Identification of approved drugs that have activity against *Plasmodium falciparum* using *in silico* and *in vitro* approaches

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MB300-0007/2015

A research thesis submitted to Pan African University Institute of Science, Technology and Innovation in partial fulfillment of the requirement for the award of the degree of Master of Science in Molecular Biology and Biotechnology

2017

DECLARATION

This research thesis is my original work and has not been presented to any other university for award of a degree.

Sign ____________________________Date_____________________________

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DEDICATION

I dedicate this thesis to my Lord and Savior, Jesus Christ. Thank you for bringing me this far.

"I can do all things through Christ who strengthens me", Philippians 4: 13.

I also dedicate this thesis to my friend Elvira Lukelesia. You are not trained in biological research, but your contributions, ideas and motivations have greatly impacted this work.

ACKNOWLEDGEMENTS

I would like to thank Dr Steven Ger Nyanjom, Prof. Hans A. Elshemy and Dr Hosea Akala for their supervision and mentorship throughout the research period.

My thanks and appreciation to Major Mathew Brown and Dr Ben Andagalu for giving me the opportunity to do my lab work at Malaria Drug Resistance (MDR) Laboratory, USAMRD-K. I would also wish to appreciate all the students and staff at MDR. I am grateful for the assistance in laboratory procedures and the invaluable contributions and insights during project presentations.

I also wish to thank Japan International Cooperation Agency (JICA) for the research grant (iCMOB/05/16) that funded part of this study.

Lastly, but most importantly, I am grateful to Pan African University for the Scholarship without which this work would not be possible.

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ABBREVIATIONS AND ACRONYMS

ABC	ATP binding cassette
ACE	Angiotensin converting enzyme
ADA	Adenosine deaminase,
ADME	Absorption, distribution, metabolism and elimination
ADMET	Absorption, distribution, metabolism, elimination and toxicity
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
CADDD	Computer Aided Drug Discovery and Development
DHFR	Dihydrofolate reductase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
GMP	Guanosine 5'-monophosphate
HDAC	Histone deacytalase,
HIV	Human immunodeficiency virus
HRPII	Histidine-rich protein II
IC_{50}	Half maximal inhibitory concentration
ImmH	Immucillin-H
IMPDH	Inosine 5'-monophosphate dehydrogenase,
IUPAC	International Union of Pure and Applied Chemistry
KEMRI	Kenya Medical Research Institute
MDR	Malaria Drug Resistance
MRP 1	Multidrug resistance protein 1

MSA	Multiple sequence alignment
MT-ImmH	5'-Methylthio-Immucillin-H
NA	Not available
NCBI acc. No	National Center for Biotechnology Institute accession number
PCR	Polymerase Chain Reaction
PK	Pharmacokinetics
pLDH	Parasitic lactate dehydrogenase
RFU	Relative florescence unit
RPMI 1640	Roswell Park Memorial Institute 1640
TDR	Tropical Disease Research
TTD	Therapeutic Target Database
USAMRD-K	United States Army Medical Research Directorate - Kenya
WHO	World Health Organization
WWARN	World Wide Antimalarial Resistance Network

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ABSTRACT

Recent reports on artemisinin resistance in Southeast Asia warrant urgent discovery of novel drugs for treatment of malaria. Search for new chemical entities often fail at safety and toxicity stages of drug development. Drug repositioning offers an alternative strategy where drugs are used to treat diseases or conditions other than the ones they have been approved for. This study screened approved drugs for antiplasmodial activity using an *in silico* chemogenomics approach prior to *in vitro* validation. All the *P. falciparum* protein sequences available at NCBI RefSeq were used to perform a similarity search between these proteins and putative target proteins of approved drugs in three databases: Therapeutic Target Database, DrugBank and STITCH. Functional residues of the drug targets were determined using ConSurf server which were used to fine tune the similarity search. Druggability indices of the potential drug targets were obtained from TDR targets database. A literature search was done to determine drugs previously been tested against malaria. Finally, drug susceptibility assays was done to validate the antimalarial activity of some of the predicted drugs. This study predicted 133 approved drugs that could target 28 *P. falciparum* proteins. Published literature search showed 99 of these drugs to have been tested against malaria, most of which had antiplasmodial activity. *In vitro* results showed 10 out of the 12 drugs tested had antiplasmodial activity with IC_{50} values below 100 μM on *P. falciparum* 3D7, these are Cladribine, Levofloxacin, Dasatinib, Clofarabine, Tacrolimus, Irinotecan, Zidovudine, Moxifloxacin, Oxaliplatin and Tadafil. Diadzin and Zafirlukast did not show any activity at concentration below 50,000 ng/ml. These results show that target similarity can be successfully used to identify approved drugs with antiplasmodial activity, validating it as a viable method for repositioning drugs for antimalarial use. The drugs that showed activity can further be analyzed for *in vivo* activity on rodents and also act as templates for synthesis of novel antimalarial drugs.

CHAPTER 1: INTRODUCTION

1.1. Background Information

1.1.1. Malaria

Malaria is an infectious disease with high morbidity and mortality. Approximately 3.3 billion people are at risk of getting malaria, with 1.2 billion of this having a higher risk of one malaria case per 1000 population (Burchard, 2014). In 2015 alone, there was 214 million new cases of malaria worldwide with about 438,000 deaths reported (World Health Organization, 2015). The disease prevalence is higher among children and pregnant women (van Eijk *et al.*, 2015). Out of the total reported malaria cases and deaths, 90 % of them occur in Africa followed by the South-East Asia (Burchard, 2014). This disease burden is further aggravated by rapid development of resistance to the currently used drugs. Already, resistance to artemisinin-based combination therapy (ACT), the recommended first-line treatment for uncomplicated malaria (White, 2004; World Health Organization, 2006), has been reported in Southeast Asia (Herlekar, 2014). This warrants an urgent discovery of novel drugs for malaria treatment.

1.1.2. *Plasmodium* genus

Malaria is caused by parasites of the genus *Plasmodium*. Those that infect humans are *P. ovale, P. falciparum, P. vivax, P. knowlesi* and *P. malariae* (Mueller *et al.,* 2007). Of these, *P. falciparum* is responsible for most deaths, followed by *P. vivax* (Sarkar *et al.*, 2009). *P. knowlesi* is common among primates but has also been shown to infect humans (Collins, 2012) making it the only species that infects higher apes that is of public health importance. *P. vivax* is more prevalent in Latin America and Southeast Asia (Kochar *et al.*, 2005), though Liu *et al.* (2014) showed it to have originated from Africa. *P. vivax* and *P. ovale* are the only species that have been shown to have relapses in their lifecycles (William *et al.*, 2005; Imwong *et al.*, 2007).

1.1.3. Life cycle of Malaria parasite

Malaria parasites have a life cycle that involve two hosts; a vertebrate and an invertebrate (Figure 1.1) as described by Shalgenahauf-Lawlor, (2000). Typically for human infections, female *Anopheles* mosquito are the definitive host while humans are the secondary host. An infected female *Anopheles* mosquito transmits sporozoites into the human during feeding. The sporozoites get to the liver through blood vessels and enter hepatocytes where they multiply asexually to form merozoites. The merozoites leave the hepatocytes and infect red blood cells. These merozoites undergo a series of asexual multiplication cycles called blood schizogony producing 8-36 new merozoites depending on infecting species. The infected red blood cells then burst releasing the merozoites into the blood stream. The merozoites infect other red blood cells repeating this cycle. Some merozoites develop into gametocytes which mature to either male or female gametocytes. During the feeding of a female anopheles mosquito, these gametocyte are taken up with blood by a female *Anopheles* mosquito. The gametocytes then mature in the mosquito gut. A male and female gametocyte fuse to form an ookinete which further matures into sporozoites. These sporozoites move to the salivary glands of the insect. As the infected mosquito feeds, it injects its saliva with sporozoites into the human skin (Shalgenahauf-Lawlor, 2000).

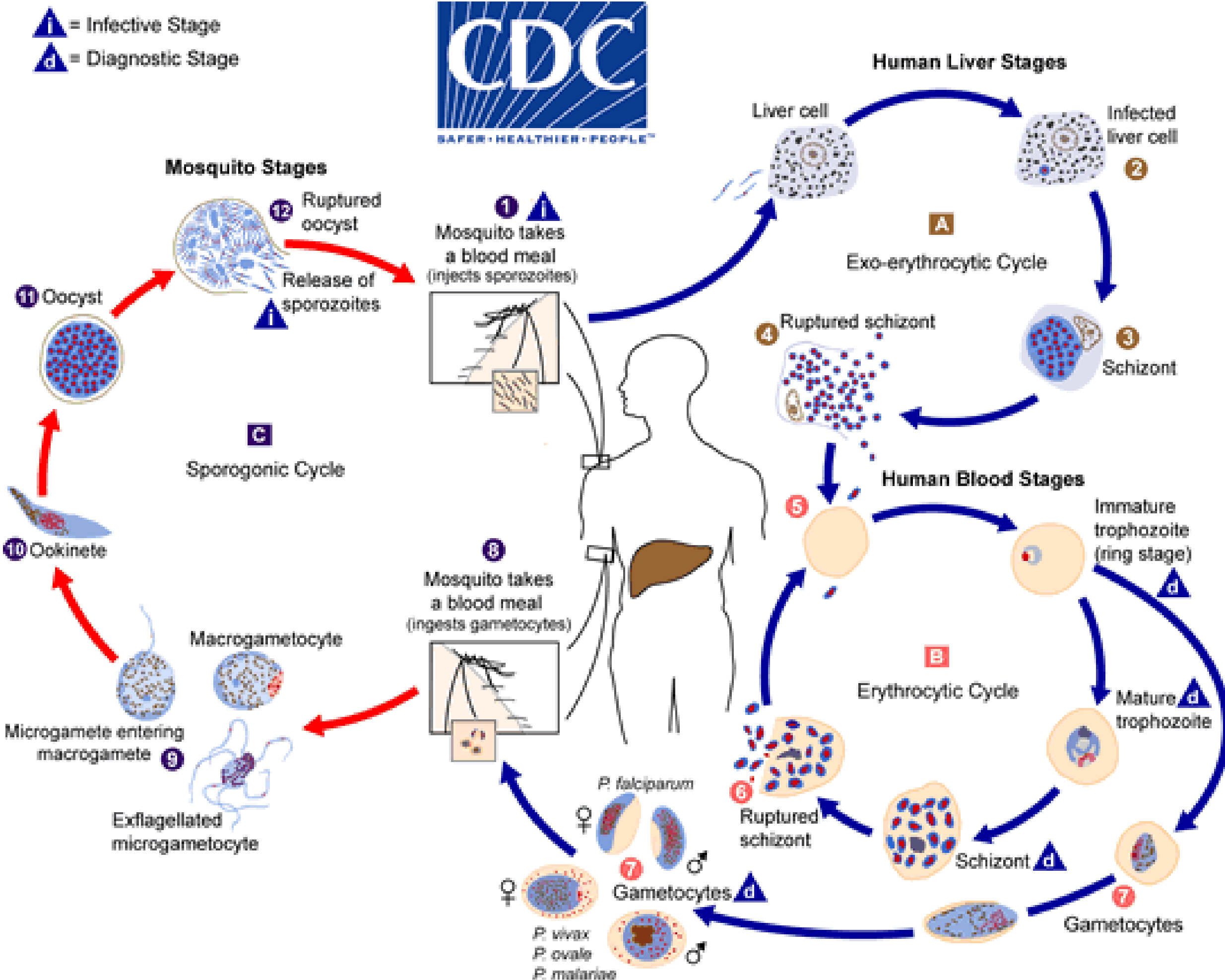


Figure 1.1: Life cycle of *P. falciparum* (www.cdc.gov)

1.1.4. Signs and symptoms of malaria

Malaria signs and symptoms fall within a window period of about 8 - 25 days from the time of infection. These commonly include fevers, convulsions, headache, shivering, joint pain and vomiting. Also jaundice, heamoglobin, heamolytic anaemia in the urine and retinal damage can occur (Beare *et al.,* 2006). Paroxysm (cycles of chills, fever and sweating) can occur every two days in *P. vivax* and *P. ovale* infections, while for *P. malariae* it occurs every three. In humans, severe malaria is caused mainly by *P. falciparum* (Bartoloni & Zammarchi, 2012).

1.1.5. Malaria diagnosis

Diagnosis of malaria is a challenge because malaria presents itself with non-specific symptoms. The suspicion that a patient has malaria is usually when the person has a fever, low platelets in the blood, enlarged liver, high levels of bilirubin and/or has traveled in a malaria endemic region (Nadjm & Behrens, 2012). The gold standard of malaria diagnosis is by preparation and examination of blood films by microscopy (Kattenberg *et al.*, 2011). Diagnosis can also be done by use of rapid diagnostic tests that are antigen or molecular based, some of which have higher sensitivity and specificity in diagnosis and species differentiation (Nadjm & Behrens, 2012).

1.1.6. Malaria control and prevention

There are many methods that have been used to prevent malaria infections, this includes prophylaxis (Castelli *et al.,* 2010), vector elimination and repulsion interventions (malERA Consultative Group on Vector Control, 2011). Research also on going to develop vaccines, though there are currently no effective vaccines against malaria (Mueller *et al.*, 2015).

1.1.7. Malaria chemotherapy

The current treatment used for *P. falciparum* are arteminisins which are combined with other antimalarial drugs to reduce the occurrence of resistance, a practice called Artemisinin-Combination Therapy (ACT) (White, 2004). Arteminisins are combined with either lumefantrine, amodiaquine, mefloquine or pyrimethamine (Kokwaro, 2009). For treatment of pregnant women, it is recommended that quinine with clindamycin be used in the first trimester, followed by ACT in the second and third trimesters (Manyando *et al.*, 2012). On the other hand, *P. vivax* infections are treated with chloroquine or ACT to clear the blood stages and while primaquine is used to clear its liver forms (Waters & Edstein, 2012).

1.2. Statement of the problem

Malaria is a disease burden to humans for many centuries (Joy *et al.*, 2003). Malaria caused an estimated 429,000 deaths in 2015 with 90 % of these deaths found in Africa, followed by the South-East Asia Region (7%) and the Eastern Mediterranean Region (2%) (World Health Organisation, 2016). The use of antimalarial drugs in treatment and prevention is one of the most effective ways to control malaria. However, reports indicate resistance has developed to all antimalarial drugs classes, including arteminisins (Sinha *et al.,* 2014). This is a major drawback threatening to reverse the gains made in the fight against malaria. As a result of this, malaria has recently been reinstated as a global health priority (Newman, 2012). Use of vector control methods has also suffered a major setback since mosquitos have developed resistance to pyrethroids. Vaccination on the other hand is not feasible since no effective vaccine has been developed to date. Development of new drugs has been greatly impeded because many discovered drugs fail to meet the ideal standards for an antimalarial drug which are: ease of synthesis, toxicity, cost, and potency (Flannery *et al.*, 2013).

1.3. Justification

Parasites are increasingly becoming resistant to current antimalarial drugs. There is an urgent need to develop new drugs that are effective against malaria. Unfortunately, development of new drugs to the point of their introduction into the market is expensive and time consuming, costing about $100 - 800 million and taking a duration of 12 -15 years on average (Morgan *et al.*, 2011). This is promising to improve with the use of *in silico* approaches to complement conventional methods in developing novel drugs against malaria (Kapetanovic, 2008). Drug repositioning is increasingly becoming appealing because of reduced risk, cost and time involved in the drug discovery process. This is because approved drugs have already been tested and approved for other diseases/conditions.

1.4. Research questions

1) What are the *P. falciparum* proteins that can be used as drug targets?

2) What approved drug targets are similar to *P. falciparum* proteins?

3) Does *P. falciparum* show *in vitro* susceptibility to drugs predicted to have antiplasmodial activity using *in silico* methods?

1.5. Objectives

1.5.1. General objective

To identify approved drugs that have unknown antimalarial activity against *Plasmodium falciparum* using *in silico* and *in vitro* methods.

1.5.2. Specific objectives

1) To identify proteins targets expressed in *P. falciparum* using bioinformatics approaches.

2) To predict approved drugs that have activity against *P. falciparum* target proteins using *in silico* approaches.

3) To validate antiplasmodial activity of predicted drugs using *in vitro* drug susceptibility assays.

1.6. Hypothesis (null hypothesis)

There are no approved drugs with undiscovered antiplasmodial activity.

1.7. Scope of the study

This study focused on searching approved drugs that have undiscovered antiplasmodial activity against *P. falciparum.*

CHAPTER 2: LITERATURE REVIEW

All malaria control and management strategies have suffered various setbacks. For instance, vaccine development is very slow, there hasn't been an effective vaccines approved (Horn & Duraisingh, 2014), resistance to all classes of antimalarials has been reported (White, 2004) and mosquitos have also developed resistance to pyrethroids in some regions (Strode *et al.*, 2014). In terms of malaria research funding in 2014, WHO report (World Health Organisation, 2016) showed research on antimalarial medicines had 35 %, followed by vaccines which heard 28 % and basic research had 27 %.

A lot of research in finding alternative antimalarial drugs is currently on-going using various strategies (Flannery *et al.,* 2013). The ultimate goal is to develop an ideal drug that would be active against all stages of the life cycle of the parasite and potent enough to work in a single dose to ensure patient compliance (Burrows *et al.,* 2013). The drug should also be cheap to manufacture since most antimalarial treatments are paid for or subsidized by charitable organizations and governments.

2.1. Strategies of developing new antimalarial drugs

Various approaches have been used to develop new antimalarial drugs. The two major ones are; modification of a currently available drug and by high-throughput screens for novel drugs parasite (Flannery *et al.,* 2013). The former is usually done to try to counteract the effect of resistance to the scaffold drug or to increase potency of the drug. On the other hand, high-throughput screens involve screening a large library of compounds that are active against the parasite of a particular target in the parasite (Flannery *et al.,* 2013).

One high-throughput method is a whole cell-based approach (Macarron *et al.,* 2011) where compounds with antiplasmodial activity are identified by incubating the *Plasmodium* parasite

in a medium containing the test compounds. If the identified compound has the ability to kill or inhibit growth the parasite, it is further evaluated for use as an antimalarial drug. The major challenge with this approach is that it is expensive to screen a large number of compounds. Nevertheless, recent technological advancement has made it more cost effective, enabling the discovery of novel antiplasmodial compounds via high-throughput screening of millions of compounds (Sharma *et al.*, 2012).

Another high throughput method that has been used in the search for new antiplasmodial drugs is target-based (Werbovetz, 1970). A target protein is a protein that is crucial in the survival of the parasites. Therefore, a search is done for compounds that could inhibit the target protein's function hence kill the parasite. These protein targets are isolated from the parasite and used in biochemical assays to screen for compounds that could have activity against it. Nevertheless, extracting the protein from a parasite is quite costly hence not economical for high-throughput screens. This explains why recombinant technology has been used to produce these target proteins for high throughput screens (Gurard-Levin *et al.*, 2011). After biochemical assays involving a group of compounds has been carried out, those that can inhibit the protein target's function are selected for further study and possible development. Examples of drugs that have been discovered in this manner include pyrimethamine whose mechanism of action is by inhibiting the enzyme dihydrofolate reductase (DHFR) hence interfering with the regeneration of tetrahydrofolic acid from dihydrofolate (Schweitzer *et al.,* 1990). If the three dimension conformation of the protein is known, then compounds that inhibit the protein functions can also be designed and used in screening using *in silico* means (Verma & Prabhakar, 2015).

Another method is used in drug discovery is based on medicinal chemistry. This method makes use of the knowledge of chemical structures of antimalarial drugs that are already in existence to develop new drugs. The existing drugs can be modified to optimize their therapeutic activity

or mitigate their side effects. An example of drugs that have been developed using this approach are the synthetic ozonides which are based on arteminisins (Tang *et al.,* 2004).

Similarly, drugs have been derived from traditionally used herbal medicines (Flannery *et al.,* 2013), examples include quinine that is derived from the *Cinchona* trees and arteminisins that is derived from the Chinese herb *Artemisia annua* (Cechinel-Filho, 2012). It is interesting to note that these two are among the most effective drugs available and some other drugs are developed from their scaffold (Tang *et al.,* 2004).

2.2. Drug repositioning

Development of new drugs to the point of introduction into the market is an expensive and time consuming process, costing about $100 - 800 million over a period of 12-15 years on average (Morgan *et al.*, 2011). In appreciating these challenges, some recent drug discovery studies have focused on drug repositioning which basically entails developing new indications for existing drugs other than those they were approved for (Sekhon, 2013). This has the advantage of maximizing the value of already existing therapeutic drugs and saving on the cost of developing new ones.

Examples of drugs that have been successfully repositioned for other diseases include duloxetine which was initially developed for depression but is now used for stress urinary incontinence; dopoxetine which was used as follow-on to fluoxetine is now used to treat premature ejaculation; thalamide, used as a sedative for morning sickness is used to treat leprosy and multiple myeloma (Ashburn & Thor, 2004). An example of a drug that has been shown to be active against *P. falciparum* is astemizole which was initially approved as an antihistamine (Chong, *et al.*, 2006).

Repositioning also includes combination of two or more drugs formerly used singly, a strategy based on synergistic or additive properties of the individual drugs (Wu *et al.*, 2013). Numerous studies have been done to determine antimalarial activity of non-antimalarial drugs (Ekins & Williams, 2011; Engel *et al.*, 2015; Lotharius *et al.*, 2014; Matthews, 2013; Sekhon, 2013; Wu *et al.*, 2013). This is attributed to the fact that the drugs to be analyzed for use have already been tested and approved for other diseases, hence preliminary stages of drug development are circumvented or even shortened.

Through target similarity approach, this study sort to predict approved drugs that have undiscovered activity against *P. falciparum* and hence reposition them for antimalarial treatment. The study is based on the principle that a drug would have similar effect on a protein that is similar to its putative target. Each of *P. falciparum* protein sequences in NCBI RefSeq database was used to check for similarity with confirmed drug targets. Functional regions of the drugs targets were determined and used to further fine tune the similarity search. This study did identify approved drugs that had antiplasmodial activity and hence could bring new antimalarial drugs into the market faster and more cost effectively.

2.3. Computer Aided Drug Design and Development

Traditional approaches in drug design have proven to be expensive and time consuming with little successful outcomes. This is why many *in silico* techniques have been employed in current steps of the drug discovery processes (Figure 2), making it less costly, faster and with less risk (Boruah *et al.,* 2013). Computer Aided Drug Discovery and Development (CADDD) has led to the discovery and development of several drugs that are approved for clinical use or are in clinical trials, examples include Captopril, Dorzolamide, Saquinavir, Zanamivir, Oseltamivir, Aliskiren, Boceprevir, Nolatrexed, TMI-005, LY-517717, Rupintrivir and NVP-AUY922 as reviewed by Talele *et al.* (2010).

Typically, steps involved in the *in silico* drug design process (Prachayasittikul, 2015) are as follows; target identification, target validation, lead identification, lead optimization, predicting drug-like properties and preclinical pharmacology and toxicology (Figure 2). After a potential drug has passed through these steps, it is then taken through preclinical and clinical stages before it is approved.

Target identification involves determining potential protein targets that are associated with the disease (in this case, the parasite proteins). These targets would be used to design drug compounds that would inhibit its activity and hence control the disease. One approach used in this step is by studying and analyzing the biological pathways involved in the disease and looking for possible points of intervention (Sakharkar *et al.*, 2004).

PharmaMapper is server that has been developed by Liu *et al.*, (2010) to identify potential target candidates for small molecules (natural products, drugs, or new compounds that have unidentified binding targets) using pharmacophore mapping approach. Target validation involves confirming if intervention at the prospective targets will be effective in controlling the disease. This was achieved traditionally by the use of reliable animal models and expression techniques, but currently computational methods have been used to complement these processes (Ekins *et al.*, 2007). Validation methods can be grouped into chemical validation methods (used to address druggability issues), genetic validation methods, gene knockouts and RNA interference (Wyatt *et al.*, 2011).

1. Target identification

- Potential protein targets associated with a disease are identified, such that when its activity is inhibited the disease is treated eg. Data mining tools

2. Target validation

- Prospective targets are confirmed if indeed intervention at this step will be effective in controlling the disease

3. Lead identification

- Screening is done for compounds that could have the desired effect on the prospective protein targets

4. Lead optimization

- Desirable characteristics of the lead compound are optimized to improve activity.

5. Predicting drug-like properties

- The lead molecule is further analyzed to determine if it has sufficiently acceptable ADMET profiles

6. Preclinical pharmacology and toxicology

- The toxicity, pharmacokinetics and metabolism of this drug is scrutinized at this stage

7. Pre-clinical trials

9. Clinical trials (phase II, II, III)

10. Drug approval

Market

Figure 2.1: Steps involved in Computer Aided Drug Discovery and Development (shown in blue). The possible "computer aided" steps in drug discovery and development are shown in blue while the rest of the steps are shown in green and orange.

One good example of a group of drugs that have been developed by CADDD are angiotensin converting enzyme (ACE) inhibitors. This started with the discovery of orally inactive peptides in snake venom and the role that ACE inhibitors in regulating blood pressure which eventually lead to the development of Captopril (Acharya *et al.*, 2003). Though it was discovered that Captopril had some adverse effects (similar to Penicillamine) and new derivatives needed to be designed with less toxicity. This led to a search for ACE inhibitors without a mercapto (SH) function (also found in penicillamine) but would have a weaker chelating function (Patchett *et al.*, 1980). The work started with an N-carboxymethyl-dipeptide scaffold with a general

structure of (R-CHCOOH-A_1-A_2), where A are amino acid residues and R is a side chain. Proline was substituted at A_2 since it was assumed from previous studies that substituting a cyclic amino acid at this position would result to higher potency, which proved to be true. Substituting hydrophobic and basic amino acid residues at the R and A1 groups was also shown to give a potent compound. Due to the enzymes specificity, it was also noted that imino acids in the position next to the carboxyl terminus will result into loss of potency. This changes to the scaffold molecule resulted in Enalaprilat and Lisinopril.

2.4. Softwares and resources used in Computer Aided Drug Discovery and Development

There are several kinds of software and other computational resources that can be used in the discover and optimizing biologically active compounds. These have made computer aided drug design an indispensable tool in the pharmaceutical industry. These tools can be classified into chemical databases, structure-based drug design (involves docking), ligand-based drug design and chemoinformatics tools (Liao *et al.*, 2011).

2.4.1. Structure-based virtual screening

Structure-based virtual screening identifies active compounds for a particular protein target from a chemical library based on docking techniques. Molecular docking determines how a compound binds to an active site of a protein target and the approximate binding energy involved. Docking is important in investigating how a ligand would interact with a protein, hence is only used if the structure of the protein is known. The main tasks of docking programs are the prediction of the correct binding poses and the correct ranking of these poses so as to predict most likely one that a protein will take in particular conditions (Liao *et al.*, 2011). Several docking programs have been described by Yuriev *et al.* (2015), these include DOCK, AutoDock, GalaxyDock, Robetta and AutoMap.

2.4.2. Ligand-based virtual screening

Ligand-based virtual screening has been used to discover active compounds for a particular target from a chemical library based on pharmacore modelling techniques. Traditionally, potential ligands have been identified using high-throughput screening assays. But currently, high-throughput screening has been complemented and in some cases substituted with CADDD techniques because they are generally more economical, faster and easier to set up than biochemical assays. Furthermore, in CADDD one can optimize ligands to give them higher binding affinity, more selectivity and better pharmacokinetic properties (before it is synthesised) which is not usually the case in high-throughput screens. Examples of common ligand-based virtual screening softwares include DISCOtech, PHASE and Catalyst (Liao *et al.*, 2011).

