# Investigating drug repositioning as a route to combat drug resistance in *Plasmodium falciparum* Malaria

**Attika Atif Munir**

School of Science, Engineering and Environment

University of Salford, Manchester

# DECLARATION

I certify that this thesis, which I submit to the University of Salford a partial fulfilment of the requirements for a Degree of Master of Science in Research, is a presentation of my own research work. Wherever contributions of others are involved, every effort is made to indicate this clearly with due reference to the literature and acknowledgement of collaborative research and discussions. The content of this thesis has not been submitted for a higher degree at this or any other university.

**Attika Atif Munir**

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ABSTRACT

Malaria is known as one of the deadliest and life-threatening parasitic diseases. It is caused by a bite of an infected female *Anopheles* mosquito transmitting *Plasmodium* parasites to humans. In 2021, there was an estimated 247 million cases of malaria: with fatalities at 619,000. Although chemotherapy and vector control measures have been the mainstay of disease control, over time there has been an emergence of drug resistance to most of the antimalarial therapies available. There is an urgent need for novel therapeutic drugs to become available. Traditional drug development pipelines and research would have significant time and cost implications, whereas a more time-efficient drug discovery pathway such as drug repositioning could yield much needed results. Drug repositioning is a novel drug discovery pathway using drugs that are used for other conditions and repurposing them for another. In this study, six compounds were selected from the LOPAC FDA-approved (Food and Drug Administration agency) drug library to test for anti-malarial activity (Fluoxetine, Mitoxantrone, Nicardipine, Propafenone, Ivermectin and Nicardipine). Antimalarial activity was defined in the multi drug resistant *Plasmodium falciparum* strain K1 using the *in vitro* SYBR Green micro-titre drug assay. The $IC_{50}$ values were as follows: Ivermectin 0.7067μM, Mitoxantrone 1.636μM, Fluoxetine 11.23μM, Nicardipine 4.388μM, Propafenone >200μM and Spironolactone >200μM using Chloroquine as a control. Cell cytotoxicity profiles were determined for the selected compound panel using MTT assays in human HepG2 cell lines. All compounds apart from Mitoxantrone showed better safety profiles than the control Cisplatin ($IC_{50}$ 0.665 μM). The calculated selectivity indices for the main two drug leads (Ivermectin SI 77.95 and Nicardipine SI 21.23) showed promising results for progression to second phase optimisation. The study provides preliminary data on a panel of compounds with antimalarial efficacy, which could be further explored through derivatisation or as combinatorial partner drugs.

# INTRODUCTION

## *1.1 History of Malaria*

Malaria is known as one of the most serious health problems faced by people of the developing world. Malaria is an ancient disease, and references date back to 2700 BC in Chinese documents and clay tablets and Egyptian papyri from 1570 BC (Cox, 2010). In about 400 BC, the Greek physician Hippocrates stated he was well-aware of the characteristics of malaria being poor health, malarial intermittent fevers and enlarged spleens of people living in marshy areas (Cox, 2010). Malaria literally translates as 'bad air', as for centuries many assumed it was a result of the inhalation of swamp vapours and toxic fumes (Chohan. et al., 2001; Desowitz, 1991). This belief was strengthened by the fact that the number of malaria cases decreased once the swamps had been drained (Desowitz, 1991). However, it was not until 1880 that Alphonse Laveran (1845-1922), a military physician in Algeria identified the crescent shaped malaria parasites in the blood of a solider suffering from intermittent fevers (Chitnis, Chauhan & Gaur, 2016). While conducting clinical examinations he concluded that there were no crescent structures present when there were no disease symptoms. He also documented at the time that microscopic organisms were cleared by quinine treatment (Chitnis et al., 2016). With time, the findings were corroborated by more scientists using eosin-containing blood stains. By 1890 it was known that malaria was caused by infection with protozoan parasites belonging to the genus *Plasmodium* that invades and multiplies in erythrocytes (Cox, 2010). The disease is transmitted by the bite of a female *Anopheles* mosquito that resides in humid, swampy areas. It is caused by the parasitic protozoan that commonly infects the *Anopheles* mosquito that feeds on humans (Targett, 1991). There are four species of malaria parasites that infect humans, part of the genus *Plasmodium*: *P. falciparum, P. vivax, P. ovale and P. malariae* (Carpenter. et al., 199; Greenwood et al., 2008). *P. vivax* was previously known to be responsible for most of the malaria infections in the world, however, the current picture has seen a predominance of the more virulent species *P. falciparum,* where the lack of prompt treatment may lead to death (Carpenter et al., 1991). *P. vivax* predominates in India, Pakistan, Bangladesh, Sri Lanka, and Central America (Dunbar et al., 1996). *Plasmodium falciparum* dominates in Africa and New

Guinea. However, both are prevalent in South- East Asia, Southeast Asia, South America, and Oceania (Dunbar et al., 1996). More recently in 2016, *Plasmodium knowlesi* has become the most common cause of malaria in Malaysia, that can usually be misdiagnosed for *P. falciparum* or *P. vivax* (Barber et al., 2017).

*1.2 Incidence*

Malaria remains among the top five most devastating diseases occurring in the world today. Malaria is a known tropical and sub-tropical disease most prevalent in Asia, Africa, and Latin America. Statistics have shown that malaria is transmitted within 108 countries inhabited by roughly 3 billion people (White et al., 2014). It is majorly responsible for the high child death rates and reduced economic production (Targett, 1991; White et al., 2014). Statistics show that incidence of malaria in United States in 1970 ranged from a high of 4230 cases, however most cases occurred mainly among military personnel returning from Vietnam (Chohan. et al., 2001). By 1973 it dropped to 222 cases, suggesting that very few cases of malaria had been contracted in the United States and in fact transmitted by blood transfusions or the use of contaminated needles (Chohan. et al., 2001). More recently in 2016, 91 countries reported a total of 216 million cases of malaria in a year, 5 million more cases than 2015 (Manguin & Dev, 2018). The estimated number of malaria fatalities was around 445,000 roughly the same as 2015 (Manguin & Dev, 2018). Further to this, the latest statistic released by the World Health Organization suggests that in 2021, there was an estimated number of 247 million cases of malaria worldwide, with the death toll standing at 619,000 (Organization, 2023). Children were the most common under 5 years of age to be affected by malaria, as they accounted for 80% of all malaria worldwide (Organization, 2023). It can then be said that even though there has been a significant reduction in malaria largely due to the extreme effective control measures in place such as vector control through insecticide-impregnated bed nets and efficacious antimalarial medicines, the rate of decline of malaria has stalled since 2014, that further suggests medications and protective measures are failing to attempt to control the deadly disease (Manguin & Dev, 2018).

*1.3 Life Cycle of Plasmodium*

It is important to understand the life cycle that is common for all human *Plasmodium* species. A bite from an infected female *Anopheles* mosquito initiates the life cycle in which the mosquito during a blood meal injects the spindle-shaped invasive stages knowns as sporozoites into the skin. The mosquito engages in repeated probing, releasing saliva and sporozoites into the subcutaneous tissue of the host. The sporozoites travel through the blood stream to the liver, where they travel through multiple cells such as the Kuppfer cell before finally residing in a hepatocyte. Within the hepatocytes they undergo a phase of asexual multiplication (exoerythrocytic schizogony) resulting in the production of many uninucleate merozoites. It is said that a single sporozoite within the hepatocyte grows and multiplies to yield around 40,000 merozoites. These are known as invasive structures that are released into the blood stream where they invade the red blood cells (erythrocytes). Once the merozoites invade the erythrocytes the parasite transforms from a ring to a trophozoite and finally because of DNA replication and asexual reproduction known as schizogony leads to a multinucleated schizont. The schizonts rupture eventually allowing the parasite to produce 16 to 32 daughter merozoites, which are released into the blood stream leading to another cycle of erythrocyte invasion and growth. The repeated cycles of invaded erythrocytes occur every 48 hours in all the human malaria species apart from *P. malariae* that has a cycle every 72 hours. This process is repeated and is responsible for the disease malaria (Biamonte et al., 2013: Chohan et al., 2001).

During the blood-stage life cycle, some of the parasites fail to progress and divide and differentiate into gametocytes. During a blood meal the male and female gametocytes are taken up by the Anopheles mosquito. These male gametocytes form motile microgametes that undergo the process of exflagellation. They swim to the macrogamete formed from the female gametocyte where they fertilize together resulting in the formation of a motile zygote known as an ookinete. The ookinetes reside in the thick-walled structure known as the oocyst under the mosquito's gut lining. Several sporozoites are formed within the oocyst through asexual multiplication that make their way to the mosquito's salivary glands. This is where the life cycle initiates as the sporozoites are what is injected into the human host through the bite of the Anopheles mosquito. The mosquito stage is completed in approximately 2 weeks (Chohan et al., 2001; Cox, 2010; Greenwood et al., 2008). The next blood meal along with the inoculation of the invasive sporozoites completes the life cycle of the *Plasmodium* life cycle (Figure 1.1).

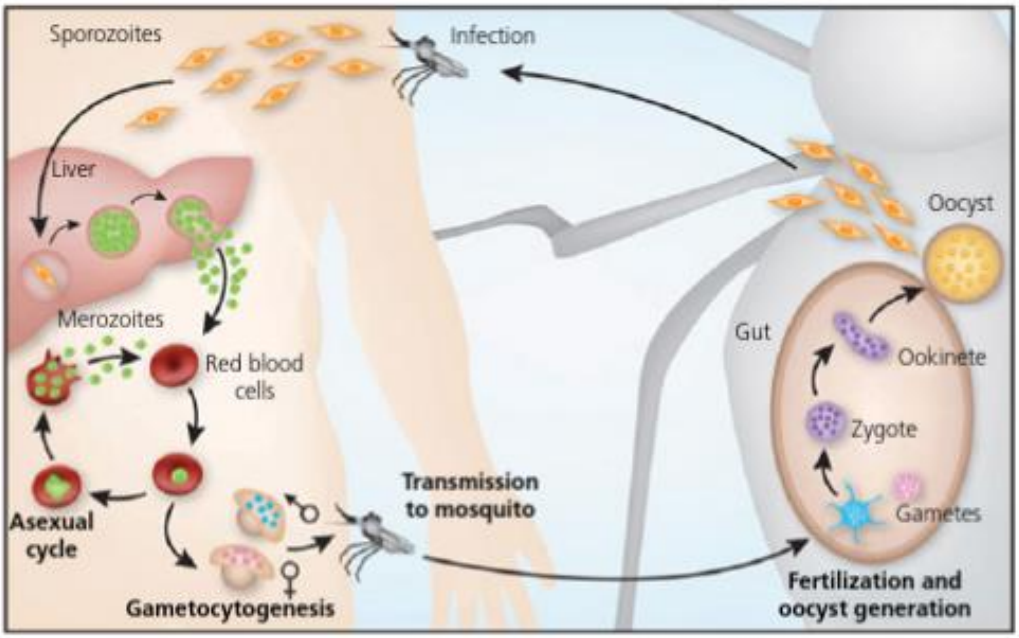


**Figure 1.1** Life Cycle of the malaria parasite from *Anopheles* mosquito into the human body. (Source: Chohan et al., 2008)

*1.4 Signs and Symptoms*

Once the mosquito has fed on the human invading its system with sporozoites, symptoms can start to occur after the incubation period. In *P. falciparum* the incubation period can be around 10- 14 days (Marcus & Babcock, 2009). The rupturing of the red blood cells and release of parasites into the blood causes the most common symptoms of fever with chills (Trampuz et al., 2003). As the parasite products are dispersed into the bloodstream following haemolysis of the mature schizonts, it triggers a reaction several hours later that culminates into the characteristic fever spikes known in malaria (Chohan et al., 2001: Marcus & Babcock, 2009). These are known as having three stages: cold stage that lasts 1-2 hours ranging from chills to extreme shaking, hot stage that last 3-4 hours with a high fever up to 41.7$^{o}$C and wet stage lasting around 2-4 hours characterized by profuse sweating (Chohan. et al., 2001).

*P. falciparum* infections can present as uncomplicated and severe malaria. If uncomplicated *falciparum* malaria is detected it must be treated immediately before developing into severe malaria. Severe *falciparum* malaria occurs when the parasite sequesters in the post capillary venules of organs, particularly the brain and shows symptoms of orthostatic hypotension, cerebral anomalies such as drowsiness,

confusion, hemiplegia, seizures, delirium, and coma (Figure 1.2) (Chohan. et al., 2001; Marcus & Babcock, 2009). It can also cause massive intravascular haemolysis leading to jaundice, haemoglobinuria, tender and enlarged spleen, acute renal failure, and uraemia, proving to be fatal in about 20% of patients (Chohan. et al., 2001). As mentioned above this severe neurological complication of P. falciparum is cerebral malaria. When investigating the pathophysiology of cerebral malaria, it's important to note that parasite sequestration, inflammatory cytokine production and vascular leakage in cerebral microvasculature are the main factors for symptoms. Sequestration is the adherence of parasite red blood cells to the endothelial lining mediated by the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) and several other parasite proteins remodelled into the host RBC membrane (Song et al., 2022). PfEMP1 is encoded by a multigene family of up to 60 var genes (Chakravorty & Craig, 2005). When the endothelial cells are exposed to parasitized red blood cells and tumour necrosis factor, they can mobilize immune and pro-adhesive responses (Chakravorty et al., 2008). These proteins mediate binding to host receptors. Intracellular adhesion molecule-1 (ICAM-1) is the most important host receptor, whose expression is upregulated adjacent to sequestered parasites (Idro et al., 2010). Alongside adherence, agglutination to other infected red bloods cells as well as platelets and white blood cells occurs which causes the mass of the sequestered parasites to increase (Idro et al., 2010). This damage to the endothelium by sequestration of infected red bloods cells leads to an increase in vascular obstruction and vasoconstriction that weakens perfusion and could aggravate a coma through hypoxia, thus cerebral malaria (Idro et al., 2010). Patients developing these symptoms must be treated immediately as these complications can rapidly develop and progress to death within hours or days (Trampuz et al., 2003). Cerebral malaria has been defined by the World Health Organisation as a coma for at least an hour after convulsion episode and asexual forms of *Plasmodium falciparum* parasites of a blood smear (Idro et al., 2010). The longer the coma, the more serious the outcome therefore it is imperative that it is treated immediately. Mortality rates of cerebral malaria ranges from 10% to 50% with treatment with over 90% patients discharged from hospital with no neurological symptoms (Trampuz et al., 2003). However, the 10% of patients discharged could have gross deficits such as blindness, ataxia, and long-term conditions such as cognitive and behaviour impairments. Furthermore, pulmonary complications can occur a few days in, that develop at a rapid rate with or without

treatment. Signs of acute lung injury are pulmonary oedema, resulting in hypoxemia and respiratory failure that could lead to intubation. Due to intense sequestration of parasitized red blood cells in the microvasculature of the myocardium, patients suffer with severe hypotension alongside pulmonary oedema, sepsis and possibly massive haemorrhage that could be fatal due to splenic rupture (Trampuz et al., 2003). Furthermore, acute kidney injury (AKI) caused by acute tubular necrosis cause kidney impairments that darken the urine due to haemoglobinuria. This is known as a complication of the *P. falciparum* infection called blackwater fever (Greenwood et al., 2008; Marcus & Babcock, 2009). However, in other malarias than *P. falciparum*, the symptoms can occur every two to three days more specifically *P. malariae* paroxysms can occur every 48-72 hours whereas when caused by *P. vivax* and *P. ovale* they can occur every 42-50 hours (Marcus & Babcock, 2009). *P. vivax* and *P. ovale* both cause hepato-splenomegaly (Finnigan & Okafor, 2023). Common symptoms can be confusion, exhaustion and extreme sweating however haemolytic anaemia is a symptom that occurs in all (Finnigan & Okafor, 2023).

