complementarity score, approximate interface area of the complex and atomic contact energy (ACE) were displayed (Table 3.4 and 3.5). Based on the binding affinity score, TAK3 and TAK6 have the highest propensity to bind to glycosyltransferase and axonemal dynein intermediate chain respectively. The PDB file for the best scoring complex for each docked complex was saved and visualized using RasMol Molecular Visualization Tool, (2009) (Figure 3.5 and 3.6). Immediately after docking, the schistosome protein-AMP complex was analyzed to ascertain the binding orientation of the ligand AMPs to the protein receptors. The right binding orientation will be the one in which the AMPs could prevent the invasion of the human system by the schistosomal worm. Additionally, the analysis would help in drawing a better conclusion on

which putative anti-schistosomal AMPs to be implemented as potent inhibitory molecule(s) to fight schistosomal infections. Therefore, the positions of the amino acid residues of the protein receptors interacting with the AMP ligands were mapped as shown in Figures 3.5 & 3.6.

Table 3.4: Geometric scores of the binding affinity obtained from the docking of the putative anti-schistosomal AMPs and glycosyltransferase protein.

Complex	Binding affinity scores	Area	ACE
Glycosyltransferase + TAK1	13374	1848.70	479.91
Glycosyltransferase + TAK2	13336	1636.70	124.23
Glycosyltransferase + TAK3	15206	2282.20	-14.95
Glycosyltransferase + TAK4	12966	2080.90	298.61
Glycosyltransferase + TAK5	14924	2244.20	169.27
Glycosyltransferase + TAK6	12706	2384.50	368.38

Table 3.5 Geometric scores of the binding affinity obtained from the docking of the putative anti-schistosomal AMPs and axonemal dynein intermediate chain protein

Complex	Binding affinity scores	Area	ACE
Axonemal dynein intermediate chain + TAK1	13946	2296.50	259.34
Axonemal dynein intermediate chain + TAK2	13918	2104.40	132.82
Axonemal dynein intermediate chain + TAK3	14292	2352.60	-63.91
Axonemal dynein intermediate chain + TAK4	15786	2241.70	73.39
Axonemal dynein intermediate chain + TAK5	15834	2419.50	379.00
Axonemal dynein intermediate chain + TAK6	15888	2701.40	-62.31
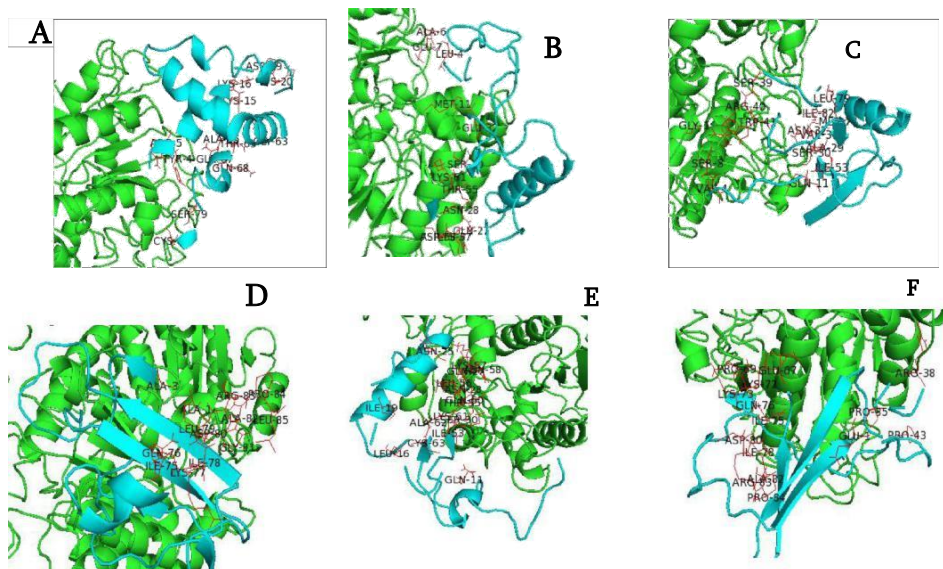
Figure 3.5 Interaction between Schistosome glycosyltransferase and putative anti-schistosomal AMPs. The cartoon representation in green is the glycosyltransferase, while represented in turquoise are the putative anti-schistosomal AMPs. Residues involved in the interaction are shown on the docked complex (A): **Glycosyltransferase-TAK1**, (B): **Glycosyltransferase-TAK2**, (C): **Glycosyltransferase-TAK3**, (D): **Glycosyltransferase-TAK4**, (E) **Glycosyltransferase-TAK5**, (F) **Glycosyltransferase-TAK6.**
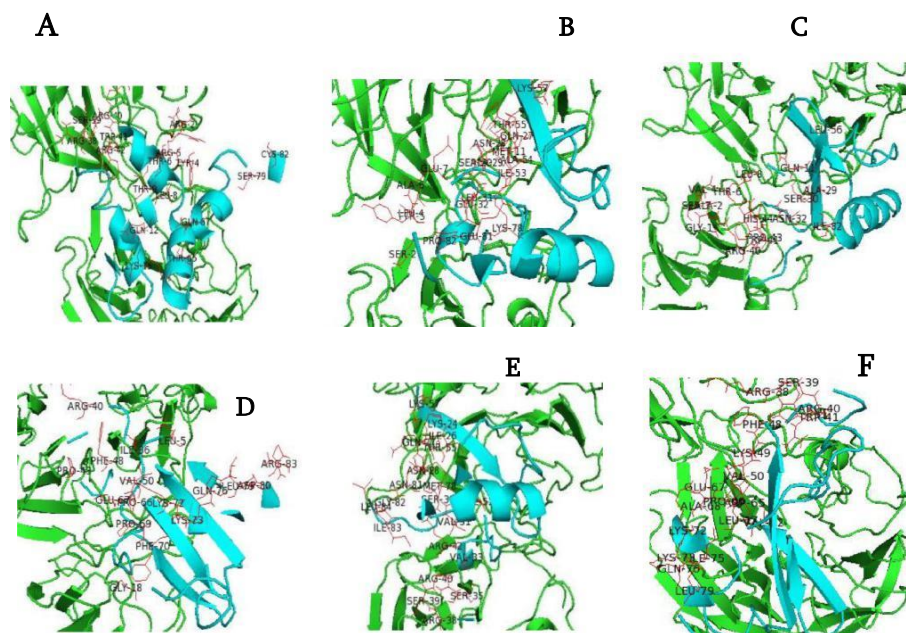
Figure 3.6 Interaction of Schistosome protein axonemal dynein intermediate chain and putative anti-schistosomal AMPs. The cartoon representation in green depicts the axonemal dynein intermediate chain, while represented in turquoise are the respective putative anti-schistosomal AMPs. Residues involved in the interaction between the two proteins are shown on the docked complex (A) **Axonemal dynein-TAK1**, (B) **Axonemal dynein-TAK2**, (C) **Axonemal dynein-TAK3**, (D) **Axonemal dynein-TAK4**, (E) **Axonemal dynein-TAK5**, (F) **Axonemal dynein-TAK6.**