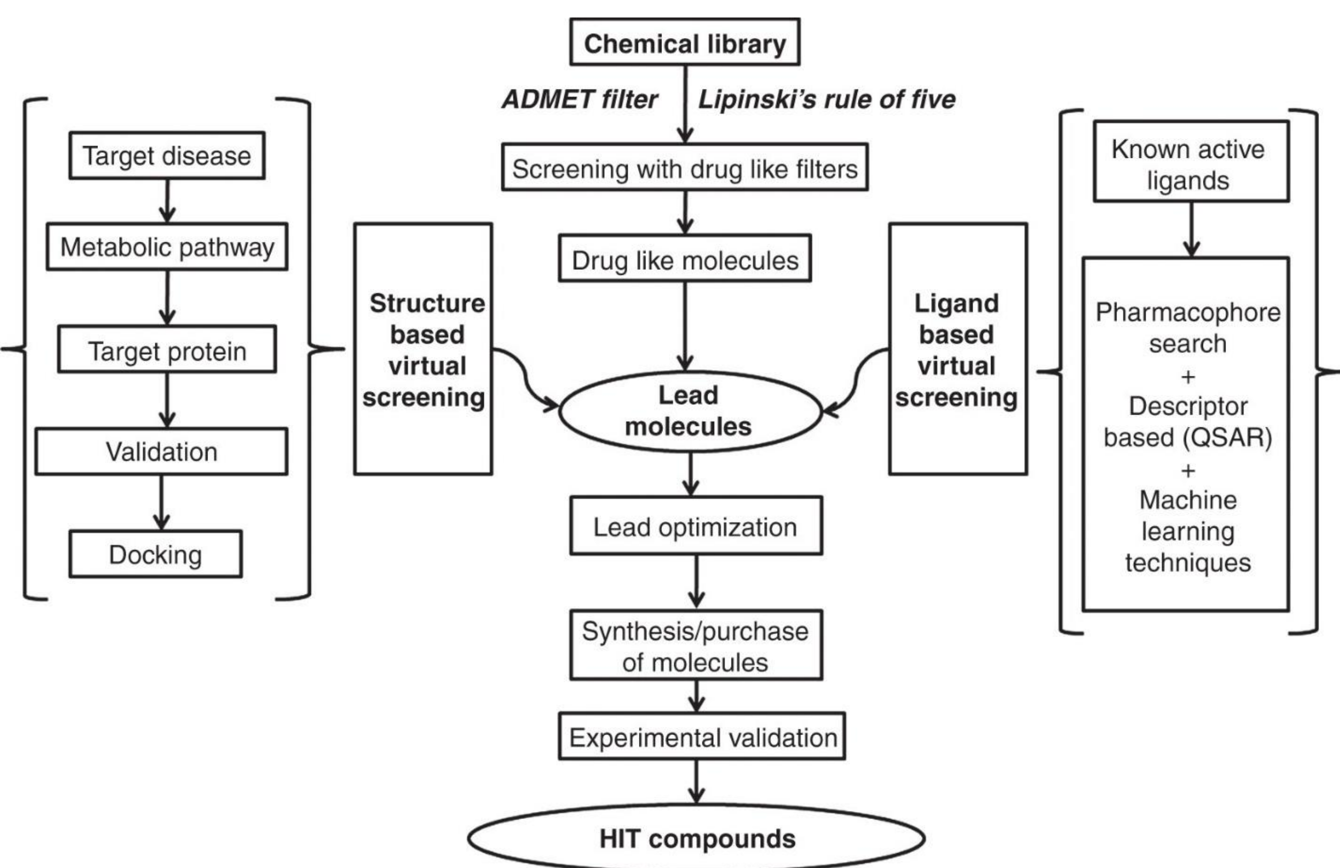


Figure 2.2: Virtual screening in CADDD (Kar & Roy, 2013).

2.4.4. Chemoinformatics tools

Cheminformatics tools are important in the acquisition, analysis and management of data and information of molecules during drug development. This minimizes the challenges involved in interchanging information between different programs which usually resulted in minimal usage of these program themselves. This has been made possible by data pipelining environments (also called visual workflow) such as Pipeline Pilot and Konstanz Information Miner (KNIME) (Warr, 2012)

2.4.5. Homology modelling

Many softwares and programs in CADD require the 3D structure of a possible protein target. Several databases exist that have the 3D conformation of proteins, the most common one is Protein Data Bank (Velankar *et al.*, 2012). This structures are either experimentally determined by techniques such as NMR or X-ray crystallography or they could be modelled using softwares as shown in Figure 4. Homology modelling involves using a related protein whose 3D structure is known to predict the 3D structure of the protein of interest using its amino acid sequence. Homology modelling typically involves four steps; retrieval of 3D structure of related protein(s), sequence alignment of the template and target proteins, building of the protein model and finally, validation of the predicted model (Liao *et al.*, 2011). Several standalone softwares exist that either specializes in one of the four steps while some (mostly servers) such as SWISS-MODEL, MODELLER and COMPOSER can do all the four steps (Vyas *et al.*, 2012).

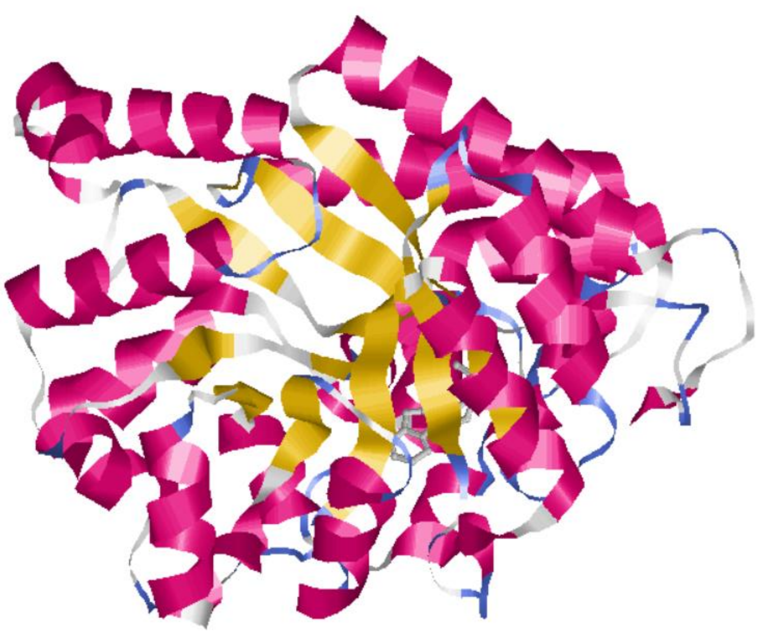


Figure 2.3: Protein model of a drug target (POO813) generated by SWISS-MODEL (Arnold *et al.*, 2006). The alpha helices are colored pink, beta sheets yellow, beta turns blue and random coils grey.

2.4.6. Bio-chemical databases

Several databases exist that have become indispensable in CADDD. Some of these databases are purely for chemical structures of small molecules while some have more information such as their putative targets, mechanism of action, bioactivity, physicochemical properties etc. A review of these databases has been done by Liao *et al.* (2011). Few examples of high profile databases include; PubChem (stores original structure records and bioactivity screens of compounds (Kim *et al.*, 2016); ChEMBL is database of bioactive drug-like small molecules with some linked with their biological targets (Gaulton *et al.*, 2012); DrugBank provides a detailed information about drugs including information on their mechanism of action (Knox *et al.*, 2011); STITCH is an integrated database of interaction connecting chemical compounds to their confirmed (or possible) protein targets from several organisms (Kuhn *et al.*, 2012); Therapeutic Target Database (TTD) was developed to provide comprehensive information about targets and their corresponding approved, clinical trial and investigative drugs (Zhu *et al.*, 2012).

2.4.7. ConSurf server

ConSurf server (Glaser *et al.*, 2003) is used to determine functionally important amino acid residues in a protein of known 3D structure. Functional regions of a protein are important in the biological role and structure of a protein hence are highly conserved by evolution. These functional residues are determined by estimating the degree of conservation of amino acids across 150 close sequence homologues obtained from UNIPROT database (Bateman *et al.*, 2015). Evolutionary conservation of amino acid positions are estimated based on phylogenetic relationship between homologous sequences which is determined by Neighbor Joining (Saitou & Nei, 1987) with Maximum Likelihood (Stamatakis, 2006) distance. Conservation scores is calculated using Bayesian method (Smith, 2001). Determination of functional residues is important in predicting other possible targets of a compound that are similar to the compounds putative target, this is the principle of this study.

2.4.8. NCBI GenBank database

Other databases that are important in CADD include the NCBI GenBank database (Benson *et al.*, 2015; Clark *et al.*, 2016) which has both nucleotide and protein (translated) sequences. NCBI Reference Sequence (RefSeq) database (Pruitt *et al.*, 2007) is a secondary database at NCBI websites that has been curated to avoid duplication of protein sequences and has the best sequence (reference sequence) available for each protein.

2.4.9. Druggability index

Druggability index (D index) describes how likely a protein is druggable i.e. the ability of a protein to bind to a small molecule with high affinity and modulate its function (Owens, 2007). Druggability index ranges from 0 to 1.0, with higher druggability indices reflecting higher odds of a protein being druggable. These scores reflect a number of factors such as how similar the

protein is to a library of targets at ChEMBL database (Gaulton *et al.*, 2012), if the protein has physiochemical features of known drug targets and empirically determined interactions with drug like compounds. This step was important in determining which of the predicted target *P. falciparum* proteins are likely to be viable targets for the corresponding drug. TDR Targets Database v5 (Magariños *et al.*, 2012; T D R Targets, 2015) was used to determine druggability indices in this study.

2.5. *In vitro* drug susceptibility assays

In vitro drug-sensitivity assays are quite useful in antimalarial drug discovery because of their ability to measure the intrinsic sensitivity of malaria parasites to drugs. This allows for exclusion of host related factors such as host immunity or even drug failure due to absorption, distribution, metabolism and elimination (ADME) profile of the drug (Noedl *et al.*, 2003).

Most of the approaches for *in vitro* drug-sensitivity assays are based on malaria parasites cultures. Techniques for culturing malaria parasites were developed in the 1970s, which can either be for short periods (Haynes *et al.*, 1976) or continuous (Trager & Jensen, 1976). Drug susceptibility assays are meant to determine the ability of the predicted drugs to kill or inhibit growth of malaria parasites. This is achieved by inhibiting the parasite's essential life functions through varied mechanisms. There are several drug susceptibility assays that are used to test for the antiplasmodial activity of a compound. These methods are mostly *in vitro* or *in vivo* (Nogueira & do Rosário, 2010) and some are *ex vivo* (Traore *et al.*, 2015).

2.5.1. Measurement of antiplasmodial activity

Drug sensitivity assays in antimalarial studies describe the effect of test compounds on the growth profile of the *Plasmodium* parasite. Common methods used in measuring parasitaemia in drug sensitivity assays include; microscopic measurement of parasitaemia after GIEMSA

staining, isotopic assays using radiolabeled hypoxanthine, Histidine-rich protein II (HRPII) assay, parasitic lactate dehydrogenase assay (pLDH) and use of PCR-based techniques and nucleic acid stains to quantify parasitic DNA.

Histidine-rich protein II (HRPII) assay is based on histidine-rich protein II (HRP II) that is rich in histidine and alanine. The production of this protein has been associated with proliferation and development of the *P. falciparum* and hence Noedl *et al.*, (2002) used it in drug susceptibility tests to measure parasite growth. This test was based on enzyme-linked immunosorbent assay (ELISA). This enzyme has been used in rapid diagnostic tests for malaria, but because it is only produced in *P. falciparum*, it gives negative results with *P. vivax*, *P. malariae* and *P. ovale* (Iqbal *et al.,* 2000).

Parasitic lactate dehydrogenase (pLDH) assay is an antigen based assay specific to the parasite's LDH. Parasite's LDH is structurally and functionally different from its human isozymes (Shoemark, 2007). LDH is one of the most abundantly expressed enzymes by *P. falciparum* (Vander Jagt *et al.*, 1981). Makler *et al.* (1993) described the use of pLDH assay in drug sensitivity assays using *P. falciparum* in 1993. The study also analyzed the effectiveness of pLDH assay compared to 3H-hypoxanthine incorporation and Giemsa microscopy in accessing inhibition of *P. falciparum* by antimalarial drugs, and the results were been found to be similar. This study also found the test to be reproducible, rapid, inexpensive and easy to interpret. The pLDH assay has been used in assessing antiplasmodial activity of ferroquine by Barends *et al.*, (2007). D'Alessandro *et al.*, (2013) developed colorimetric screening method for anti-gametocyte compounds based on the pLDH assay. Orjuela-Sánchez *et al.*, (2012) also used pLDH assay in analysis of drug sensitivity of *Plasmodium bergei* in rodents. A study by Maltha *et al.*, (2014) showed pLDH assay to have similar sensitivity but higher specificity as

compared to HRPII assay. pLDH assay has also been used to determine drug inhibition profiles for *P. falciparum* (Makler *et al.*, 1993).

PCR based methods have been developed to quantify *Plasmodium* parasite's DNA (Kamau *et al.*, 2011; Lee *et al.*, 2002), some of which are species specific (Rougemont *et al.*, 2004). Corbett *et al.*, (2004) described PCR-based methods to measure growth inhibition profiles of *Plasmodium* parasite in drug sensitivity assays. This has also been used in quantification of *Plasmodium* parasite for routine clinical diagnosis (Perandin *et al.*, 2004).

Nucleic acid stains have also been used in quantification of *Plasmodium* parasite, especially in high throughput studies. This work in principle by quantifying the parasite's DNA. SYBR Green I has been shown to be ideal for high throughput studies by giving identical and similar results with radioisotopic assays (Bennett *et al.*, 2004; Smilkstein *et al.*, 2004). For purpose of this study, SYBR Green I-based assay was used based on an described protocol by Johnson *et al.*, (2007). A study evaluating the performance of 22 other DNA dyes in quantification of DNA in real-time PCR has been done (Eischeid, 2011), this study showed EvaGreen and the SYTO dyes 80, 16, 13 and 82 performed better than SYBR Green in general.

2.5.2. *P. falciparum* reference strains

There exist different of *P. falciparum* reference strains with different characteristics. These have been used to achieve different objectives in *in vitro* cultures. The most characterized *P. falciparum* strain (and most commonly used) is the chloroquine sensitive 3D7 strain. 3D7 was derived from NF54 isolate that was initially obtained from a patient living near Amsterdam Netherlands (Walliker *et al.*, 1987). Indochina III/CDC strain (one of the first strains to be isolated) was obtained from a Lao refugee in Aug 1980 (Campbell *et al.*, 1982). Both chloroquine resistant strain W2 and the mefloquine resistant W2-mef were derived from the

Indochina III/CDC strain (Oduola *et al.*, 1988). Multidrug resistant strain DD2 was obtained from W2-mef (Guinet *et al.*, 1996). Artemisinin resistant F32 ART (obtained by *in vitro* exposure to artemisinin for 3 years) and its sister strain (artemisinin sensitive F32 TEM) are isolates from Tanzania (Witkowski *et al.*, 2010). For purposes of these study, 3D7 will be used for *in vitro* cultures. F32 TEM and field isolates were also used because of their availability in the laboratory where this work was done.

CHAPTER 3: MATERIALS AND METHODS

This study involved *in silico* chemogenomics in prediction of drugs with antiplasmodial activity and validation using *in vitro* assays. A summary of the steps involved is shown in Figure 3.1. The *in silico* procedures were carried out at Pan African University, Juja while the *in vitro* techniques were done at KEMRI-Walter Reed Project, Malaria Drug Resistance (MDR) laboratories in Kisumu which has an established antimalarial drug resistance surveillance study. In MDR, resistance to a panel of antimalarial drugs are tested both *in vitro* and immediate *ex vivo*. In addition, this study tests were carried out under a well-established standard operating procedures that are ethically approved by KEMRI (KEMRI/RES/7/3/1, Appendix 2) and Walter Reed Army Institute of Research (WRIAR) (MCMR-UWZ, Appendix 3).

1. *P. falciparum* proteome compilation

Compilation of all the proteins expressed in *P. falciparum* available at NCBI RefSeq

2. STITCH, DrugBank and Therapeutic Target Database search

Sequence similarity search between the *P. falciparum* proteins and putative protein targets of approved drugs

3. TDR Targets Database

Determination of druggabillity indices for successful *P. falciparum* targets

4.ConSurf server

Funtional residues were determined in drug targets and compared to corresponding *P. falciparum* proteins

5. PubMed and Google Scholar search

Search for all the drugs in lead drug list that have been previously tested for antimalarial activity

6. Drugs susceptibility assays

Validation of antimalarial activity

Figure 3.1: Workflow involved in prediction and validation of approved drugs that have activity against *P. falciparum*

3.1. Chemogenomics screening (*in silico*)

The methodology used in this study is modified from two previous studies; one used to search for approved drugs with activity against *P. falciparum* apicoplast (Bispo *et al.,* 2013) and another that was used to search for approved drugs with schistosomicidal activity (Neves *et al.*, 2015).

3.1.1. Compilation of *P. falciparum* proteome

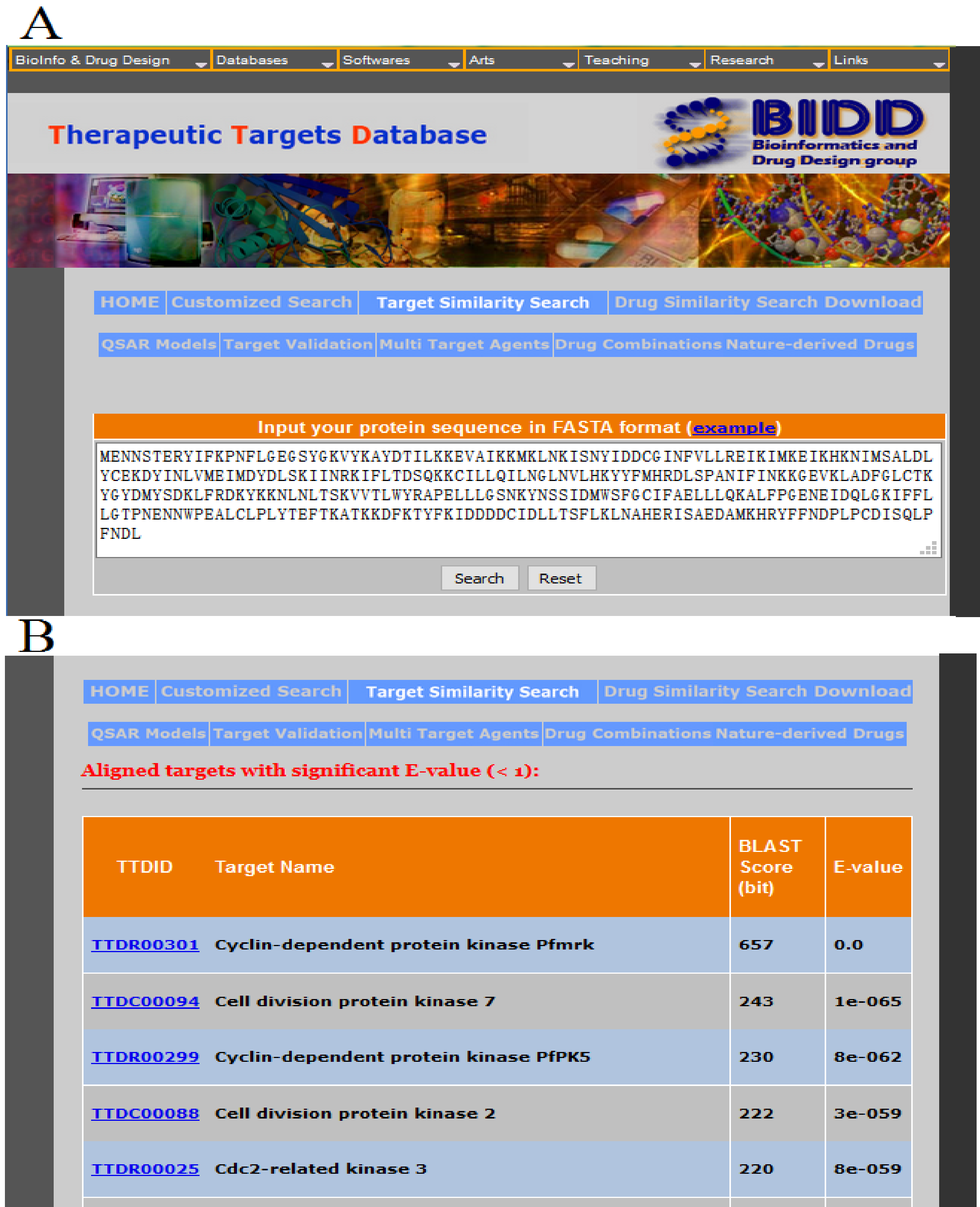
A list of all available *P. falciparum* proteins expressed in all life stages of was obtained from NCBI Reference Sequence (RefSeq) database, release 75. The search at NCBI webpage was made by keying in the words "*Plasmodium falciparum*" in the search box and selecting "Protein" database in the dropdown menu before clicking the search button. To filter the results, "*Plasmodium falciparum*" was selected in the organisms section and RefSeq as the source database. These sequences were sorted by their submission dates, starting with the newest. Then all the protein sequences were downloaded in Multi-FASTA format.

For easy manipulation of the large number of sequences, the downloaded sequences were converted into a CSV spreadsheet using R statistical programming software (Team, 2004). The spreadsheet had the description line of the FASTA sequence entries in the first column and the protein sequences in the second. More columns were added to accommodate additional information concerning the proteins as the study progressed.

3.1.2. Identification of putative drug targets using STITCH, DrugBank, and the Therapeutic Target Database (TTD) databases

Using the *Plasmodium* protein sequences as queries, a search was done for similar putative drug targets on publicly available databases namely; STITCH 4.0 (http://stitch.embl.de/), DrugBank (https://www.drugbank.ca/biodb/search/bonds/sequence) , and Therapeutic Target

Database, TTD (http://bidd.nus.edu.sg/group/ttd/TTD_Blast.asp). At the time of writing this thesis, STITCH was updated from version 4.0 to 5.0. Each of the protein sequences was used to search for sequence similarity against known drug target sequences. For TTD and DrugBank, homologous proteins with output expectation values (E-values) lower than 1e-20 were considered for further analysis. Here, the E value describes the number of times one can expect to see a match by chance, thus the lower the E value, the better. For the STITCH database, a confidence score above 0.7 was considered. Predicted protein targets were keyed into a spreadshe*et al*ongside their corresponding homologous *P. falciparum* proteins. In another column, all the approved drugs that interact with these targets were also included for further analysis.



TTDID	Target Name	BLAST Score (bit)	E-value
TTDR00301	Cyclin-dependent protein kinase Pfmrk	657	0.0
TTDC00094	Cell division protein kinase 7	243	1e-065
TTDR00299	Cyclin-dependent protein kinase PfPK5	230	8e-062
TTDC00088	Cell division protein kinase 2	222	3e-059
TTDR00025	Cdc2-related kinase 3	220	8e-059

Figure 3.2: Therapeutic Targets Database (TTD) target similarity search submission and results webpages. A protein sequence is pasted in FASTA format and searched (A). The output results comprised of a list of proteins targets similar to the query protein sorted according to their E values in descending order (B).

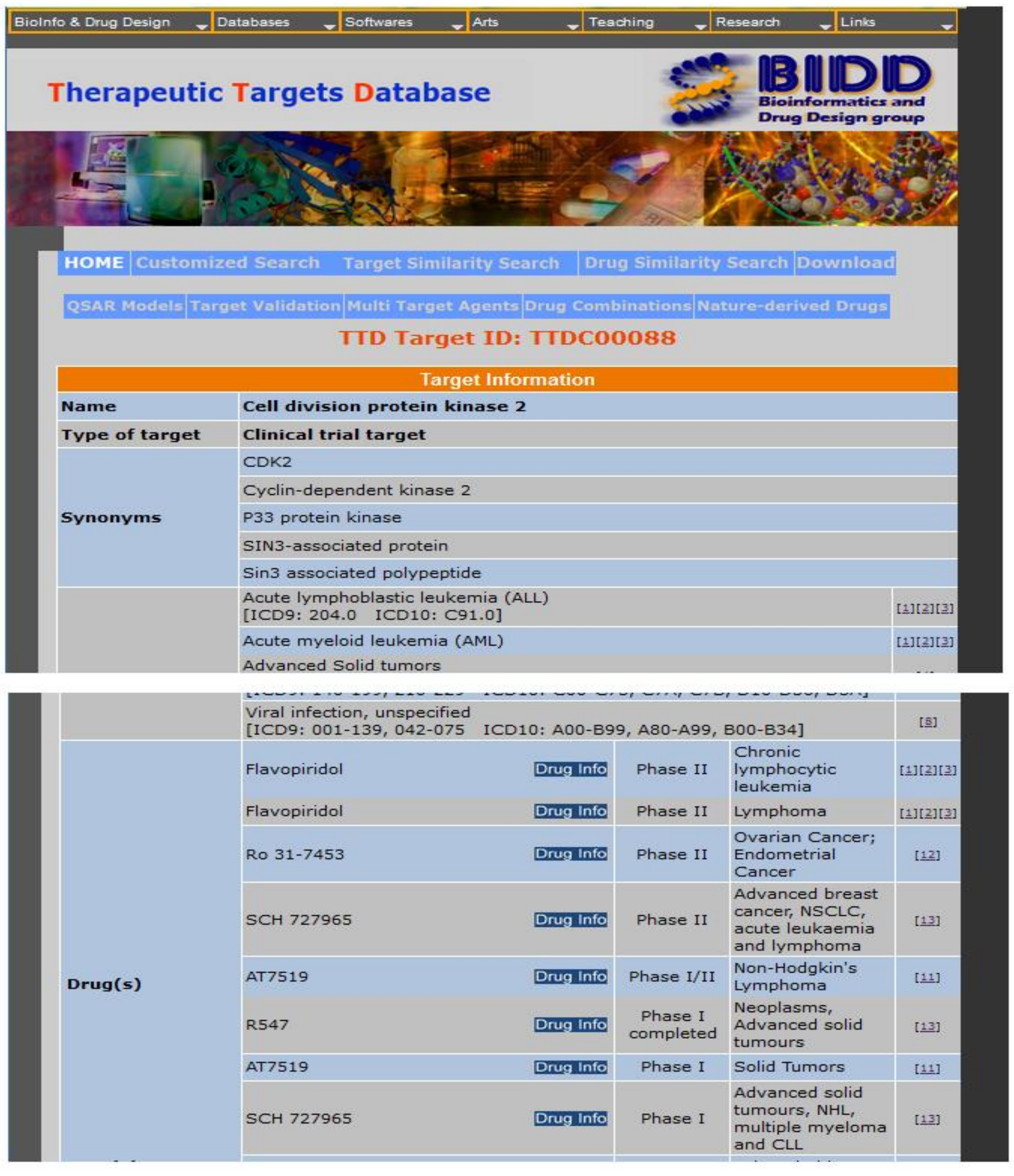


Figure 3.3: Therapeutic Targets Database (TTD) protein target details webpage. Output results consisted of a list of proteins similar to the query sequence with their E values (A). When a protein of interest (those with E values lower than 1e-20) was selected, it directed to another webpage showing more details about the protein, this included drugs it is a targets to (B).