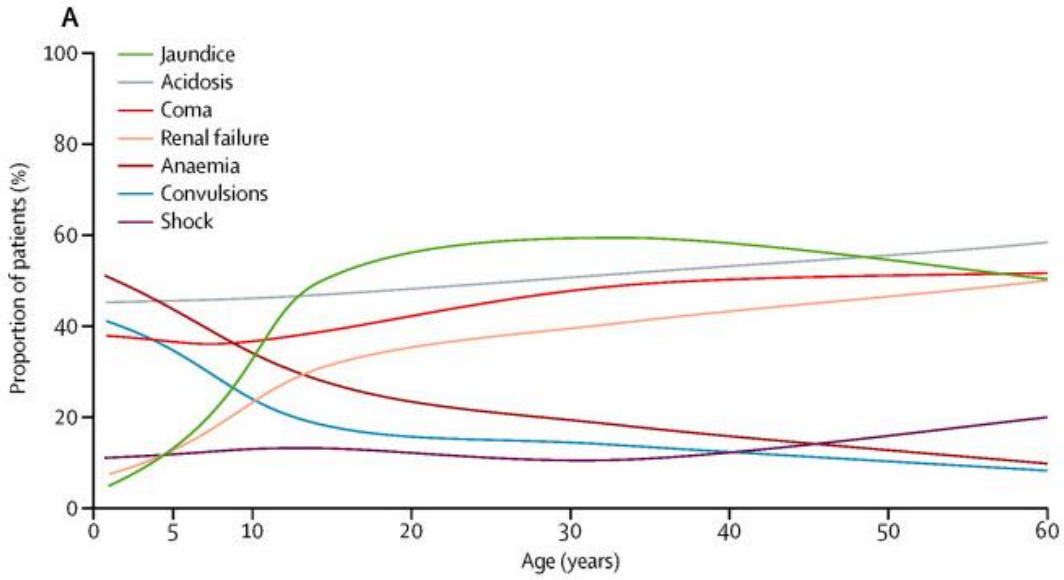


**Figure 1.2.** Manifestations of severe malaria (*P. falciparum*) by age. (Source: Chohan et al., 2001)

*1.5 Diagnosis*

Diagnosis of malaria can be done in many ways. As malaria symptoms mimic many diseases such as hepatitis, meningitis, and typhoid; non-specific features supporting malaria diagnosis must be considered such as consistent clinical and travel history, splenomegaly, and thrombocytopenia (Dunbar et al., 1996). A history showing travel

to endemic areas, recent blood transfusions, or drug abuse in a person with high fever of unknown origin could suggests malaria (Bria et al., 2021). Knowing the intensity of transmission in the community would help towards the correct diagnosis of the patient.

However, as clinical features of severe and uncomplicated malaria are not specific more accurate diagnosis is required. Laboratory identification of the parasites in RBCs of peripheral blood smears would be ideal. Microscopic analysis of stained thick and thin blood smears would be a diagnostic reference standard. The presence of parasitized RBCs in the patients' blood (parasitaemia) is measured as a percentage when looking at the severity of malaria (Jong & Netter, 2015). When using thick-smear diagnosis, it allows sensitive parasitaemia quantification usually as low as 30 parasites per microlitre (Plewes et al., 2019). Whereas thin-smear diagnosis permits speciation and prognostic assessment via parasite staging and proportion of pigment containing neutrophils (Plewes et al., 2019). These methods have been always deemed as the gold standard (White et al., 2014).

Another method of detection are rapid diagnostic tests (RDTs) that can be said to be a lifesaving method of *P. falciparum* (Jong & Netter, 2015). These tests are lateral flow immune chromatographic antigen detection tests and can be conducted on patients showing signs and symptoms of malaria and who either live or have returned from travel in endemic areas. These tests are meant to aid in the help of diagnosis in primary care settings, as it can demonstrate consistent detection of malaria at both low parasite densities and high parasite densities (Jong & Netter, 2015). It is relatively easy to use and has a low false-positive rate, detecting both *P. falciparum* and *P. vivax* infections. Research data has shown that for *P. falciparum*, sensitivity and specificity for this test was 99.7% and 94.2% (Jong & Netter, 2015). An example of an RDT is aldolase antigens in finger prick blood test that are now widely used as a faster simpler diagnostic method (White et al., 2014).

To support the diagnosis, supplementary laboratory values can be used. In the case of malaria, the haemoglobin levels would be decreased, normal to decreased leukocyte count (as low as 3,000/µl) and protein and leukocytes in urine sediment (Chohan. et al., 2001). Moreover, in the case of *P. falciparum*, serum levels reflect disseminated intravascular coagulation (DIC): reduced number of platelets (20,000 to

50,000/µl), prolonged prothrombin time (18 to 20 seconds), prolonged partial thromboplastin time (60 to 100 seconds), and decreased plasma fibrinogen (Chohan. et al., 2001).

Polymerase chain reaction (PCR) may be used to diagnose malaria, as they show a high degree of sensitivity and accurate speciation including *P. knowlesi* (Walker, Nadjm & Whitty, 2014). The patients' blood can be sent off to a reference laboratory for species confirmation by molecular testing (Jong & Netter, 2015). It can detect the *Plasmodium*-specific nucleic acid sequences however are limited by speed, cost, and availability of technology (Walker, Nadjm & Whitty, 2014).

### *1.6 Prevention*

Across the world, with upcoming technology, drugs and equipment, prevention is the main priority as the drugs currently being used are becoming resistant. Prevention methods include vector control using insecticides and larvicides, impregnated bed nets, vaccinations and chemoprophylaxis and chemoprevention (Organization, 2023).

#### 1.6.1 Environmental Prevention (Vector Control)

Firstly, environmental interventions such as draining swampy areas could be a prevention method. The areas where the mosquitoes breed, the standing fresh water is collected and then introducing fish into the fresh-water ponds to eat the mosquito larvae is a known prevention method (Jong & Netter, 2015). Other methods used previously can include, applying larvicidal chemicals into ponds, lakes, and slow-moving streams where the mosquitoes breed (Jong & Netter, 2015). The use of indoor residual sprays (IRS) for example residual insecticides such as dichlorodiphenyltrichloroethane (DDT) were used to spray the inside of human houses (Jong & Netter, 2015), however negative environmental impacts of such methods have now minimised their uses. Human intervention methods include using insect sprays, mosquito bed-nets, insect repellents, mosquito coils, sensible clothing covering limbs and avoiding exposure at night especially (Walker, Nadjm & Whitty, 2014). These methods are used as a preventative measure against being bit by mosquitoes. Statistics have shown that wide scale deployment of pyrethroid-insecticide-treated mosquito nets has contributed to the substantial fall in malaria morbidity and mortality (White et al., 2014). Studies have shown that applying insect repellent, such as N, N-

diethyl-meta-toluamide (DEET), to all exposed skin areas and having clothes that have been treated with insecticides such as permethrin, can greatly decrease exposure to mosquito bites (Jong & Netter, 2015). However, the use of insect repellent depends largely on the behaviours of the local *Anopheles* vector and whether resistance has emerged (White et al., 2014).

### 1.6.2 Chemoprophylaxis

Another prevention method would be drug prophylaxis (chemoprophylaxis), that protects against developing disease when given for four weeks after exposure to catch any blood-stage infections that emerge from liver (Dunbar et al., 1996; White et al., 2014). It is to be taken under consideration when deciding chemoprophylaxis, the traveller's medication allergies, itinerary, activities, length of travel to endemic areas and medication costs. Malarial chemoprophylaxis functions by targeting the liver schizont, blood schizont or hypnozoite stages of the plasmodium life cycle (DeVos & Dunn, 2019). This is essential for travellers going to high transmission countries, especially Africa. The most up to date advice can be found at the Public Health England Malaria Reference (Walker, Nadjm & Whitty, 2014). Most prescribed medications for chemoprophylaxis are atovaquone, doxycycline and mefloquine. Studies have shown over time that these medications have all been equally effective in the prevention of diseases in short-term travellers, with atovaquone and doxycycline having the fewest side effects on the patients (DeVos & Dunn, 2019). For travellers spending less than 3 weeks in areas where malaria exists, weekly prophylaxis includes oral chloroquine beginning 2 weeks before the exposure and ending 6 weeks after it (Chohan. et al., 2001 Again, recommendations of chemoprophylaxis depend on local patterns of susceptibility to antimalarials and likelihood of acquisition of malaria, duration of the stay and the prevalence of chloroquine-resistant *P. falciparum* (White et al., 2014).

## *1.7 Treatment*

There are four different types of drug classes that are used to treat malaria. These include quinoline, antifolates, artemisinin and antimicrobial compounds. Due to rapid drug resistance, the use combination therapy has become more popular with the use of three or more compounds with different mechanisms of action to target and

eradicate malaria (Chen & Hsiang, 2022). Treatment is dependent on location of the infection and the types of plasmodium species and the severity of the disease (Bennett & Herchline, 2023).

### 1.7.1 Quinoline derivative compounds

Chemotherapy has played an important role in treating malaria, with Quinoline containing antimalarial components being some of the most effective. This group of compounds evolved from structural modifications of quinine, including 4-aminoquinoline compounds such as mefloquine and chloroquine (Figure 1.3) (Farooq & Mahajan, 2004). These are both cheap and highly potent as antimalarial in addition to being safe and commonly available drugs. These drugs work by interfering particularly with the digestion of haemoglobin in the blood stages of the malaria life cycle (Foley & Tilley, 1997). The effectivity of quinolines derivatives is further enhanced by the ability of the drug to concentrate in the plasmodial food vacuole. Specifically, chloroquine increases pH and accumulates in the food vacuole of the parasites (Zhou et al., 2020). The host red blood cell haemoglobin therefore degrades leading to the release of the toxic products (Zhou et al., 2020). Chloroquine works by inhibiting the polymerisation of the toxic haemoglobin digestion biproduct haem and preventing the parasite from converting it to inert haemozoin, killing the parasite with its own waste (Foley & Tilley, 1997). Due to the accumulation of free hematin that is highly toxic to *Plasmodium*, the cell membrane is broken leading to death of parasites (Zhou et al., 2020). Predictably resistance to this family of antimalarials is widespread, limiting their use in chemotherapy and prophylaxis. Chloroquine resistance directly relates to mutation in the *Plasmodium falciparum* chloroquine- resistance transporter (PfCRT) gene of the parasite. Evidence suggests that PfCRT is a transporter protein that removes chloroquine out of the food vacuole in resistant PfCRT mutants, therefore causing failure of the drug to accumulate in the plasmodial food vacuole (Van Schalkwyk & Egan, 2006).

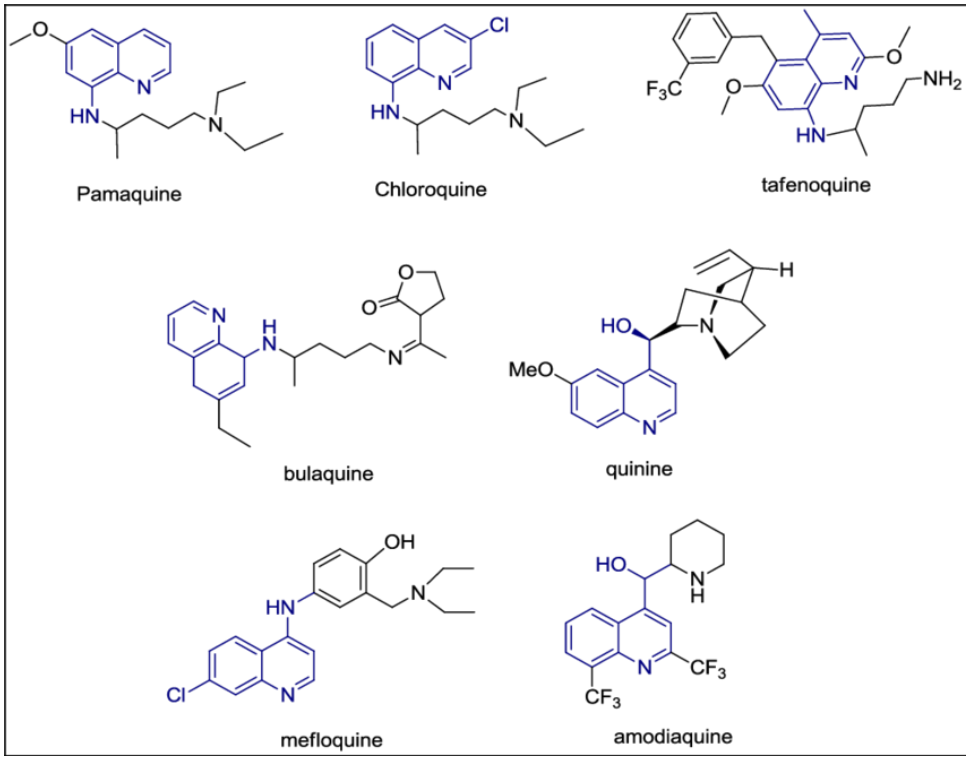


**Figure 1.3.** Examples of Quinoline anti-malarial marketed drugs. (Patel et al., 2017).

### 1.7.2 Antifolate Compounds

Antifolate drugs are used as mainline agents in some sub-Saharan African countries. This type of antimalarial drug interferes with the folate metabolism that is key to the survival of the parasite (Gregson & Plowe, 2005). Antifolates are known antimalarials due to their inhibition of dihydrofolate reductase (P*f*DHFR) and dihydropteroate synthase (P*f*DHPS) (Henriquez & Williams, 2020). Most common antifolate drugs are Pyrimethamine (PYR), Proguanil (PG), Cycloguanil (CG) and the sulpha drugs. These most known as Sulphonamide and Sulphodoxine (Müller & Hyde, 2010). Drugs such as pyrimethamine and proguanil target (P*f*DHFR), whereas sulpha drugs act as competitive inhibitors of P*f*DHPS (Henriquez & Williams, 2020). Inhibitors of both enzymes P*f*DHPS and (P*f*DHFR) are synergistic when used together in combination therapy (Henriquez & Williams, 2020). However, over the years antifolates have become resistant to malaria parasite by point mutations in dihydrofolate reductase and dihydropteroate synthase. These two enzymes are key in the folate biosynthetic pathway that are targeted by the antifolates (Gregson & Plowe, 2005).