3.5 Discussion

The HMMER algorithm was used to identify six putative anti-schistosomal AMPs; the best peptide has an E-value of 3.20E-55, which is indicative of the very low probability of the peptide to be a falsely predicted anti-schistosomal AMP. The lowest E-value observed among the six selected peptides is 1.7E-4, meaning that there is only 1.7E-4 % possibility for the peptide to be predicted as a false anti-schistosomal AMP. Thus, all the HMMER predicted putative anti-schistosomal AMPs had excellent probability scores to be considered true anti-schistosomal AMPs. The physico-chemical parameters showed that all the putative AMPs are novel since none of them matched any existing AMPs related to schistosome inhibition in various AMPs database libraries as shown in Table 3.2a. All the peptides had a positive net charge due to the abundance of positively charged (basic side chain) amino acid residues and total hydrophobicity greater than 30 %, which is standard for known AMPs as shown by Tincho and co-workers [26]. The binding potentials of antimicrobial peptides as depicted by the Boman index is known to range between 2.53-3.04 kcal/mol, which denotes peptides with multifunctional and high binding potentials. Conversely, a low Boman index signifies an antibacterial drug target with minimal negative effects

[42]. In this study, five of the AMPs showed values below the 2.53 cut-off index, except TAK1, which showed a value above the cut-off. This meant that majority of the AMPs tested possessed anti-bacterial activity [42]. Moreover, all the 20 amino acids were present in the proteins as shown in Figure 3.2 which contributes to their respective average molecular weight of 56833.82 Dalton (Da) and 71781.50 Da. Leucine was the most abundant amino acid in the primary sequence of glycosyltransferase with an abundance of 10 % and glutamate in axonemal dynein intermediate chain protein with an abundance of 11 %. Their aliphatic indexes of the two proteins were 93.93 and 73.34 respectively; these high values signified the proteins would stay stable over a range of

temperatures [43]. Finally, it was revealed that the proteins have the ability to remain intact without being degraded for more than 30 hours in mammalian cells. This information is particularly useful because it shows that protein degradation will be limited during protein structure-function studies [44].

3.5.1 Modelling of putative AMPs and Schistosome proteins

The parameters C-score, TM-score and RMSD were used to measure the quality of the predicted models by I-TASSER, thereby enabling the validation of the structural model. The C-score is a confidence score used in evaluating the quality of the predicted model and ranges from -5 to 2, where a higher C-score value indicates a model with a high confidence and the reverse is the case for a model with a lower C-score [45-47]. TM-score and RMSD are known criteria for assessing the structural similarity between two structures and are generally employed to evaluate the accuracy of structural modelling when the native structure is known. Apart from TAK1, all the predicted structures had a low C-score, which could be indicative of the lack of available templates for their modelling [47]. With all TM-scores that were above or close to 0.5, the predicted structures are structurally similar to the templates used for prediction [45]. Also, all the putative antimicrobial peptides, TAK1 to TAK6 had good RMSD.

3.5.2 Docking study of the interaction of putative AMPs with Schistosome proteins

Molecular docking was done to ascertain the interaction of the putative anti-schistosomal AMPs with glycosyltransferase. This could be a new approach in the inhibition of glycosyltransferase; an enzyme responsible for the biosynthesis of glycan, which helps in the maintenance of cell membrane integrity in Schistosomes [48, 49]. This approach also helps to establish how the interaction of these putative anti-schistosomal AMPs with axonemal dynein intermediate chain could be a new strategy in the prevention of the protein by acting as a competitive inhibitor.

Axonemal dynein intermediate chain protein presumably powers the cilia [50]; the cilia are linked to cell cycle development, proliferation, as well as play a major role in the Schistosomes development and everyday life. The docking results displayed a very high binding affinity score above 8731, which indicates good binding for the putative anti-schistosomal AMPs [51]. TAK3 was shown to have the best geometric shape complementarity score (15206) for glycosyltransferase, which can be attributed to its good Boman index (1.82 kcal/mol). Interestingly, the high abundance of lysine in this putative anti-schistosomal AMP makes it an excellent drug target because documented evidence has shown that poly-L-lysine destroys the surface membrane of adult schistosomes during perfusion [52, 53]. TAK5, another peptide with a high abundance of lysine had the second highest binding affinity to glycosyltransferase. Further analysis revealed that TAK6 had the highest geometric shape complementarity score of 15888 for axonemal dynein intermediate chain, with an approximate interface area size of 2701.40 and an atomic contact energy of -62.31 kJ/mol for axonemal dynein intermediate chain (Table 3.5). The high score can be accredited to its net charge of +6 and good Boman index of 2.08 kcal/mol; added to this, the AMP has high lysine content. Therefore, TAK3 and TAK6 are highly probable and energetically favourable model for glycosyltransferase and axonemal dynein intermediate chain respectively. All the interaction between the proteins and the putative anti-schistosomal AMPs are shown in Figure 3.5 and 3.6 respectively. On the whole, the realization from this study is that the binding affinity of the putative AMPs with the proteins does not show a parallel decrease from putative anti-schistosomal AMPs with the lowest E-value to the putative anti-schistosomal protein with the highest E-value. This is in an accordance with the study by Tincho and colleagues [26], where ten AMPs where retrieved using *in silico* methods and two were further utilized in developing lateral flow device prototype that can accurately detected both HIV-1 and HIV-2 [54].