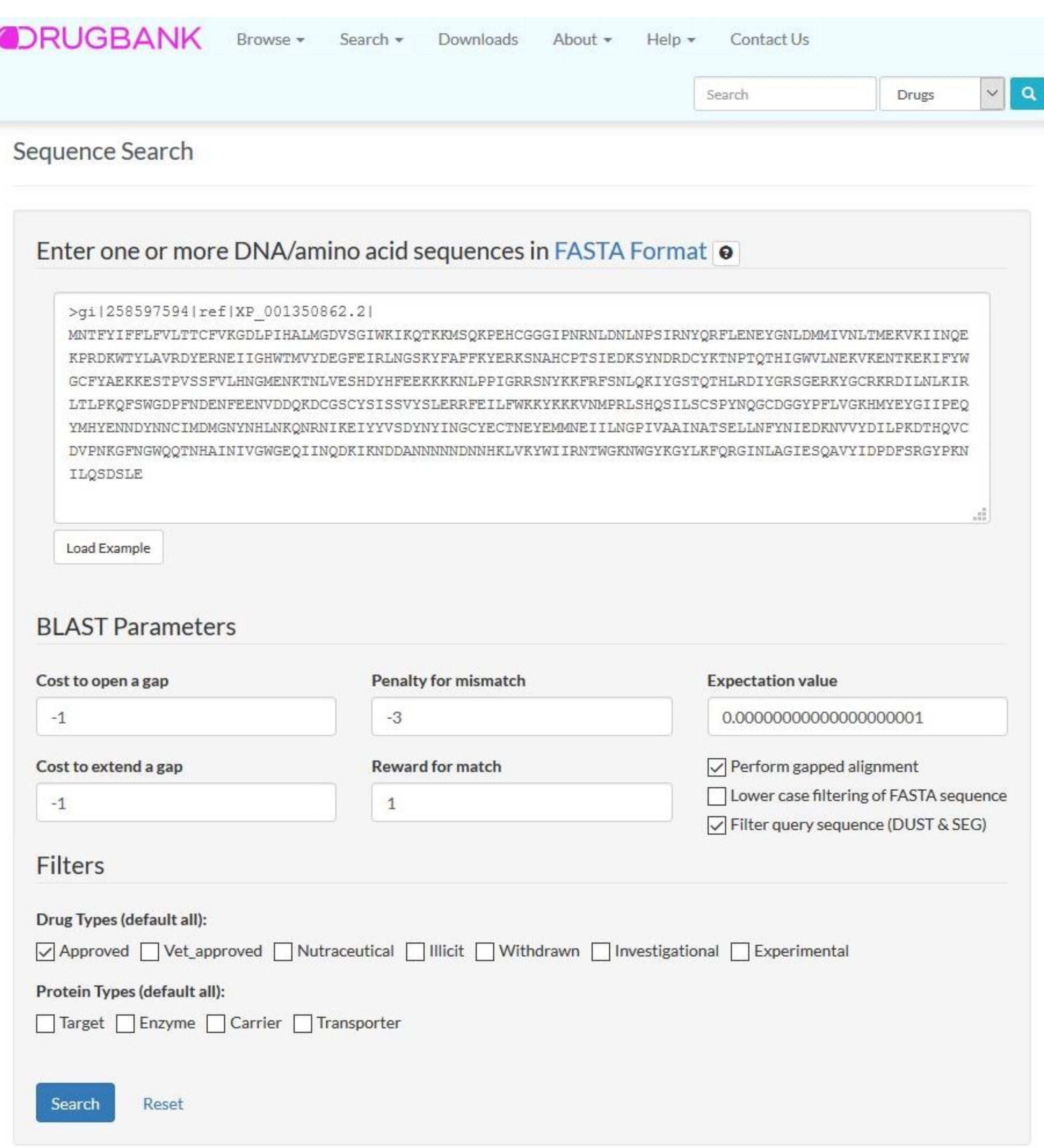


Figure 3.4: DrugBank database target sequence search submission webpage. A protein sequence was pasted in FASTA format, Expectation value set at 10e-20, filters set for approved drugs only and the rest of the parameters were left in their default values before searching.

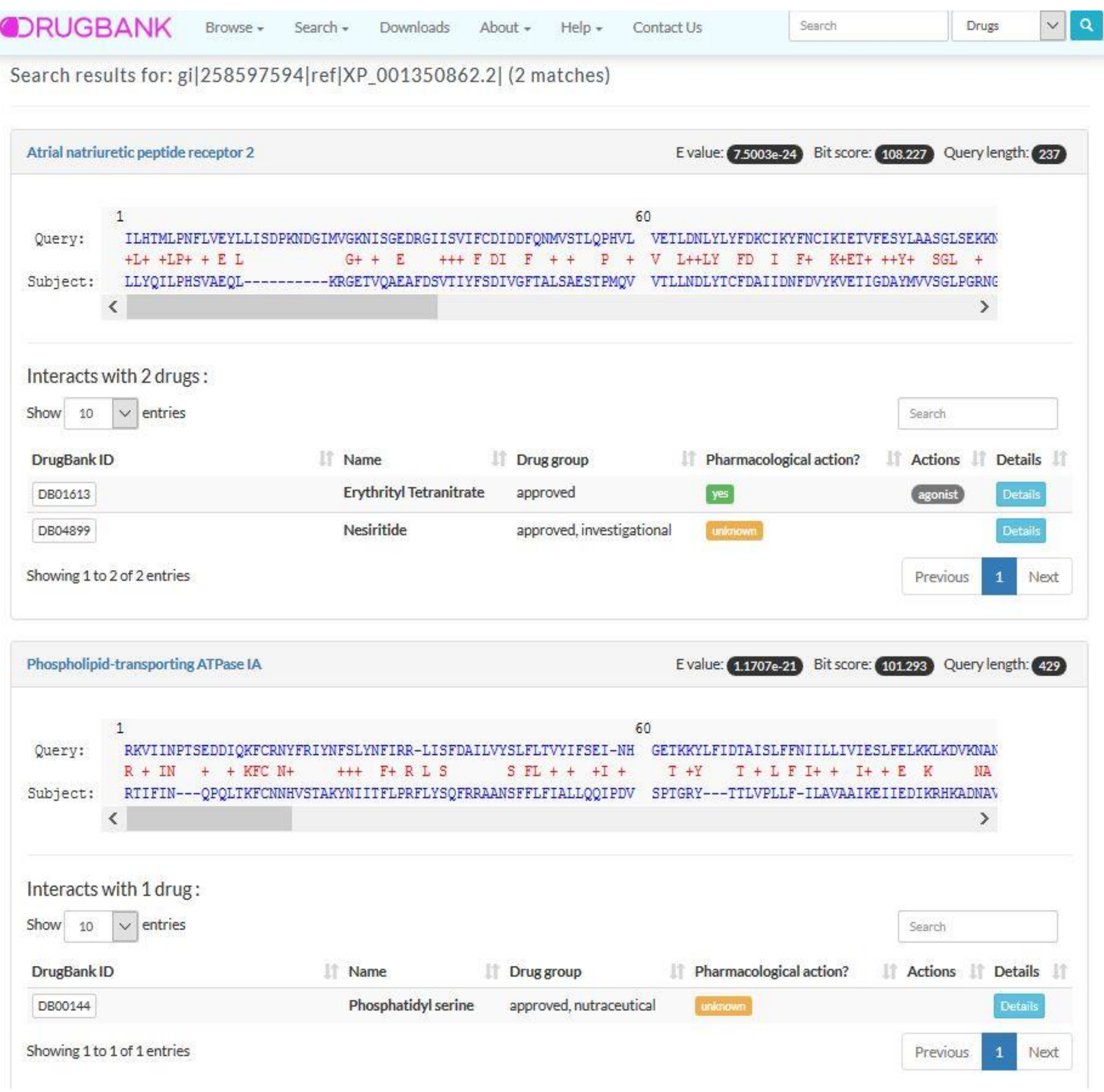


Figure 3.5: DrugBank database sequence search results webpage. Output results comprised of approved drugs targets (similar to the protein query) with approved drugs they interact with.

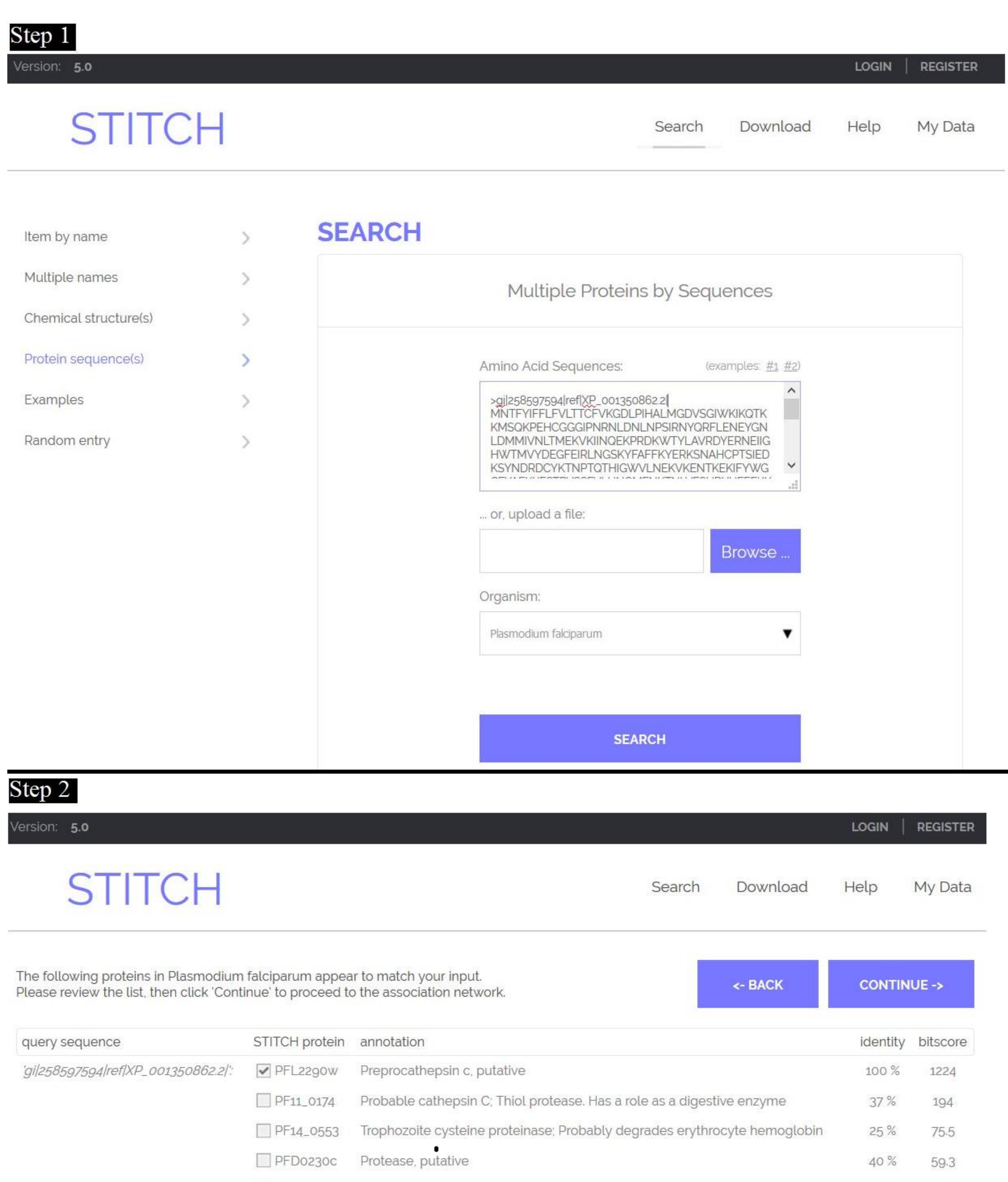


Figure 3.6: STITCH similarity search submission webpage. A protein sequence was pasted in FASTA format or uploaded from a FASTA file, the source organism selected and then the search initiated. The next step involved confirming the identity of the query protein sequence before finishing the search.

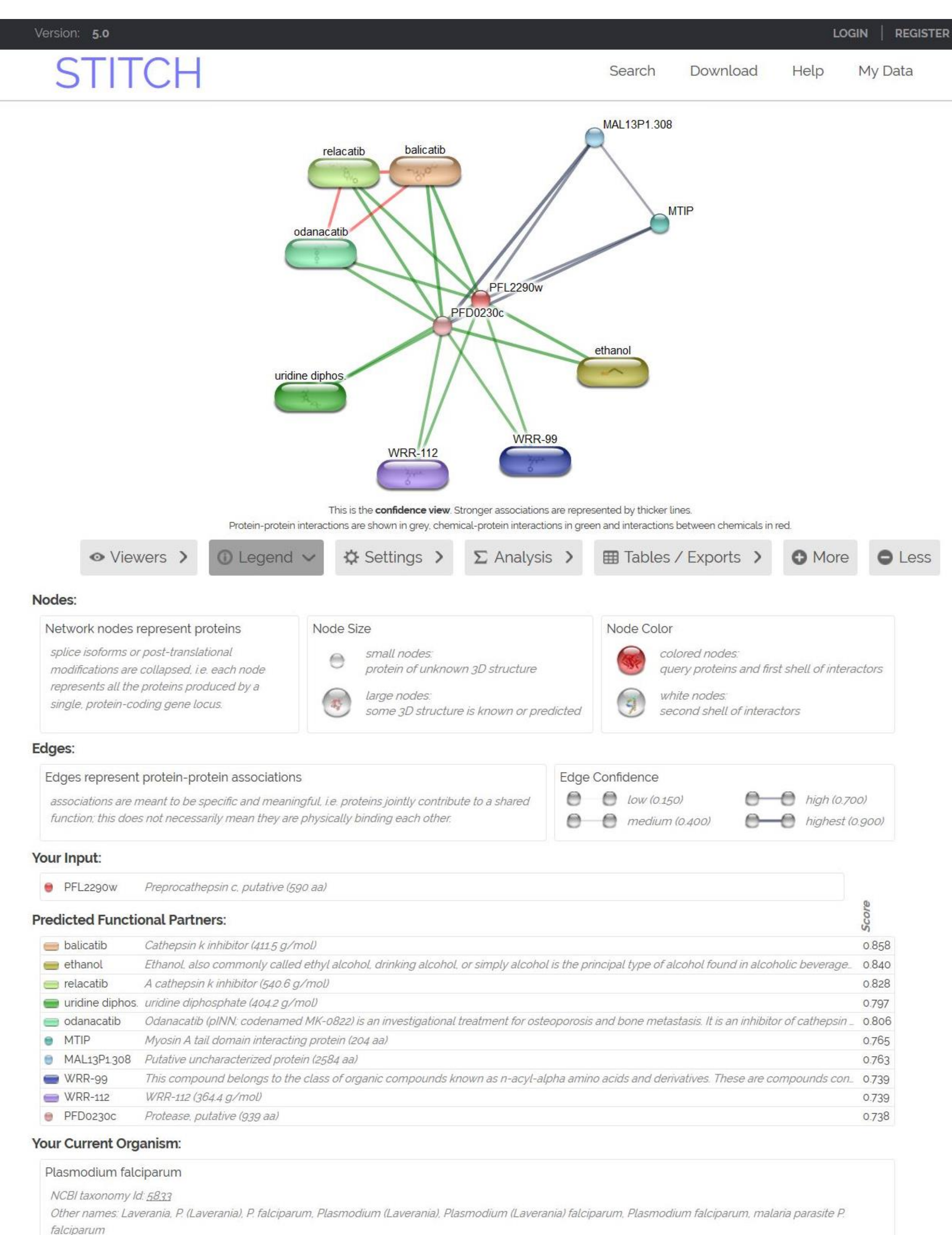


Figure 3.7: STITCH results output for a *P. falciparum* protein (XP_001350316.2). The results output showed the protein of interest with biomolecules and other compounds that interact with it. Only approved drugs with at least a score of 7 was selected for further analysis.

3.1.3. Determination of druggability index

Druggability indices of all the *P. falciparum* possible drug targets successful in the similarity search (from step 3.1.2.) were obtained. These druggability indices were retrieved from TDR Targets Database v5 (http://www.tdrtargets.org/) as shown in Figure 3.9 and Figure 3.10.

The TDR Targets Database v5
A chemogenomics resource for neglected tropical diseases
Search
Login | Register | Documentation | Contact | FAQ
home | targets | compounds | my history | posted lists | targets survey | manual | my queries: 1

Search for genes/targets
Use this form to search for proteins/targets using any of the following criteria. Note that all selected criteria will be evaluated as the intersection (boolean 'AND') of the respective result sets. You can read more about this SEARCH page in the User's Manual.

1. Select pathogen species of interest
Mycobacterium leprae | *Mycobacterium tuberculosis*
Wolbachia endosymbiont of Brugia malayi | *Brugia malayi*
Caenorhabditis elegans | *Schistosoma mansoni*
Plasmodium falciparum | *Plasmodium vivax*
Toxoplasma gondii | *Leishmania major*
Trypanosoma brucei | *Trypanosoma cruzi*
search | reset

2. Filter targets based on:
Name / Annotation
Search for targets using keywords (names, functions, identifiers).
Name: MO15-related protein kinase (MRK) [e.g. farnesyl, kinase, pyrophosphatase]
Identifier/Accession [e.g. LmjF22.1360, PF11_0295, tbr3370]
EC number: [e.g. 2.5.1.10, or use '*' or type 'any']
Gene Ontology: [GO id/term (GO:0020011, apicoplast)]

Figure 3.8: TDR Targets Database search submission webpage. "Targets" option was selected, pathogen species box checked (in this case *P. falciparum*) and the name of the protein (in this case adenosine deaminase) was used to filter the targets before searching.

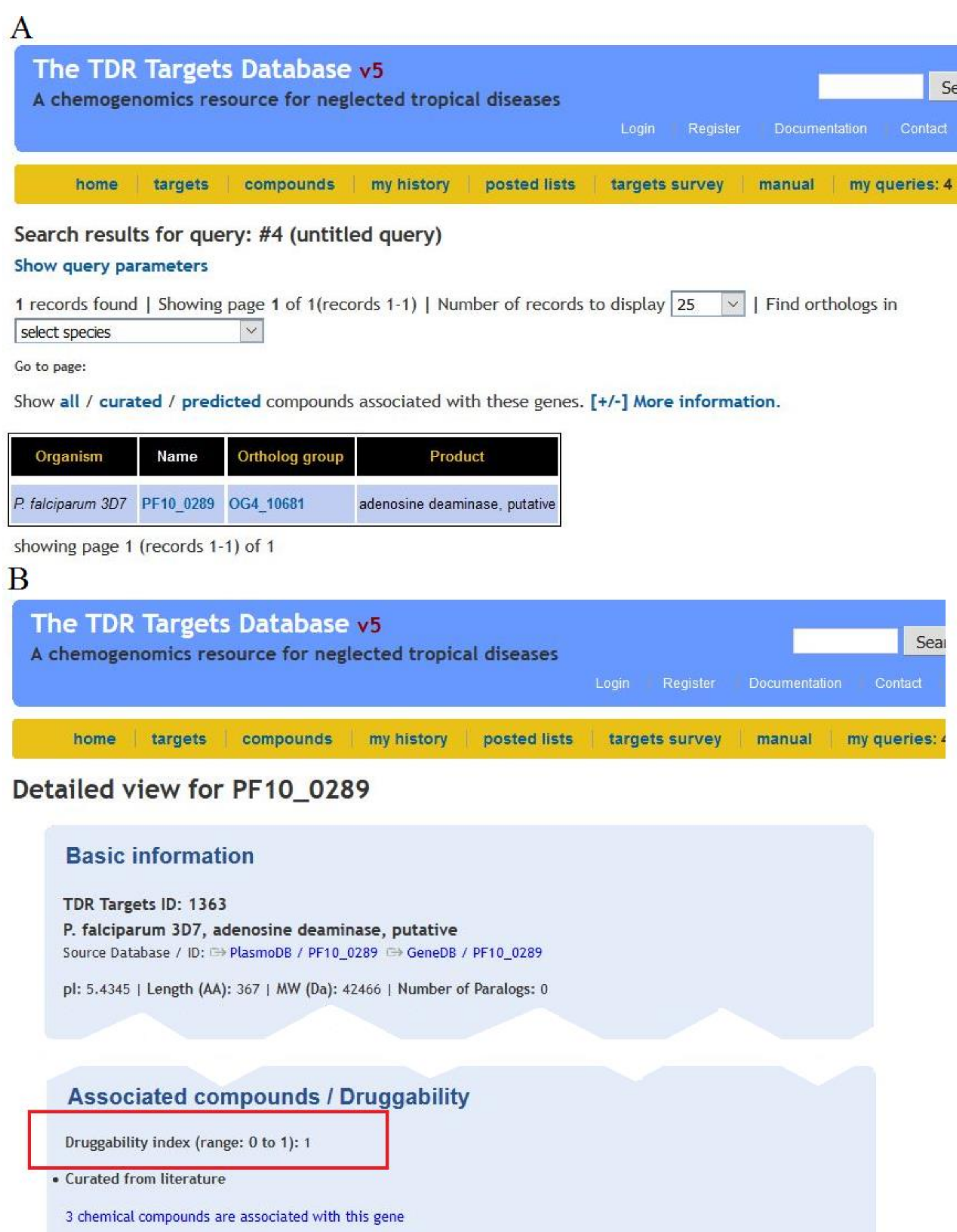


Figure 3.9: TDR Targets database targets results webpage. First, the possible protein targets similar to the query (adenosine deaminase) were shown, if one of interest was present it was selected (A) then several information about the protein including the druggability index (highlighted in red) is displayed (B).

3.1.4. Determination and comparison of functional regions

All the successful *P. falciparum* protein targets from the step 3.1.2 were also used to perform a comparison analysis to determine if they share functional regions of their corresponding putative drug target. Their analysis in this study is important since a drug is more likely to have a similar effect on a protein that shares functional regions with its confirmed target. ConSurf server (http://consurf.tau.ac.il/2016/index_proteins.php) was used to determine these functional regions.

Before determining the functional residues, a protein-protein pairwise alignment using BLAST (Altschul *et al.*, 1990) was done at NCBI with the drug target as the query sequence and its corresponding *P. falciparum* homolog as the subject as shown in Figure 3.11. A sample of the BLAST results is shown in Figure 3.12. Only proteins pairs that had more than 80 % query coverage were considered for ConSurf server analysis since this increased the likelihood of these proteins sharing functional regions.

The ConSurf server required the 3D structure of the proteins in PDB format, this was obtained from Protein Data Bank in Europe, PDBe (Velankar *et al.*, 2012). Proteins whose 3D image were not available were modelled using SWISS-MODEL server (Arnold *et al.*, 2006).

The multiple sequence alignment (MSA) results from ConSurf server is color coded according to conservation scores, this was snipped using Windows® "Snipping tool" and overlaid over the BLAST protein alignment results for visual comparison as shown in Figure 4.4. The functional residues of *P. falciparum* protein were then visually compared between its corresponding homologous drug target and the percentage of shared functional residues calculated. Similarity of conserved functional residues were categorized into those with high similarity (more than 80 %), moderate similarity (50-79 %) and low similarity (less than 50

%), this was modified from classification done by Neves *et al.* (2015). Those with low similarity were excluded from further analysis.

3.1.5. List of drugs to be tested against malaria

Drugs that are applied topically, nutraceuticals and protein based drugs (e.g. insulin) were excluded from drug lead list. This is because they are less likely to be used as antimalarial drugs or even have any antiplasmodial activity because of their physicochemical properties. Duplicate entries that had not yet been detected up to this point were also eliminated.

A literature search was then carried out at PubMed and Google Scholar to identify if any of the approved drugs in the lead list had undergone antimalarial analysis of any kind. Drugs with no such records were considered as not tested and were evaluated further, the rest were excluded. Drugs that had been tested but their IC_{50} not indicated were not excluded. The search was made by keying in the "name of the drug" with either "malaria", "malaria *in vitro* testing" or "*plasmodium*". For *in vitro* assays, drugs were selected both from those that had not been tested previously and few that had been tested, the latter was for comparison purposes.

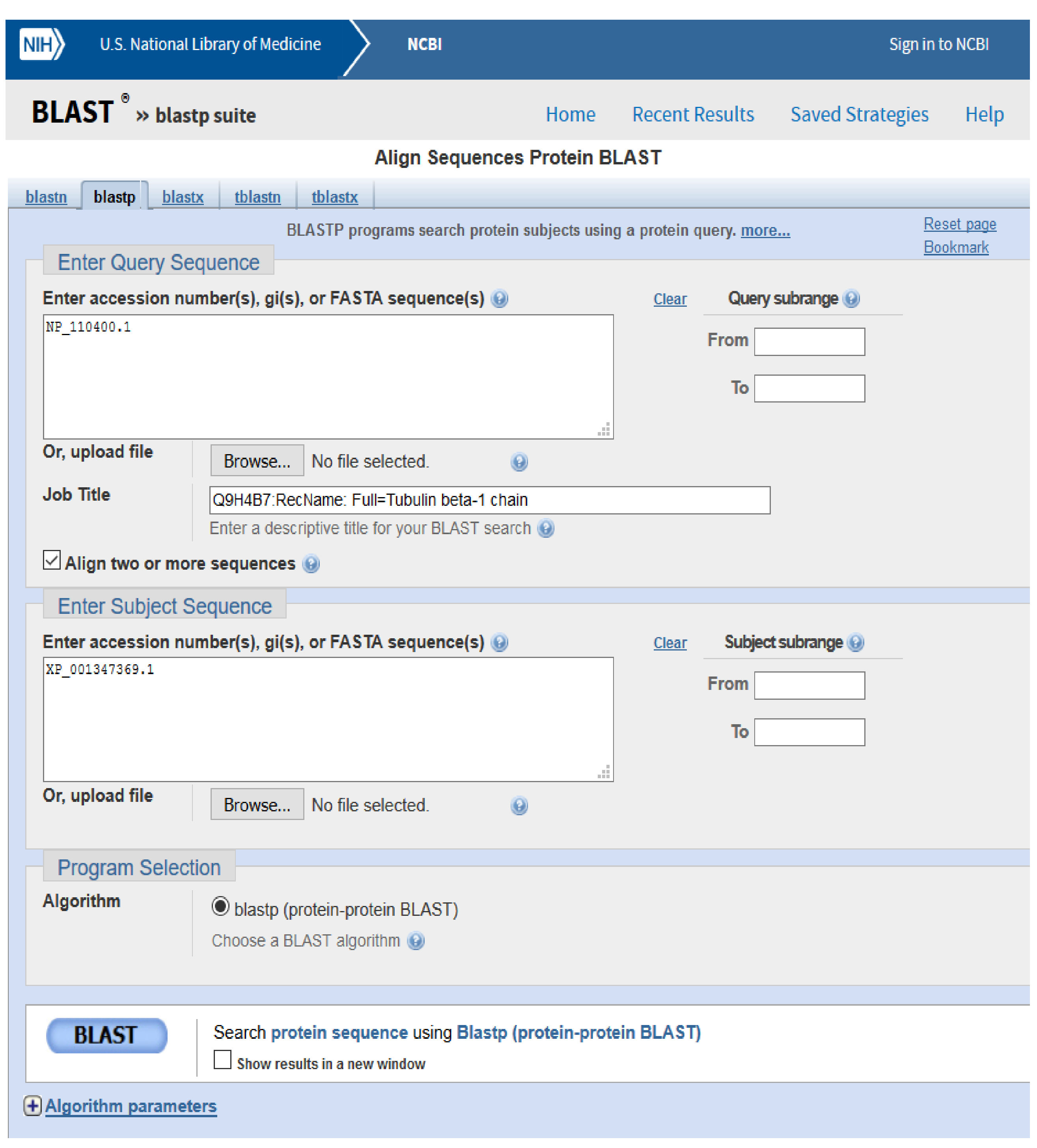


Figure 3.10: Protein-protein pairwise alignment using NCBI BLAST (submission webpage). Analysis involved pasting the putative drug target NCBI Accession number (in this case NP_110400.1) as the query sequence and the *P. falciparum* homolog as the subject sequence (in this case XP_001347369.1) and the search was initiated by clicking the “BLAST” button. Algorithm parameters were left in their default settings.