### 1.7.3 Artemisinin Compounds

Artemisinin compounds are synthesized from a plant *Artemisia annua* (Farooq & Mahajan, 2004). These drugs have been used in traditional Chinese medicine for more than 2000 years (Maude et al., 2009). Compounds such as artesunate, artemether and arteether are most effective antimalarials that seem to influence protein synthesis by the malaria parasite. In comparison to quinine compounds, these have shown a very rapid parasite clearance and are used for the treatment of severe malaria (Farooq & Mahajan, 2004). They can kill parasite stages at a safe concentration. Artemisinin compounds have a very short half-life and known to have a low risk of resistance. However, to further reduce risk of resistant artemisinin combination therapies is preferred.

### 1.7.4 Treatment Combinations

Moreover, before treatment can start, the patient initially needs to be diagnosed as to which malaria they have, whether it is *P. falciparum* and if so whether it is uncomplicated or severe.

Benign relapsing malaria known to be consistent in *P. vivax, P. ovale* and *P. malariae* strains, chloroquine is known as the most common drug of choice used for 3 days. Followed from this, primaquine is used for 14 days to eliminate the hepatic reservoir of infection and prevent relapses in *P. vivax* and *P. ovale* (Chohan et al., 2001; Dunbar et al., 1996; Walker, Nadjm & Whitty, 2014). However, in South- East Asia, the primaquine must be given for 3 weeks because of the relative resistance. Before primaquine is administered as a precaution levels of the enzyme glucose-6-phosphate dehydrogenase (G-6-PD) must be checked, as haemolysis may develop (Dunbar et al., 1996).

Uncomplicated malaria usually has low parasitaemia in patients and although it is uncomfortable, the patients are fully conscious (Jong & Netter,2015). For uncomplicated falciparum malaria artemisinin combination treatment (ACT) would be recommended as first line therapy that would be given over three days with a slowly eliminated antimalarial. It is known to be curative in around 90% cases and is a rapid and highly effective course of treatment for uncomplicated falciparum (White et al., 2014). Symptoms and parasitaemia tend to decrease within 24 hours after the

artemisinin/ chloroquine is administered, and the patient should recover within 3 to 4 days (Chohan. et al., 2001).

However severe malaria most caused by *P. falciparum,* is when there is more than 5% parasitaemia. They must have no other recognized disease cause and have one of the following clinical problems such as impaired consciousness, coma and renal failure (Jong & Netter, 2015). Malignant malaria known commonly as *P. falciparum* requires a different level of treatment due to its severe symptoms. Malaria caused by *P. falciparum*, known to be resistant to chloroquine must be treated with oral quinine for at least 10 days given concurrently with pyrimethamine and a sulfonimine, however side effects expected can be nausea, vomiting and deafness (Chohan. et al., 2001). As treatment is given, patients should be monitored for hypoglycaemia and cardiac arrythmias (Idro et al., 2010). In a study in more recent years, children suffering from severe falciparum malaria in Africa reacted significantly to artesunate reducing the mortality rate from 10.9% to 8.5% compared to quinine (White et al., 2014). The main benefit of the drug is the rapid reduction of parasitaemia (Walker, Nadjm & Whitty, 2014). Toxicity also proved that artesunate had no severe side effects, only when high cumulative doses are given, which can suppress bone marrow temporarily (White et al., 2014). The only drug available in the United States effective against the hepatic stage is primaquine phosphate given daily for 14 days, however the drug can induce haemolytic anaemia, especially with patients with glucose-6-phosphate dehydrogenase deficiency (Chohan. et al., 2001). In the case that the patient is vomiting, has a high parasitaemia of more than 4% then intravenous (IV) quinine should be given.

### *1.8 Drug Resistance*

Drug resistance is defined as the ability of a parasite strain to survive or multiply despite the administration and absorption of a drug when given in does equal or higher than the recommended limits (Farooq & Mahajan, 2004). Drug resistance can be determined in the parasite by either *in vivo* or *in vitro* drug susceptibility assays (Farooq & Mahajan, 2004).

Resistance in *P. falciparum* has been emerging and has been a major contributor to the global resurgence of malaria in the last thirty years. In particular, *P. falciparum* in most malaria-affected areas is highly resistant to chloroquine (White, 2004). Resistance to chloroquine was noticed as early as 1957, observed in Southeast Asia and Colombia. Artemisinin have been known when combined with antimalarial drug to enhance complete cure rates and shorten the duration of therapies and delay the selection and spread of resistant parasites (DeVos & Dunn, 2019). However, artemisinin resistance has now been reported in 5 countries around Far East Asia (DeVos & Dunn, 2019). Over a decade ago, reports showed that strains of *P. falciparum* were showing early resistance to artemisinin-based combination therapy (ACT) (Pazhayam, Chhibber-Goel and Sharma, 2019). *P. Vivax* malaria is becoming increasingly resistant to chloroquine however is still very sensitive to the artemisinin combinations (Krishna and Staines, 2012). Resistance usually develops about 15 years later after the antimalarial is introduced (Figure 1.4). In Africa, a most affordable, effective, practical, and well tolerated alternative to 4-aminoquinolines is sulfadoxine-pyrimethamine (SP) (Wongsrichanalai et al., 2002). This combination was used as a drug of choice to successfully treat chloroquine resistant malaria for a period (Farooq & Mahajan, 2004).) They are both antifolates and act on different targets in the folate pathway of the parasite with synergistic efficacy (Gregson & Plowe, 2005). However, there was a development of resistance occurring in *P. falciparum* starting from the Thai-Cambodian border in the 1960s to Delhi and Africa in the 1980s.

Mefloquine targets blood schizonts and has side effects such as seizures and psychosis (DeVos & Dunn, 2019). Although it is said to be effective against chloroquine-resistant *P. falciparum,* mefloquine resistance is becoming more prominent in parts of Southeast Asia such as Thailand, Cambodia, and Vietnam (DeVos & Dunn, 2019; White, 2004). Moreover, Quinine resistance was reported about a century ago; however, it has been used in combination with tetracycline or doxycycline to enhance its effectiveness (Farooq & Mahajan, 2004). Doxycycline targets blood schizonts and is effective against chloroquine-resistant *P. falciparum.* Evidence has shown that doxycycline has an efficacy rate of 92% to 96% for *P. falciparum* however, side effects can usually include GI upset, esophagitis, and photo sensitivity.

Due to malarial resistance to drugs becoming increasingly more prevalent, urgent efforts are required to discover effective alternative and expand the anti-malarial drug repertoire. Figure 1.4 shows the many antimalarials available and the first report of resistance against them. The major effect of drug resistance is felt mostly in areas of low herd communities, as well as in infants and young children in areas with high malaria endemicity (Wernsdorfer, 1994). In countries that have a lower demographic, effective, affordable, and alternative antimalarial regimens are required. However, this is an increase in costs, therefore the complete elimination of mortality from the disease may become impossible for economic reasons only (Wernsdorfer, 1994).

Understanding the mechanisms of resistance is also a crucial step to working toward reversing resistance and developing more robust antimalarials. Molecular markers have been discovered for antimalarial resistance; including *pfmdr-1* and *pfcrt* polymorphisms associated with chloroquine, mefloquine and artemisinin resistance and *dhfr* and *dhps* polymorphisms associated with sulfadoxine- pyrimethamine (SP) resistance (Farooq & Mahajan, 2004). Using this genetic information, early detection of resistance and future monitoring of drug resistant malaria can be easily detected (Farooq & Mahajan, 2004).

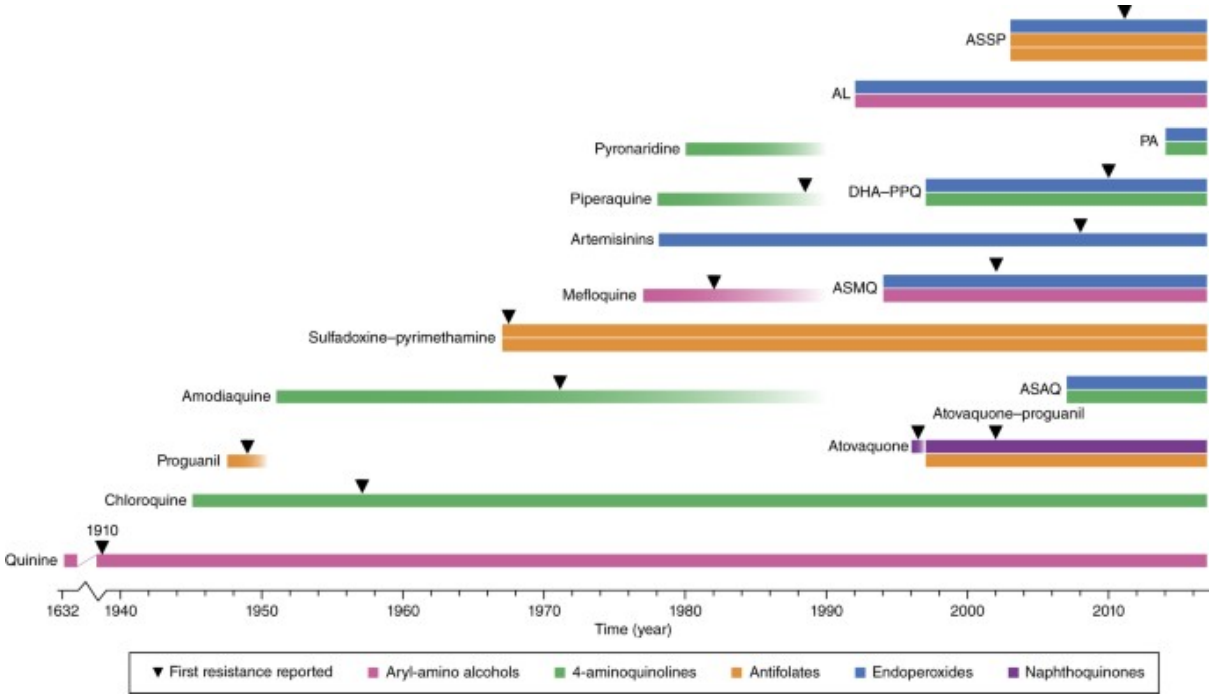

**Figure 1.4.** Dates of introduction and first reports of antimalarial drug resistance against each drug. (Source: Blasco et al., 2017)

## *1.9 Modern Day Malaria*

### 1.9.1 Vaccination Development

A lot of research, funding and time has gone into searching for an effective vaccine for malaria. The most advanced vaccine in development in 2014 is the RTS, S subunit vaccine, which is boosted with potent ASO adjuvant, that targets the circumsporozoite protein of *P. falciparum* (White et al., 2014). Studies using this vaccination showed promising results in older children aged 5-17 months with 55% protection against all falciparum malaria and 35% protection against severe malaria for 14 months (White et al., 2014).

However most recently, the University of Oxford and Serum Institute of India PvT Ltd developed and manufactured R21/Matrix-M malaria vaccine (Datoo et al., 2022). This vaccine received regulatory clearance to be used in Ghana. It has been approved for children aged between 5-36 months who are at the highest risk of death from malaria. The R21/Matrix-M malaria vaccine is a low dose vaccine that can be manufactured at mass scale, which will allow millions of doses to be distributed around African countries that are drastically affected by malaria (Datoo et al., 2022). The World Health Organisation managed to reach their goal of 75% of greater efficacy over the last 12 months (Datoo et al., 2022).

## *1.10 Drug Discovery*

Drug discovery has always been a long, time and money consuming procedure. Human civilisation has been experimenting and consuming drugs for many centuries. Developing a new drug from an original idea to the launch of a finished product costing up to 1 billion dollars and 12 to 15 years (Hughes et al., 2011). It has only been the last 100 years where a foundation has been laid for systemic research and development of drugs. Multi-disciplinary teams comprising of scientists, clinicians, medical practitioners, and legal attorneys are all involved in the process of drug discovery and development (Ng, 2009). Previously the main scientific personnel in drug discovery were synthetic chemists however recently molecular biologists, biochemist and microbiologists all have equally important roles in the drug discovery

and development timeline (Ng, 2009). Drug discovery process usually consists of finding out the target that causes the disease. Chemical or biological compounds are then screened and tested against the known targets. They can also be tested against assays which are representative of the known targets, to find leading drug candidates for further development.

Drug discovery for antimalarials has been prominent since the 1600s, when quinine was first introduced. Quinine was first isolated from the bark of the cinchona tree, a natural product (Tse et al., 2019). Although it was one of the most effective treatments for malaria, resistance was reported in the early 1900s and as of 2006 was no longer used as a front-line treatment for malaria (Tse et al., 2019). Mepacrine is a derivative of methylene blue which was another antimalarial discovered in 1891 by Paul Ehrlich and found to be an effective treatment for malaria (Rubin, 2007). Mepacrine was used throughout the Second World War as a prophylactic sold under the name Atabrine (Tse et al., 2019). Although Mepacrine's use has declined due to the high chance of side effects such as toxic psychosis, methylene blue and its derivatives are subject to increasing interest (Tse et al., 2019).

More recent studies have shown hope in a newly discovered drug based on the 1,2,4-trixolane pharmacophore (Umumararungu et al., 2023). This is a fully synthetic ozonide better known as an advanced derivative of the ring system artemisinin (ART). This compound was said to be as clinically effective as the semi-synthetic artemisinin derivative as well as being well tolerated in trials. However, to avoid resistance it was suggested that a long-acting antimalarial partner drug should be used (Umumararungu et al., 2023).