3.6. Conclusion

The emergence of drug resistance to anti-schistosomal drugs reveals a clear necessity to design and develop novel therapeutic agents. AMPs possess broad-spectrum anti-parasitic, anti-fungal, anti-protist, anti-viral and anti-bacterial activities. Therefore, exploration of the inhibition of schistosome proteins using AMPs will provide proper insights into the discovery of new schistosomicides hence, *in silico* methods that are less expensive and not time-consuming will hasten this discovery. However, additional experimental investigation will be done to establish the validity of these AMPs. Taking all findings together, it was clearly revealed that the identified AMPs in this study have a strong binding affinity to the schistosome proteins and can be utilized for the design of novel anti-schistosomal therapy with high efficacy.

Disclosure: The authors declare no conflict of interests.

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CHAPTER FOUR

Manuscript already submitted for publication in the journal: Advances and Applications in Bioinformatics and Chemistry

Optimization of putative anti-schistosomal AMPs against Schistosome enzymes towards drug development

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4.1 Abstract

Knowing the structural basis of protein-protein association and ability to ascertain the key residues involved in their interactions is a vital issue with vast practical application in functioning and regulation of metabolic networks, signal transduction and may assist in rational drug design (structure-based design of ligands) and protein engineering. Glycosyltransferase and axonemal dynein intermediate chain have been identified using STITCH as druggable protein targets in schistosomiasis. Hence, to inhibit the enzymatic activity of these proteins, we have employed computational methods to simulate the interaction between mutated anti-schistosomal AMPs and these proteins. The KFC server was used for site-directed mutagenesis on the putative anti-schistosomal AMPs to increase their biological activities, homology modelling of the mutated AMPs using I-TASSER showed they are identical with the parental AMPs. More so, *in silico* validation of the quality of both the parental and mutated AMPs demonstrated that the models are of good quality and results from molecular docking using PatchDock showed that these mutated AMPs are capable of interacting with the schistosome proteins. Thus, with the efficient interactions between the mutated AMPs and proteins, we propose that these peptides maybe promising in schistosomiasis therapy and could as well prove effective against PZQ-resistant schistosome strains.

4.2 Introduction

Schistosomiasis, also called bilharzia, is a disease afflicting over 243 million people. Its causative organisms are trematodes belonging to the genus Schistosoma. Already, this disease has been reported to be affecting people in 78 countries and approximately 800 million are at risk of getting infected (Weerakoon *et al*., 2015). In human, three *Schistosoma* species which live an average 3-

10years but up to 40years in some cases has been identified to cause this disease; namely, *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma japonicum* (Colley *et al*., 2014). Host of the parasite suffers hypertension (Raia *et al*., 1994; Simonneau *et al*., 2009; Graham *et al*., 2010), cancer (Mostafa *et al*., 1999; Honeycutt *et al*., 2014; Botelho *et al*., 2015), renal disorder (Duarte *et al*., 2015; Mpondo *et al*., 2016), hepatic damage (Angelico *et al*., 1997; Wynn *et al*., 2004; ANDRADE, 2009; Olveda *et al*., 2017) amongst other chronic indications. However, with the severity of this disease, chemotherapy remains the sole means for tackling it. The large-scale use of Praziquantel (PZQ) as the only drug in treating schistosomiasis has raised the fear of the development of resistance to this drug (Lee and Fairlie 2015). Although, various compounds have been tested against Schistosomes in the past but PZQ remains the drug of choice. It is therefore of paramount importance to develop a new drug not only against drug resistance strains but also against the juvenile worm which PZQ does not show activity against (Wu *et al*., 2011).

Presently, the techniques and methods involved in the discovery and design of anti-parasitic compounds requires the identification of novel targets from various online repositories on the parasite genome and biological pathways. In a previous study, glycosyltransferase and axonemal dynein intermediate chain have been identified using STITCH (Kuhn *et al*., 2008) as druggable protein targets in schistosomiasis. Several studies have documented that schistosomes glycoconjugates are localized at the surface of the parasite and these glycoconjugates and glycans play a vital role in the biology of parasite, specifically host-pathogen interactions despite this their main function remains a misery (Cummings and Nyame, 1996; Prasanphanich *et al*., 2013, Mickum *et al*., 2014). More so, Schistosomes glycans are involved in the stimulation of Th2-type immunological responses and immunomodulation particularly in eggs (Okano *et al*., 2001; Faveeuw *et al*., 2003; Pearce *et al*., 2004). The Th2 response produces pro-inflammatory response

which can be fatal if not controlled early. Hence, having more insight into how glycosyltransferase (enzyme responsible for biosynthesis of glycans) acts as immunomodulators could assist in the design and development of diagnostics tools and drugs that could potentially act as inhibitor of glycosyltransferase biosynthesis, as well as glycan-based vaccine discovery.

However, of all the motor proteins present in schistosomes, the dynein light chains are expressed abundantly in the tegument of the schistosomes and have been the core of several research (Jones *et al*., 2004). These dyneins are complexes of proteins that network with microtubules of the cytoskeleton, they assist in the movement of vesicles and molecules. The two classes of dynein are: axonemal and cytoplasmic dyneins (Milisav, 1998). Axonemal dyneins are responsible for the movement of the cilia and flagella, while the cytoplasmic dyneins trafficks the vesicles in interphase cells, arrangement of mitotic spindle poles, reverse axonal transport in neurons and nuclear migrations in fungi.

Keeping in mind the vast therapeutic potentials of AMPs, in the present study, we have mutated the previously identified anti-schistosomal AMPs in chapter three. These AMPs can be used as substitute or adjuvant in schistosmiasis remedy because several studies have evidenced that AMPs can act as signalling molecules, mitogenic and antitumor agent, immunomodulatory agent and drug delivery vector (Oyinloye *et al*., 2014; Aruleba *et al*., 2018). Therefore, this study employed *in silico* techniques to increase the biological activities of the previously identified anti-schistosomal AMPs that could aid the design and development of schistosomicide with transient negative effects, high efficacy and tolerance.