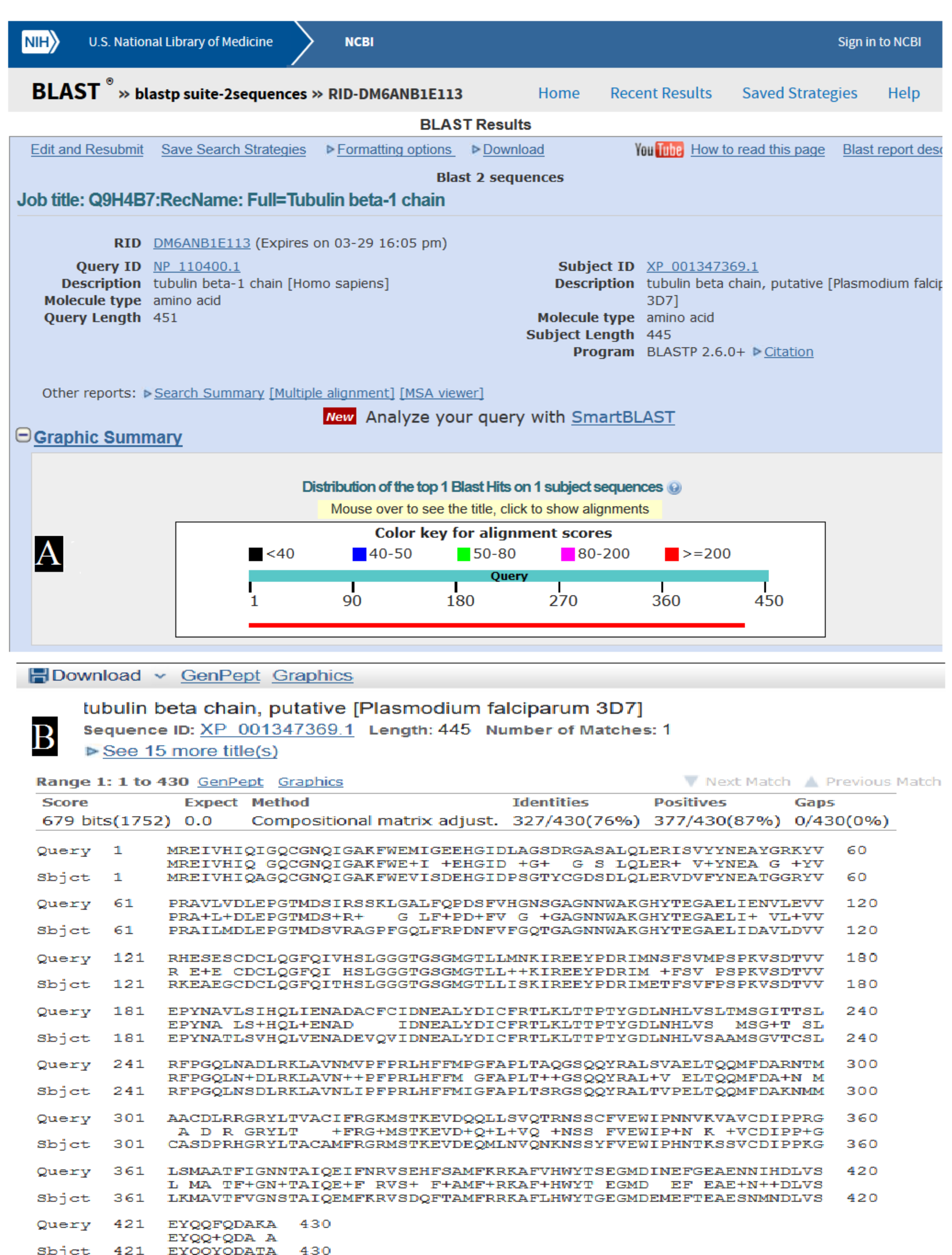


Figure 3.11: Protein-protein pairwise alignment using NCBI BLAST (results webpage). Results of protein-protein pairwise alignment included among other things graphical representation of the coverage (A) and an alignment of the two proteins (B).

A

The ConSurf Server

Server for the Identification of Functional Regions in Proteins

HOME GALLERY OVERVIEW QUICK HELP FAQ CITING & CREDITS OLD VERSIONS CONSURF-DB TERMS OF USE

Load an Example

I am an expert user, Please show me all options

Analyze Nucleotides or Amino Acids?

Amino-Acids

Is there a known protein structure?

YES Enter the PDB ID: ?

NO OR

Upload your own PDB file: Browse... No file selected. ?

Next

B

Is there a known protein structure?

YES Chain Identifier: A

Do you have a Multiple Sequence Alignment (MSA) to upload? (If not, ConSurf will make an MSA for you.)

YES

NO

Choose parameters to homolog search algorithm

Homolog search algorithm HMMER

Number of iterations 1

E-value cutoff 0.0001

Proteins database UNIREF-90 ?

Select homologs for ConSurf analyses: automatically (recommended) manually

C

Is there a known protein structure?

YES Chain Identifier: A

Do you have a Multiple Sequence Alignment (MSA) to upload? (If not, ConSurf will make an MSA for you.)

YES

NO

Choose parameters to homolog search algorithm

Homolog search algorithm HMMER

Number of iterations 1

E-value cutoff 0.0001

Proteins database UNIREF-90 ?

Select homologs for ConSurf analyses: automatically (recommended) manually

Choose parameters to select the sequences for the analysis out of homolog search algorithm results

Select 150 sequences that sample the list of homologues to reference sequence ?

Maximal %ID between sequences 95 ?

Minimal %ID for homologs 35 ?

Choose alignment method to build the Multiple Sequence Alignment (MSA)

Alignment method MAFFT-L-INS-i

Calculation method Bayesian ?

Evolutionary substitution model Best model (default)

Job title Q9UNQ0

Optional

Enter a descriptive job title for your ConSurf query

User E-Mail reaganmoseti@gmail.com

Optional

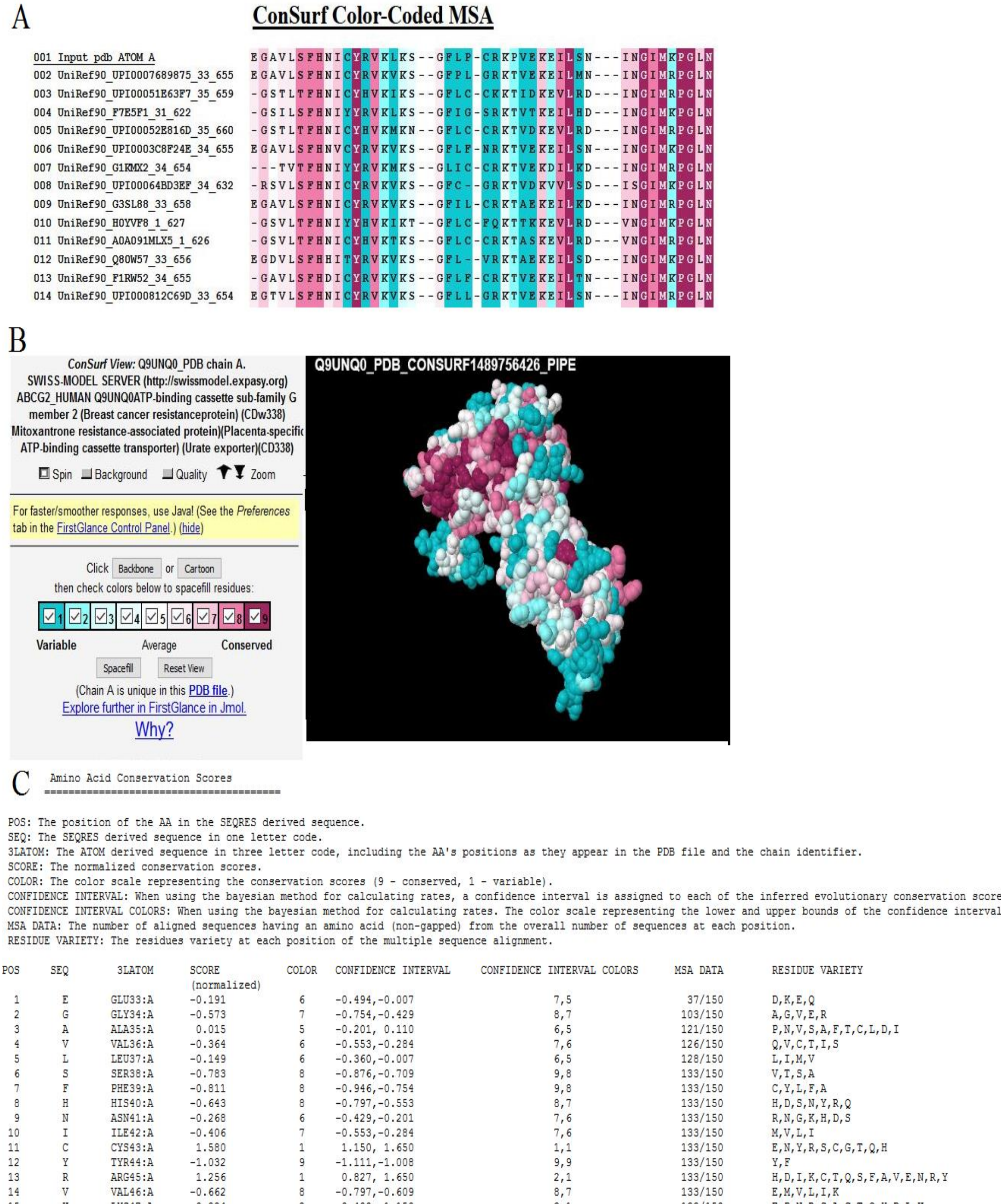
We will use this address to report you when the job has finished or if error has occurred.

Send a link to the results by e-mail

Providing your e-mail address ensures that you don't lose the URL to your results and enables you to view your results in the future.

Submit

Figure 3.12: ConSurf Server submission webpages. Analysis first involved selecting the type of sequence i.e. amino acids and uploading a 3 D structure of the protein of interest (A). The next step involved identifying its chain identity (for multimeric proteins) and selecting the mode of MSA (B), the rest of parameters were left on their default settings. Lastly, an email address and title of the protein was keyed in and the work submitted (B).



Amino Acid Conservation Scores
=======================================

POS: The position of the AA in the SEQRES derived sequence.
SEQ: The SEQRES derived sequence in one letter code.
3LATOM: The ATOM derived sequence in three letter code, including the AA's positions as they appear in the PDB file and the chain identifier.
SCORE: The normalized conservation scores.
COLOR: The color scale representing the conservation scores (9 - conserved, 1 - variable).
CONFIDENCE INTERVAL: When using the bayesian method for calculating rates, a confidence interval is assigned to each of the inferred evolutionary conservation scores.
CONFIDENCE INTERVAL COLORS: When using the bayesian method for calculating rates. The color scale representing the lower and upper bounds of the confidence interval.
MSA DATA: The number of aligned sequences having an amino acid (non-gapped) from the overall number of sequences at each position.
RESIDUE VARIETY: The residues variety at each position of the multiple sequence alignment.

POS	SEQ	3LATOM	SCORE (normalized)	COLOR	CONFIDENCE INTERVAL	CONFIDENCE INTERVAL COLORS	MSA DATA	RESIDUE VARIETY
1	E	GLU33:A	-0.191	6	-0.494,-0.007	7,5	37/150	D,K,E,Q
2	G	GLY34:A	-0.573	7	-0.754,-0.429	8,7	103/150	A,G,V,E,R
3	A	ALA35:A	0.015	5	-0.201, 0.110	6,5	121/150	P,N,V,S,A,F,T,C,L,D,I
4	V	VAL36:A	-0.364	6	-0.553,-0.284	7,6	126/150	Q,V,C,T,I,S
5	L	LEU37:A	-0.149	6	-0.360,-0.007	6,5	128/150	L,I,M,V
6	S	SER38:A	-0.783	8	-0.876,-0.709	9,8	133/150	V,T,S,A
7	F	PHE39:A	-0.811	8	-0.946,-0.754	9,8	133/150	C,Y,L,F,A
8	H	HIS40:A	-0.643	8	-0.797,-0.553	8,7	133/150	H,D,S,N,Y,R,Q
9	N	ASN41:A	-0.268	6	-0.429,-0.201	7,6	133/150	R,N,G,K,H,D,S
10	I	ILE42:A	-0.406	7	-0.553,-0.284	7,6	133/150	M,V,L,I
11	C	CYS43:A	1.580	1	1.150, 1.650	1,1	133/150	E,N,Y,R,S,C,G,T,Q,H
12	Y	TYR44:A	-1.032	9	-1.111,-1.008	9,9	133/150	Y,F
13	R	ARG45:A	1.256	1	0.827, 1.650	2,1	133/150	H,D,I,K,C,T,Q,S,F,A,V,E,N,R,Y
14	V	VAL46:A	-0.662	8	-0.797,-0.609	8,7	133/150	E,M,V,L,I,K
15	K	LYS47:A	0.824	2	0.400, 1.150	3,1	133/150	E,P,N,R,S,A,G,T,Q,H,D,L,K
16	L	LEU48:A	1.164	1	0.589, 1.650	3,1	134/150	L,D,I,K,T,Q,S,A,F,E,P,M,V

Figure 3.13: ConSurf server results for protein target Q9UNQ0. Results from ConSurf server are in several forms: multiple sequence alignment (only 14 of 150 homolog sequences are shown) with amino acids color coded according to conservation (A), 3 D image of the protein showing the protein with the amino acids similarly color coded (B) and a spreadsheet with a more comprehensive analysis of the protein's amino acids (6 of 622 amino acids are shown), also shown in Appendix 6 (C).

A

Score	Expect	Method	Identities	Positives	Gaps
679 bits(1752)	0.0	Compositional matrix adjust.	327/430(76%)	377/430(87%)	0/430(0%)

```
Drug target            1    MREIVHIQIGQCGNQIGAKFWEMIGEEHGIDLAGSDRGASALQLERISVYYNEAYGRKYV   60
                            MREIVHIQ GQCGNQIGAKFWE+I +EHGID +G+   G S LQLER+ V+YNEA G +YV
P. falciparum protein  1    MREIVHIQAGQCGNQIGAKFWEVISDEHGIDPSGTYCGDSDLQLERVDVFYNEATGGRYV   60
Drug target            61   PRAVLVDLEPGTMDSIRSSKLGALFQPDSFVHGNSGAGNNWAKGHYTEGAELIENVLEVV   120
                            PRA+L+DLEPGTMDS+R+    G LF+PD+FV G +GAGNNWAKGHYTEGAELI+ VL+VV
P. falciparum protein  61   PRAILMDLEPGTMDSVRAGPFGQLFRPDNFVFGQTGAGNNWAKGHYTEGAELIDAVLDVV   120
Drug target            121  RHESESCDCLQGFQIVHSLGGGTGSGMGTLLMNKIREEYPDRIMNSFSVMPSPKVSDTVV   180
                            R  E+E CDCLQGFQI HSLGGGTGSGMGTLL++KIREEYPDRIM +FSV PSPKVSDTVV
P. falciparum protein  121  RKEAEGCDCLQGFQITHSLGGGTGSGMGTLLISKIREEYPDRIMETFSVFPSPKVSDTVV   180
Drug target            181  EPYNAVLSIHQLIENADACFCIDNEALYDICFRTLKLTTPTYGDLNHLVSLTMSGITTSL   240
                            EPYNA LS+HQL+ENAD     IDNEALYDICFRTLKLTTPTYGDLNHLVS  MSG+T SL
P. falciparum protein  181  EPYNATLSVHQLVENADEVQVIDNEALYDICFRTLKLTTPTYGDLNHLVSAAMSGVTCSL   240
Drug target            241  RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTAQGSQQYRALSVAELTQQMFDARNTM   300
                            RFPGQLN+DLRKLAVN++PFPRLHFFM GFAPLT++GSQQYRAL+V ELTQQMFDA+N M
P. falciparum protein  241  RFPGQLNSDLRKLAVNLIPFPRLHFFMIGFAPLTSRGSQQYRALTVPELTQQMFDAKNMM   300
Drug target            301  AACDLRRGRYLTVACIFRGKMSTKEVDQQLLSVQTRNSSCFVEWIPNNVKVAVCDIPPRG   360
                             A D R GRYLT    +FRG+MSTKEVD+Q+L+VQ +NSS FVEWIP+N K +VCDIPP+G
P. falciparum protein  301  CASDPRHGRYLTACAMFRGRMSTKEVDEQMLNVQNKNSSYFVEWIPHNTKSSVCDIPPKG   360
Drug target            361  LSMAATFIGNNTAIQEIFNRVSEHFSAMFKRKAFVHWYTSEGMDINEFGEAENNIHDLVS   420
                            L MA TF+GN+TAIQE+F RVS+ F+AMF+RKAF+HWYT EGMD  EF EAE+N++ DLVS
P. falciparum protein  361  LKMAVTFVGNSTAIQEMFKRVSDQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVS   420
Drug target            421  EYQQFQDAKA   430
                            EYQQ+QDA A
P. falciparum protein  421  EYQQYQDATA   430
```


Figure 3.14: Determination and comparison of functional residues. (A) For visual comparison of functional residues, ConSurf server MSA results (color coded according conservations scores) of one of the drug targets, the human tubulin beta-1 (NCBI Accession Number NP_110400.1), is overlaid above its BLAST pair-wise alignment with its *P. falciparum* homolog (NCBI Acc. No.: XP_001347369.1). The percent of the shared conserved residues was then determined. This was done for all the 26 other protein pairs.

3.2. *In vitro* drug susceptibility assays

3.2.1. Test drugs and controls

Out of the 34 drugs, a representative group of drugs were selected for *in vitro* testing due to limitation of resources. Selection of the drugs was based on their indications, representative drugs were selected depending on their indications, mostly from those that had not been tested previously and a few from those had been tested. Nonetheless, the final drug list tested *in vitro* was also subject to their availability in the market since some drugs were not available in pharmaceutical standard.

Drugs that were selected for testing included; two anticancer drugs, two antibiotic, one antidipsotropic, one immunosuppressive, one antiasmatic, one antiretroviral and two antileukemic. Details on indications, putative drug target, predicted *P. falciparum* drug target, percentage of shared conserved regions and druggability indices of tested drugs are shown in Table 3.1. Functional regions of predicted target of Tacrolimus, FK506-binding protein (FKBP) were not analyzed since it was identified directly from STITCH database and therefore did not have a corresponding putative target to compare to.

Table 3.1: Details of drugs tested *in vitro* showing their indications, putative drug target, and predicted target on *P. falciparum*, percentage of shared conserved regions and druggability indices.

Drug	**Indication**	**Putative drug target (UNIPROT ID)**	***P. falciparum* target (NCBI Acc. No.)**	**Conserved functional residues (%)**	**Druggability index**
Cladribine	Hairy cell leukemia	Adenosine deaminase (P00813)	Adenosine deaminase (XP_001347573.1)	55 %	1

Table 3.1 cont.

Diadzin	Anti-dipsotropic	ATP-binding cassette sub-family G member 2 (Q9UNQ0)	ABC transporter (XP_001348418.1)	51 %	0.5
Zafirlukast	Asthma	ATP-binding cassette sub-family G member 2 (Q9UNQ0)	ABC transporter (XP_001348418.1)	51 %	0.5
Levofloxacin	Antibacterial	DNA topoisomerase 2-alpha (P11388)	DNA topoisomerase II (XP_001348490.1)	61 %	0.8
Dasatinib	Anticancer	ATP-binding cassette sub-family G member 2 (Q9UNQ0)	ABC transporter (XP_001348418.1)	51 %	0.5
Clofarabine	Antileukemia	ATP-binding cassette sub-family G member 2 (Q9UNQ0)	ABC transporter (XP_001348418.1)	51 %	0.5
Tacrolimus	Organ transplant	NA	FK506-binding protein (FKBP)-type peptidyl-propyl isomerase (XP_001350859.1)	-	0.6
Irinotecan	Colorectal cancer	ATP-binding cassette sub-family G member 2 (Q9UNQ0)	ABC transporter (XP_001348418.1)	51 %	0.5
Zidovudine	Anti-retroviral	ATP-binding cassette sub-family G member 2 (Q9UNQ0)	ABC transporter (XP_001348418.1)	51 %	0.5
Moxifloxacin	Antibacterial	DNA topoisomerase 2-alpha (P11388)	DNA topoisomerase II (XP_001348490.1)	61 %	0.8

Table 3.1 cont.

Oxaliplatin	Colorectal cancer	ATP-binding cassette sub-family G member 2 (Q9UNQ0)	ABC transporter (XP_001348418.1)	51 %	0.5
Tadafil	Erectile dysfunction	CGMP-specific 3',5'-cyclic phosphodiesterase (O76074)	3',5'-cyclic nucleotide phosphodiesterase (XP_001349954.1)	> 5 %	-

All the 12 candidate drugs were bought from Sigma-Aldrich Inc., while standard malaria drugs i.e. chloroquine, dihydroartemisinin and mefloquine, were obtained from World Wide Antimalarial Resistance Network (WWARN). The 12 drug candidates and standard malaria drugs were assayed using a non-radioactive assay technique (Smilkstein *et al.*, 2004) with modifications (Cheruiyot *et al.*, 2016; Johnson *et al.*, 2007) to determine 50 % growth inhibition of cultured parasites.

Two different reference clones, chloroquine-sensitive (3D7) and F32 TEM were cultured as described by Johnson *et al.* (2007). For each of the test drugs, the drug susceptibility assays were carried out in 3 replicates for the 3D7 strain. For F32 TEM strain and field isolates, one assay was done for each of the drug tested due to limitations of laboratory consumables. Drugs and compounds were dissolved in 99.5 % dimethylsulfoxide (DMSO) (Sigma-Aldrich) and diluted in complete Roswell Park Memorial Institute 1640 series of Cell Culture Medium (RPMI 1640) prepared as described by Akala *et al.* (2011), the procedure for preparation of all reagents and media are enumerated in the step 3.2.2.

3.2.2. Preparation of reagents

3.2.2.1. Preparation of 1.45 mM hypoxanthine

Hypoxanthine solution was prepared by dissolving 40 milligrams of hypoxanthine in 200 ml of distilled water. This was boiled for 10 min and volume adjusted to 200ml. It was then cooled to 30℃, the solution filter sterilized using 0.2 µM filtration unit and stored at 2-8℃.

3.2.2.2. Preparation of RPMI basic medium (1 litre)

RPMI basic medium was prepared by adding 1 packet of RPMI 1640 powdered medium (10.4 g) in 850 ml of distilled water. At that time, 2 g of glucose and 5.95 g of HEPES was added and the mixture gently shaken until the contents are dissolved. This was filter sterilized using 0.2 µM filtration unit and stored at 2 - 8℃. This is used within one month.

3.2.2.3. Preparation of complete RPMI medium (50 ml)

Complete RPMI medium was prepared by adding 5.0 ml of pooled human plasma (blood group A, B and O) into 43.3 ml RPMI basic medium (prepared in step 3.2.2.2. above). 1.0 ml of 1.45 mM hypoxanthine and 1.6 ml of sodium bicarbonate (7.5 %) was added into the solution and mixed. The solution was filter sterilized using 0.2 µM filtration unit and stored at 2-8℃. The solution is good for use within 8 months.

3.2.2.4. Preparation of MSF lysis buffer (1L)

MSF lysis buffer was prepared by dissolving 15.76 g of Tris base in 1 litre of distilled water and mixed using a magnetic stirrer. The pH was adjusted using concentrated HCl to neutral range. 20 ml of 0.5 M EDTA, 160 mg saponin and 16 ml Triton X-100 was added into the solution and mixed thoroughly.

3.2.2.5. MSF lysis buffer containing SYBR Green 1 (11ml)

SYBR Green 1 (10,000 x) stock solution was thawed in room temperature in the dark. SYBR Green (10 µl) was added into 11 ml of MSF lysis buffer and mixed gently by swirling. This

was enough for termination of one 96-well plate. This solution is used fresh and away from direct light.

3.2.2.6. Quality control of Medias and solutions

The colour of the basic media was checked and should be pale straw. Lysis buffer should be clear with no bubbles. Both media should have no turbidity. To ensure sterility for the basic media, they were filtered using 0.2 µM filtration unit and stored done at 2-8℃ when not in use. All the preparations of reagents and *in vitro* procedures (other than termination) is carried out in a biosafety level 2 hood to ensure sterility and safety.

3.2.3. Optimization

Since the IC_{50} of the drugs were not known, the drug concentration range to be used for the cultures was determined by carrying out prescreening experiments. Starting concentration of 500 ng/ml and 50,000 ng/ml were used in the prescreen parasite cultures, for those that did not show any activity were done with a starting concentration of 100,000 ng/ml. A concentration above 100,000 ng/ml was not used since it would result in the final culture having dimethylsulfoxide (DMSO) concentration above 2 % which has been shown to inhibit the parasite growth *per se*. A 1:2 dilution was done across 12 well plates for each of the selected drug preparation in a 96-well plate. This resulted in a concentration range of 24 ng/ml to 50000 ng/ml and 0.24 ng/ml to 500 ng/ml as shown below in Table 3.2 below. Also a starting concentration of 100,000 ng/ml was used for those drugs that did not show any activity with 50,000 ng/ml.

Table 3.2: Concentrations of each drug used across 12 wells in a 96-well plate after a 1:2 dilution for starting concentrations of 50000 ng/ml and 500 ng/ml

Well plate position	Concentration (ng/ml)	
1	50000	500
2	25000	250
3	12500	125
4	6250	62.5
5	3125	31.25
6	1562.5	15.63
7	781.25	7.81
8	390.63	3.91
9	195.31	1.95
10	97.66	0.98
11	48.83	0.49
12	24.41	0.24

Two types of negative control cultures were done; one with DMSO and another without (had plain complete culturing media with serum). A control with DMSO was used because it was the solvent used to dissolve all the tested drugs including the drug controls. Therefore, it was imperative to access if DMSO had any inhibitory effects on parasite growth at the concentrations used. For reconstitution of the DMSO control, it was done as if it had a placebo drug with 10 mg/ml, just as the rest of the drugs.

3.2.4. Malaria parasites

Two reference clones of *P. falciparum* were use in this study; chloroquine sensitive 3D7 and artemisinin sensitive F32 TEM. All were obtained from Kenya Medical Research Institute-Walter Reed Project (KEMRI-WRP), Kisumu. Field isolates were also used, these were obtained from western Kenya under a study ethically approved by the Kenya Medical Research Institute (KEMRI) and Walter Reed Army Institute of Research (WRAIR) Institutional Review Boards (protocol numbers: KEMRI 1330 and WRAIR 1384 respectively), approval letters are shown in Appendix 2 and 3 respectively.

Inclusion criteria for the subjects who donated *Plasmodium* infected blood samples were; are patients attending outpatient clinics, at least 6 months old and suspected of having non-complicated *P. falciparum* malaria. Written informed consent was obtained from adult subjects ($\geq$ 18 years of age) or legal guardians for subjects $<$ 18 years of age. A copy of the consent form is included in Appendix 5. Persons treated for malaria within the last 2 weeks were excluded.

3.2.5. Parasite cryopreservation

Parasites were cryopreserved if they were not needed for immediate cultures. Parasites were first confirmed to have 4 - 6 % parasitaemia and be in the ring stage before cryopreservation. The parasite cultures were centrifuged at 2000 revolutions per minutes (rpm) for 10 minutes. The supernatant was discarded and the volume (V) of the pellet estimated. $\frac{1}{3}$ V of Glycerolyte 57 solution was then added dropwise and the tube gently shaken. Further mixing was done by pippeting in and out twice. The mixture was let to stand at room temperature for 5 minutes. A further $\frac{4}{3}$ V of Glycerolyte 57 solution was added and mixed with pippeting. This was then

transferred to previously labelled cryotube vials and transferred immediately to -20 °C and left overnight. For long term storage, it was transferred to a liquid nitrogen storage tank.

3.2.6. Parasite reviving

Parasite reviving is done to *P. falciparum* cultures that have been cryopreserved before they are cultured. Before removing vials of cryopreserved parasites from freezer, the solutions were prewarmed in 37 °C incubator. The vials containing the cryopreserved parasites were removed and thawed using warm tap water. The contents were then transferred into a 15 ml tube and their volume (V) noted. One fifth volume (V) of 12 % NaCl solution was added while swirling the tube. The contents were left to stand at room temperature for 5 minutes. Nine volumes of 1.6 % NaCl was then added and mixed gently. The contents were centrifuged at 1500 rpm for 3 minutes. The supernatant was aspirated and discarded. Nine volumes of 0.9 % NaCl with 0.2 % dextrose solution was then added and mixed gently. The contents were then centrifugued at 1500 rpm for 3 minutes and the supernatant discarded. The pellet was resuspended in 4.5 ml basic culture media and 0.5 ml of 50 % washed red blood cells to make a 5 ml culture. The culture was finally gassed with a gas mixture comprising 5 % CO_2, 5 % O_2, and 90 % N_2. Incubation was done at 37 °C.

Parasite viability was determined by doing blood smears of the cultures and staining with GIEMSA dye. This was done after every 72 hours for 3D7 and field isolates and 48 hours for F32 TEM. Total red blood cells infected with schizonts and those not infected were determined by microscopy and the percentage of those infected is calculated. The culture was used in drug susceptibility assays when the parasitaemia reached 3-8 %.

3.2.7. Parasite culturing

Complete RPMI 1640 culture medium was prepared as described in protocol 3.2.2.3. The media (150 µl) was pipetted into each well of the 96-well plate, first well of each row was excluded. Drugs to be tested were dissolved (from the stock solution of the drugs in DMSO) in complete RPMI 1640 culture medium to obtain a starting concentration of 1,000 ng/ml for Chloroquine, 250 ng/ml for mefloquine, 100 ng/ml for dihydroartemisinin and 50,000 ng/ml for the drug candidates. The prepared solutions was then pipetted (330 µl) into the first wells. The row of the specific drugs was noted.

Two-fold serial dilutions was then done by picking 150 µl from the first well, pippeting it to the second. This was mixed by pippeting eight times before transferring 150 µl to the next well. This was repeated to the 12th well. This resulted in a concentration range of chloroquine (1.953 to 1,000 ng/ml), mefloquine (0.488 to 250 ng/ml), dihydroartemisinin (0.2 to 100 ng/ml) and drug candidates (24 to 50,000 ng/ml). The amount of DMSO was equal or less than 0.5236 %. This 96-well plate was called a “mother plate” because it was used to prepare other plates called “daughter plates”.