### 1.11 *Drug Repositioning*

In the last decade, more drugs are becoming resistant to malaria, therefore there is an urgent need for new treatments. With the slow pace of new drug discovery and substantial costs, it is becoming increasingly hard to source new cures. Therefore, repurposing (also known as drug repositioning) is a new and upcoming innovative way that can discover 'old drugs' to treat both common and rare diseases and fast track drug discovery (Pushpakom et al., 2018). This is an attractive proposition as it involves the use of de-risked compounds, preferably patent expired, with potentially lower

overall development costs and shorter development timelines (Pushpakom et al., 2018). As mentioned previously drug repositioning decreases the time/cost of the drug development process significantly, that researchers say they only need 1-2 years to identify new drug targets and 8 years to develop a repositioned drug on average (Xue et al., 2018). Using the traditional drug development strategy can cost up to 15 billion dollars whereas repositioning costs around 2 billion dollars maximum (Shameer et al., 2015). This extreme decrease in costs is beneficial for countries such as Africa who have such a small budget for healthcare and towards treating malaria.

Traditionally, drug development strategies consist of five stages: discovery and pre-clinical, safety review, clinical research, FDA review and FDA post- market safety monitoring (Xue et al., 2018). However, drug repositioning only consists of four stages: compound identification, compound acquisition, development, and FDA post-market safety monitoring (Xue et al., 2018) (Figure 1.5). There have been many success stories about drug repositioning such as the drug Sildenafil, originally intended for treating Angina, however, now has indication of treating erectile dysfunction and pulmonary arterial hypertension (Li & Jones, 2012). Another example is Duloxetine, that originally taken for depression to target Serotonin, norepinephrine, and reuptake. After drug repurposing, there was indication that this drug can be explored to treat stress urinary incontinence, fibromyalgia, and chronic musculoskeletal pain (Li & Jones, 2012). Clearly all these examples prove that this is not an opportunity to be missed by researchers to develop and discover new drugs.

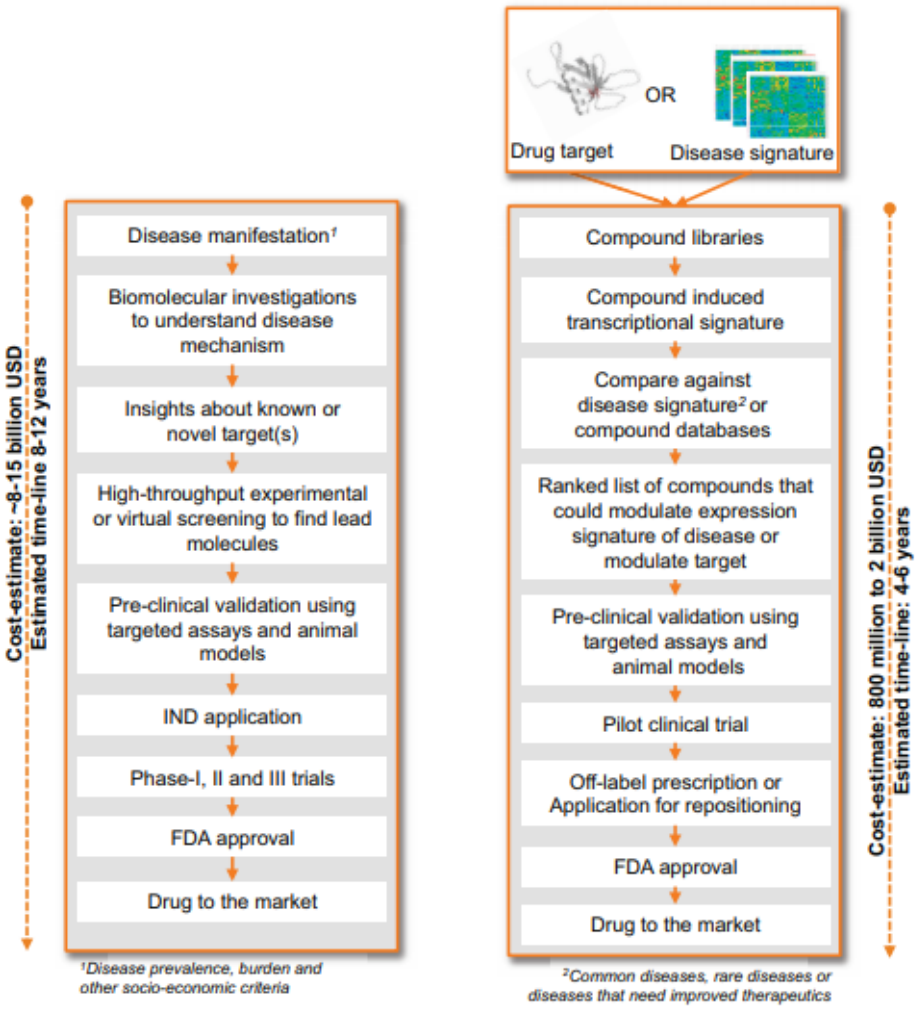


**Figure 1.5.** A comparison of traditional drug discovery and drug repositioning timelines along with cost estimate and timeline from initial findings to the market. (Source: Shameer et al., 2015)

### 1.11.1 Malaria Drug Repositioning

Drug repositioning for malaria has been of interest since the early 1900s. The first drugs known as Prontosil were developed initially as industrial azo dyes, that were said to have possessed antibacterial activity and could treat a wide range of bacterial infections (Nzila, Ma & Chibale, 2011). However, due to unacceptable toxicity and low efficacy, their use was abandoned. Similarly in the 1970s, methotrexate (MTX), an anticancer drug that disrupts folate metabolism showed promising result to block malaria parasite (Nzila et al., 2011). However, these results were never continued into investigation due to concern over the MTX toxicity.

There are selected compound libraries already approved by the Food and Drug Administration agency, therefore the pace of drug repositioning (repurposing) is much more efficient. The pharmacological advantage of having these libraries is that it narrows down to chemical entities that either act as novel or potential targets in the malaria parasite. Known bioavailability and safety profiles mean that developmental pipeline could be shorter. Moreover, diseases such as malaria have major significant factors such as time and development cost, therefore new acquired methods of repositioning can not only offer a solution but also identify suitable combinatory regimes with existing anti-malarial that can be further developed (Matthews, 2015).

*1.12 Testing Compound Drugs*

This study is a follow on from previous work published by Matthews et al., 2015 in our laboratory where ~1200 drug leads from the LOPAC FDA-approved, patent-expired compound library was interrogated for antimalarial efficacy. The LOPAC library consisted of 1,280 pharmacologically active compounds that cover major target classes such as kinases, gene regulators and neurotransmitters (Merck, 2024). 6 compounds were selected to show mild to moderate potency *in vitro* antimalarial activity in preliminary studies.

This project seeks to carry out dose response profiles and *in vitro* safety experiments to investigate the selected compounds in view of progressing them for second phase optimisation. $IC_{50}$ values were obtained from these experiments to understand potency and toxicity of the drugs. $IC_{50}$ values determined drug concentration that can inhibit growth of parasites by half when compared to the control, Chloroquine. In context, the lower $IC_{50}$ value the more potent and effective the drug and therefore a lower dosage would be required. The effects of a higher dosage of drug can be associated with toxicity due to a surplus within the blood.

A brief description of the drugs and their current uses is given below:

| **Drug** | **Main use of Drug** |
|---|---|
| Propafenone | Anti-arrhythmic Drug |
| Spironolactone | Mineralocorticoid receptor antagonist |
| Mitoxantrone | Doxorubicin Analogue |
| Ivermectin | Anti-viral, antimicrobial |
| Fluoxetine | Selective serotonin reuptake inhibitor |
| Nicardipine | Calcium Channel blocker |

**Table 1.** 6 drug compounds selected and their main use.

### 1.12.1 Propafenone

Propafenone is a known anti-arrhythmic drug that can be given both intravenously and orally (Harron & Brogden, 1987). It is a medication that is used to treat a range of arrhythmias including atrial fibrillation and supraventricular tachycardia. It works by restoring the heartbeat to a steady and regular state and restoring to a normal heart rhythm (Harron & Brogden, 1987). Propafenone showed significant potency therefore further development of this drug for antimalarial purposes could prove successful.

### 1.12.2 Spironolactone

Spironolactone is a mineralocorticoid receptor antagonist used in the management and treatment of hypertension and heart failure. Spironolactone works by promoting magnesium and potassium retention, an increase in uptake of myocardial norepinephrine, decreases the formation of myocardial fibrosis and reduces mortality associated with malignant ventricular arrythmias and progressive ventricular dysfunction (Soberman & Weber, 2000).

### 1.12.3 Mitoxantrone

Mitoxantrone was developed in the 1980s as a doxorubicin analogue in a hope to find a cytotoxic agent with decreased cardiotoxicity compared to doxorubicin (Fox, 2004). As both mono and combination therapy, Mitoxantrone has displayed therapeutic efficacy in advanced breast cancer, non-Hodgkin's lymphoma, acute non-lymphoblastic leukaemia, and chronic myelogenous leukaemia (Faulds et al., 1991). It works by inhibiting DNA synthesis by intercalating DNA, inducing DNA strand breaks, and causing DNA aggregation and compaction (Faulds et al., 1991).

### 1.12.4 Ivermectin

Ivermectin was discovered in the late 1970s, a dihydro derivative of Avermectin (Figure 1.7). Although originally introduced as a veterinary drug, Ivermectin has become known for its antimicrobial, antiviral and anti-cancer properties. It is said to be highly effective against many microorganisms including some viruses. It has been

approved for treatment in humans for only certain tropical diseases (Heidary & Gharebaghi, 2020). But it is most openly used to treat infections such as parasitic worms, head lice and skin conditions such as rosacea.

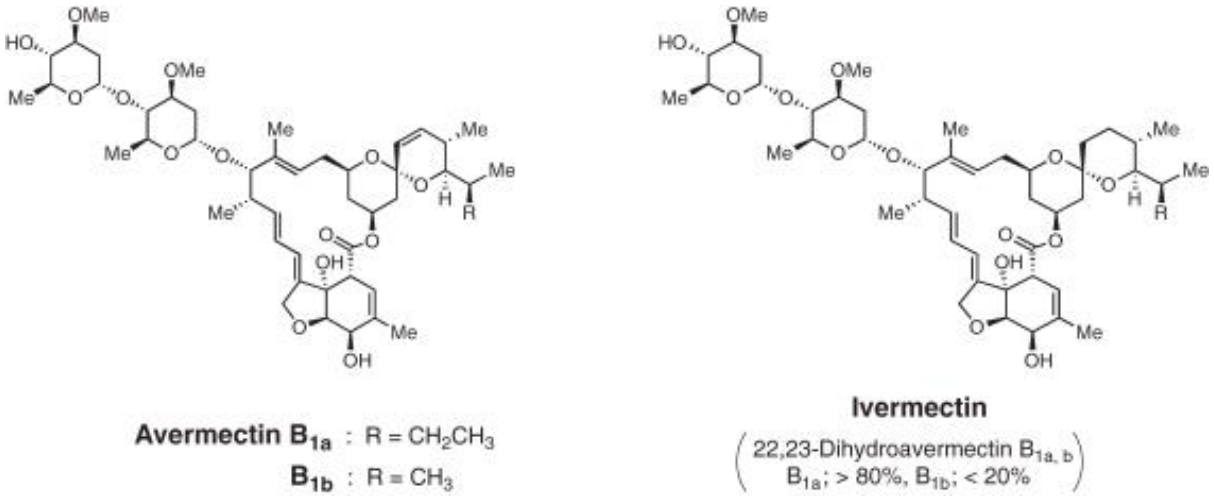


**Figure 1.6.** Molecular diagrams of Avermectin and the di-hydro derivative, Ivermectin. (Source: Crump & Omura, 2011).

### 1.12.5 Fluoxetine

Fluoxetine is known as a selective serotonin reuptake inhibitor (SSRI). It is an FDA approved drug for conditions such as major depressive disorder, obsessive-compulsive disorder, panic disorder, bulimia, bipolar depression, and treatment resistant depression. Fluoxetine works by blocking the reuptake of serotonin into presynaptic serotonin neurons by blocking the reuptake transporter protein located in the presynaptic terminal (Sohel et al., 2022). It also has many drug-drug interactions due to its metabolism at the CYP2D6 isoenzyme.

### 1.12.6 Nicardipine

This drug is a dihydropyridine calcium channel blocker that interferes with the inward displacement of calcium ions through active cell membranes. Myocardial contractility is reduced as they influence the myocardial cells therefore its primary physiologic action is vasodilation (Tobias, 1995). This drug has proven clinical efficacy in managing hypertension, angina pectoris and ischaemic related cerebrovascular disease. Nicardipine has shown to be useful as initial monotherapy as well as in combination with other antihypertensive drugs (Sorkin & Clissold, 1987).

1.13 Aims and Objectives of project:

1. To identify out of the 6 compounds chosen from FDA approved-drug libraries, which drugs show potency *in vitro* antimalarial activity against *P. falciparum* K1 strain
2. To obtain $IC_{50}$ levels of parasite growth inhibition profiles for the compounds selected
3. To obtain compound safety profiles using MTT toxicity results
4. Using $IC_{50}$ values to obtain selectivity indices for all drug compounds

## 2. General Materials and Methods

### *2.1 In vitro culture of Plasmodium falciparum*

All procedures were carried out in a sterile hood (ESCO class II biological safety cabinet), using pre sterilised equipment and aseptic techniques. All waste material was disinfected with 10 % Chemgene before autoclaving at 121$^{o}$C for over two hours and disposed of in an airtight heavy duty bio waste bag.

#### *2.1.1 Preparation of complete media*

RPMI 1640 1x (+) L-Glutamine (+) 25mM HEPES (GIBCO, Life Technologies, UK) was used to prepare the culture media. The incomplete media was supplemented with four additives: 2.5 g Albumin bovine serum fraction V (Sigma, UK), 2.5ml (1mg/1ml) hypoxanthine (Sigma, UK) in phosphate buffered saline (PBS) (Fisher Chemical, UK), (40%) glucose (Dextrose Anhydrous, Fisher Scientific, UK) in sterile water and 0.5ml (50mg/ml) gentamycin (Sigma, UK) in PBS which was added in a 50ml falcon tube and syringe filtered in the 500 ml RPMI incomplete media. The bottle was then mixed gently and stored at 2-8$^{0}$C.

#### *2.1.2 Washing Media*

RPMI 1640 1x (+) L-Glutamine (+) 25mM HEPES media without additives was used as washing media and stored at 2-8$^{o}$C for 2 weeks.