4.3 METHODOLOGY

4.3.1 Site-Directed Mutagenesis

All the six putative anti-schistosomal AMPs were subjected to site-directed mutagenesis in order to increase their binding affinity for glycosyltransferase and axonemal dynein intermediate chain. This was achieved by substituting an amino acid on the putative anti-schistosomal AMPs, this substitution will strengthen the electrostatic attraction between the protein and the mutated AMPs in their complex formation, which subsequently will lead to increased binding affinity of the complex. Vital amino acids residue interacting in the complex were identified so that the ligand does not shift conformation after performing mutation on it. These amino acids residues are in the biomolecular interface and are called hotspots, they provide most of the free energy of binding for protein-protein interaction. There are many web servers and tools for predicting protein-protein interaction and interfaces but, the importance of a user-friendly server or tool cannot be over emphasized. Hence, we used the KFC (Knowledge-based FADE and contacts) (Darnell *et al*., 2008) to delineate the protein-protein interfaces in this study. KFC server is a machine learning algorithm that forecast binding hot spots or the subset of amino acids residues that account for most of the protein interface binding free energy (Darnell *et al*., 2008). To achieve its aim, this web-based algorithm considers the shape specificity and surrounding structural elements of the residues (Tuncbag *et al*., 2010). In order to determine the potential 'hotspot' amino acids in the two proteins and the parental putative anti-schistosomal AMPs complexes, the PDB coordinates of each complexes derived from the previous study were uploaded onto the KFC online server. The resulting file generated a list of amino acid residues on the interface, which contributes to the interaction of the ligand and the receptor. Now, the substitution of amino acids residues in the parental AMPs were done putting the individual residue physicochemical parameters into

consideration. Positive charged, R-group amino acids of the class, hydrophobic amino acids were parameters used in the mutation.

4.3.2 Physicochemical properties

Physicochemical properties including; Boman index, half-life, instability index, extinction coefficient, hydrophobic residues, net charge, and the isoelectric point of the mutated AMPs were computed using the Bactibase (http://bactibase.hammamilab.org/physicochem) (Hammami *et al*., 2007) and the APD (http://aps.unmc.edu/) (Wang *et al*., 2009) interfaces.

4.3.3 *De-novo* 3-D structure prediction and validation

All the 3-D structural elements of the mutated anti-schistosomal AMPs were predicted using I-TASSER (Zhang, 2008; Roy *et al*., 2010; Yang *et al*., 2015), an online algorithm that employs a *de-novo* or *ab-initio* approach. The amino acid sequences of the mutated AMPs were inputted into the online server I-TASSER. The resulting file gave PDB file containing the structural element of each AMP with a score which grades accuracy of each predicted structure. The generated 3-D structures were visualized using PyMol. The structure of the parental and the mutated anti-schistosomal AMPs were validated and compared using the program PROCHECK (Laskowski *et al*., 1993). PROCHECK gives information on the stereo chemical quality of the predicted structure and other statistical parameters. Finally, the structures of the proteins (glycosyltransferase and axonemal dynein intermediate chain) derived in the previous study were refined by a single run of 3-D$^{\text{refine}}$ server (http://sysbio.rnet.missouri.edu/3-Drefine/) (Bhattacharya *et al*., 2016); a server that optimizes hydrogen-bonding and minimizes energy at atomic-level.

4.3.3 Protein-protein docking of mutated AMPs and Schistosome proteins.

To understand the binding mode of the mutated anti-schistosomal AMPs and the proteins, molecular docking studies were performed. PatchDock (Duhovny *et al*., 2002) was used to dock all the mutated anti-schistosomal AMPs onto the proteins, in the interest to ascertain the probable binding conformation of these inhibitors. The PDB file of each mutated AMP (Ligand) were submitted with each of the refined proteins (receptor) to the PatchDock server to have a deeper understanding on how these AMPs would act as selective inhibitors of these proteins towards the development of drugs with good tolerability and high efficacy. The PatchDock server is built on the foundation of a rigid-body geometric investigating algorithm. The result generated shows the top 20 transformations ranked based on the PatchDock score and atomic contact energy and the redundant solutions were removed by 4.0A RMSD clustering during the docking process. The best transformation was selected, and the interaction was visualized using PyMol.

4.4 RESULTS

4.4.1 Hotspot Analysis

Amino acids that provide most of the free energy of binding for protein-peptide interactions are known as "hotspots". All the parental AMPs were subjected to analysis using the KFC2 server to identify the amino acid residues that form vital energetically contacts when interacting with the proteins. The predicted hotspots were analysed using the embedded Jmol viewer, these hotspots contributed maximum to energy.

4.4.2 Computation of the physico-chemical parameters of the mutated AMPs

As displayed in Table 4.1, physicochemical parameters experienced a slight change in one or two parameters for all the putative AMPs upon site-directed mutagenesis. All the putative AMPs had a insignificant decrease in their respective molecular weight. The net charges remained positive,

however, TAK2 and TAK4 had a decreased in their net charges which is indicative of the replacement of glutamine (Q) with glutamic acid (E) in TAK2 and substitution of Asparagine (N) for Aspartate (D) in TAK4. With exception of TAK6, all the mutated AMPs exhibited increase Boman index. Hydrophobicity for all mutated AMPs remained the same with the parental AMPs but TAK5 decreased, hydrophobicity is an important parameter because it contributes to the mode of action and binding affinity of AMPs.

Table 4.1: Physicochemical properties for the 6-mutated putative anti-schistosomal AMPs.