From the mother plates, 12.5 µl was picked from each well using a multichannel micro-pippette and released into another 96-well “daughter plate”. This was done column by column, starting with the last column with the lowest concentration of the drugs. *In vitro* drug testing was initiated when 100 µl of culture-adapted *P. falciparum* (5 % haematocrit and greater than 3 % parasitaemia were adjusted to 2 % haematocrit and 0.5 % parasitaemia) was aliquoted to each well in the daughter plates containing a dose range of drugs. This was incubated in gas mixture comprising 5 % CO_2, 5 % O_2, and 90 % N_2 at 37 °C. The assay was terminated after 72 hours with addition of SYBR Green dye in lysis buffer (prepared as shown in protocol 3.2.2.5) and kept in dark for 24 hours at room temperature before reading (Cheruiyot *et al.*, 2016).

Table 3.3: Arrangement of drugs and their concentration ranges (in ng/ml) across a 96-well plate.

Concentrations (ng/ml)													
	<>	1	2	3	4	5	6	7	8	9	10	11	12
Chloroquine	A	2000	1000	500	250	125	62.5	31.25	15.625	7.8125	3.9063	1.9531	0.977
Mefloquine	B	500	250	125	62.5	31.25	15.63	7.813	3.90625	1.95313	0.9766	0.4883	0.244
Diadzin	C	50000	25000	12500	6250	3125	1563	781.3	390.625	195.313	97.656	48.828	24.41
Zafirlukast	D	50000	25000	12500	6250	3125	1563	781.3	390.625	195.313	97.656	48.828	24.41
Clofaribine	E	50000	25000	12500	6250	3125	1563	781.3	390.625	195.313	97.656	48.828	24.41
Cladribine	F	50000	25000	12500	6250	3125	1563	781.3	390.625	195.313	97.656	48.828	24.41
Dasatinib	G	50000	25000	12500	6250	3125	1563	781.3	390.625	195.313	97.656	48.828	24.41
DMSO negative control	H	50000	25000	12500	6250	3125	1563	781.3	390.625	195.313	97.656	48.828	24.41

3.2.8. Reading and analysis of the results

The fluorescence intensity was measured from the bottom of the plate with a GENios Plus plate reader, with excitation wavelengths of 485 nm, emission wavelengths of 535 nm, gain set at 60 and number of flashes at 10. IC_{50} values were obtained by analysis parasite growth inhibition using GraphPad Prism software version 5.02 from GraphPad Software, Inc., CA, USA , this is described by Johnson *et al.* (2007). Statistical analysis to determine possible errors of the fluorescence readings and their significance at 95 % confidence using a T test was done using the same software.

CHAPTER 4: RESULTS

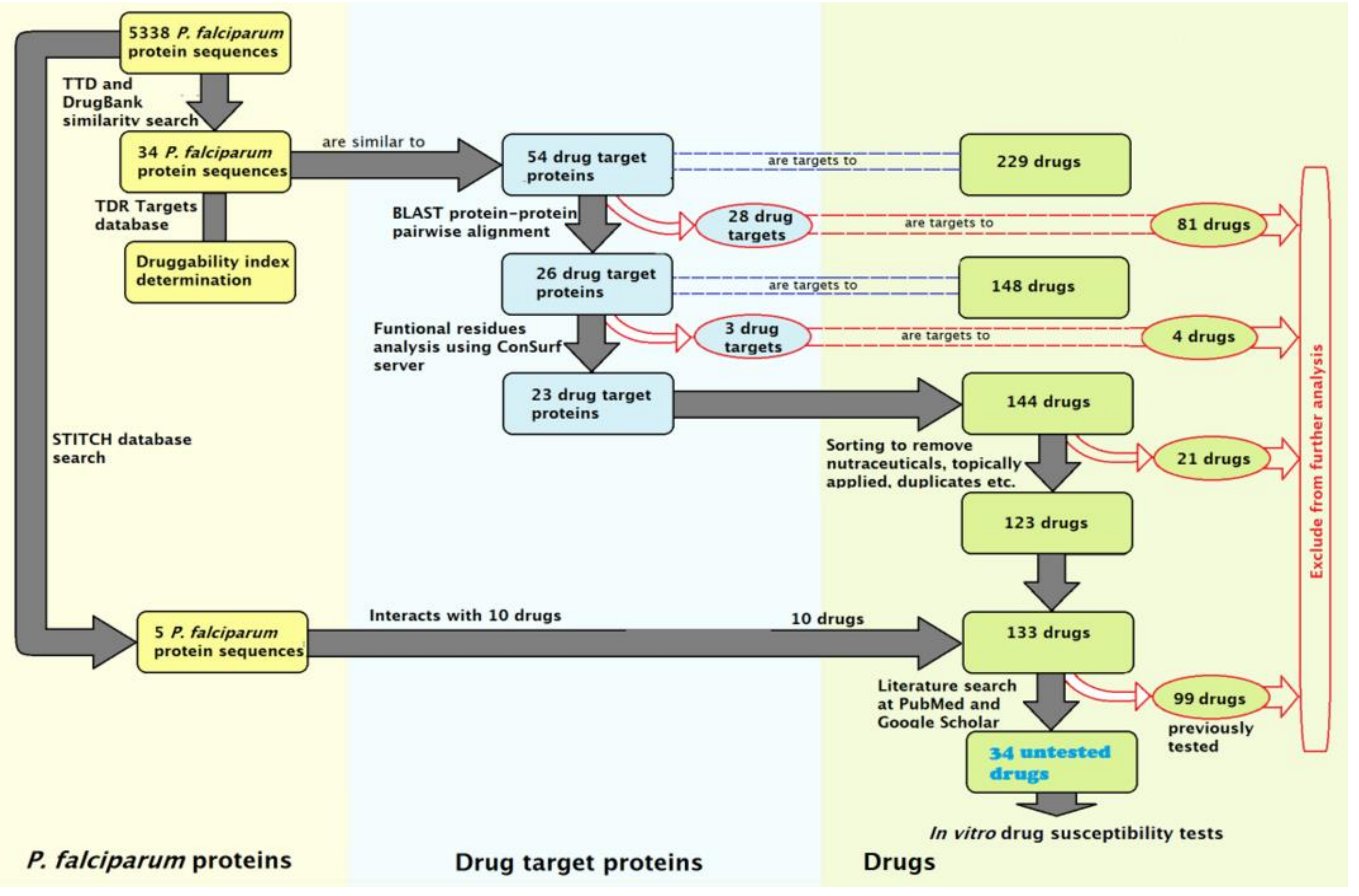


Figure 4.1: Summary of chemogenomics repositioning workflow and the corresponding results. The yellow boxes represents *P. falciparum* sequences, drug targets are shown in blue boxes and drugs are shown in green. Excluded drugs and proteins they target have red box outlines

The overall results for the steps in the study are illustrated in Figure 4.1. More comprehensive results for each step are described in consecutive sections.

4.1. Compilation of *P. falciparum* proteome

A total of 5338 protein sequences were obtained from RefSeq. This number represents all the *P. falciparum* protein sequences available at RefSeq release 75.

4.2. Identification of putative drug targets using drug databases

A total of 54 drug targets were associated with 229 approved drugs (Appendix 1). It is worth noting that some results were very much alike for many target searches. Some drugs had multiple targets, hence appeared more than once.

4.3. Druggability index

Druggability indices of 34 predicted drug targets was searched in TDR database and summarized in Table 4.1. Druggability ranges from 0 (least druggable) to 1 (most druggable). The least druggable drug target in the study had an index of 0.3 while 5 had the highest possible index of 1. Eight possible drug targets did not have their D index in TDR database.

Table 4.1: Druggability indices of predicted *P. falciparum* drug targets obtained from TDR targets database. They are sorted according to their indices in descending order.

***P. falciparum* protein**	**Accession number**	**Druggability index**
Inosine-5'-monophosphate dehydrogenase	XP_001352079.1	1
Tubulin beta chain	XP_001347369.1	1
Adenosine deaminase	XP_001347573.1	1
ADP/ATP transporter on adenylate translocase	XP_001347650.1	1
Ribonucleotide reductase small subunit	XP_001348226.1	1

Table 4.1 cont.

MO15-related protein kinase	XP_001347426.1	0.9
DNA topoisomerase II	XP_001348490.1	0.8
Histone deacetylase	XP_001352127.1	0.8
Ribonucleotide reductase small subunit	XP_001347439.2	0.8
Histone deacetylase	XP_001347363.1	0.7
CGMP-dependent protein kinase	XP_001348520.1	0.7
M1-family alanyl aminopeptidase	XP_001349846.1	0.6
FKBP type peptidyl-propyl isomerase	XP_001350859.1	0.6
Serine/threonine protein phosphatase	XP_001348315.1	0.6
Preprocathepsin c precursor	XP_001350862.2	0.6
Acetyl-coA acetyltransferase	XP_001348658.1	0.6
Cyclic nucleotide phosphodiesterase	XP_001348846.2	0.5
ABC transporter	XP_001348418.1	0.5
Calcium/calmodulin-dependent protein kinase	XP_001348401.2	0.5
Guanylyl cyclase	XP_001348065.1	0.5
Transporter	XP_001349605.2	0.5
Stromal-processing peptidase	XP_001348556.2	0.5
Acyl coA:diacylglycerol acyltransferase	XP_001351293.1	0.4
Guanylyl cyclase beta	XP_001350316.2	0.4
Cysteine proteinase falcipain-1	XP_001348727.1	0.3

Table 4.1 cont.

Flavodoxin-like protein	XP_002808949.1	0.3
Centrin-3	XP_001347555.2	Not available
CGMP-specific phosphodiesterase	XP_001350504.2	Not available
Delta-aminolevulinic acid dehydratase	XP_001348555.1	Not available
Ornithine aminotransferase	XP_966078.1	Not available
RNA binding protein	XP_001347313.1	Not available
3',5'-cyclic nucleotide phosphodiesterase	XP_001349954.1	Not available
Heat shock protein 110	XP_001349002.1	Not available
Ferrochelatase	XP_001350360.2	Not available

4.4. Determination and Comparison of functional regions

Protein pairwise alignment using BLAST revealed that 26 of 54 approved drug targets had more than 80 % cover with their corresponding *P. falciparum* proteins. These were analyzed using ConSurf while the rest (28) were excluded from further analysis. ConSurf server had a variety of results (Figure 4.3) which included multiple sequence alignment (MSA) with residues color coded according to their conservation scores, 3 dimension image of the protein with residues also color coded according to their conservation scores (Figure 4.2) and a spreadsheet with a more comprehensive analysis of the occurance of amino acids across the homologs (Appendix 6). This was snipped using the Windows 10 ® "Snipping tool" and aligned above the BLAST alignment results for visual comparison as illustrated in Figure 4.4**.** Percentage of shared functional residues (those with conservation score of more than six) was then calculated as shown in Figure 4.4. Eight drug targets shared more than 80 % of the functional regions with their homologous *P. falciparum,* this was categorized as high similarity. Eleven had moderate similarity (50-79 %), three had low similarity (less than 50 %) while four

had inconclusive outcomes since their ConSurf analyses couldn't be completed. Those with low similarity were eliminated from further analysis.

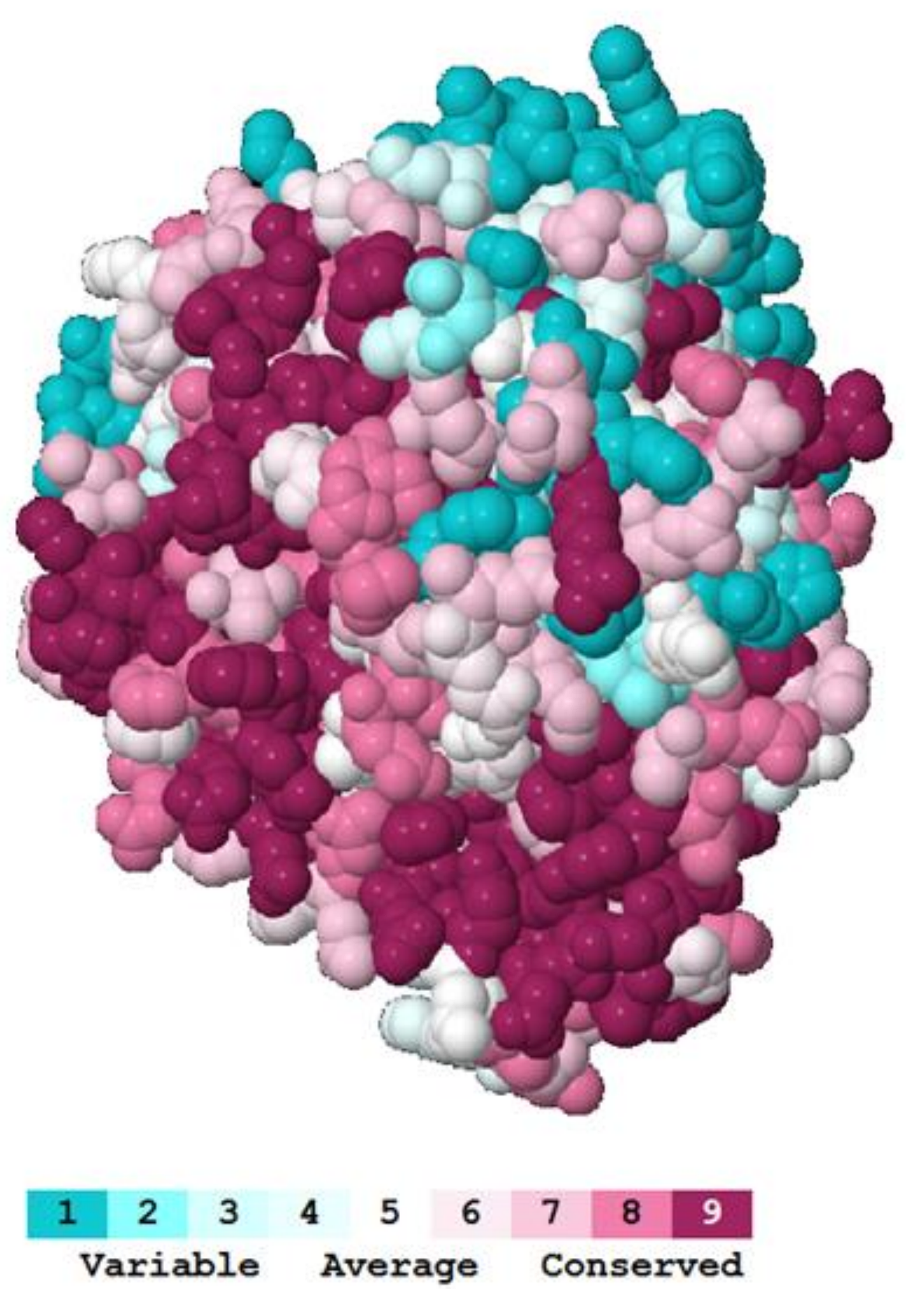


Figure 4.2: Three dimension image of a putative protein target (Q9UNQ0). This image was generated by Consurf server with amino acid residues color coded according to conservation scores.

4.5. Final drug lead list

A total of 154 drugs were predicted by *in silico* methods to target a 28 protein targets. Twenty one drugs were eliminated from drug lead list that were applied topically, protein based drugs and pure elements such as sodium. A literature search of the 133 remaining drugs revealed that 99 drugs to have undergone testing for antimalarial activity. These were excluded from the final drug lead list. Some of the previously tested drugs and their IC_{50} are summarized in Table 4.3. Table 4.2 shows 34 drugs out of the 133 that have not been tested. Drugs such as Cladribine, which had been tested but the IC_{50} not indicated in the study (Bobbala *et al.*, 2009) was also included among the 34 drugs. For validation of antiplasmodial effect, some of the drugs were earmarked for *in vitro* drug susceptibility testing to validate their antiplasmodial activity. Twelve of the 34 drugs were taken through *in vitro* drug susceptibility tests using *P. falciparum* 3D7, F32 TEM and field isolates.

Table 4.2: List of all the drugs predicted to have antimalarial activity that have not been tested

DRUG	UNIPROT ID	Accession number	ConSurf Results	Druggability
Cladribine	P00813	XP_001347573.1	55 %	1
Fludarabine	P00813	XP_001347573.1	55 %	1
Moxifloxacin	P11388	XP_001348490.1	61 %	0.8
Epirubicin	P11388	XP_001348490.1	61 %	0.8
Levofloxacin	P11388	XP_001348490.1	61 %	0.8
Finafloxacin	P11388	XP_001348490.1	61 %	0.8
Palbociclib	P11802	XP_001347426.1	54 %	0.9

Table 4.2 cont.

Capridine-beta	P24941	XP_001347426.1	70 %	0.9
Motexafin gadolinium	P31350	XP_001347439.2	60 %	0.8
Aprindine	P62158	XP_001347555.2	76 %	-
Venlafaxine	Q9UNQ0	XP_001348418.1	51 %	0.5
Zidovudine	Q9UNQ0	XP_001348418.1	51 %	0.5
Oxaliplatin	Q9UNQ0	XP_001348418.1	51 %	0.5
Erlotinib	Q9UNQ0	XP_001348418.1	51 %	0.5
Zafirlukast	Q9UNQ0	XP_001348418.1	51 %	0.5
Clofarabine	Q9UNQ0	XP_001348418.1	51 %	0.5
Sumatriptan	Q9UNQ0	XP_001348418.1	51 %	0.5
Irinotecan	Q9UNQ0	XP_001348418.1	51 %	0.5
Buprenorphine	Q9UNQ0	XP_001348418.1	51 %	0.5
Idelalisib	Q9UNQ0	XP_001348418.1	51 %	0.5
Cobicistat	Q9UNQ0	XP_001348418.1	51 %	0.5
Lenvatinib	Q9UNQ0	XP_001348418.1	51 %	0.5
Daclatasvir	Q9UNQ0	XP_001348418.1	51 %	0.5
Osimertinib	Q9UNQ0	XP_001348418.1	51 %	0.5
Alectinib	Q9UNQ0	XP_001348418.1	51 %	0.5
Pitavastatin	Q9UNQ0	XP_001348418.1	51 %	0.5
Rilpivirine	Q9UNQ0	XP_001348418.1	51 %	0.5

Table 4.2 cont.

Apixaban	Q9UNQ0	XP_001348418.1	51 %	0.5
Pazopanib	Q9UNQ0	XP_001348418.1	51 %	0.5
Vandetanib	Q9UNQ0	XP_001348418.1	51 %	0.5
Biricodar dicitrate	Q9UNQ0	XP_001348418.1	51 %	0.5
Dasatinib	Q9UNQ0	XP_001348418.1	51 %	0.5
Daidzin	Q9UNQ0	XP_001348418.1	51 %	0.5
Cabazitaxel	Q9UNQ0	XP_001348418.1	51 %	0.5
Tacrolimus	STITCH	XP_001350859.1	-	0.6

The details of 36 previously tested drugs are shown in Table 4.3. In addition to their IC_{50}, this table also shows their approved indications, putative drug targets, druggability index (ranges from 0.5 to 1) and references of studies in which the drugs were tested for antiplasmodial activity.

Table 4.3: Examples of previously tested drugs correctly predicted in this study with their indication, IC_{50}, (all IC_{50} values have been converted to µM), putative drug targets, druggability index and references to their corresponding publication

***P. falciparum* target, druggability index, % similarity of the functional regions**	**Drug**	**Indication**	**Antiplasmodial activity (IC_{50})**	**References**
ABC transporter Druggability index: 0.5 Moderate conservation (51 %)	Dactinomycin	Antibiotic	0.0009 µM	(Lotharius *et al.*, 2014)
	Cisplatin	Anticancer	0.02067 µM	(Nair & Bhasin, 1994)
	Cyclosporine	Immunosuppressant	0.032 µM	(Bell *et al.*, 1994)
	Docetaxel	Anticancer	~0.01 µM	(Sinou *et al.*, 1996)

Table 4.3 cont.

	Doxorubicin	Antibiotic	0.21 µM	(Lotharius *et al.*, 2014)
	Ivermectin	Antiparasitic	9.14 µM	(Nasveld *et al.,* 2003)
	Lamivudine	Antiretroviral	> 50 µM	(Nsanzabana & Rosenthal, 2011)
	Saquinavir	Antiretroviral	5 µM	(Nsanzabana & Rosenthal, 2011)
	Vincristine	Anticancer	0.00205 µM	(Mahmoudi *et al.*, 2003)
DNA Topoisomerase II Druggability index: 0.8 Moderate conservation (61 %)	Amsacrine	Cutaneous T Cell Lymphoma	0.1 to 2.8 µM	(Figgitt *et al.*, 1992)
	Ciprofloxacin	Antibiotic	19.92 µM	(Shanks *et al.*, 1991)
	Enoxacin	Antibiotic	121.1 µM	(Mahmoudi *et al.*, 2003)
	Fleroxacin	Antibiotic	93.68 µM	(Mahmoudi *et al.*, 2003)
	Lomefloxacin	Antibiotic	>284.62 µM	(Mahmoudi *et al.*, 2003)
	Lovastatin	Hypolipidemic	>200 µM	(Pradines *et al.*, 2007)
	Norfloxacin	Antibiotic	55.74 µM	(Mahmoudi *et al.*, 2003)
	Ofloxacin	Antibiotic	177.10 µM	(Mahmoudi *et al.*, 2003)
	Pefloxacin	Antibiotic	258.88 µM	(Mahmoudi *et al.*, 2003)
	Sparfloxacin	Antibiotic	135.06 µM	(Mahmoudi *et al.*, 2003)
	Trovafloxacin	Antibiotic	27.5 µM	(Hamzah *et al.,* 2000)
	Dactinomycin	Antibiotic	0.0009 µM	(Lotharius *et al.*, 2014)
Histone deacetylase Druggability index: 0.8 High conservation (99 %)	Trichostatin A	Antifungal, Antibiotic	--	(Andrews *et al.*, 2000)
	Valproic Acid	Epilepsy And Seizures Treatment	209.34 µM	(Mahmoudi *et al.*, 2003)

Table 4.3 cont.

	Vorinostat	Cutaneous T Cell Lymphoma	0.12 µM	(Engel *et al.*, 2015)
Inosine-5'-Monophosphate Dehydrogenase Druggability index: 1 No conservation %	Azathioprine	Immunosuppressant	≥ 1 µM	(Bobbala *et al.*, 2009)
Inosine-5'-monophosphate dehydrogenase Druggability index: 1 Moderate conservation (79 %)	Mycophenolic Acid	Immunosuppressant	5.4 µM	(Veletzky *et al.*, 2014)
Ribonucleotide reductase small subunit Druggability index: 0.8 Moderate conservation (60 %)	Cladribine	Leukemia	⩾0.01 µM	(Bobbala *et al.*, 2009)
Serine/threonine protein phosphatase Druggability index: 0.6 High conservation (99 %)	Cantharidin	Warts	3 µM	(Bajsa *et al.*, 2010)
Tubulin beta chain Druggability index: 1 High conservation (99 %)	Albendazole	Anthelmintic	2 µM	(Lotharius *et al.*, 2014)
	Vinblastine	Anticancer	0.007175 µM	(Mahmoudi *et al.*, 2003)
	Vindesine	Anticancer	0.00599 µM	(Mahmoudi *et al.*, 2003)
	Vincristine	Anticancer	0.00205 µM	(Mahmoudi *et al.*, 2003)
Cyclic nucleotide phosphodiesterase Druggability index: 0.5, NA	Dipyridamole	Anticoagulants	0.03 µM	(Akaki *et al.*, 2002)
Adenosine deaminase Druggability index: 1 Moderate conservation (55%)	Dipyridamole	Anticoagulants	0.03 µM	(Akaki *et al.*, 2002)
Centrin-3 Druggability index: NA Moderate conservation (76 %)	Trifluoperazine	Antipsychotic, Antiemetic	0.46626 µM	(Menezes *et al.*, 2002)

Table 4.3 cont.

Calcium/calmodulin-dependent protein kinase Druggability index: 0.5 Low conservation (47 %)	Bosutinib	Chronic myelogenous leukemia (CML)	0.22 μM	(Lotharius *et al.*, 2014)

4.6. *In vitro* drug susceptibility assays

A total of 12 drugs predicted to have antiplasmodial activity using *in silico* methods were subjected to *in vitro* drug susceptibility tests. The florescence readings for the 96 well-plates were in a table form, Table 4.4 show one of the readings. These readings were analyzed using Graphpad Prism generating graphs that displayed the IC_{50} and R square values (Figure 4.5 to Figure 4.9).

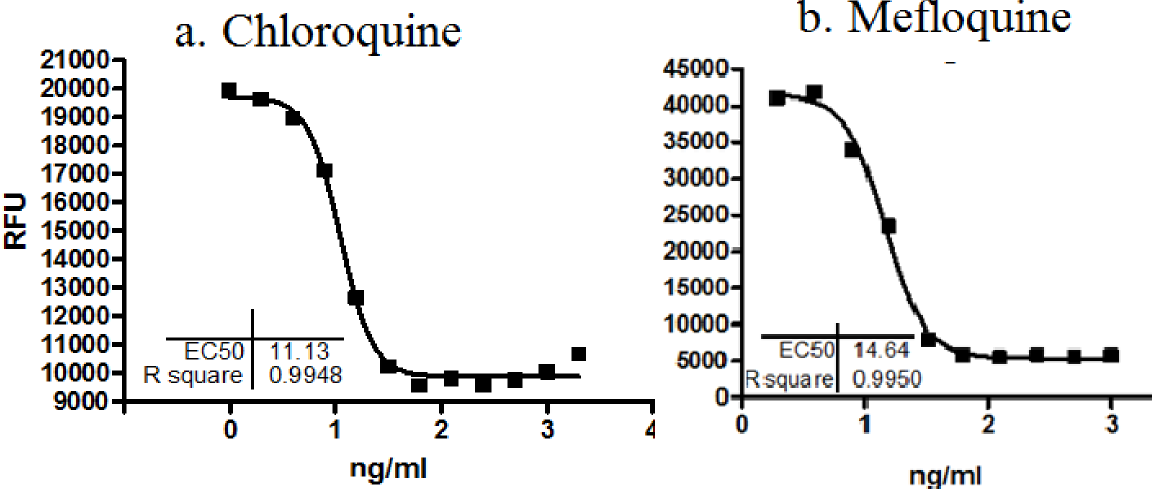
The IC_{50} values in triplicates for the drugs tested on *P. falciparum* 3D7 are summarized in Table 4.5 for. Table 4.6 showed IC_{50} values for F32 TEM and field isolates (was run once). Errors in the 3D7 readings were determined by statistical analysis using Graphpad Prism and are shown in Table 4.7. This showed all readings were significant, with their P values less than 0.05.

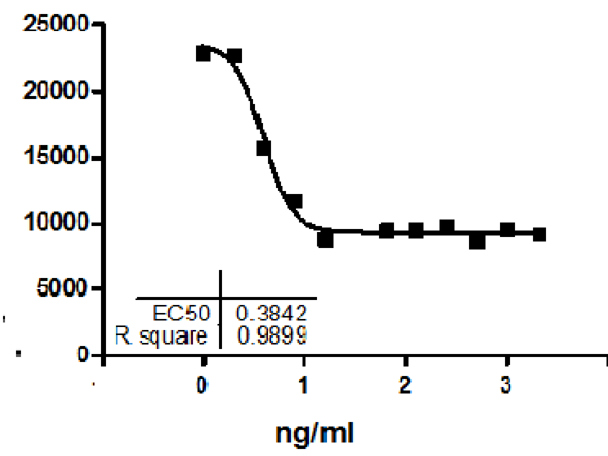
Chloroquine, artemisinin and mefloquine IC_{50} results are also shown in the same tables. These controls had their IC_{50} within acceptable ranges with Chloroquine having an IC_{50} of 0.01240 μM, Mefloquine 0.05893 μM and Dihydroartemisinin 0.002610 μM. Only Diadizin and Zafirlukast did not show significant activity at starting concentrations of 50,000 ng/ml, i.e. their activity was similar to negative controls. All drugs other than Oxaliplatin did not show any activity with a starting concentration of 500 ng/ml either. Oxaliplatin showed the highest antiplasmodial activity with an IC_{50} of 1.156 μM. There was no significant difference in the negative control growth profiles, with or without DMSO.