#### *2.1.3 Preparation of the Red blood cells (RBCs)*

An O+ blood purchased from Manchester human blood bank centre was washed to remove leukocytes and preservatives added on donation. Equal volumes of whole blood (~40 ml) were aliquoted in the falcon tube and stored at 2-8$^{o}$C for no longer than 4 weeks. Immediately before use the in culturing the parasites, the blood was washed with incomplete media, centrifuging the falcon tubes containing whole blood for 5 minutes at 3400 rpm. Following centrifugation, the blood plasma, and the top pale layer (buffy coat containing white blood cells) was removed. This process was repeated 3 times ensuring all leukocytes were removed from the whole blood. This was followed by a wash in complete media. Finally, the whole blood was at 100% haematocrit and was re-suspended with an equal amount of RPMI 1640 complete media to gain a 50% haematocrit.

*2.1.4 Plasmodium falciparum continuous culture*

The *Plasmodium* culture was initiated by aliquoting 10ml of complete media (RPMI 1640) and 0.5ml of washed blood into 50ml culture. Thereafter, 0.5ml of the parasitized blood (retrieved from the liquid nitrogen) was added to the flask to gain 5 % haematocrit. The culture flask was gassed with 5% $CO_2$, 5% $O_2$ and 90% $N_2$ gas mixture (BOC Limited, UK). The culture flask was placed back into the incubator (Leec culture safe touch 190 $CO_2$ Leec Limited, UK) at 37$^{o}$C.

*2.1.5 Maintenance of Plasmodium falciparum culture*

Regular maintenance of the culture flasks was required to prevent overgrowth and cell death. Parasites were normally cultured in 10 ml volumes in a 50 ml culture flask. The culture was maintained at 5% haematocrit assessed by using a microscope. The media was changed approximately every 48-72 hours, by carefully removing the media in the flask without disrupting the parasitised blood layer settled down at the bottom of the flask. New media was then added to the flask to give it a final 5% haematocrit. A blood film was prepared for the estimation of the level of parasitaemia. Following parasitaemia count, the culture was diluted, gassed, and incubated as previously described.

*2.1.6 Estimating the parasitaemia.*

The parasitaemia was estimated by preparing a thin blood film. A concentrated parasitised blood was placed on a glass slide and smeared across to obtain a thin film. The slide was air dried and fixed with 100% methanol and stained with the Giemsa stain. The Giemsa stain was prepared by diluting Gurr's Giemsa stain solution (BDH/VWR International limited, UK) to 10% using Giemsa buffer. The Giemsa buffer was prepared by dissolving one buffer tablet of pH 6.4 (BDH Laboratory Supplies, England) into 1 litre of distilled water. The stained slides were left to air dry for 20 minutes and washed with water. Using a Leica DM 500 compound microscope, viewed under the oil immersion x100 lens, the parasitaemia was estimated. The parasitaemia was estimated by counting the number of red blood cells (RBCs) in 3 different fields that could range from 100-250 cells. Then the number of infected RBCs within the same view was counted, taking into consideration that even RBCs that contained two parasites within were counted as one parasite. Thereafter, the parasitaemia level was

estimated by calculating the average of infected RBCs divided by the total number of RBCs and multiplied by hundred to obtain a parasitemia percentage.

*2.1.7 Preserving the Plasmodium falciparum in liquid nitrogen.*

The culture with approximately 10% parasitaemia, majorly a high level of ring stage. The culture was transferred into falcon tube and centrifuged for 5 minutes at 3400 rpm. The supernatant was carefully removed, thereafter, an equal amount of media was added ensuring a 50 % haematocrit. 0.5ml of the resuspension were transferred to two 2ml sterile cryovials. A Ringer's solution prepared by adding 9g NaCl, 0.42g KCl and 0.25g $CaCl_2$ in a litre) alongside adding 20 % of dimethyl sulphoxide (sterile filtered DMSO, Sigma, UK). 2ml of the solution was added into sterile cryovials. These cryovials were immediately frozen in the liquid nitrogen for preservation and storage for further use.

*2.1.8 Retrieval/ Thawing the Plasmodium falciparum from the liquid nitrogen.*

Two cryovials were removed from the liquid nitrogen and placed into a water bath at 37$^{o}$C. The culture was transferred into an Eppendorf and centrifuged at 12,000 rpm for 2 minutes using the minispin (Eppendorf, UK). The supernatant was removed, and the culture was gently resuspended in 1ml of 10% sorbitol (Fisher Scientific, UK) solution (in distilled water ($dH_2O$)) and further centrifuged. Once centrifuged the supernatant was removed again and resuspended in 1ml of 5% sorbitol solution (in $DH_2O$) and further centrifuged. Once the supernatant was removed, the culture was washed three times in complete media. The pellet was resuspended with 1ml complete media and placed in the culture flask with 10ml complete media with 0.5 ml washed blood. The culture flask was gassed and placed in the incubator at 37$^{o}$C until required.

*2.1.9 Plasmodium Culture Synchronisation*

Synchronisation was performed on a predominantly ring parasite stage. Briefly, the culture was transferred into a falcon tube and centrifuged at 3400 rpm for 5 minutes and the media was removed. Thereafter, 5% sorbitol made up in distilled water (DH2O) that had been filtered using Millipore filter of 0.22µm (Sartorius Stadium Biotech GmbH, Germany) was added and incubated for 5 minutes at room temperature before centrifugation. The supernatant was discarded, and the culture pellet was then washed with complete media at least three times before final

resuspension with complete media added to make up a 50% haematocrit. This synchronised culture is then used in the same way to set up a new culture as described previously (section 2.1.5).

*2.2. Drug Assays*

*2.2.1. Preparation of drug stock solutions*

All drugs used in the study were obtained from Sigma Aldrich UK. All stock solutions for each drug were prepared in accordance with the manufactures instructions that were found on the manufacturers site. In this experiment Chloroquine was used as a control. A 20mM stock of chloroquine (MW= 515.86) was prepared using sterile water. 2mg of powder stock was diluted with 1.9ml of sterile water.

For each of the compounds being tested, the same method was used, however different concentration and dissolving agent. Table 2 shows the amount of compound weighed out and DMSO added to achieve an 25mM drug stock concentration.

| **Drug** | **Molecular Weight (MW)(g/mol)** | **Weighed out (mg)** | **DMSO added (µL)** |
|---|---|---|---|
| Propafenone | 377.90 | 2 | 211 |
| Spironolactone | 416.57 | 2 | 192 |
| Mitoxantrone Dihydrochloride | 517.40 | 2 | 155 |
| Ivermectin | 875.1 | 2 | 91 |
| Fluoxetine | 345.8 | 2 | 231 |
| Nicardipine | 515.99 | 2 | 155 |

**Table 2**. Molecular weights of each drug compound against how much was weighed out and DMSO added to achieve an 25mM concentration.

All these drug stock solutions were passed through a 0.22µm filter and have a concentration of 25mM. These stock solutions were labelled appropriately and stored at 2$^{o}$C until required for assay use.

*2.2.2 SYBR Green Microtitre plate Assay*

SYBR Green assay is an easy fluorescence-based assay which is used for monitoring the susceptibility of *P. falciparum* (Rason et al., 2008). SYBR Green assay was used in this study as a malaria drug screening assay to accurately determine the drug concentration that inhibits parasite growth by 50% ($IC_{50}$) (Cheruiyot et al., 2016). $IC_{50}$ is a quantitative measure that indicates the concentration of drug that is required for 50% inhibition (Swinney, 2011).

1% infected RBCs at trophozoites stage was exposed to 9 dilutions (4-fold serial dilution) of compounds from 20 mM stock solutions in DMSO. 100 µl of culture volume was added in 96-well plates with 100 µl drug dilutions. Plates were incubated for 48 hours o at 37 °C, 5% $CO_2$, 5% $O_2$ in $N_2$. Following the incubation, 150 µl of medium was carefully removed and 150 µl of 5X SYBR Green solution (prepared by adding 2 µl of 10 000 X SYBR Green in 4 ml of PBS) was added to each well. Plates were kept in the dark for 45 minutes. Fluorescence intensity was measured using a micro plate reader (Genius Tecan) with an excitation of 485 nm and emission of 535 nm.

### *2.2.4 MTT assay*

#### *2.2.4.1 Preparation of cell growth medium for HepG2*

Following the SYBR Green assay, the compounds were further screened for cytotoxicity using the MTT (3-[4,5-dimethylthiazol-2-yl] – 2,5 diphenyl tetrazolium bromide) assay. MTT is a yellow dye that is water-soluble and can be readily taken up by viable cells and reduced by the action of mitochondrial reductase (Sittampalam & Markossian, 2013). The principle of the MTT is that for viable cells, mitochondrial action is steady, and consequently, an increase or decline in the number of viable cells is directly identified with mitochondrial activity (Sittampalam & Markossian, 2013). The mitochondrial activity of the cells is reflected by the transformation of the tetrazolium salt MTT into formazan. Therefore, any increase or decrease in viable cells can be detected by measuring formazan concentration using a plate reader.

RPMI culture media and supplemented with 10% Foetal Bovine Serum (FBS), 1% glutamine and 1% Penicillin/Streptomycin (Pen/Strep). Briefly, 60ml was removed from the RPMI bottle and then 50ml of FBS, 5ml of L-glutamine and 5ml of Pen/Strep was added in the 500ml bottle. Human hepatoma (HepG2) cells were cultured in a

culture flask using the complete media, described above. Following 48 hours, a media change was carried out. Once HepG2 cells were 80% confluent, the cells were washed three times with PBS. Thereafter 2 ml trypsin-EDTA reagent was added to the flask and incubated at 37 ° C for 2 minutes. Once the cells have detached, ~ 5ml of complete media was added and the cells were transferred into a falcon flask and centrifuged for 5 minutes at 31 000 rpm. The supernatant was discarded, and the pellet was resuspended in ~ 3ml media. The cell count was conducted using haemocytometer. Cells were then diluted to seed in 96-well plates with 5 x $10^3$ cells per well with a final volume of media of 100 µl. The cells were then incubated for 24 hours at 37 °C to allow cell adherence. After the incubation, cells were treated with different drug concentrations (dispensed in additional 100 µl of media) and further incubated for 5 days at 37 °C. Following the incubation period, 50 µl of the MTT solution (Thiazolyl Blue Tetrazolium Bromide, Sigma, UK) was added, and the plates were further incubated for 3 hours at 37 °C. The solution from the plate was removed, 150 µl of DMSO was added. This was added to dissolve the formazan crystals that were created in the experiment. After this, plates were immediately analysed on the Ascent plate reader. The optical density was determined spectrophotometrically with a 570 nm filter and a background at 630nm. Cisplatin was included as a control cytotoxic drug. The positive control was untreated cells.

## 3. Results

### 3.1 Dose Response

A total of 6 compounds (Ivermectin, Mitoxantrone, Fluoxetine, Nicardipine, Propafenone and Spironolactone) alongside the positive drug control were screened for the malaria efficacy and cytotoxicity. A 1% parasitized culture at the trophozoite stage was treated with the compounds for 48 hours at concentrations 5µM for the initial point test and 3.05 nM – 200 µM (4-fold serial dilution) for $IC_{50}$ against the *P. falciparum* K1 strain and the growth was measured using the SYBR Green-based plate reader assay.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed to investigate the toxicity of 4-aminoquinoline hydrazone compounds. HepG2 cells were incubated with 4- fold serial dilution at dose ranges from 3.05 nM - 200 µM for 5 days (120 hrs). Cisplatin was used as a control drug to validate the MTT assay due to its DNA damaging anti-cancer properties.

#### *3.1.1 Chloroquine dose response*

Chloroquine, a known antimalarial was tested alongside all compounds to validate the assay. The $IC_{50}$ value of 0.1971µM obtained from using the SYBR green assay (Figure 1.7) was observed which agreed with the known IC50 value of chloroquine in K1 strain.

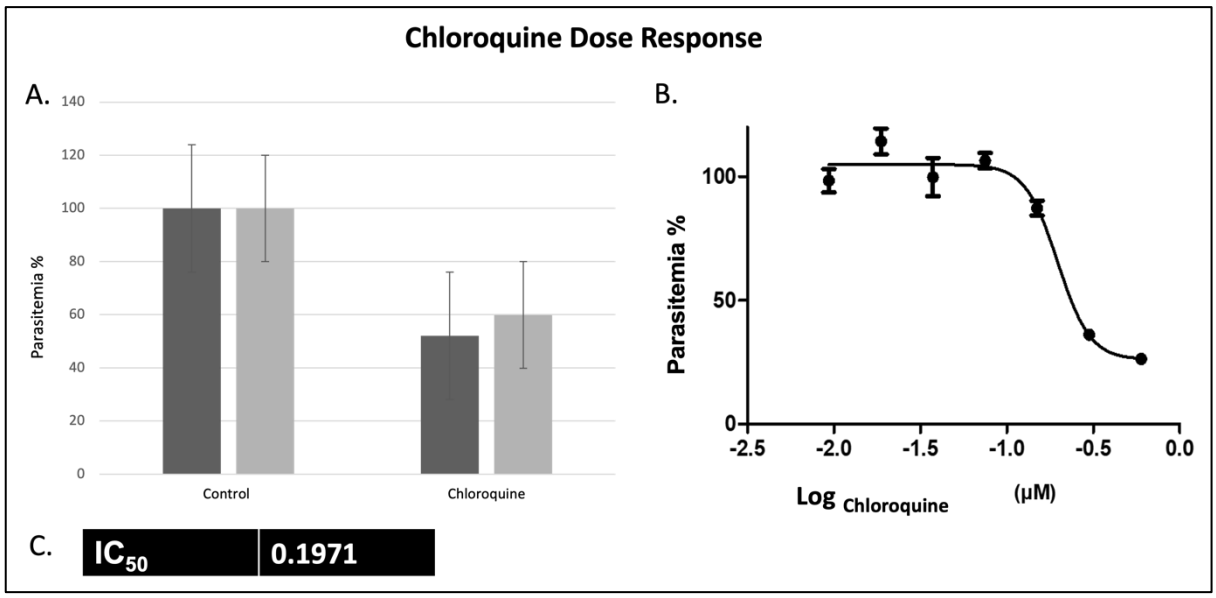

**Figure 1.7 Dose Response of Chloroquine against *P. falciparum*, strain K1.** The image is complied with A.) A point exposure of 5μM Chloroquine against *P. falciparum.* The data in (A) shows the results of two repeats. The graph beside shows (B.) $IC_{50}$ Chloroquine Dose response curve for log transformed drug concentrations (C). Nonlinear regressions analysis for an accurate $IC_{50}$ inhibition value using GraphPad prism 9.0.