	Mass(Da)	Most common amino acids	Isoelectric point	Net charge	Total hydrophobic ratio(%)	Boman index (kcal/mol)	Half-life (hrs)	percentage and (%) Sequence similarity with other molecules
TAK1	9553.29	Arg: 16.87	11.1	12	34	3.16	30	AP01161: 89.15
TAK2	9595.47	Lys: 11.76	9.8	8	35	2.32	1.1	AP01373: 75.29
TAK3	9394.18	Lys: 9.52	11.2	13	39	1.87	30	AP02076: 80
TAK4	9447.32	Leu: 10.59	10.08	7	45	1.44	4.4	AP02185: 61.79
TAK5	9330.48	Lys: 10.73	10.73	12	39	1.5	30	AP02077: 78.82
TAK6	9568.98	Lys/Leu/Val: 9.41	10.83	6	40	2.07	1	AP02185: 73.03

4.4.3 3-D structure and validation

All structures predicted by I-TASSER (Figure 4.1) are consistent with the structures of known AMPs. With the exception of TAK2 and TAK5, all other mutated AMPs had the same number of structural elements with the parental AMPs structure. Mutated TAK2 added a 3_{10} helix and a β sheet, also, mutated TAK5 added a 3_{10} helix. Consequently, PROCHECK validation was done on the modelled parental (Chapter three) and mutated AMPs. For PROCHECK statistic, less than 10 bad contacts for each 100 residues, an average hydrogen bond energy in the range of 2.5-4.0kJ mol^{-1} and Goodness factor (G-factor) evaluates how unusual or out of the ordinary a property is, where values below – 0.5 is unusual and values below -1.0 is termed highly unusual (Laskowski *et al*., 1993). Thus, for a good model the G-factor should be greater than -0.5. Ramachandran plot calculations computed with PROCHECK was used to validate the quality of the models. As displayed in Table 4.2, mutated TAK2, TAK3 and TAK6 had an increase in the most favoured region and numbers of residues in the most favoured region reduced for TAK4 and TAK5 respectively. The G-factor shows the quality of covalent and overall bond/angle distances. The observed G-factor (Table 4.2) for the modelled structure of mutated TAK3, TAK5 and TAK6 increased but TAK2 and TAK4 decreased. Still in this context, only mutated TAK5 has a Goodness factor above -0.5, however parental TAK4 and TAK5 had G-factor above -0.5.

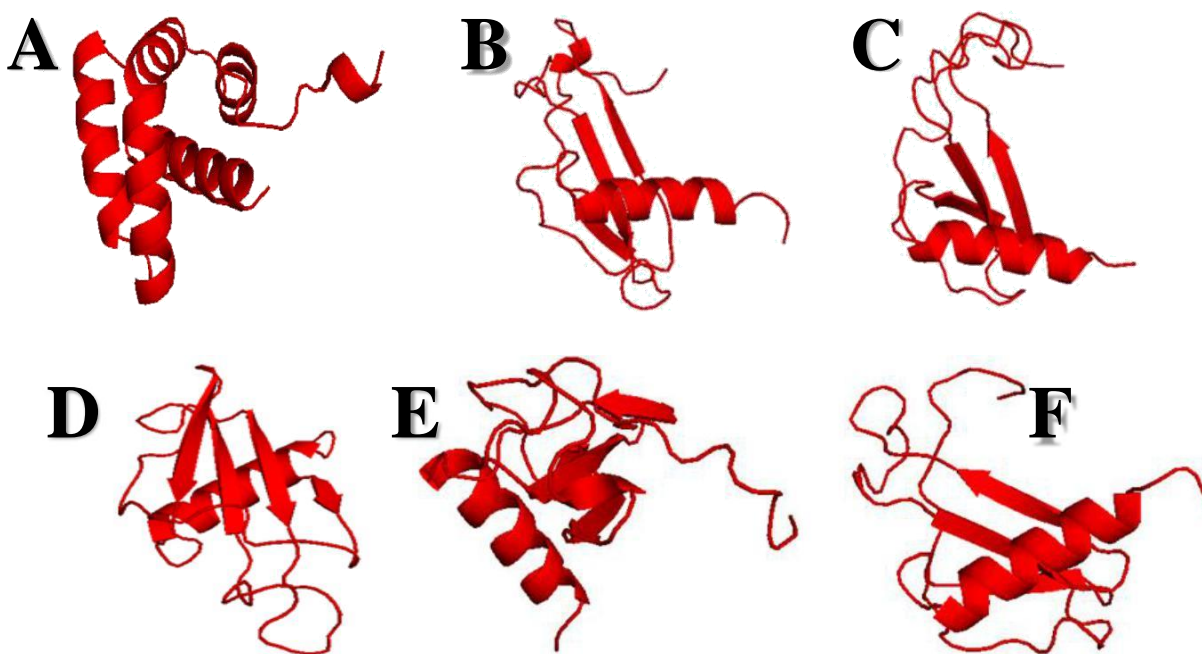**Figure 4.1**- **I-TASSER predicted 3-D structures of the mutated AMPs**. (A): **TAK1**, (B): **TAK2**, (C): **TAK 3**, (D): **TAK4**, (E): **TAK5**, (F): **TAK6**. The AMPs 3-D structures were predicted using an online server I-TASSER and they were visualized using PyMol.

Table 4.2: **Validation of the parental and mutated AMPs using PROCHECK program**. Parental AMPs are as followed: (A) **TAK1** (B) **TAK2** (C) **TAK3** (D) **TAK4** (E) **TAK5** (F) **TAK6**; Mutated AMPs are as followed: (A) **TAK1.1** (B) **TAK2.1** (C) **TAK3.1** (D) **TAK4.1** (E) **TAK5.1** (F) **TAK6.1**

Molecules	Residues in most favoured region (%)	Dihedral G-factor
TAK1	59	-0.45
TAK1.1	68	-0.17
TAK2	58.7	-0.58
TAK2.1	60.0	-0.74
TAK3	63.5	-0.58
TAK3.1	66.2	-0.52
TAK4	67.6	-0.49
TAK4.1	63.5	-0.57
TAK5	68.5	-0.48
TAK5.1	67.6	-0.46
TAK6	66.2	-0.63
TAK6.1	68.9	-0.55

4.4.4 Docking

Glycosyltransferase and axonemal dynein intermediate chain plays a significant role in the parasite development and everyday life, hence, it's mandatory to inhibit the expression of these proteins. PatchDock was used to investigate the interaction between the mutated AMPs and these proteins as displayed in figure 4.2 and 4.3. Results showed that mutated AMPs had an increased binding affinity to both proteins in exception of TAK5 for glycosyltransferase (Table 4.3) and TAK4-TAK6 for axonemal dynein intermediate chain (Table 4.4). Mutated type of TAK2 has the highest binding score, highest interface area of the complex and lowest Atomic Contact Energy. More so,

mutated TAK1 showed the highest binding score to axonemal dynein intermediate chain. After the documentation of the binding affinities of each of the mutated peptide and visualization of each of the resulting complexes were studied. All the mutated AMPs are selected as good candidate towards the development of vaccine or drug to remedy schistosomiasis.