Table 4.4: Fluorescence readings of a 96-well plate *in vitro* culture with excitation wavelengths of 485 nm and emission wavelengths of 535 nm.

Flourescence readings													
	<>	1	2	3	4	5	6	7	8	9	10	11	12
Chloroquine	A	5072	5351	5303	5198	5677	5289	5342	6284	18965	22732	21091	24031
Mefloquine	B	5058	5326	5368	5450	6249	11539	19950	19301	24450	25133	25386	22741
Diadzin	C	24109	23667	20757	23289	20276	21054	21385	19914	23009	21999	23474	19119
Zafirlukast	D	5529	15251	19933	22068	22228	23046	21983	20178	23678	21484	24231	25022
Clofaribine	E	5214	6891	12044	19463	23794	20464	21966	19707	23524	22467	22781	16691
Cladribine	F	6100	12819	17393	21884	21720	23198	20265	21798	22847	23656	25767	20330
Dasatinib	G	6316	5968	5682	15493	19804	23597	21121	21642	22642	27581	30544	21710
DMSO	H	17879	21263	17133	21312	22548	21946	20214	21331	21319	19624	18994	20675

Positive controls





Negative controls

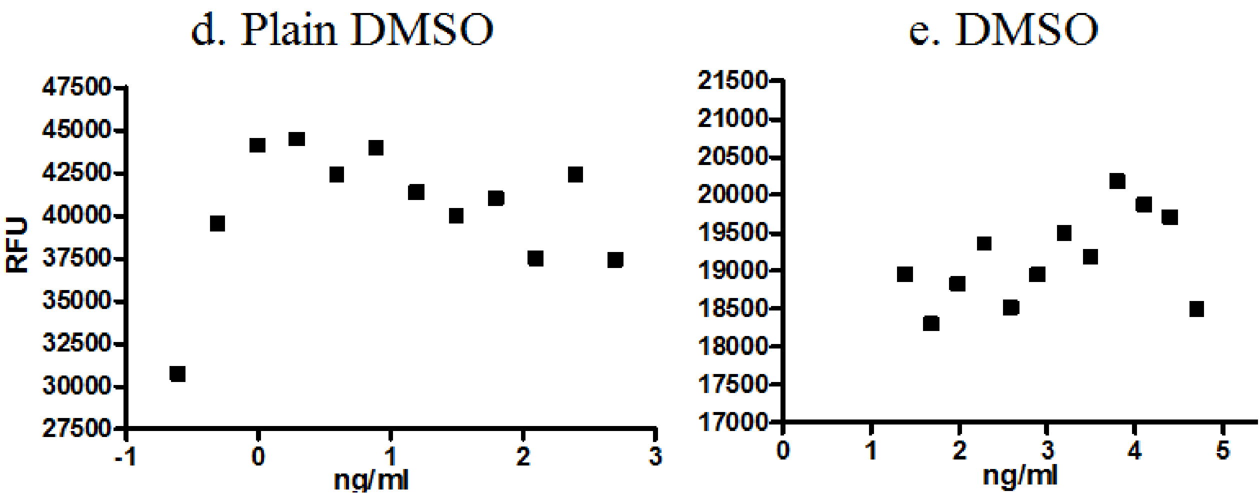


Figure 4.3: ***In vitro*** **assay analysis graphs for controls tested on** ***P. falciparum*** **3D7.**

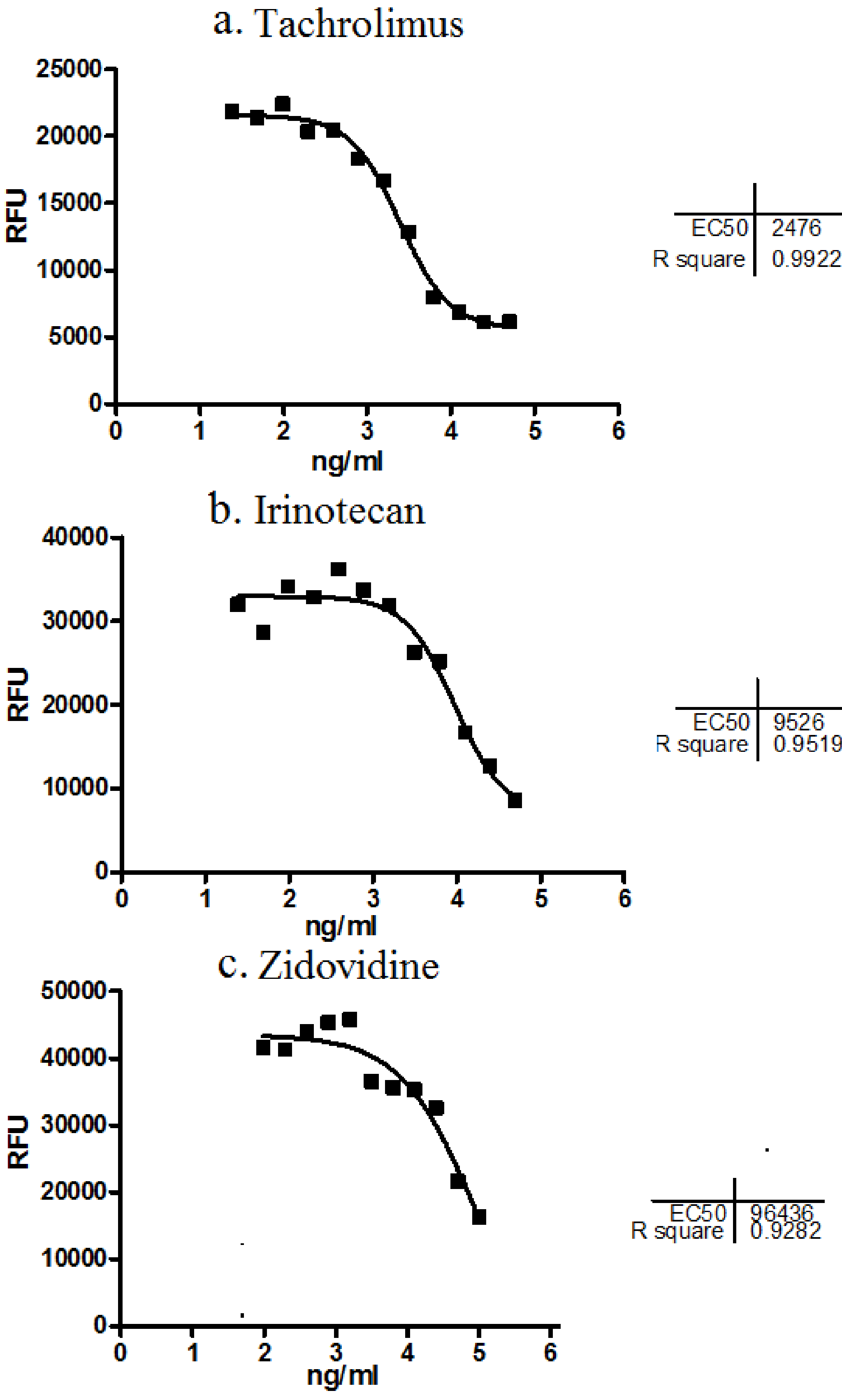


Figure 4.4: ***In vitro*** **assay analysis graphs for Tacrolimus (a), Irinorectan (b) and Zidovudine (c) tested on** ***P. falciparum*** **3D7.**

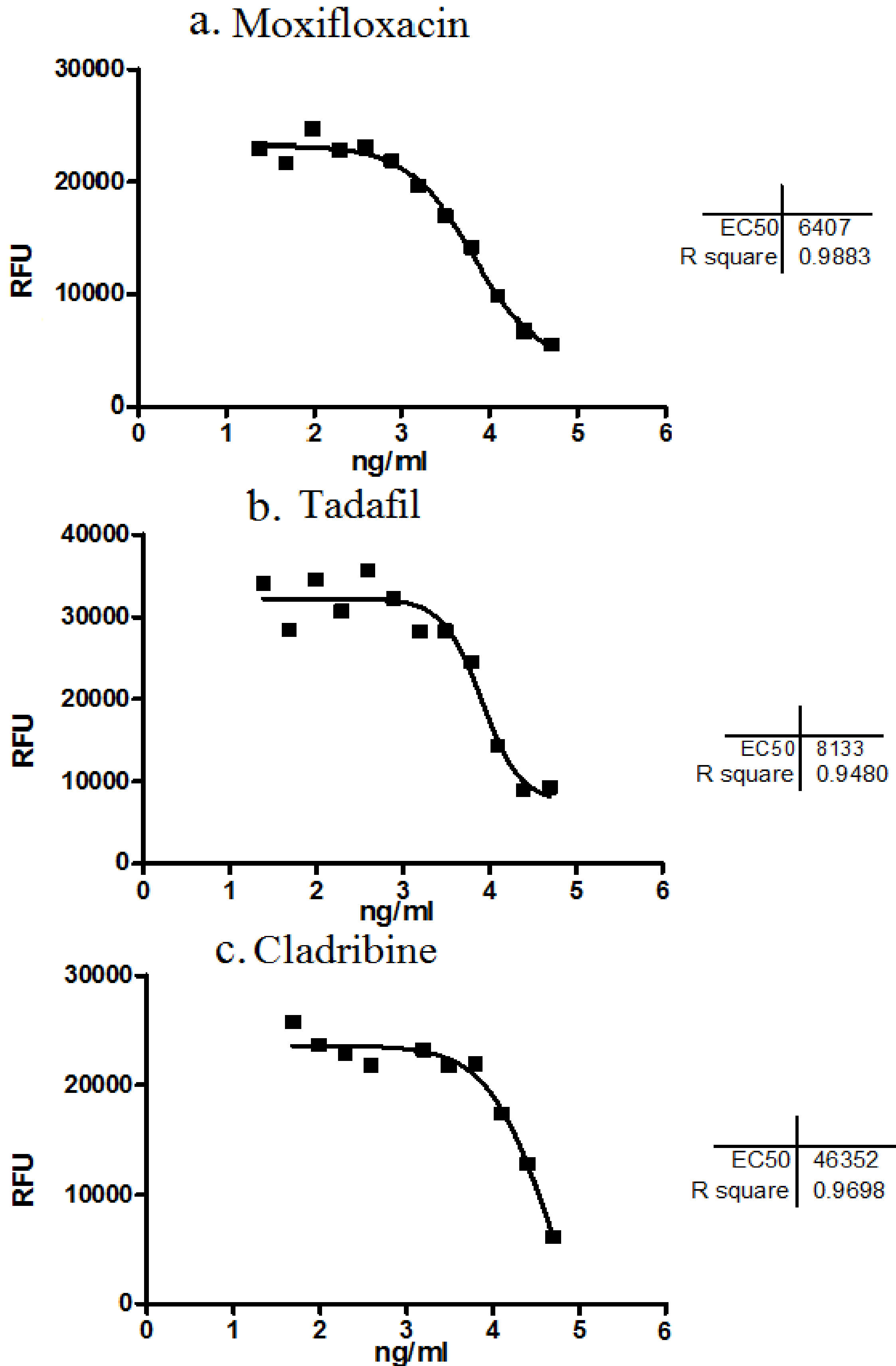


Figure 4.5: ***In vitro*** **assay analysis graphs for Moxifloxacin (a), Irinorectan (b) and Cladrine (c) tested on** ***P. falciparum*** **3D7.**

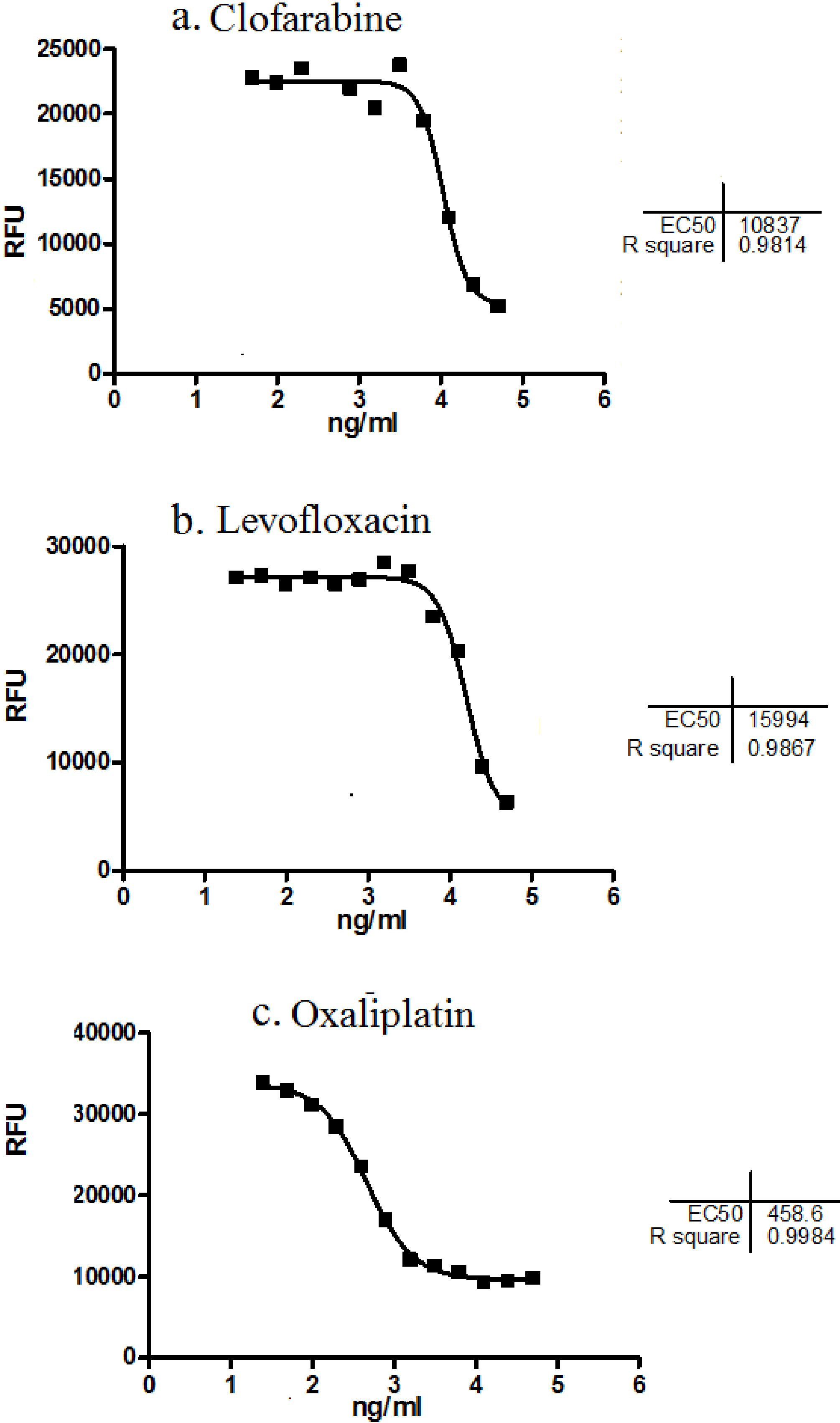


Figure 4.6: ***In vitro*** **assay analysis graphs for Clofarabine (a), Levofloxacin (b) and Oxaliplatin (c) tested on** ***P. falciparum*** **3D7.**

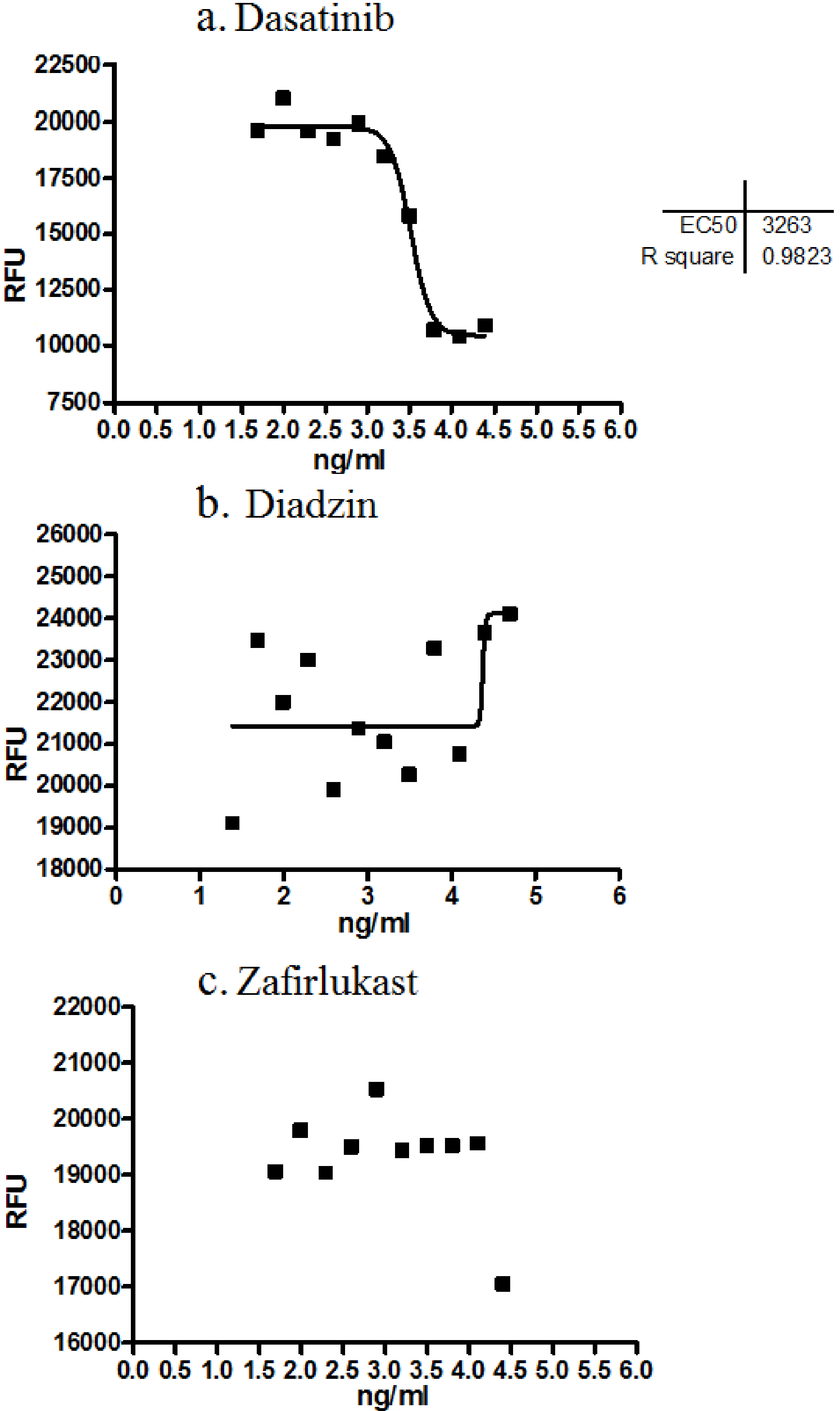


Figure 4.7: ***In vitro*** **assay analysis graphs for Dasatinib (a), Diadzin (b) and Zafirlukast (c) tested on** ***P. falciparum*** **3D7.**

Table 4.5: Antiplasmodial activity of drugs tested against *P. falciparum* 3D7 strain (IC_{50} are in µM)

	IC_{50} in µM			
REPLICATES	**1**	**2**	**3**	**Mean ± SD**
Tadafil	25.71109	20.88576	23.27917	23.2 ± 2.413
Irinotecan	12.94236	16.23719	13.8611	14.35 ± 1.7
Levofloxacin	44.25959	34.16462	41.87144	40.1 ± 5.276
Oxaliplatin	1.154321	1.057162	1.257771	1.156 ± 0.1003
Zidovudine	198.251	191.9608	195.0854	195.1 ± 3.145
Clofarabine	46.89193	50.95546	48.99943	48.95 ± 2.032
Moxifloxacin	11.70811	11.18748	15.9604	12.95 ± 2.618
Tacrolimus	4.614325	4.497412	4.452637	4.521 ± 0.08348
Cladribine	94.78205	113.0258	80.24516	96.02 ± 16.43
Dasatinib	7.954755	11.07149	6.770353	8.599 ± 2.222
Reference drugs				
Chloroquine	0.011326	0.011289	0.012055	0.01156 ± 0.000432
Dihydroartemisinin	0.002619	0.002741	0.002559	0.00264 ± 0.0000929
Mefloquine	0.033702	0.038698	0.046126	0.03951 ± 0.006251

Table 4.6: Antiplasmodial activity of drugs tested against *P. falciparum* in F32 TEM strain and field isolates, IC_{50} are in μM

Drug	F32 TEM	Field isolates
Cladribine	109.402	55.585
Diadzin	NA	NA
Zafirlukast	NA	NA
Levofloxacin	45.687	64.795
Dasatinib	6.641	9.719
Clofarabine	82.709	27.029
Tacrolimus	4.253	3.808
Irinotecan	28.507	13.861
Zidovudine	201.676	91.744
Moxifloxacin	19.703	11.187
Tadafil	41.871	-
Oxaliplatin	1.035	-
Chloroquine	0.01287	0.01081
Mefloquine	0.08326	0.05930
Dihydroartemisinin	0.01461	0.007498

Table 4.7: Statistical analysis for significance and errors in IC_{50} values (3D7)

	Significant (alpha=0.05) ?	P value (two tailed)	T value, degree of	One sample t test	Upper 95% CI of mean	Lower 95% CI of mean	Std. Error	Std. Deviation	Mean (µM)
Tadafil	Yes	0.0036	t=16.72 df=2		29.29	17.3	1.393	2.413	**23.29**
Irinotecan	Yes	0.0046	t=14.61 df=2		18.57	10.12	0.9817	1.7	**14.35**
Levofloxacin	Yes	0.0057	t=13.16 df=2		53.2	26.99	3.046	5.276	**40.1**
Oxaliplatin	Yes	0.0025	t=19.97 df=2		1.406	0.9072	0.05792	0.1003	**1.156**
Zidovudine	Yes	< 0.0001	t=107.4 df=2		202.9	187.3	1.816	3.145	**195.1**
Clofarabine	Yes	0.0006	t=41.72 df=2		54	43.9	1.173	2.032	**48.95**
Moxifloxacin	Yes	0.0134	t=8.568 df=2		19.46	6.448	1.512	2.618	**12.95**
Tacrolimus	Yes	0.0001	t=93.81 df=2		4.729	4.314	0.0482	0.08348	**4.521**
Cladribine	Yes	0.0096	t=10.13 df=2		136.8	55.22	9.483	16.43	**96.02**
Dasatinib	Yes	0.0215	t=6.704 df=2		14.12	3.08	1.283	2.222	**8.599**
Chloroquine	Yes	0.0005	t=16.72 df=3		0.01263	0.01048	0.000249	0.000432	**0.01156**
Dihydroartemisinin	Yes	0.0004	t=14.61 df=3		0.00287	0.002409	5.36E-05	9.29E-05	**0.00264**
Mefloquine	Yes	0.0082	t=13.16 df=3		0.05504	0.02398	0.003609	0.006251	**0.03951**

CHAPTER 5: DISCUSSION

The likelihood of identifying new approved drugs with antiplasmodial activity could reduce the time and cost in developing new drugs for antimalarial chemotherapy. This study used target similarity to correctly predict drugs that had activity against *P. falciparum*. This is made possible several databases with information on drugs; this includes already approved drugs, some in the process of gaining approval while others are still under investigation. There is a wealth of information on approved drugs because they have undergone several studies. Consequently, many approved drugs have their putative target and mechanisms of actions well elucidated, making their repurposing easier (Huang *et al.*, 2011).

In this study, the full proteome sequences for *P. falciparum* available at RefSeq was used to do a sequence similarity search on targets of approved drugs. Previously individual targets at Protein Data Bank have been used for repurposing (Moriaud *et al.*, 2011). Out of the 5338 *P. falciparum* protein sequences, only 34 possible drug targets (associated with 229 approved drugs) met similarity threshold set by this study, i.e. E values below 1e-20 for DrugBank and TTD. 5 drugs had a confidence score above 0.7 for STITCH database. Using the same parameters in similar study for *Schistosomiasis mansoni*, 49 protein targets associated with 276 approved drugs were identified (Neves *et al.*, 2015). Bispo *et al.*, (2013) used an E value threshold of 1e-5 to identify drug targets similar to *P. falciparum* apicoplast proteins in DrugBank and TTD databases. In this study, they identified 72 possible targets. Bispo *et al.*, (2013) used only part of the *P. falciparum* proteome as queries (595 apicoplast proteins sequences), but they identified more similar proteins because of the larger E value. The use of a lower E value threshold had the benefit of increasing the likelihood of finding protein targets that had shared higher similarity to *P. falciparum* proteins .This had the advantage of increasing the likelihood of discovering drugs that could have similar effects. This also reduced the

number of proteins that would be analyzed in downstream processes such as the ConSurf analysis, these processes could be very time and resource consuming.

During the sequence similarity search at TTD and DrugBank databases, there was repetition in some results; some *P. falciparum* proteins produced similar results. Interestingly, a study by Suthram *et al.*, (2005) showed 60% *Plasmodium* proteins to lack significant similarity to most eukaryotes. Similarity in some proteins search results could be attributed to paralogous nature of these *P. falciparum* proteins and the homologous relationship with some drug targets. Of the *Plasmodium* proteins, *Plasmodium* merozoite surface protein 1 (PMSP1) was the most common. One reason why PMSP1 came up quite often in the searches could be probably due to paralogous relationship between it and some *Plasmodium* proteins. Therefore, if one used *P. falciparum* proteins as queries, it is not a strange for PMSP1 to come up severally. The PMSP1 antigen was among the drug targets because it is as a target for vaccines in clinical trials (Malkin *et al.*, 2007; Stowers *et al.*, 2001). Other examples of proteins targets that showed up quite frequently include troponin C, heat shock protein 40, calmodulin, centromeric protein E and Rho-associated protein kinase 1.

The drugs that we tested have been shown to target different proteins from the antimalarials currently being used. Mechanism of action of these drugs are shown in Table 5.1, this data was obtained from DrugBank (http://www.drugbank.ca). This implies that they are less likely to be affected by resistance that is affecting the current antimalarials. Though it is important to note that the antiplasmodial activity shown by these predicted drugs could be due to side targets not identified yet. Their mechanism of action would need to be validated by biochemical assays

Table 5.1: Mechanism of action, physiochemical properties and selected details of tested drug, information got from DrugBank (Knox *et al.*, 2011).

Name And DrugBank Accession Number, IUPAC Name	**Molecular formula and mass And Water solubility**	**Structure**	**Mechanism of action**
Cladribine (DB00242) **IUPAC Name:** (2R,3S,5R)-5-(6-amino-2-chloro-9H-purin-9-yl)-2-(hydroxymethyl) oxolan-3-ol	**Formula:** $C_{10}H_{12}ClN_5O_3$ **MW:** 285.687 g/mol **Water Solubility:** 6.35 mg/mL		Cladribine is phosphorylated to nucleotidecladribine triphosphate (CdATP; 2-chloro-2′-deoxyadenosine 5′-triphosphate) by enzyme deoxycytidine kinase. This accumulates and is incorporated into DNA in cells such as lymphocytes that contain high levels of deoxycytidine kinase and low levels of deoxynucleotidase, resulting in DNA strand breakage and inhibition of DNA synthesis and repair
Moxifloxacin (DB00218) **IUPAC Name:** 7-[(4aS,7aS)-octahydro-1H-pyrrolo[3,4-b]pyridin-6-yl]-1-cyclopropyl-6-fluoro-8-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid	**Formula:** $C_{21}H_{24}FN_3O_4$ **MW:** 401.175 g/mol **Water Solubility:** 0.168 mg/mL		Moxifloxacin acts by inhibition of the enzymes DNA gyrase, (an essential enzyme involved in replication, transcription and repair of bacterial DNA) and topoisomerase IV (plays a key role in the partitioning of the chromosomal DNA during bacterial cell division).

Table 5.1 cont.