*3.1.2 Ivermectin dose response*

An initial point test at 5 μM for the 6 compounds was conducted on Plasmodium falciparum K1 strain (Figure 1.8, (A.). The experiment was repeated twice. Ivermectin was observed to inhibit just about 10 -15% growth. When screened to assess the 50 % inhibition, the compound was found to inhibit 50% of the growth only at high concentration of 200 μM (Figure 1.8). A similar trend was observed when assessing the toxicity of this compound (Figure 1.9). Table 3 shows $IC_{50}$ data obtained from the MTT assay for both the control Cisplatin and drug compound Ivermectin. Results show that over the course of 120 hours, cisplatin had a significantly lower $IC_{50}$ value in comparison to Ivermectin (Table 3).

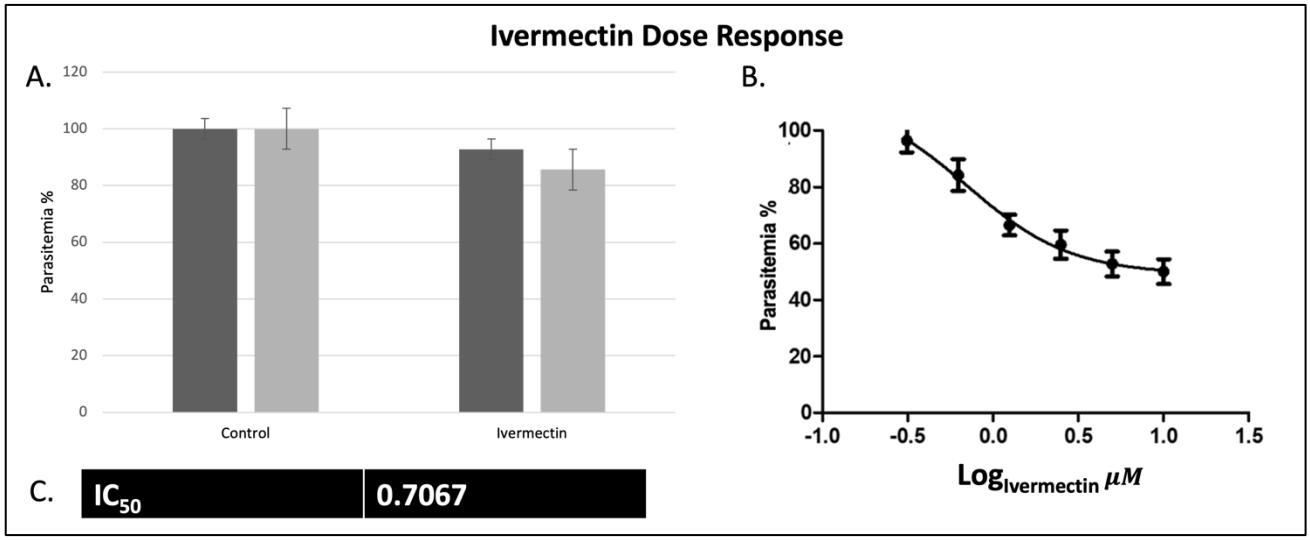


**Figure 1.8 Dose Response of Ivermectin against *P. falciparum*, strain K1.** The compiled image shows (A.) A point exposure of 5μM Ivermectin against *P. falciparum.* The data in (A) shows the results of two repeats. The graph beside shows (B.)

Ivermectin dose response for log transformed drug concentrations (C). Nonlinear regressions analysis for an accurate $IC_{50}$ inhibition value using GraphPad prism 9.0.

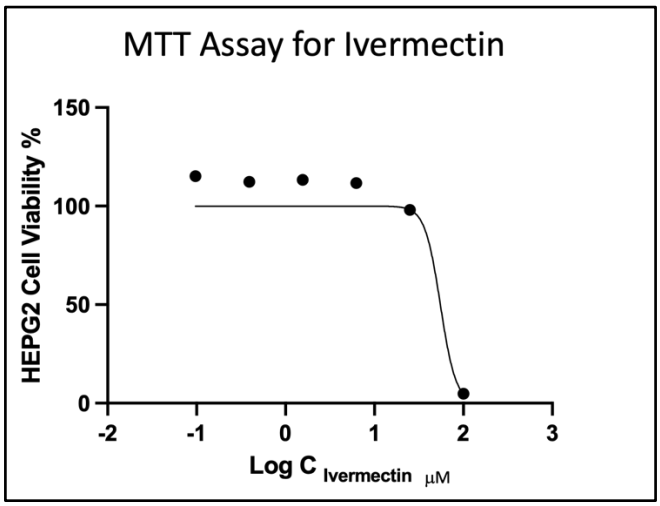


**Figure 1.9 Dose Response of Ivermectin against *HepG2 cells* following 120 hours of exposure.** Cell viability was established using the MTT standard assay using triplicate data.

| | **Cisplatin $IC_{50}$** | **Ivermectin $IC_{50}$** |
|---|---|---|
| 120 Hours | $0.6655\mu M$ | $55.09\mu M$ |

**Table 3** $IC_{50}$ values for Cisplatin (positive control) and the test compound Ivermectin at exposure times of 120 hours. $IC_{50}$ values were determined using GraphPad Prism 9.0, using the nonlinear regression variable slope.

*3.1.3 Mitoxantrone dose response*

When assessing the antimalarial activity of Mitoxantrone, the compound was observed to inhibit over 50 % growth of the parasite (Figure 1.10). The cell viability data is illustrated in Table 4. The data obtained (Figure 1.11) shows that the compound is toxic on the HepG2 cells with an $IC_{50}$ of 0.0093 µM, lower than the obtained control value of Cisplatin at 0.6655µM (Table 4).

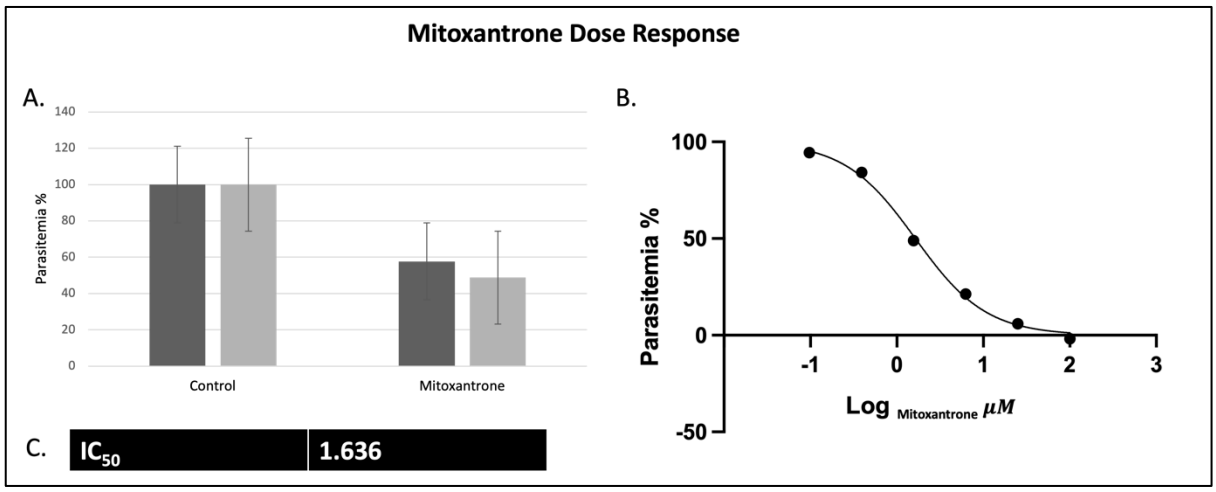


**Figure 1.10 Dose Response of Mitoxantrone against *P. falciparum*, strain K1.** The compiled image shows (A.) The point exposure of 5μM Mitoxantrone against *P. falciparum.* The data in (A) shows the results of two repeats. The graph beside shows (B.) Mitoxantrone dose response for log transformed drug concentrations (C). Nonlinear regressions analysis for an accurate $IC_{50}$ inhibition value using GraphPad prism 9.0.

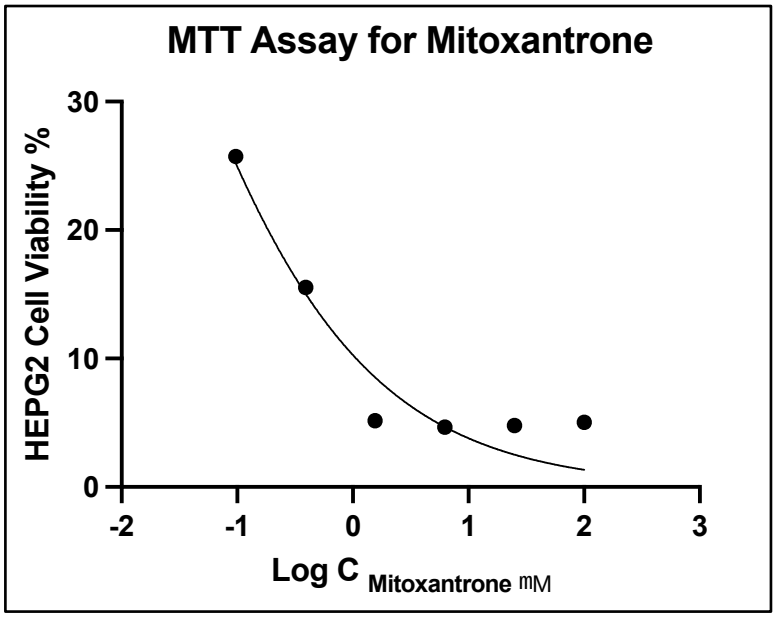


**Figure 1.11 Dose Response of Mitoxantrone against *HepG2 cells* following 120 hours of exposure.** Cell viability was established using the MTT standard assay using triplicate data.

| | **Cisplatin** $IC_{50}$ | **Mitoxantrone** $IC_{50}$ |
|---|---|---|
| 120 Hours | $0.6655\mu M$ | $0.0093\mu M$ |

**Table 2** $IC_{50}$ values for Cisplatin (positive control) and the test compound Mitoxantrone at exposure times of 120 hours. $IC_{50}$ values were determined using GraphPad Prism 9.0, using the nonlinear regression variable slope.

3.1.4 Fluoxetine Dose Response

The initial dose exposure at 5μM for Fluoxetine on *P. falciparum K1 strain indicated that the compound was not inhibiting the parasite growth with parasitemia growth* between 105% to 111%. Although this indicated that Fluoxetine is not active, the $IC_{50}$ assay was carried out which showed that the compound was only active at high concentrations (Figure 1.12). On the other hand, the compound was observed to be toxic and completely inhibiting all the HepG2 cells at high concentrations (Figure 1.13). Furthermore, $IC_{50}$ values show that Fluoxetine is not as toxic as Cisplatin as shown when both values are compared against each other (Table 5).

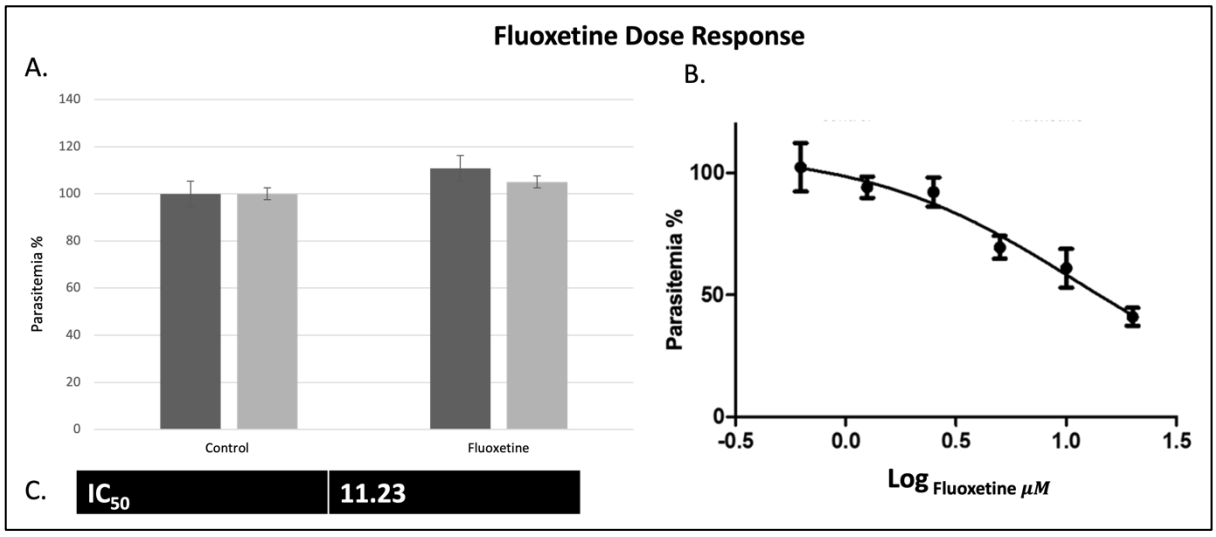


**Figure 1.12 Dose Response of Fluoxetine against *P. falciparum*, strain K1.** The compiled image shows (A.) the point exposure of 5μM Fluoxetine against *P. falciparum.* The data in (A) shows the results of two repeats. The graph beside shows

(B.) Fluoxetine dose response for log transformed drug concentrations (C.). Nonlinear regressions analysis for an accurate $IC_{50}$ inhibition value using GraphPad prism 9.0.

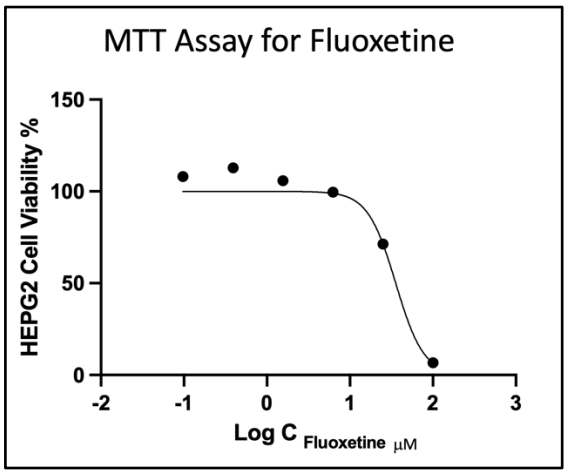


**Figure 1.13 Dose Response of Fluoxetine against *HepG2 cells* following 120 hours of exposure.** Cell viability was established using the MTT standard assay using triplicate data.

| | **Cisplatin $IC_{50}$** | **Fluoxetine $IC_{50}$** |
|---|---|---|
| 120 Hours | 0.6655$\mu M$ | 35.63$\mu M$ |

**Table 5** $IC_{50}$ values for Cisplatin (positive control) and the test compound Fluoxetine at exposure times of 120 hours. $IC_{50}$ values were determined using GraphPad Prism 9.0, using the nonlinear regression variable slope.