Table 4.3: Binding affinity of the mutated AMPs with glycosyltransferase

Complex	Binding affinity score	Area	ACE
Glycosyltransferase + TAK1	13570	1999.10	452.50
Glycosyltransferase + TAK2	16600	2550.10	-146.59
Glycosyltransferase + TAK3	15050	1399.30	-74.10
Glycosyltransferase + TAK4	13638	2028.40	94.18
Glycosyltransferase + TAK5	13752	1800.80	184.19
Glycosyltransferase + TAK6	14544	2408.30	-74.10

Table 4.4: Binding affinity of the mutated AMPs with axonemal dynein intermediate chain

Complex	Binding affinity score	Area	ACE
Axonemal dynein intermediate chain + TAK1	16942	2193.60	233.69
Axonemal dynein intermediate chain + TAK2	16336	3065.00	-187.04
Axonemal dynein intermediate chain + TAK3	16462	2503.60	145.07
Axonemal dynein intermediate chain + TAK4	14148	1990.50	343.53
Axonemal dynein intermediate chain + TAK5	15272	2615.50	-104.70
Axonemal dynein intermediate chain + TAK6	14960	2701.40	-62.31

Figure 4.2: Interaction of Schistosome protein glycosyltransferase and the mutated anti-schistosomal AMPs. The cartoon representation in blue colour is the glycosyltransferase, represented in red are the mutated anti-schistosomal AMPs. **(A)** Glycosyltransferase-TAK1, **(B)** Glycosyltransferase-TAK2, **(C)** Glycosyltransferase-TAK3, **(D)** Glycosyltransferase-TAK4, **(E)** Glycosyltransferase-TAK5 **(F)** Glycosyltransferase-TAK6.
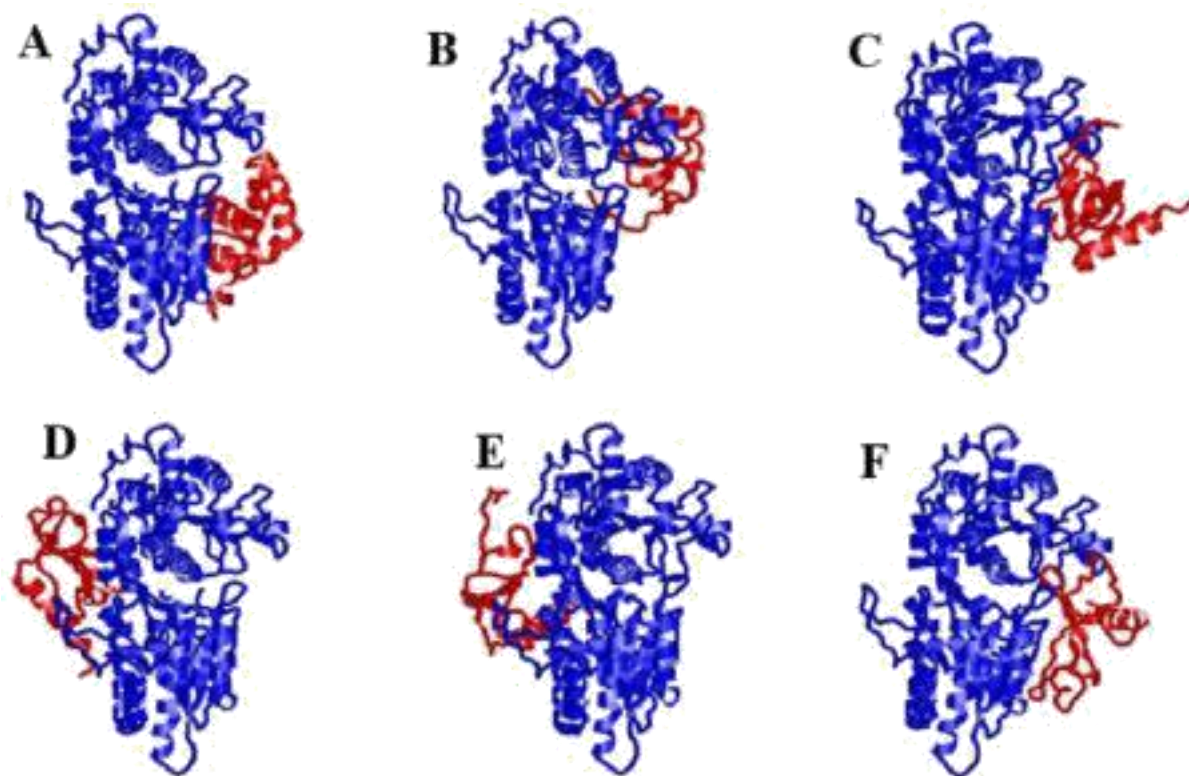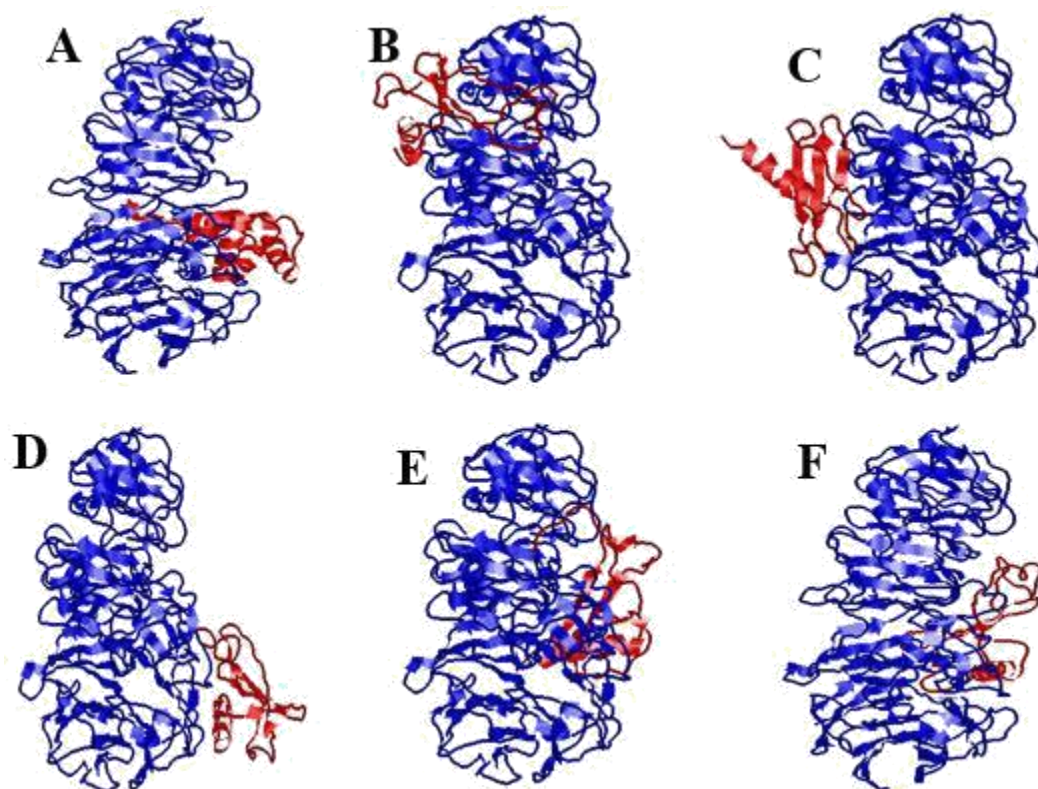
Figure 4.3: Interaction of Schistosome protein glycosyltransferase and the mutated anti-schistosomal AMPs.
The cartoon representation in blue colour is the axonemal dynein intermediate chain, represented in red and the mutated anti-schistosomal AMPs. **(A)** axonemal dynein intermediate chain-TAK1, **(B)** axonemal dynein intermediate chain-TAK2, **(C)** axonemal dynein intermediate chain-TAK3, **(D)** axonemal dynein intermediate chain -TAK4, **(E)** axonemal dynein intermediate chain -TAK5 **(F)** axonemal dynein intermediate chain-TAK6.