Levofloxacin (DB01137) **IUPAC Name:** (2S)-7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo[7.3.1.$0^{5,13}$]trideca-5(13),6,8,11-tetraene-11-carboxylic acid	**Formula:** $C_{18}H_{20}FN_3O_4$ **MW:** 361.368 g/mol **Water Solubility:** 1.44 mg/mL		Levofloxacin inhibits bacterial type II topoisomerases, topoisomerase IV and DNA gyrase, resulting in strand breakage on bacterial chromosomes, supercoiling, and resealing; this leads to inhibition of DNA replication and transcription.
Aprindine (DB01429) **IUPAC Name:** N-[3-(diethylamino)propyl]-N-phenyl-2,3-dihydro-1H-inden-2-amine	**Formula:** $C_{22}H_{30}N_2$ **MW:** 322.487 g/mol **Water Solubility:** 0.00782 mg/mL		Not available
Zidovudine (DB00495) **IUPAC Name:** 1-[(2R,4S,5S)-4-azido-5-(hydroxymethyl)oxolan-2-yl]-5-methyl-1,2,3,4-tetrahydropyrimidine-2,4-dione	**Formula:** $C_{10}H_{13}N_5O_4$ **MW:** 267.2413 g/mol **Water Solubility:** 16.3 mg/mL		Zidovudine is a structural analog of thymidine. It is first phosphorylated to its active 5′-triphosphate metabolite; zidovudine triphosphate (ZDV-TP). Zidovudine inhibits the activity of HIV-1 reverse transcriptase (RT) causing DNA chain termination after incorporation of ZDV-TP. Zidovudine competes with the natural substrate dGTP and incorporates itself into viral DNA. Zidovudine is also a weak inhibitor of cellular DNA polymerase α and γ.

Table 5.1 cont.

Oxaliplatin (DB00526) **IUPAC Name:** (3aR,7aR)-octahydro-2',5'-dioxaspiro[cyclohexa[d]1,3-diaza-2-platinacyclopentane-2,1'-cyclopentane]-3',4'-dione	**Formula:** $C_8H_{14}N_2O_4Pt$ **MW:** 397.294 g/mol **Water Solubility:**		Oxaliplatin is first activated by displacement of the labile oxalate ligand. It then binds preferentially to the guanine and cytosine moieties of DNA. This leads to cross-linking of DNA, hence inhibiting DNA synthesis and transcription.
Zafirlukast (DB00549) **IUPAC Name:** cyclopentyl N-[3-({2-methoxy-4-[(2-methylbenzenesulfonyl) carbamoyl]phenyl}methyl)-1-methyl-1H-indol-5-yl]carbamate	**Formula:** $C_{31}H_{33}N_3O_6S$ **MW:** 575.675 g/mol **Water Solubility:** 0.000962 mg/mL		Zafirlukast is a selective and competitive receptor antagonist of leukotriene D4 and E4 (LTD4 and LTE4) which are components of slow-reacting substance of anaphylaxis.
Clofarabine (DB00631) **IUPAC Name:** (2R,3R,4S,5R)-5-(6-amino-2-chloro-9H-purin-9-yl)-4-fluoro-2-(hydroxymethyl)oxolan-3-ol	**Formula:** $C_{10}H_{11}ClFN_5O_3$ **MW:** 303.677 g/mol **Water Solubility:** 4.89 mg/mL		Clofarabine is metabolized to 5'-monophosphate metabolite by deoxycytidine kinase and 5'-triphosphate metabolite by mono- and di-phospho-kinases, it inhibits DNA synthesis through an inhibitory action on ribonucleotide reductase, and by terminating DNA chain elongation and inhibiting repair through competitive inhibition of DNA polymerases.

Table 5.1 cont.

Irinotecan (DB00762) **IUPAC Name:** (19S)-10,19-diethyl-19-hydroxy-14,18-dioxo-17-oxa-3,13-diazapentacyclo[11.8.0.$0^{2,11}$.$0^{4,9}$.$0^{15,20}$]henicosa-1(21),2,4(9),5,7,10,15(20)-heptaen-7-yl 4-(piperidin-1-yl)piperidine-1-carboxylate	**Formula:** $C_{33}H_{38}N_4O_6$ **MW:** 586.678 g/mol **Water Solubility:** 0.107 mg/mL		Irinotecan inhibits the action of topoisomerase preventing religation of the DNA strand by binding to topoisomerase I-DNA complex. This ternary complex interferes with the moving replication fork, which induces replication arrest and lethal double-stranded breaks in DNA. Due to this, DNA damage is not efficiently repaired and apoptosis (programmed cell death) is initiated.
Dasatinib (DB01254) **IUPAC Name:** N-(2-chloro-6-methylphenyl)-2-({6-[4-(2-hydroxyethyl)piperazin-1-yl]-2-methylpyrimidin-4-yl}amino)-1,3-thiazole-5-carboxamide	**Formula:** $C_{22}H_{26}ClN_7O_2S$ **MW:** 488.006 g/mol **Water Solubility:** 0.0128 mg/mL		Dasatinib is predicted to bind to multiple conformations of the ABL kinase and has been shown to inhibit a number of kinases: BCR-ABL, SRC family (SRC, FYN, YES, LCK), c-KIT, PDGFRβ, and EPHA2.

Table 5.1 cont.

Daidzin (DB02115) **IUPAC Name:** 3-(4-hydroxyphenyl)-7-{[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-4H-chromen-4-one	**Formula:** $C_{21}H_{20}O_9$ **MW:** 416.3781 g/mol **Water Solubility:** NA		NA
Tacrolimus (DB00864) **IUPAC Name:** (1R,9S,12S,13R,14S,17R,18E,21S,23S,24R,25S,27R)-1,14-dihydroxy-12-[(1E)-1-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]prop-1-en-2-yl]-23,25-dimethoxy-13,19,21,27-tetramethyl-17-(prop-2-en-1-yl)-11,28-dioxa-4-azatricyclo[22.3.1.$0^{4,9}$]octacos-18-ene-2,3,10,16-tetrone	**Formula:** $C_{44}H_{69}NO_{12}$ **MW:** 804.018 g/mol **Water Solubility:** 0.00402 mg/mL		NA

Many drug target searches yielded kinases with very low E values. Genomic analysis of the *P. falciparum* genome identified 99 protein kinases and other related proteins (Anamika *et al.,* 2004). The essential role that protein kinases play in cell signaling and cell cycle in eukaryotes has led to them being considered as potential drugs targets in *P. falciparum* in some studies (Doerig *et al.*, 2002; Ward *et al.*, 2004).

Some of the drugs identified by this study had more than one target. An example is the anticancer drug vincristine which is predicted to target both ABC transporter (D index: 0.5, moderate conservation: 51 %) and tubulin beta chain (D index: 1, high conservation: 99 %). Previous studies showed higher activity of vincristine (IC_{50} of 0.00205 µM) (Mahmoudi *et al.*, 2003) compared to its sister drug, vinblastine that had only tubulin beta chain as its target, (IC_{50} of 0.007175 µM) (Mahmoudi *et al.*, 2003). Though it is important to note that Vinblastine having one target could probably be due to an oversight by the curators of these databases. Another drug that had more than one target is dipyridamole which has been was predicted to target both adenosine deaminase and DNA Topoisomerase II, it has an IC_{50} of 0.03 µM as tested by Akaki *et al.* (2002).

Since the whole proteome of *P. falciparum* was used, drugs that could targ*et al*l stages of its life cycle was considered. Most drug development efforts focuses on erythrocytic parasites because they cause the symptoms of the disease and are easier to manipulate in the laboratory. In fact, current antimalarials were discovered on the basis of their activity against red blood cell stage parasite. Conversely, developing drugs targeting the exo-erythrocytic cycle and sporogonic cycle are increasingly drawing interest in the quest for malaria elimination (Buchholz *et al.*, 2011; Derbyshire *et al.*, 2011; Gandhi *et al.*, 2003; Graewe *et al.*, 2011). The effect of the current antimalarials on these stages has also been studied (Delves *et al.*, 2012).

Similarity of a prospective protein target to a putative protein drug target has been used as basis for repositioning attempts (Bispo *et al.*, 2013; Li & Lai, 2007; Neves *et al.*, 2015). This also explains why most of the drugs we predicted to have antimalarial activity are already tested, confirming use of this approach in identifying new drugs against malaria and other diseases. This study was based on the principle that if a *P. falciparum* protein has high similarity to a confirmed drug target, then by inference, the drug in question would have a similar effect to the protein. All the proteins expressed in *P. falciparum* in RefSeq database were used to search for similar drug targets in TTD, DrugBank and STITCH databases. Most of the 99 previously tested drugs that we predicted to have antimalarial activity were shown to have some activity in *in vitro* studies, as shown in Table 4. These findings shows that this approach can correctly be used to identify antimalarial drugs from those already approved.

The *P. falciparum* protein ATP binding cassette (ABC) transporter (NCBI acc. no: XP_001348418.1, D index: 0.5, functional residues: 51 % conservation) was predicted as a potential target of 5 drugs that were tested in this study; Clofaribine, Irinorectan, Diadizin, Zafirlukast and Dasatinib. These drugs target the human ABC homolog, ATP-binding cassette sub-family G member 2 (NCBI accession number; Q9UNQ0.3) also called multidrug resistance protein 1 (MRP 1). The ATP-binding cassette sub-family G member 2 protein is rightly called "multidrug resistance protein" because of its role in drug resistance and treatment failure in trypanosomatid, apicomplexan and amitochondriate parasites of clinical significance (Leprohon *et al.*, 2011). This is mainly because they actively translocate a wide range of structurally and functionally diverse amphipathic compounds across cellular membranes (Koenderink *et al.*, 2010). Grant *et al.* (1994) associeted resistance to several drugs with overexpression of multidrug resistance protein 1. ABC transporters have been implicated with high IC_{50} values in response to chloroquine and quinine in *P. falciparum* field isolates (Raj *et*

al., 2009). Because of the crucial role they play , thy have been considered as targets of antibacterial vaccines and chemotherapies (Garmory & Titball, 2004).

Members of multidrug resistance protein (MRP) family have also been shown to play a vital role during blood stage multiplication in *Plasmodium* species (Rijpma *et al.*, 2016), yet another reason why they should be considered as potential targets for antimalarial drugs. The *P. falciparum* protein has E value of 2e-61 when aligned with its human homolog drug target using protein-protein BLAST suggesting their strong similarity and hence the high likelihood that the former could be a target to drugs targeting the latter. This could explain the moderate activity of Clofaribine, Irinorectan and Dasatinib. However, Zafirlukast and Diadizin did not show any activity at concentrations below 50000 ng/ml.

Adenosine deaminase, ADA (NCBI Acc. no: XP_001347573.1) is predicted to be a target of the Cladribine. This protein has an E value of 2e-29 when aligned with its human homolog drug target (NCBI Acc no. P00813.3), has a D index of 1 (high druggability) and 55 % of functional residues are shared. ADA is an attractive drug target since *P. falciparum* is unable to synthesize purine bases and hence relies upon purine salvage and purine recycling to meet its purine needs. *P. falciparum* ADA is unique because it catalyzes the deamination of both adenosine and 5‘-methylthioadenosine while the human form cannot deaminate 5‘-methylthioadenosine. It is because of this difference together with many others that Sriram *et al. (*2009) used a bioinformatics approach to show how quinine and primaquine could bind to the ADA protein. 5‘-methylthio coformycins have been shown to inhibit the *P. falciparum* ADA without inhibition of its human homolog (Tyler *et al.*, 2007). Examples of 5‘-methylthio coformycins are 5'-Methylthio-Immucillin-H (MT-ImmH) and Immucillin-H (ImmH) which had IC_{50} values of 63 nM and 50 nM on 3D7, respectively (Ting *et al.*, 2005). This was comparable to Dipyridamole’s IC_{50} of 30 nM , which also targets ADA (Akaki *et al.*, 2002).

Cladribine, also predicted in this study to target ADA, had an IC_{50} of 86.906 µM (this study) which is much higher than that of the MT-ImmH, ImmH and Dipyridamole. This could be attributed to lower activity of Cladribine in inhibiting ADA or differences in drug susceptibility assays.

Levofloxacin is a broad spectrum fluoroquinolone antibacterial agent that acts by inhibiting two type II DNA topoisomerase enzymes in bacteria; DNA gyrase and topoisomerase IV (Davis & Bryson, 1994; Drlica & Zhao, 1997). DNA topoisomerases enzymes are involved in the overwinding or underwinding of DNA during DNA replication and transcription. Several types of DNA topoisomerases have been characterized, these have been classified into two major classes depending on how they change the topology of DNA: topoisomerase I and topoisomerase II (Champoux, 2001). Garcia-Estrada *et al.* (2010) explained in a review how DNA topoisomerase could make targets for drug development because of their structural differences between host and apicomplexan, differential expression patterns and lack of orthologous topoisomerases in mammals (e.g. there are no apicoplast DNA gyrases in mammals).

Levofloxacin was predicted in this study to inhibit the activity of *P. falciparum's* DNA topoisomerase II (D index: 0.8, functional residues: 61 % conservation). It has an E value of 0.0 when aligned to its homologous drug target (NCBI Acc no: P11388.3) suggesting very high similarity. Levofloxacin showed activity against *P. falciparum* 3D7 with an IC_{50} of 14.17 µg/ml. Camptothecin, also predicted in this study to inhibit the same protein has previously been shown to inhibit the plasmodial DNA topoisomerase I, but due to its toxicity it is less likely to be used as an antimalarial (Bodley *et al.*, 1998), but its derivatives with less toxicity can.

Plasmodium histone deacytalase, HDAC (D index: 0.8, functional residues: 99 % conservation) was also predicted to be a target for drugs such as Vorinostat (used to treat cutaneous T cell lymphoma), valproic acid (approved for epilepsy and seizures treatment) and trichostatin A (used to treat fungal and bacterial infections). A recent study has used HDAC inhibitors to impede growth of *P. falciparum* both *in vivo* and *in vitro* (Andrews *et al*, 2009). Vorinostat has displayed high antiplasmodial activity *in vitro* with an IC_{50} of 0.12 μM (Engel *et al.*, 2015). HDAC inhibitors have also been investigated as drugs for a range of diseases such as trypanosomiasis, schistosomiasis, leishmaniasis, toxoplasmosis, HIV/AIDS and even cancer (Katherine *et al.*, 2012). Apicidin, a novel fungal metabolite, has been identified to inihibit HDAC in apicomplexan parasites including malaria (Darkin-Rattray *et al.*, 1996). The main challenge about the *in vivo* use of most evaluated HDAC inhibitors is that their zinc-binding hydroxamate group tends to be broken down rendering it less active (K. Andrews *et al.*, 2009).

Inosine 5'-monophosphate dehydrogenase, IMPDH (has a D index of 0.8 and 99 % of functional residues are shared with putative target homolog) plays a crucial role in catalyzing the first committed step of guanosine 5'-monophosphate (GMP) biosynthesis. It is an attractive target for drug interventions since most parasites depend on the salvage pathway due to their inability to synthesize purine nucleotides *de novo*. IMPDH inhibitors such as ribavirin and mycophenolic acid have been used as immunosuppressives, antivirals and anticancer drugs with little side effects to host cells (Bentley, 2000; Chen & Pankiewicz, 2007; Tam *et al.*, 2001). Nevertheless, little has been done about its application in treating microorganisms (Hedstrom *et al.,* 2011). Mycophenic acid, predicted in this study to inhibit IMPDH has been shown to be active against *P. falciparum* with an IC_{50} of 5.4 μM (Veletzky *et al.*, 2014).

Bosutinib, a drug approved for chronic myelogenous leukemia treatment showed some significant antiplasmodial activity with an IC_{50} of 0.22 μM (Lotharius *et al.*, 2014). This is

despite its predicted *P. falciparum* protein target, the calcium/calmodulin-dependent protein kinase sharing 47 % of its functional amino acids with its confirmed target, which is below the minimum threshold of 50 % used in this study. Its drug target was eliminated from further analysis at the ConSurf server analysis stage. This is an example of the drugs that could be identified by this approach but due to the use of stringent parameters, they were not found in the search. We therefore recommend that some steps of these study could be repeated, such as searching the TTD, DrugBank and STITCH databases with a higher E value. This could reveal other drugs that have antiplasmodial activity that were not identified in this study.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1: Conclusion

There is an urgent need to develop new drugs to counteract the effect of resistance on the current antimalarials. Unfortunately, the success rate of bringing new drugs to the market is quite low, mainly because of the cost and time involved in drug development and the fact that most bioactive compounds do not get approved because they fail to meet safety requirements. Therefore, repositioning of already approved drugs can be sustainable since these have passed safety tests, at least in treatment of some diseases.

In this study, the full proteome of *P. falciparum* was used to do a similarity search on targets of approved drugs. Results showed 133 approved drugs could target key *P. falciparum* proteins. A literature search revealed most of these drugs to have having being tested before with most of them showing antiplasmodial activity. *In vitro* assays confirmed the antiplasmodial activity of 10 drugs out of 12 that were tested, these include: Cladribine, Levofloxacin, Dasatinib, Clofarabine, Tacrolimus, Irinotecan, Zidovudine, Moxifloxacin, Oxaliplatin and Tadafil.

In conclusion, this study validates the use of target-similarity in identifying approved drugs that have activity against the *Plasmodium* parasite and hence reposition them for antimalarial treatment. This approach would circumvent many challenges involved in preliminary stages of drug discovery and development hence could save on cost and time spent in introducing new drugs into the market.

6.2 Recommendations

1. Conduct preclinical *in vivo* studies using animal models on the drugs that have shown *in vitro* antiplasmodial activity, these are Cladribine, Levofloxacin, Dasatinib, Clofarabine, Tacrolimus, Irinotecan, Zidovudine, Moxifloxacin, Oxaliplatin and Tadafil.
2. Conduct drug susceptibility tests on the remaining 22 drugs against reference *P. falciparum* clones and field isolates to ascertain their antiplasmodial activity.
3. Use drugs that have shown antiplasmodial activity as templates for synthesis of novel antimalarial drugs.
4. Validate the mechanism of action of the drugs that have shown antiplasmodial activity. Using the possible protein targets predicted in this study, these can be validated using target-based biochemical assays.
5. Use the target-similarity approach used in this study to find activity of approved drugs against other pathogens such as leishmaniasis, trypanosomiasis, onchocerciasis, helminthiasis etc.

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APPENDICES

Appendix 1: Complete predicted drug list

Complete predicted drugs list showing their putative target Uniprot ID with its homologous *P. falciparum* predicted target protein NCBI Acc no. Those obtained directly from STITCH database do not have putative targets. Some drugs had multiple targets hence occur in replicates.

Drug	Predicted *P. falciparum* target NCBI Acc no.	Putative drug target Uniprot ID
1. 3,6-diol, L-azetidine-2-carboxylic acid	XP_001350859.1	STITCH
2. AC1LA1XN	XP_001347426.1	STITCH
3. Acetonitrile	XP_001347573.1	STITCH
4. Albendazole	XP_001347369.1	P07437
5. Alectinib	XP_001348418.1	Q9UNQ0
6. Aminolevulinic acid	XP_001348555.1	P13716
7. Aminophylline	XP_001352127.1	Q92769
8. Aminophylline	XP_001348846.2	P27815
9. Aminophylline	XP_001348846.2	P27815
10. Aminophylline	XP_001348846.2	Q14432
11. Amrinone	XP_001350504.2	Q07343
12. Amrinone	XP_001348846.2	Q14432
13. Amsacrine	XP_001348490.1	P11388
14. Anagrelide	XP_001348846.2	Q14432
15. Apixaban	XP_001348418.1	Q9UNQ0
16. Apremilast	XP_001350504.2	Q07343

Appendix 1 cont.

17. Apremilast	XP_001348846.2	P27815
18. Aprindine	XP_001347555.2	P62158
19. Arsenic trioxide	XP_001347426.1	P28482
20. Arsenic trioxide	XP_001348401.2	P31749
21. Avanafil	XP_001349954.1	O76074
22. Azathioprine	XP_001352079.1	P20839
23. Bacitracin	XP_001348556.2	P14735
24. Benzphetamine	XP_002808949.1	P16435
25. Bepridil	XP_001350504.2	Q01064
26. Bestatin	XP_001349846.1	P09960
27. Biricodar dicitrate	XP_001348418.1	Q9UNQ0
28. Boceprevir	XP_001350862.2	P43235
29. Boceprevir	XP_001348727.1	P07711
30. Bosutinib	XP_001348401.2	Q13555
31. Buprenorphine	XP_001348418.1	Q9UNQ0
32. Cabazitaxel	XP_001347369.1	P68366
33. Cabazitaxel	XP_001348418.1	Q9UNQ0
34. Caffeine	XP_001350504.2	Q01064
35. Camptothecin	XP_001348418.1	Q9UNQ0
36. Cantharidin	XP_001348315.1	P08129
37. Capridine-beta	XP_001347426.1	P24941
38. Carboplatin	XP_001348418.1	Q9UNQ0
39. Celecoxib	XP_001348520.1	O15530
40. Celecoxib	XP_001348401.2	O15530

Appendix 1 cont.

41. Cerivastatin	XP_001348418.1	Q9UNQ0
42. Cholic Acid	XP_001350360.2	P22830
43. Cilomilast	XP_001350504.2	Q07343
44. Cilostazol	XP_001348846.2	P27815
45. Cilostazol	XP_001348846.2	Q14432
46. Ciprofloxacin	XP_001348490.1	P11388
47. Cisplatin	XP_001348418.1	Q9UNQ0
48. Cladribine	XP_001347573.1	P00813
49. Cladribine	XP_001347439.2	P31350
50. Cladribine	XP_001347439.2	Q7LG56
51. Clodronate	XP_001347650.1	P12235
52. Clofarabine	XP_001348418.1	Q9UNQ0
53. Cobicistat	XP_001348418.1	Q9UNQ0
54. Coformycin	XP_001347573.1	STITCH
55. Conjugated Estrogens	XP_001348418.1	Q9UNQ0
56. Cyclosporine	XP_001348418.1	Q9UNQ0
57. Daclatasvir	XP_001348418.1	Q9UNQ0
58. Dactinomycin	XP_001348418.1	Q9UNQ0
59. Dactinomycin	XP_001348490.1	P11388
60. Dactinomycin	XP_001348490.1	Q02880
61. Daidzin	XP_001348418.1	Q9UNQ0
62. Dasatinib	XP_001348418.1	Q9UNQ0
63. Daunorubicin	XP_001348418.1	Q9UNQ0
64. Daunorubicin	XP_001348490.1	P11388

Appendix 1 cont.

65. Daunorubicin	XP_001348490.1	Q02880
66. Daunorubicin	XP_002808949.1	P16435
67. Dexrazoxane	XP_001348490.1	Q02880
68. Diethylstilbestrol	XP_001348418.1	Q9UNQ0
69. Dipyridamole	XP_001347573.1	P00813
70. Dipyridamole	XP_001348846.2	P27815
71. Divalproex sodium	XP_966078.1	P80404
72. Docetaxel	XP_001348418.1	Q9UNQ0
73. Doxorubicin	XP_001348418.1	Q9UNQ0
74. Doxorubicin	XP_001348490.1	P11388
75. Doxorubicin	XP_002808949.1	P16435
76. Drotaverine	XP_001348846.2	P27815
77. Dyphylline	XP_001350504.2	Q07343
78. Dyphylline	XP_001348846.2	P27815
79. Elacridar	XP_001348418.1	Q9UNQ0
80. Enoxacin	XP_001348490.1	P11388
81. Enoximone	XP_001348846.2	Q14432
82. Enprofylline	XP_001350504.2	Q07343
83. Enprofylline	XP_001348846.2	P27815
84. Epirubicin	XP_001348490.1	P11388
85. Erlotinib	XP_001348418.1	Q9UNQ0
86. Erythrityl Tetranitrate	XP_001350316.2	Q9Y2Q0
87. Ethylmorphine	XP_002808949.1	P16435
88. Etoposide	XP_001348418.1	Q9UNQ0

Appendix 1 cont.

89. Etoposide	XP_001348490.1	P11388
90. Etoposide	XP_001348490.1	Q02880
91. Ezetimibe	XP_001351293.1	O75907
92. Felodipine	XP_001350504.2	Q01064
93. Finafloxacin	XP_001348490.1	P11388
94. FK-228	XP_001347363.2	P56524
95. Flavopiridol	XP_001348418.1	Q9UNQ0
96. Fleroxacin	XP_001348490.1	P11388
97. Fludarabine	XP_001347573.1	P00813
98. Fluorouracil	XP_001348418.1	Q9UNQ0
99. Fluoxetine	XP_001349605.2	STITCH
100. Gallium nitrate	XP_001347439.2	P31350
101. Gefitinib	XP_001348418.1	Q9UNQ0
102. Genistein	XP_001348418.1	Q9UNQ0
103. Gentamicin	XP_001349002.1	P11142
104. GSK2696273	XP_001347573.1	P00813
105. Hesperetin	XP_001351293.1	O75907
106. Hesperetin	XP_001348418.1	Q9UNQ0
107. Hydrocortisone	XP_002808949.1	P35228
108. Ibudilast	XP_001350504.2	Q07343
109. Ibudilast	XP_001348846.2	Q14432
110. Icrf-187	XP_001348490.1	Q02880
111. Idarubicin	XP_001348490.1	P11388
112. Idelalisib	XP_001348418.1	Q9UNQ0

Appendix 1 cont.

113. Iloprost	XP_001350504.2	Q07343
114. Iloprost	XP_001348846.2	P27815
115. Irinotecan	XP_001348418.1	Q9UNQ0
116. Isoprenaline	XP_001347426.1	P28482
117. Isosorbide Mononitrate	XP_001348065.1	P33402
118. Ivermectin	XP_001348418.1	Q9UNQ0
119. Ixabepilone	XP_001347369.1	Q13509
120. Ketotifen	XP_001350504.2	Q07343
121. Lamivudine	XP_001348418.1	Q9UNQ0
122. Leflunomide	XP_001348418.1	Q9UNQ0
123. Lenvatinib	XP_001348418.1	Q9UNQ0
124. Levofloxacin	XP_001348490.1	P11388
125. Levosimendan	XP_001348846.2	Q14432
126. Lomefloxacin	XP_001348490.1	P11388
127. Losmapimod	XP_001347426.1	Q16539
128. Lovastatin	XP_001352127.1	Q92769
129. Lucanthone	XP_001348490.1	P11388
130. MD-1100	XP_001350316.2	P25092
131. Mebendazole	XP_001347369.1	P07437
132. Mercaptopurine	XP_001352079.1	P20839
133. Methotrexate	XP_001348418.1	Q9UNQ0
134. Methyl aminolevulinate	XP_001350360.2	P22830
135. Milrinone	XP_001348846.2	P27815
136. Milrinone	XP_001348846.2	Q14432

Appendix 1 cont.

137. Mitomycin	XP_002808949.1	P16435
138. Mitoxantrone	XP_001348418.1	Q9UNQ0
139. Mitoxantrone	XP_001348490.1	P11388
140. Motexafin gadolinium	XP_001347439.2	P31350
141. Motexafin gadolinium	XP_001348226.1	P31350
142. Moxifloxacin	XP_001348490.1	P11388
143. Mycophenolate mofetil	XP_001352079.1	P12268
144. Mycophenolic acid	XP_001352079.1	P12268
145. Naringenin	XP_001348418.1	Q9UNQ0
146. Nelfinavi	XP_001348418.1	Q9UNQ0
147. Nesiritide	XP_001350316.2	P16066
148. Nicardipine	XP_001350504.2	Q01064
149. Nilotinib	XP_001348418.1	Q9UNQ0
150. Nilutamide	XP_002808949.1	P16435
151. Nitrofurantoin	XP_002808949.1	P16435
152. Norfloxacin	XP_001348490.1	P11388
153. Novobiocin	XP_001348418.1	Q9UNQ0
154. Ofloxacin	XP_001348490.1	P11388
155. Oral paclitaxel	XP_001347369.1	Q9H4B7
156. Osimertinib	XP_001348418.1	Q9UNQ0
157. Oxaliplatin	XP_001348418.1	Q9UNQ0
158. oxindole	XP_001347426.1	STITCH
159. Oxtriphylline	XP_001352127.1	Q92769
160. Oxtriphylline	XP_001348846.2	P27815

Appendix 1 cont.