*3.1.5 Nicardipine Dose response*

The initial done response of Nicardipine showed that the compound was only inhibiting ~ 20 % of the parasite growth at 5 µM. The IC50 assay indicated that that the compound does inhibit the 50% growth, however at high concentrations with an IC50 value of 4.388 µM (Figure 1.14). The same trend was observed upon testing the toxicity of the compound, which showed to be toxic only at high concentration with an $IC_{50}$ value of 93.19 µM (Figure 1.15). Results obtained from the MTT assay were used and compared against the control Cisplatin. Nicardipine clearly shows that it is a much less toxic drug compared to Cisplatin that displayed an $IC_{50}$ of 0.6655 µM (Table 6).

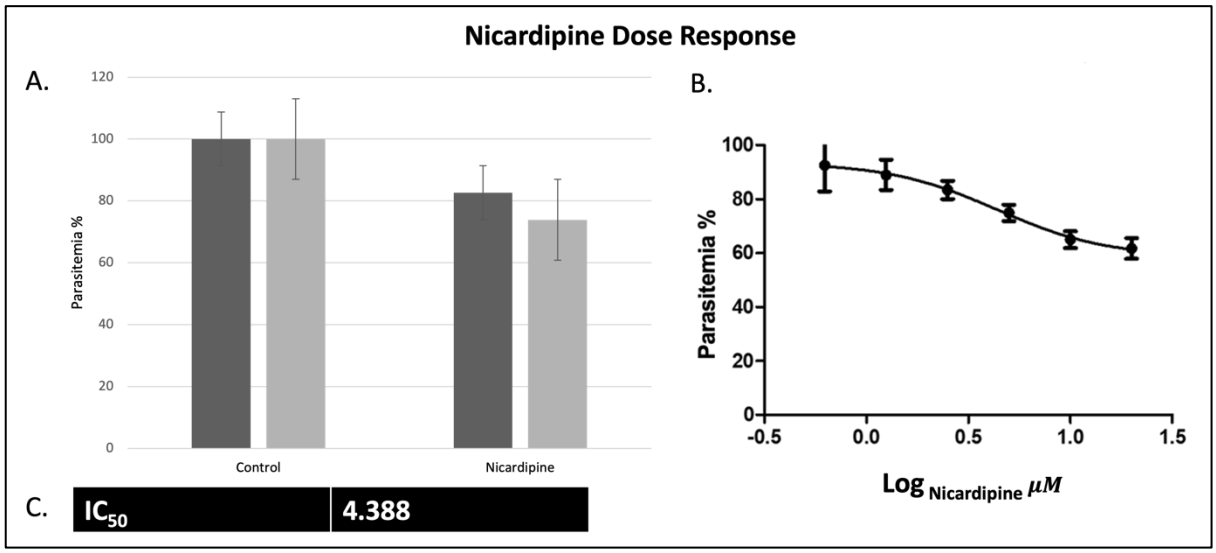


**Figure 1.14 Dose Response of Nicardipine against *P. falciparum*, strain K1.** The compiled image shows (A.) the point exposure of 5μM Nicardipine against *P. falciparum.* The data in (A) shows the results of two repeats. The graph beside shows (B.) Nicardipine dose response for log transformed drug concentrations (C.). Nonlinear regressions analysis for an accurate $IC_{50}$ inhibition value using GraphPad prism 9.0.

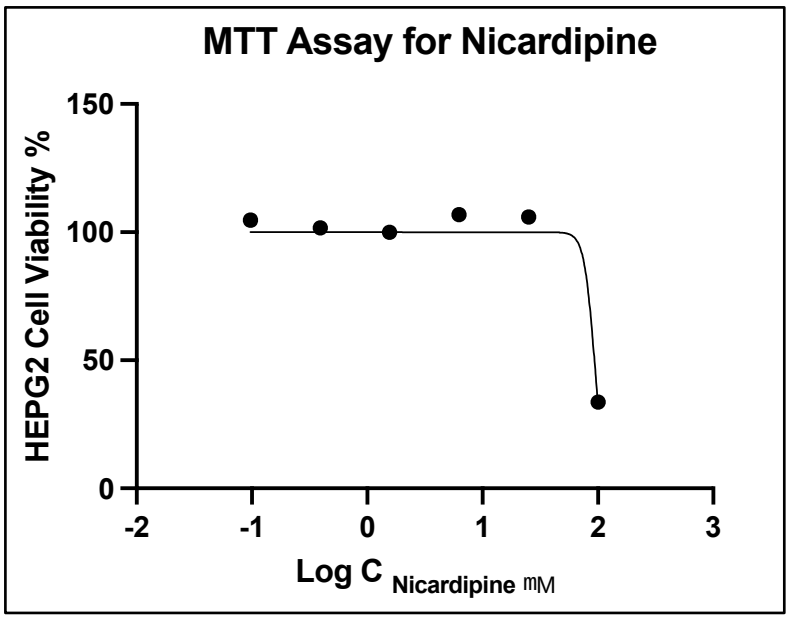


**Figure 1.15 Dose Response of Nicardipine against *HepG2 cells* following 120 hours of exposure.** Cell viability was established using the MTT standard assay using triplicate data.

| | Cisplatin $IC_{50}$ | Nicardipine $IC_{50}$ |
|---|---|---|
| 120 Hours | $0.6655\mu M$ | $93.19\mu M$ |

**Table 6** $IC_{50}$ values for Cisplatin (positive control) and the test compound Nicardipine at exposure times of 120 hours. $IC_{50}$ values were determined using GraphPad Prism 9.0, using the nonlinear regression variable slope.

*3.1.6 Propafenone Drug Response results*

The initial done response of propafenone showed similar activity to nicardipine with the compound was only inhibiting ~ 20 % of the parasite growth at 5 µM. The IC50 assay indicated that the compound is not active ($IC_{50}$ >200 µM) against *P. falciparum* K1 strain (Figure 1.16). The cytotoxicity assay indicated that compound was only toxic at high concentration and the IC50 value was observed to be 44.84 µM in comparison to the control Cisplatin (Figure 1.17, Table 7).

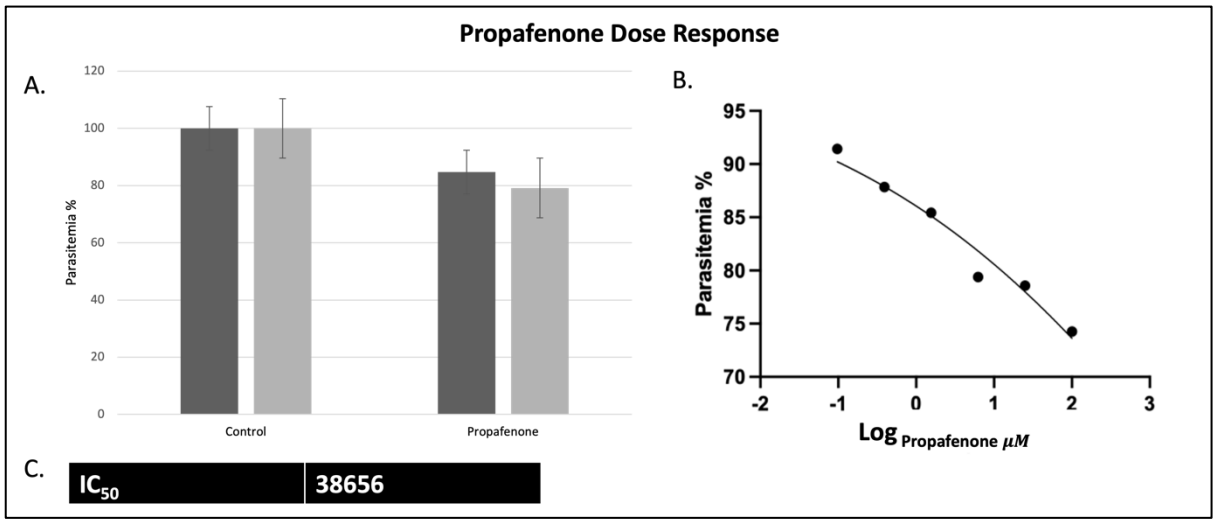


**Figure 1.16 Dose Response of Propafenone against *P. falciparum*, strain K1.** The compiled image shows (A.) the point exposure of 5µM Propafenone against *P. falciparum.* The data in (A) shows the results of two repeats. The graph beside shows (B.) Propafenone dose response for log transformed drug concentrations (C). Nonlinear regressions analysis for an accurate $IC_{50}$ inhibition value using GraphPad prism 9.0.

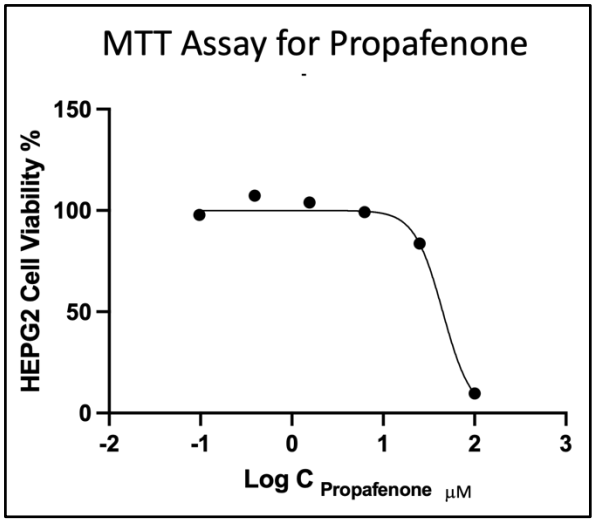


**Figure 1.17 Dose Response of Propafenone against *HepG2 cells* following 120 hours of exposure.** Cell viability was established using the MTT standard assay using triplicate data.

| | **Cisplatin $IC_{50}$** | **Propafenone $IC_{50}$** |
|---|---|---|
| 120 Hours | $0.6655\mu M$ | $44.84\mu M$ |

**Table 5** $IC_{50}$ values for Cisplatin (positive control) and the test compound Propafenone at exposure times of 120 hours. $IC_{50}$ values were determined using GraphPad Prism 9.0, using the nonlinear regression variable slope.

*3.1.7 Spironolactone Dose Response results*

The initial dose response of Spironolactone at 5 µM on K1 strain *P. falciparum* showed the compound was only inhibiting 10-20 % parasite growth. The compound further screened at concentration ranging from 200 µM - 3.05 nM, which showed that the compounds was still not active with an $IC_{50}$ > 200 µM (Figure 1.18). The cytotoxicity assay showed that compound was only active at high concentration with an $IC_{50}$ value of 100.3 µM. This suggested that the compound was not toxic in comparison to the control Cisplatin with an $IC_{50}$ of 0.6655 µM (Figure 1.19, Table 8).

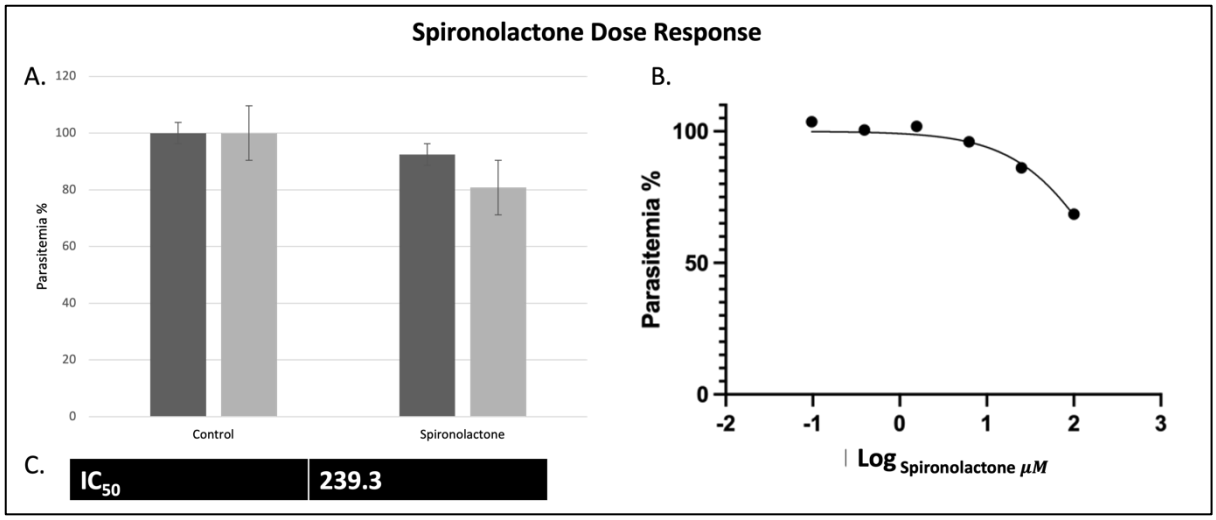


**Figure 1.18 Dose Response of Spironolactone against *P. falciparum*, strain K1.** The compiled image shows (A.) the point exposure of 5μM Spironolactone against *P. falciparum*. The data in (A) shows the results of two repeats. The graph beside shows (B) Spironolactone dose response for log transformed drug concentrations (C). Nonlinear regressions analysis for an accurate $IC_{50}$ inhibition value using GraphPad prism 9.0.

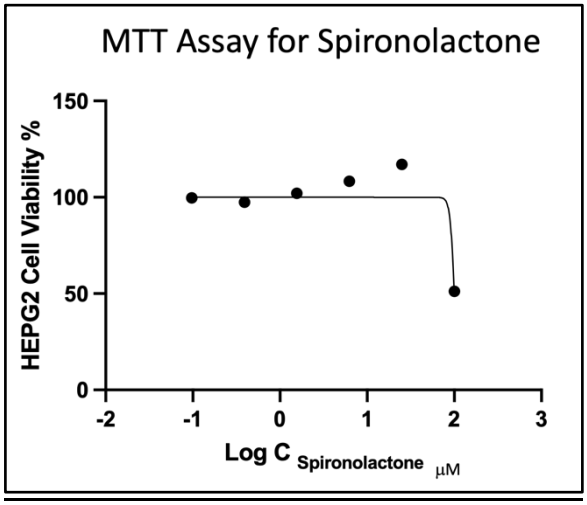


**Figure 1.19 Dose Response of Spironolactone against *HepG2 cells* following 120 hours of exposure.** Cell viability was established using the MTT standard assay using triplicate data.

| | Cisplatin $IC_{50}$ | Spironolactone $IC_{50}$ |
|---|---|---|
| 120 Hours | $0.6655\mu M$ | $100.3\mu M$ |

**Table 8** $IC_{50}$ values for Cisplatin (positive control) and the test compound Spironolactone at exposure times of 120 hours. $IC_{50}$ values were determined using GraphPad Prism 9.0, using the nonlinear regression variable slope.