4.5 DISCUSSION

The high capital involved in structural studies and waste of time is a continual hurdle to examine functional characteristics of biological complexes and molecules. However, *in silico* studies remains one of the credible alternatives for conventional structural studies (Mohammadpour *et al*., 2016), circumventing unavoidable difficult trials lie ahead of experimental techniques. In drug development and design, the three-dimensional structure of the target is crucial for delineating the active site, designing and docking of small ligands to the target. Hence, various bioinformatics techniques have been effectively used in the identification and discovery of new drug and vaccine candidates in treating several diseases (Tyagi *et al*., 2013, Adekiya *et al*., 2017, Dash *et al*., 2017).

In this study, six anti-schistosomal AMPs were mutated and after computing the physicochemical parameters, it was shown that all the mutated AMPs were positively charged and Hancock and Chapple (1999) have documented that AMPs generally contain two to nine positively charged amino acids. More so, all the mutated AMPs are made up of the 20 common amino acids which adds up to their molecular weight, it has been established that peptides performance is strongly associated to its residue order (Tyagi *et al*., 2013).

The structures of the glycosyltransferase and axonemal dynein intermediate chain proteins have been predicted using I-TASSER in a previous study (Chapter three). All the structural elements of the six-mutated putative anti-schistosomal AMPs were predicted using I-TASSER server (Zhang, 2008). PyMol molecular graphics was utilized in visualizing the resulting file from the I-TASSER algorithm as shown in Figure 4.2. It was revealed that all the 6 mutated AMPs structures are in consistent with known AMP structures. Plethora studies has proposed that the structural elements are responsible for the interaction and insertion of membrane-active peptides such as cell

penetrating peptides, AMPs amongst others (Eiriksdottir *et al* 2010; Huang *et al* 2011), which can be linked to the distribution of residues.

However, PROCHECK Ramachandran plot was employed to ascertain the structural validity of this AMPs (parental and mutated). The plot gets the stable conformations of N–Cα and Cα–C main chain bonds rotations in the AMPs by torsion angles. Torsion angles of the residues in the most favored regions for TAK1 increased from 59 (parental) to 68.0 (mutated), TAK2 had an increase from 58.7 (parental) to 60.0 (mutated), TAK3 increased from 63.5 (parental) to 66.2 (mutated) and TAK6 increased from 66.2 (parental) to 68.9 (mutated). Conversely, TAK4 had a decrease from 67.6 (parental) to 63.5 (mutated) and TAK5 decreased from 68.5 (parental) to 67.6. The overall G-factor which analyses the normality of main chain length and bond angles were calculated by PROCHECK (Harvey *et al*., 2000). The quality of the covalent and the bond/angle distance increased for TAK3 from -0.58 (parental) to -0.52 (mutated), also, TAK5 increased from -0.48 to -0.46 and finally TAK6 G-factor increased from -0.63 (parental) to -0.55 (mutated). G-factor for TAK2 decreased from -0.74 (parental) to -0.74 (mutated) and TAK4 decreased from -0.49 (parental) to -0.57 (mutated). The whole main-chain and side-chain characteristics analysed by PROCHECK, are all favourable. The comparable G-factors and Ramachandran plot validated the quality of the generated model. In order to find how these AMPs will act has inhibitor for the glycosyltransferase and axonemal dynein intermediate chain, we tried docking study. This is imperative because it will assist in the design and development of novel schistosomicide that will curb the emergence of drug-resistant schistosomes. PatchDock was utilized here, the algorithm works by going through three key phases: Firstly, molecular shape demonstration via a segmentation algorithm to maintain the hotspot residue patches; Secondly, complementing surface patch by applying geometric hashing and clustering matching methods to complement the patches

discovered in the previous stage based on complementarity; thirdly; filtering and scoring is performed by investigating the candidate complexes and removing the improper complexes. The remaining candidates are ranked according to a geometric shape complementarity. Mutated TAK1, TAK2, TAK3, TAK4 and TAK 6 had an increase in their binding affinity to glycosyltransferase which could be as a result of substitution of non-polar and uncharged amino acids for both positively and negatively charged amino acids residues. Basically, positively charged amino acids are vital for AMPs binding to their targets which subsequently could lead to high performance (Tincho *et al*., 2016). Conversely, TAK1, TAK2 and TAK3 had a significant increase in their binding affinity to axonemal dynein intermediate chain which could be because of the aforementioned reason for glycosyltransferase. In conclusion, based on the strong interactions between the mutated AMPs and the schistosomal proteins, we propose that these peptides maybe potential "drug leads" in the design and development of alternative schistosomal therapy and could as well prove effective against PZQ-resistant schistosome strains.