161. Oxtriphylline	XP_001348846.2	Q14432
162. Oxtriphylline	XP_001350504.2	Q08499
163. Paclitaxel	XP_001348418.1	Q9UNQ0
164. Palbociclib	XP_001347426.1	P11802
165. Panobinostat	XP_001352127.1	Q92769
166. Papaverine	XP_001350504.2	Q07343
167. Papaverine	XP_001349954.1	O76074
168. Pazopanib	XP_001348418.1	Q9UNQ0
169. Pefloxacin	XP_001348490.1	P11388
170. Pentostatin	XP_001347573.1	P00813
171. pentostatin	XP_001347573.1	STITCH
172. Pentoxifylline	XP_001350504.2	Q07343
173. Pitavastatin	XP_001348418.1	Q9UNQ0
174. Podofilox	XP_001347369.1	P68366
175. Podofilox	XP_001348490.1	P11388
176. Pravastatin	XP_001348418.1	Q9UNQ0
177. Prazosin	XP_001348418.1	Q9UNQ0
178. PXD101	XP_001352127.1	Q13547
179. Pyridoxal Phosphate	XP_966078.1	P80404
180. Quercetin	XP_001348418.1	Q9UNQ0
181. Rabeprazole	XP_001348418.1	Q9UNQ0
182. Rapamycin	XP_001350859.1	P20071
183. Regorafenib	XP_001347426.1	Q15759
184. Resveratrol	XP_001347426.1	P19138

Appendix 1 cont.

185. Ribavirin	XP_001352079.1	P20839
186. Rilpivirine	XP_001348418.1	Q9UNQ0
187. Riluzole	XP_001348418.1	Q9UNQ0
188. Riociguat	XP_001348065.1	P33402
189. Riociguat	XP_001348418.1	Q9UNQ0
190. Roflumilast	XP_001350504.2	Q07343
191. Rolapitant	XP_001348418.1	Q9UNQ0
192. Romidepsin	XP_001352127.1	Q13547
193. Romidepsin	XP_001352127.1	Q92769
194. Rosuvastatin	XP_001348418.1	Q9UNQ0
195. Saquinavir	XP_001348418.1	Q9UNQ0
196. Sildenafil	XP_001348846.2	STITCH
197. Sildenafil	XP_001349954.1	O76074
198. Sofosbuvir	XP_001348418.1	Q9UNQ0
199. Sparfloxacin	XP_001348490.1	P11388
200. Sphingosome	XP_001347369.1	P07437
201. Sulfasalazine	XP_001348658.1	P24752
202. Sulindac	XP_001347426.1	P27361
203. Sumatriptan	XP_001348418.1	Q9UNQ0
204. Sunitinib	XP_001348418.1	Q9UNQ0
205. tacrolimus	XP_001350859.1	STITCH
206. Tadalafil	XP_001349954.1	O76074
207. Tamoxifen	XP_001348401.2	P05771
208. Taurocholic Acid	XP_001348418.1	Q9UNQ0

Appendix 1 cont.

209. Telmisartan	XP_001348418.1	Q9UNQ0
210. Teniposide	XP_001348490.1	P11388
211. Teriflunomide	XP_001348418.1	Q9UNQ0
212. Theobromine	XP_001350504.2	Q07343
213. Theophylline	XP_001352127.1	Q92769
214. Theophylline	XP_001350504.2	Q07343
215. Tofisopam	XP_001350504.2	O00408
216. Tofisopam	XP_001348846.2	P27815
217. Topotecan	XP_001348418.1	Q9UNQ0
218. trichostatin A	XP_001352127.1	Q92769
219. Trifluoperazine	XP_001347555.2	P62158
220. Trovafloxacin	XP_001348490.1	P11388
221. Udenafil	XP_001349954.1	O76074
222. Valproic Acid	XP_001347363.1	Q9UKV0
223. Valproic Acid	XP_001352127.1	Q92769
224. Valrubicin	XP_001348490.1	P11388
225. Vandetanib	XP_001348418.1	Q9UNQ0
226. Vardenafil	XP_001349954.1	O76074
227. Venlafaxine	XP_001348418.1	Q9UNQ0
228. Verapamil	XP_001348418.1	Q9UNQ0
229. Vesnarinone	XP_001348846.2	P27815
230. Vigabatrin	XP_966078.1	P80404
231. Vinblastine	XP_001347369.1	P05217
232. Vincristine	XP_001347369.1	P68366

Appendix 1 cont.

233. Vincristine	XP_001348418.1	Q9UNQ0
234. Vindesine	XP_001347369.1	P07437
235. Vorinostat	XP_001347363.1	Q13547
236. Vorinostat	XP_001352127.1	Q92769
237. Zafirlukast	XP_001348418.1	Q9UNQ0
238. zaprinast	XP_001348846.2	STITCH
239. Zidovudine	XP_001348418.1	Q9UNQ0

Appendix 2: Kenya Medical Medical Research Institute ethical clearance letter

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840 - 00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 (020) 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2720030
Email: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **December 2, 2015**

TO: **DR. HOSEAH AKALA,**
PRINCIPAL INVESTIGATOR

THROUGH: **ACTING DIRECTOR, CCR,**
NAIROBI

8/12/15

Dear Sir,

RE: **PROTOCOL NO. KEMRI/SERU/CCR/0018/3126 (*RESUBMISSION 2 OF INITIAL SUBMISSION*): ASSESSMENT OF *IN VITRO* ANTIPLASMODIAL ACTIVITIES OF THE COMPOUND LIBRARIES FOR MALARIA DRUG DISCOVERY AND DEVELOPMENT (*VERSION 4.0 DATED 28 OCTOBER 2015*)**

Reference is made to your letter dated 16 November 2015. KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised protocol on 1st December 2015.

This is to inform you that the Committee notes that the issues raised at the 242nd meeting of the KEMRI ERC held on August 18, 2015 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **2nd December, 2015** for a period of one year. Please note that authorization to conduct this study will automatically expire on **December 1, 2016.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **October 20, 2016**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

Appendix 3: Walter Reed Army Institute of Research ethical clearance letter

MCMR-UWZ-C 22 December 2015

MEMORANDUM FOR Hoseah Akala, PhD, Kenya Medical Research Institute (KEMRI)/Walter Reed Project (WRP), PO Box 54-40100, Kisumu, Kenya.

SUBJECT: WRAIR's Participation Qualifies as Research Not Involving Human Subjects, **WRAIR #2257**

1. A determination was made that WRAIR's participation on the protocol **WRAIR #2257**, entitled "Assessment of *In Vitro* Antiplasmodial Activities of Compound Libraries for Malaria Drug Discovery and Development," (Version 4.0, dated 28 October 2015), submitted by Hoseah Akala, PhD, Kenya Medical Research Institute (KEMRI)/Walter Reed Project (WRP), does not require review by the WRAIR Institutional Review Board (IRB) in accordance with WRAIR Policy Letter #12-09, as WRAIR personnel will support specimen analysis for which they will have no access to subject/patient identifiers. Therefore, WRAIR's participation in this research activity does not meet the definition of research involving human subjects and 32 CFR 219 does not apply.

2. The primary objective of the study protocol is to establish *in vitro* antiplasmodial activities of lead synthetic compounds and natural products for malaria drug discovery and development. This study will use specimens approved for future use under the human subjects research protocol WRAIR #1384 and WRAIR #1919.

3. Candidate compounds will be provided from local universities' assembled libraries of synthetic and natural compounds that are suspected to have antimalarial properties. Blood specimens collected under WRAIR protocol #1919, which is conducted at the same facility, will be used for *P. falciparum* culture. The specimens have been permanently anonymized at the time of collection. Type O blood cells will be added to a solution of parasite samples, stabilizing agents, and tissue culture media to form the parasitemia-infected culture. The culture will be incubated until 3 to 8% parasitemia is obtained, at which point drug testing will commence. SYBR Green I-based assay will be used to test for antiplasmodial *in vitro* activity in various experimental compounds applied to the infected specimens, using an established reference drug and plasma as controls.

4. The Commander, US Army Medical Unit – Kenya , determined the project to be militarily relevant, scientifically valid and feasible, and appropriately resourced on 15 July 2015.

5. The KEMRI Scientific and Ethics Review Unit reviewed and approved this protocol on 2 December 2015; the approval will expire on 1 December 2016.

6. This project is sponsored and funded by the Division of Global Emerging Infections Surveillance and Response System Operations, Armed Forces Health Surveillance Center.

7. Per the WRAIR Education Policy Letter #11-49, all individuals covered under the WRAIR Human Research Protection Program (HRPP) are required to complete Collaborative Institutional Training Initiative (CITI) training; documentation of completed CITI training has been provided for the investigators.

8. The WRAIR point of contact (POC) has the responsibility to obtain all business agreements <u>prior</u> to initiation of any work with partners/collaborators or contracted services under this project. This includes any transfer of data or specimens. All relevant business agreements are to remain current throughout the duration of the study and must be maintained by the WRAIR POC. Failure to obtain business agreements prior to initiation could result in sanctions or disciplinary actions for

Appendix 4: Consent form for blood collection

Informed consent form

Study Title:

Human blood collection for the in vitro culture of malaria parasites

Who is the doctor in charge of the project?

Dr. Ben Andagalu., Kenya Medical Research Institute/U.S. Army Medical Research Unit-Kenya, Kisumu, Kenya P.O. Box 54-40100. Telephone 0716004851, 0733-333-530.

Purpose of the project

This is a research project that aims to collect blood from healthy volunteers. The blood collected will be used to grow malaria parasites in the laboratory, and therefore study how the parasite responds to malaria drugs.

Which institution is managing the project?

The Kenya Medical Research Institute - Centre for Clinical Research and the U.S. Army Medical Research Unit-Kenya (together known as The Walter Reed Project) are managing this project. The study will be done at Walter Reed project Kericho District Hospital and KEMRI staff clinic located within KEMRI campus in Nairobi

What are the project procedures?

1. If you agree to participate, one of the project clinicians will examine you to see whether you can participate. In order to participate in this project, you must be aged between 18 and 50 years, and be in good health. You cannot participate if you have any of the following:
    - Blood pressure that is not normal.
    - Low blood levels
    - Chronic illness
    - Frequent blood donation
    - Use of narcotic drugs
    - Frequent travel to malaria zones
    - Prior prolonged residence in a malaria zone
    - Frequent malaria episodes in the past
    - Pregnancy or breastfeeding mothers

2. If you are found fit to join the project, you will be one of the group of donors, who will be contacted from time to time to donate blood. A test to know your blood group will then be done, and you will be informed of which blood group you belong to.

3. When contacted to donate blood, you will be requested to re-consent in order to donate blood. Once you have signed the consent form, one of the project clinicians will examine you again to make sure that you are healthy enough to donate blood. You will not be

allowed to donate blood if you have donated blood in the past 6 months or taken antimalarials or antibiotics in the last 2 weeks. A blood test will be done to ensure you have good blood levels. Then depending on the type of blood required for the project, you will be requested to donate either

a. Up to 60 mls (equivalent to the volume of a small a tea cup) of blood, or

b. Up to 400 mls (equivalent to the volume of approximately one and a half small bottles of soda) of blood.

The 60 mls of blood is used to prepare blood cells in which the malaria parasites will grow, while the 400mls is used to make food for the parasites. Blood will be used for the purpose explained in this informed consent document only. No blood will be stored for future use. These red blood cells will be used to grow parasite strains for research under other two related studies (WRAIR #1384, KEMRI SSC#1330 and a sub-protocol). The red blood cells may also be used to culture parasites under basic science research.

You can only donate blood once every 6 months.

4. Approximately 1300 persons will be screened into this study to enroll a maximum of 432 participants per year.

Your participation and rights

Your participation is of your free will. You can refuse to participate for any reason and at anytime. If you agree to participate, you may withdraw at any point without anybody feeling bad about it. Any collection of your blood will require an additional consent form.

If you have any questions or concern about complications that arise after your blood is taken, you can call or visit our clinician at the location where your blood is taken and evaluated in a timely manner.

Duration of participation

The project will run for a period of 10 years. If you agree, you will be contacted from time to time throughout the duration of the project to donate blood, but not more than 2 times in 1 year. The blood collection process would take approximately 4 hours.

Risks and discomforts

You may suffer pain at the place where the lancet or needle was inserted to take blood and the pain may be there for a few days after blood is taken. Other complications of taking a sample of your blood from a vein include infection at the point of taking the blood, bleeding, bruising, or blood clot formation. Donation of blood is usually well tolerated, but some healthy individuals may experience transient dizziness, nausea, or mild headache. All precautions to minimize the risk of infection will be applied. The study personnel who will perform the blood draws are well trained and experienced, and will take every precaution to minimize the risk of the above mentioned complications. Additionally, you will be observed for 1 hour after blood donation to

make sure that any problems that may arise immediately are treated promptly. Refreshments will also be offered.

Direct benefits

There is no direct benefit to you for participating in this project as a blood donor. However all study participants will be provided with their laboratory results which will include blood level and blood group. If laboratory results show that you have low blood levels you will be offered treatment at no cost to you.

Indirect benefits

You will potentially benefit your community and people worldwide by donating blood to be used in the study of malaria parasites.

Are there any payments for participation?You will receive Kshs 750 to partly cover the time you missed work to participate in this study. You will also receive transport reimbursement for each visit you are requested to make to the project center. The amount of money to be given for transport reimbursement will depend on the distance from the project center to the location where you usually reside. No other monetary compensation will be given.

Termination of participation

Your participation in the study may be stopped if the investigator decides that it is in your best interest to discontinue, or occurrence of circumstances that might make your participation dangerous or other reasons not currently known.

What should I do if I have an illness or injury?

If you think you have an illness, or injury related to donation of blood in this study, please report to the nearest health facilityand contact Dr. Ben Andagalu, Walter Reed Project by telephone: 0716004851 or 0733-333-530

Who should I contact for information or answers to questions concerning the rights of a research participant?

You may contact the Secretary of the Kenya National Ethical Review Committee, c/o Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya tel.020-2722541.

Who should I contact for information or answers to questions concerning the study

If you have any questions or need more information concerning this study you may contact Dr Ben Andagalu Walter Reed Project P.O BOX 54, 40100, Kisumu, Kenya, Telephone 0716004851 or 0733-333-530

Safety and Confidentiality – (who will have access to my information and blood sample).

The project personnel will ensure your safety and confidentiality during and after blood donation. Your name will neither appear on the blood sample nor history sheet. Your name and contact information will be kept separate from all study documents and will only be used to contact you for future blood donations. A unique number will be assigned to each sample to avoid your direct

Appendix 4 cont.

identification. Authorized representatives from regulatory agencies may access your records as part of their responsibility of overseeing research activities.

Informed Consent

I hereby confirm that I have read or have had the consent information explained to me and questions related to this study have been answered satisfactorily. A signed copy of this informed consent document will be given to me for my personal records.

Thumb print if Volunteer is unable to sign

____

Printed name of subject

______________________________ ________________

Signature of subject (Thumb print if unable to read and write) Date:

____

Participants address

____

Name of person administering consent

______________________________ ________________

Signature of person administering consent Date:

This section should only be used if the subject is unable to read and write

Impartial witness statement

I confirm that I was present when this form was being read to the person whose name is listed above, all his/her questions have been answered satisfactorily and he/she gave verbal consent willingly

____

Name of impartial witness

______________________________ ________________

Signature of impartial witness Date:

Appendix 5: Consent form for blood collection (for field isolates) from malaria patients

WRAIR 1384: Epidemiology of malaria and drug sensitivity patterns in Kenya
Version 8.1, 22 August 2013

Consent to Participate in Research
Adult Consent Form (includes parental or legal guardian consent)

TITLE OF STUDY: Epidemiology of malaria and drug sensitivity patterns in Kenya

INSTITUTIONS: Kenya Medical Research Institute, Nairobi, Kenya; Walter Reed Project (United States Army Medical Research Unit – Kenya), Nairobi, Kenya; Walter Reed Project, Kisumu, Kenya

PRINCIPAL INVESTIGATORS: Dr. Ben Andagalu, MAJ Edwin Kamau and MAJ Jacob Johnson

---

INTRODUCTION

You (your child) are being asked to participate in this research study. Participation in this study is voluntary. Refusal to participate will involve no penalty or loss of benefits to which you (your child) are otherwise entitled. You (your child) may discontinue participation at any time without penalty or loss of benefits.

PURPOSE OF THE STUDY

Your doctor suspects that you have malaria and are being asked to participate in this study to learn about your malaria germs and what drugs best treat the malaria found in your part of Kenya.

You (your child) will receive the malaria medicine prescribed by the hospital provider if it is necessary, and this testing will in no way affect your (your child's) treatment today.

WHO CAN PARTICIPATE IN THIS STUDY

Any person at least 6 months old who comes to the hospital and is suspected to have malaria can participate.

APPROXIMATE NUMBER OF VOLUNTEERS TAKING PART IN THIS STUDY

Up to 50 total volunteers per week, from all of the sites we are collecting from.

Appendix 5 cont.

PROCEDURES TO BE FOLLOWED

If you (your child) agree to participate in this study, the procedures involve answering questions, having a brief physical examination, and donating blood. We will also ask you (your child) to visit us again on day 7 so we can see if the treatment was successful. Some volunteers who are willing will be asked to return 3 additional times, on days 14, 28 and 42.

Blood samples will be taken in 2 ways. For the larger volume blood sample, which will be 2.5 ml (1/2 teaspoon), will be drawn from a vein in your (your child's) arm. For the smaller volume, 2-3 drops, a "finger stick" will be done.

Today's procedures: after signed informed consent, you (your child) will be asked some questions about your (your child's) age, occupation, village, residence, history of your sickness, symptoms, and antimalarial drug use. Then, you (your child) will undergo a brief provide a blood sample from the arm vein (2.5 ml or about 1/2 teaspoon) to test your (your child's) malaria germs in the laboratory and see what drugs will effectively treat your (your child's) malaria. The questions and blood drawing will take about 1 hour.

Day 7 procedures: you (your child) will be asked to return to this site 7 days from now, to answer questions only about whether the medication given improved the malaria illness. You will be asked to donate 1-2 drops of blood by finger-stick for a Parascreen malaria test. If the malaria test is positive, you (your child) will then be asked to donate 2.5 ml blood sample of blood from the arm vein for more laboratory testing. If the test is negative, you will not be asked for any more blood. This visit will take approximately 1 hour.

"Finger-stick " samples will be obtained from a fingertip using an automatic lancet device that minimizes pain, and makes only a small line cut (about 2-3 millimeter in length) in the finger tip skin. The finger tip is then squeezed, allowing a few drops of blood to be obtained for the laboratory tests.

Days 14, 28 and 42 procedures: if you (your child) agree to return up to 4 times, at each visit, you (your child) will be asked questions only about whether the medication given improved the malaria illness. At each visit, you will be asked to donate 1-2 drops of blood by finger-stick for a Parascreen malaria test. If the malaria test is positive, you will then be asked to donate a 2.5 ml blood sample from the arm vein for more laboratory testing. If the test is negative, you will not be asked for any more blood. Each return visit will take approximately 1 hour.

All blood samples, except those used to diagnose your malaria by rapid diagnostic test, will be sent to a research laboratory in Kisumu, Kenya to test which drugs can kill your malaria germs. This is an experimental procedure.

Appendix 5 cont.

POTENTIAL RISKS AND DISCOMFORTS

The risk from participation in this study is small. There is some inconvenience associated with one or more clinic visits. There is the possibility of mild discomfort, bruising and very rarely infection at the arm or fingerstick site where the blood is obtained. The technician will use care to cause as little pain as possible and minimize the chance of infection after the blood draw. If the site should become infected, we will treat you (your child) with medication.

ANTICIPATED BENEFITS TO VOLUNTEERS

If you (your child) agree to be in the study, on the return visit(s), you (your child) will be tested for malaria to determine response to treatment. The study team will tell you the results. The test will show if the medication you (your child) received worked. If it did not, you (your child) will see the hospital clinician for an effective alternate anti-malaria medicine.

An indirect benefit to you (your child) is knowing that you (your child) have helped with a scientific study that may benefit other persons who become infected with malaria in the future by allowing us to test what drugs work best for treatment.

ALTERNATIVE TO PARTICIPATION

The only alternative to participation in this research is to not participate.

PAYMENT FOR PARTICIPATION

There is no charge to participate in this study. In accordance with Kenyan custom, there is no direct compensation to volunteers for their participation. Support for public transportation to return to the study site for each follow up visit, and return home, will be provided to you (your child). The amount will be 300 Kenyan shillings per visit.

USE OF YOUR (YOUR CHILD'S) BLOOD SAMPLES

Your (your child's) blood samples will be stored and used only for the tests associated with this study. However, you (your child) may grant permission for the malaria germs in your blood to be used for other studies in the future. You (your child) will be given a separate form to fill out which will allow you (your child) to say whether you (your child) will or will not allow your (your child's) blood samples to be used for future studies. The stored malaria germs for future studies will not have any items that could identify you as the original source, such as your name. No genetic testing will ever be done. There is no possibility that your samples could be used for developing a commercial product.

DURATION OF PARTICIPATION

Today, you (your child) will answer questions and donate blood today. This will take about 1 hour. On day 7, you (your child) will answer some questions and donate blood. If you agree to be in the group that returns up to 4 times, you will answer questions and give a blood sample on days 7, 14, 28 and 42 days after treatment. Each visit will last approximately 1 hour. There will be no further clinic visits needed from either group.

Appendix 5 cont.

ASSURANCE OF CONFIDENTIALITY

Records relating to your (your child's) participation in the study will remain confidential. Research records will be kept in a locked file at Walter Reed Project, Kisumu, Kenya. Your (your child's) name will not be used in any report resulting from this study. All computerized records and laboratory specimens will contain only a unique study number for you (your child), not your (your child's) name, or any other personal identifying information. Computer records will be password protected and accessed by authorized study personnel only. Research records will be kept until all data analyses are completed.

PARTICIPATION AND WITHDRAWAL

Your (your child's) participation in this study is voluntary. You (your child) have the right to leave this study at any time. Refusal to participate or study discontinuation will not result in a penalty, a compromise of your medical care, or a loss of benefits to which you (your child) are otherwise entitled.

In the event that you (your child) exit the study before its completion, regardless of the reason, we encourage you to participate in the scheduled blood sampling for follow up malaria testing.

WITHDRAWAL OF PARTICIPATION BY THE INVESTIGATOR

The investigator may withdraw you (your child) from participating in this research if circumstances arise which warrant doing so. If you (your child) become ill during the research, beyond what would be expected from a malaria infection, you (your child) may have to drop out, even if you (your child) would like to continue. The investigator will make the decision and let you (your child) know if it is not possible to continue. The decisions may be made either to protect your (your child's) health and safety, or because it is part of the research plan that volunteers who develop certain conditions may not continue to participate.

You (your child) may also be removed from this study without consent if: a)
you (your child) do not follow the study procedures
b) in the opinion of the study physicians, it is in your (your child's) best interest,

NEW FINDINGS

You (your child) will be informed of all malaria test results, as they relate to your current treatment. Results obtained in the laboratory testing will not be made available to you, but will be made available to the Kenyan Ministry of Health.

During the study, you (your child) will, however, be informed of any significant new findings (good or bad) such as changes in the risks or benefits resulting form participation in the research or new alternatives to participation, which might cause you (your child) to change your (your child's) mind about continuing in this study. If new information is provided to you (your child), consent to participate in this study will be re-obtained

Appendix 5 cont.

REVIEW OF RESEARCH RECORDS

It should be noted that representatives of the US Army Medical Research and Materiel Command and KEMRI are eligible to review research records as a part of their responsibility to protect human subjects in research. The research records will be made available only to investigators and clinical hospital personnel who may need this information to treat you (your child), or to members of the Ministry of Health who require this information for administrative reasons.

MEDICAL CARE FOR RESEARCH RELATED INJURY

Should you (your child) be injured as a direct result of participating in this research project, you (your child) will be provided medical care, at no cost to you (your child), for that injury. You (your child) will not receive any injury compensation, only medical care. You (your child) should also understand that this is not a waiver or release of your (your child's) legal rights. You should discuss this issue thoroughly with the study staff before you (your child) enroll in this study.

PERSONS TO CONTACT FOR ANSWERS TO RESEARCH RELATED QUESTIONS

If you think you (your child) has a medical problem related to this study, you may report this to Dr. Ben Andagalu, MAJ Edwin Kamau and MAJ Jacob Johnson, Walter Reed Project, Kisumu, Kenya, Tel: 0716004851 or +254202023858.

PERSONS AND PLACES FOR ANSWERS REGARDING YOUR RIGHTS AS A RESEARCH SUBJECT

If during the course of this study, you have questions concerning the nature of the research or you believe you have sustained a research-related injury, you (your child) should contact Dr. Ben Andagalu, MAJ Edwin Kamau or MAJ Jacob Johnsonat the Walter Reed Project, telephone 0716004851 or +254202023858. If you are not satisfied, you may also contact the Secretary of the Kenya National Ethical Review Committee, c/o Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya, tel. 020-272-251 or 0722205901.

RIGHTS OF RESEARCH SUBJECTS

You (your child) may withdraw consent at any time and discontinue participation without penalty. You (your child) are not waiving any legal claims or rights because of your participation in this research study. If you have questions regarding your rights as a research subject, you may contact: The Director, Regulatory Affairs Office, Walter Reed Project, PO Box 54, Kisumu, Kenya or telephone +254202023858 and/or the Secretary of the Kenya National Ethical Review Committee, c/o Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya, tel. 020-272-251 or 0722205901.

CONSENT: By signing this form, you agree that you have read the information provided above, or that it has been explained to you. You have talked to a member of the study team about the study. You have also been given an opportunity to ask questions and these have

Appendix 5 cont.

been answered to your satisfaction. You agree that we have talked to you about the risks and benefits of the study, and about other choices. You (your child) may drop out of the study at any time, and nothing will change about your (your child's) medical care. A copy of this form will be given to you (your child).

If Subject is a minor Name of Subject: ________________________________

Thumbprint of adult subject/parent/ guardian if unable to sign

Printed name of participant/parent/guardian: ____________________

Signature of participant/parent/guardian: ______________________

Address of participant/parent/guardian:________________________

Date: _____________

WITNESS: I have witnessed the explanation of the research study to the participant/parent/guardian. The participant was given an opportunity to ask questions, and the participant's questions, if any, were answered.

Printed name of witness: _________________________________

Signature of witness: ____________________________________

Date: ____

INDIVIDUAL OBTAINING CONSENT: I certify that I have explained to the above participant/parent/guardian the nature and purpose of this study, potential benefits, and possible risks associated with participation in this study. I have answered any questions that have been raised.

Printed name of individual obtaining consent: ______________________________

Title: ____________________________________

Signature: ________________________________

Date: ____________________________________

INVESTIGATOR

Printed name: ______________________________

Signature: _________________________________

Appendix 6: ConSurf results; amino acid conservation analysis spreadsheet

The table details the residue variety in % for each position (position 1-20) in the query (drug target NP_001277159.1) sequence. Each column shows the % for that amino-acid, position it is found in the MSA. In case there are amino acid residues which are not standard, they are represented under column 'OTHER'

		Amino acid position on the protein									
		1	2	3	4	5	6	7	8	9	10
Amino acids one letter symbol	**A**		0.714	2.797		2.667					
	C					0.667					
	D		43.571	14.685		2			1.333		
	E		52.857			18.667		1.333	96		
	F	0.794			46						
	G			1.399							
	H					2					
	I	0.794		0.699		4.667		40			84.667
	K			9.091			94		0.667	99.333	
	L			0.699		3.333		14			8
	M	97.619		0.699		2.667		1.333			
	N		2.143	27.972		0.667					
	P										
	Q		0.714	17.483		44.667	2.667		0.667		
	R			6.993		2	3.333			0.667	
	S			2.797		2.667			1.333		
	T			3.497		5.333		1.333			
	V	0.794		11.189		8		42			7.333
	W										
	Y				54						
	OTHER										
	MAX AA	M 97.619	E 52.857	N 27.972	Y 54.000	Q 44.667	K 94.000	V 42.000	E 96.000	K 99.333	I 84.667
	ConSurf score	9	1	2	8	1	8	4	8	9	8

Appendix 6 cont.

		Amino acid position on the protein									
		11	12	13	14	15	16	17	18	19	20
Amino acids one letter symbol	**A**						2.667				
	C										
	D										
	E		100					0.667			
	F									4.667	
	G	100		100			97.333				
	H					0.667					
	I							4.667			
	K										98.667
	L										
	M				0.667						
	N										
	P										
	Q										
	R										1.333
	S										
	T				99.333			4			
	V							90.667	100		
	W										
	Y					99.333				95.333	
	OTHER										
	MAX AA	G 100.000	E 100.000	G 100.000	T 99.333	Y 99.333	G 97.333	V 90.667	V 100.000	Y 95.333	K 98.667
	ConSurf Score	9	9	9	9	9	9	9	9	8	9