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CHAPTER FIVE

General Discussion and Conclusion

5.1 General Discussion

Schistosomiasis is an infectious tropical disease caused by parasitic worms belonging to the genus *Schistosoma*. Human schistosomiasis is caused mainly by three *Schistosoma* species; *S. mansoni*, *S. haematobium* and *S. japonicum*. This infection continues to constitute a major problem in public health, especially in regions of endemicity. This parasite chronically infects over 200 million of the world's population and kills approximately 280 000 people annually (Van der Wert *et al*., 2003). The life cycle of this parasitic worm comprised of two parasitic stages and two free-living stages. At the parasitic stage, the schistosomes take over an intermediate snail host or a definite vertebrate host, while the free-living stage occurs in fresh water, thereby presenting a nexus between the two parasitic stages breaking the physical differences between the two hosts.

Indications of schistosomiasis can be mild, subsequently resulting to long lasting infections often left undiagnosed. However, eggs laid by the parasite accumulate in the liver and causes hepatomegalia and liver failure. Moreover, the eggs trapped in the bladder (*S. haematobium*) or liver (*S. mansoni* and *S. japonicum*) stimulates the production of SEA that causes the formation of granulomas (Cook *et al*., 2003). Infected people are treated with praziquantel, which destroys the matured parasites and stops egg lying. This drug is administered orally, highly tolerated with few or transients side effects and cheap to access. Sadly, treatment with PZQ does not kill the juvenile worm and does not prevent re-infection, which leads to long term schistosomiasis infections with the associated chronic inflammation (Pearce and MacDonald, 2002). According to King (2010), this has a direct outcome on morbidity thus contributing to increasing penury of the affected regions. Additionally, some strains of *Schistosoma* have been reported to show signs of resistance to PZQ due to drug pressure (Doenhoff *et al*., 2002; Oyinloye *et al*., 2014). Hence, there is an urgent need to develop a new human schistosomiasis regimen.

Presently, peptide-based therapy is gaining remarkable interest (Thundimadathil, 2012; Siegel *et al*., 2013). In tumour remedy, numerous peptide-based approaches for targeting and conveying therapeutics to different tumour types have been employed over the years and a few have translated well into the clinics (Thundimadathil, 2012). In 2016, Williams and co-workers successfully developed an AMP based Lateral Flow Device prototype that accurately detected both HIV-1 and HIV-2. Hence, exploration of AMPs towards Schistosomiasis remedy using *in silico* techniques is imperative since these techniques have been successfully used in identifying, analyzing, interpreting and validating potential drug candidates (Wilshart, 2005; Gill *et al*., 2016).

AMPs are natural antibiotics produced by all living species; they have multifunctional properties and are currently explored as a vital source for the development of new drugs. In this study, six putative AMPs possessing anti-schistosomal ability were identified using Hidden Markov Model and all of them were positively charged. These findings correlate with a study by Hancock and Chapple (1999), which reported that AMPs generally contain two to nine excesses positively charged amino acids (e.g. histidine, lysine and arginine).

Moreover, the 3-D structural elements of these peptides were modelled using I-TASSER and the analysis of the structures revealed that they are in accordance with known 3-D structures of AMPs (Reddy *et al*., 2004). Subsequently, glycosyltransferase and axonemal dynein intermediate chain proteins were retrieved as key proteins in the parasite (*Schistosoma*) survival. Docking studies revealed the binding score and interaction patterns of these schistosome proteins with the putative anti-schistosomal AMPs. All the putative anti-schistosomal AMPs possessed a high and efficient binding affinity for both proteins. The strong interaction between the AMPs and schistosome proteins could be as a result of their good hydrophobicity; hydrophobic interactions are considered to be the main forces that keep a protein structure intact (Biro, 2006). Studies have documented

that AMPs are made up of over 50% hydrophobic amino acid residues which are vital in the increase of the binding affinity of peptides (Wieprecht *et al*., 1997; Hancock and Chapple, 1999).

Furthermore, site-directed mutagenesis was done to increase the electrostatic attraction of the peptides and their binding affinity to the schistosome proteins. The standard to mutate the non-hotspot amino acid in the peptides was based on group similarity of the substituted amino acid with that of the parental peptides since these amino acids have the same physicochemical parameters. Generally, hotspot amino acids increase the binding affinity of a peptide or protein. In this study, positively charged amino acids were used to substitute other amino acids since this parameter allows AMPs to have selective activity for an organism rather than the host (Lee *et al*., 2011). Physicochemical parameters of the mutated anti-schistosomal AMPs were very similar to that of the parental anti-schistosomal AMPs. All the mutated anti-schistosomal AMPs possessed good binding affinity for Glycosyltransferase and Axonemal dynein intermediate chain. Hence, all could be implemented as potential schistosomicides since peptides can selectively bind to the organism's molecules rather than the host cells.

5.2 Conclusion

Conclusively, it is of paramount importance to design novel structure-based medications with high efficacy and few or no side effects to tackle schistosomiasis. Thus, the current dissertation has explored various bioinformatics techniques to identify antimicrobial peptides with sought therapeutic/functional activities that differ from praziquantel, thereby, knocking out resistance and remedy this debilitating disease. More so, this study may serve as a template for discovering hits cost effectively by applying the computational approaches against various diseases at the same time. Overall, findings from this work revealed that the identified parental and mutated AMPs are druggable targets for schistosomiasis remedy.

5.3 Future Work

The results from this study are not however conclusive on the mechanism of action of the anti-schistosomal AMPs and the schistosome proteins. Hence, further validation is needed using various structural and cell biology techniques in order to have a deeper understanding on how these AMPs will elicit their anti-schistosomal properties.

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