Overall, we were able to obtain $IC_{50}$ values for both the SYBR Green assay and the MTT assay (Table 9). All values were collated and used to determine selective indices (SI) using *Plasmodium* $IC_{50}$ values over the cytotoxicity $IC_{50}$ values.

| **SYBR Green Assay** | |
|---|---|
| **Drug** | **$IC_{50}$ (μM)** |
| Chloroquine (Control) | 0.1971 |
| Ivermectin | 0.7067 |
| Mitoxantrone | 1.636 |
| Fluoxetine | 11.23 |
| Nicardipine | 4.388 |
| Propafenone | 38656 |
| Spironolactone | 239.3 |
| **MTT Assay** | |
| Cisplatin (Control) | 0.6655 |
| Ivermectin | 55.09 |
| Mitoxantrone | 0.0093 |
| Fluoxetine | 35.63 |
| Nicardipine | 93.19 |
| Propafenone | 44.84 |
| Spironolactone | 100.3 |

**Table 9** $IC_{50}$ values for all 6 compounds from both SYBR Green and MTT assays, including both Chloroquine and Cisplatin as the controls respectively.

# DISCUSSION

## *4.1 Introduction*

The ongoing resistance against anti-malarial treatment has increased the urgency and importance of drug repositioning research. Previous work done in our library as a front end to this investigation carried out the preliminary single point screening of the LOPAC patent expired, FDA approved (Food and Drug Approved) library and indicated 56 compounds with potential antimalarial efficacy (Matthews, 2015) in the K1 *Plasmodium falciparum* parasite strains. For the purposes of this dissertation, 6 compounds identified in the Matthews et al., 2015 study were chosen to be further interrogated through dose response IC50 studies in the KI *Plasmodium falciparum* strains and MTT safety studies on HepG2 cell lines with appropriate controls. The six compounds chosen were Propafenone (anti-arrhythmic), Ivermectin (antiparasitic), Mitoxantrone (anti-cancer), Nicardipine (calcium channel blocker), Spironolactone (competitive antagonist of aldosterone receptor) and Fluoxetine (selective serotonin inhibitor).

Following selection of the six compounds, a preliminary single dose 5μm efficacy test was carried out to define reduction in parasitaemia. Synchronised parasite culture at trophozoite stage was used in this preliminary single point test with plates analysed 48 hours after drug exposure. Although 5 out of the 6 drug compounds showed potency, the most potent drug compound proved to be Mitoxantrone. It showed that when parasites were exposed to 5μM of drug, there was around 50% to 58% parasitemia remaining. This was important to confirm there was a reduction in parasitemia when exposed to the selected drugs.

## *4.2 SYBR Green Plate assay*

$IC_{50}$ values obtained for all six compounds were, Ivermectin 0.7067μM, Mitoxantrone 1.636μM, Fluoxetine 11.23μM, Nicardipine 4.388μM, Propafenone >200 μM and Spironolactone >200 μM.

From this, results showed out of the 6 compounds that Mitoxantrone, Ivermectin Nicardipine and Fluoxetine were the most active compounds against multi-resistant *P. falciparum* K1 strain. Preliminary $IC_{50}$ results obtained from the study for Spironolactone and Propafenone showed no potency against the strain *P. falciparum*. Although there is evidence that Spironolactone was a significantly promising drug candidate for parasitic infections like Leishmaniasis (*L. amazonensis)* there is no evidence that doses of spironolactone work against *P. falciparum* (Andrade-Neto et al., 2021). The only reports of the use of Spironolactone used in *Plasmodium Vivax* infections was to treat arrhythmias caused as a result of myocarditis symptoms secondary to malaria (Khan, 2019). After use of Spironolactone the myocarditis gradually improved as well as the malaria. However, this study cannot be used for comparison as it was a different strain of malaria that was concluded in the study. Overall evidence confirms that there was no potency for Spironolactone as an antimalarial repurposed drug.

Initially Propafenone showed potential in the first $5\mu M$ point test for repurposing as an antimalarial, however when further dose response experiments were conducted, the drug failed to supress parasitaemia to enable obtaining $IC_{50}$ values (> 200μM). Due to such a large $IC_{50}$ it would be deemed not right to take this compound further. However, other studies have shown that Propafenone inhibits the growth of *P. falciparum* (Lowes et al., 2011). However, the study did indicate that further research and development would be required to improve the pharmacokinetic profile of the drug (Lowes et al., 2011). Propafenone was advanced using rodent models and revealed orally bioavailable compounds that are non-toxic (Lowes et al., 2012). Our failure to corroborate the anti-parasitemia evidence in triplicate experiments suggests further experiments be conducted with a new stock of the compound preferably from a new supplier.

Fluoxetine $IC_{50}$ results (11.23μM) showed mild potency for antimalarial properties. Although in the initial 5μM single dose test, there was no antimalarial activity, this $IC_{50}$ value can be used to progress further research into using Fluoxetine, or a more effective derivatised version of the drug as a repurposed antimalarial treatment or as

a combinatorial partner drug to existing antimalarial. There is some published evidence that Fluoxetine exhibits synergistic activity as a combinatorial partner drug with chloroquine and mefloquine response on *P. falciparum* (Khairul., 2006). Fluoxetine is a serotonin uptake inhibitor, and its mode of action in reversing chloroquine resistance is not fully understood. Therefore, further research and optimisation is required to explore its use in malaria.

Ivermectin in this study also showed antimalarial drug efficacy with an $IC_{50}$ of 0.7067μM. Although Ivermectin is known to be a parasitic treatment for filariasis and onchocerciasis, studies were conducted to see if treating communities with Ivermectin would reduce malaria transmission. Although not specifically testing a treatment drug and rather a control drug, studies were inconclusive if Ivermectin had any effect on reducing malaria and that further studies were required (De Souza et al., 2021). Another study by Foy et al., concluded that more frequent repeated mass administration of Ivermectin during the malaria transmission season can reduce malaria episodes (Foy et al., 2019). This study provided evidence of the effect of mass Ivermectin administration showing measurable reduction in malarial incidence in children aged 5 or younger (Chaccour & Rabinovich, 2019). Further studies on Ivermectin would be needed to test it either alone or in combination with other drug-based strategies against malaria (Chaccour & Rabinovich, 2019).

Mitoxantrone showed *P. falciparum* growth inhibition both initially in the 5μM single point test and $IC_{50}$ values from dose response studies (1.636μM). There has been hardly any evidence that supports this finding that Mitoxantrone displays antimalarial properties. However, Mitoxantrone has been known to work in combination with known antimalarials such as quinine to treat acute myeloid leukaemia (Solary et al., 1996).

Results for Nicardipine show that it could be potentially used as a repurposed antimalarial drug for the strain *P. falciparum*. Nicardipine had an $IC_{50}$ value of 4.388μM. Although this isn't as potent as mitoxantrone, this compound still showed potency against inhibiting *P. falciparum*. Further to our study, there is not much research about Nicardipine and its potency, however Nicardipine was said to reverse the resistance to chloroquine in resistant parasites (Tanabe et al., 1990). Although this is a promising

study that could indicate calcium channel blockers as increasing the susceptibility to chloroquine; this study was done against the sensitive line of *Plasmodium Chabaudi* (Tanabe et al., 1990). We report here the activity in KI *Plasmodium falciparum* strains and the potential development.

*4.3 MTT Assay*

Further to this investigation into antiparasitic efficacy, to define drug safety profiles for drug leads, the MTT assay was carried out on HepG2 mammalian cells lines to determine the cell viability and compound toxicity. Cisplatin a known anticancer drug was used as a control against the HepG2 cell line. Cisplatin obtained an $IC_{50}$ value of 0.6655μM in line with expected values. $IC_{50}$ values for all six compounds were as followed, Ivermectin 55.09μM, Mitoxantrone 0.0093μM, Fluoxetine 35.63μM, Nicardipine 93.19μM, Propafenone 44.84μM and Spironolactone 100.3μM. Drug leads being carried forward from the SYBR green plate assay were Mitoxantrone, Nicardipine, Ivermectin and Fluoxetine.

All $IC_{50}$ values except for Mitoxantrone had better safety profiles in comparison to the control Cisplatin. Therefore, although results displayed in this investigation show Mitoxantrone as a significantly potent anti-plasmodial compound, when the MTT assay was performed it proved Mitoxantrone to be toxic. However, this result can be explained by the fact that Mitoxantrone is an anthracenedione that demonstrates anti-tumour activity (Posner et al., 1985). It is known as an antineoplastic antibiotic used for acute leukaemia, lymphoma and prostate and breast cancer and in this use, a range of toxic side effects are well documented (e.g. febrile neutropenia, cardiac toxicity and dose-dependent myelosuppression (Posner et al., 1985).

Furthermore, the MTT assay results for Nicardipine showed that except at the highest concentration all other log concentrations had a cell viability for HepG2 of 100%. This means that the cell growth was not inhibited therefore the compound is not toxic to the human mammalian cells. The toxicity results can be confirmed with previous research that as with other calcium channel blockers Nicardipine is well tolerated and its side effects on the body is due to its vasodilating activities (Hoofnagle et al., 2012). Mild

side effects documented for its current use include headache, flushing, dizziness and fatigue (Hoofnagle et al., 2012).

MTT data for Ivermectin (55.09μM) and Fluoxetine (35.63μM), similarly to Nicardipine showed that except at highest concentrations, cell viability was at 100%, indicating that these compounds have better safety profiles in comparison to Cisplatin. These compounds could be taken further for more analysis.

Further to the toxicity results, the selectivity index was calculated and compared for the most potent leads; Mitoxantrone, Nicardipine, Ivermectin and Fluoxetine. A selectivity index is defined as a ratio of cytotoxicity to biological activity. A SI value that is more than 10 is deemed to be a potential drug compound that could be further investigated. Using the four main drug leads, the calculated selectivity indices were Mitoxantrone SI .006, Ivermectin SI 77.95, Nicardipine SI 21.23 and Fluoxetine SI 3.172. For Mitoxantrone and Fluoxetine, this precluded further progression to second phase optimisation as it fell below the expected progression cut off. Nicardipine with a selectivity Index of 21.23, despite its higher antiplasmodial IC50 value of 4.388μM, may provide a safe molecule amenable to further chemical derivatisation in a bid to improve antimalarial efficacy. However, Ivermectin (IC50 0.7067μM, Selectivity Index 77.95), in this preliminary study shows promising results for further validation and second phase optimisation. Both Nicardipine and Ivermectin could have value as standalone, combinatorial partner drugs such as Artemisinin or through further derivatisation, like studies that have proven derivatisation of artemisinin to have been successful in displaying antimalarial properties. These would contribute to expanding the currently dwindling antimalarial drug repertoire and aid in tackling the fast-spreading impact and drug resistance.

*4.4 Limitations*

The limitations of the fluorescent SYBR green DNA staining method needs to be acknowledged. This method has been deemed by many sources as a fast and inexpensive method, however SYBR Green 1 binds to any double stranded DNA, including DNA that is present in whole blood samples, therefore this method can result in high background readings (Vossen et al., 2010). However, this potential drawback

was minimised in our experiment by washing and centrifugation steps to remove the buffy coat layer and verification through microscopy for efficacy of WBC removal (Vossen et al., 2010). Further to this the presence of haemoglobin in the parasite causes low fluorescence readings (Dery et al., 2015). However, the introduction of controls will negate this effect in our experiments.

The HepG2 MTT assay has been traditionally employed as the 'gold standard' for cytotoxicity because of its sensitivity and high throughput screening (Van Tonder et al., 2015). However, the increasing finding that many antimalarial compounds possess anti-cancer activity, raises the valid question if data from this model underestimates the safety profile for compounds possessing dual antimalarial and anticancer efficacy. There are also many variables that need to be considered such as the seeding number, concentration of MTT reagent that is added to the cells, the time the cells are left for incubation with MTT solutions and wavelengths at which the optical density is measured (Ghasemi et al., 2021). Another limitation is that the MTT solution is light sensitive, therefore long exposure to MTT solution to light can result in production of formazan and higher background absorbance (Riss, T.L.et al., 2013). Hence when carrying out this assay, appropriate storage and routine tests for compound efficacy were critical to ensure reproducible results.

An alternative method is the enzyme-linked immunosorbent assay (ELISA). Using human serum and plasma, this assay uses recombinant antigens to produce a test that is highly specific and sensitive (Doderer et al., 2007). The antigens will detect *P. falciparum* enabling the test to detect antibodies during all stages of infection (Doderer et al., 2007). The colour reactions are quantified using a spectrophotometer- based ELISA plate reader. Although it has a 72-hour incubation, it allows for testing of slow-acting drugs. Similarly, western blot analysis has also been used to diagnose vivax malaria using stage specific recombinant antigens (Son et al., 2001).

Furthermore, a Trypan blue exclusion assay could also be used as an alternative (Barnabe, 2017). Based on the principle that the viable cells with intact membranes do not take up Trypan blue stain, the dead cells can be distinguished from the viable ones due to their blue colour, whereas live cells would be white (Barnabe,2017).

## 4.5 *Further Work*

Given that this was a preliminary study to test a panel of compounds from a screening of the larger LOPAC drug library, the two compounds Ivermectin and Nicardipine have shown potential for further in vitro validation. Follow on experiment to map stage specific activity could indicate the asexual erythrocytic target of the compound. In this study we used a predominately trophozoites stage whereas using the SYBR Green assay we could look at both ring and schizont stages. Similarly strain specific efficacy will need to be carried out to map activity in wider *Plasmodium falciparum* strains. For nicardipine, further chemical derivatisation to potentiate anti-malarial activity could provide a safe antimalarial alternative. Examples of this has been done with previous antimalarials, such as artemisinin and quinoline compounds. Mode of action studies would further provide mechanistic insights on potential target pathways such as using the 2D Tanimoto chemical fingerprints (Kuwahara & Gao, 2021). This is widely used for the quantification of structural similarity of chemical compounds. Following mode of action studies, inform the selection of effective combinatorial partner drugs to test using objective Calcusyn-based synergy experiments. Calcusyn is a software program developed to analyse combined drug effects and quantitys synergism and inhibition. This programme could be used to further our work and provide a different perspective on the interaction between combinatorial antimalarial drugs.

## *4.6 Conclusion*

Overall, given the increasing drug resistance to *P. falciparum*, drug repositioning could provide fast-tracked options to deliver new antimalarial drugs. This study supports the strategy of drug repositioning as a valid route to antimalarial drug discovery. The study has recognised that out of 6 compounds, Nicardipine and Ivermectin, primarily a calcium channel blocker and anti-arrhythmic have shown inhibition for *P. falciparum*. HepG2 cellular toxicity results exhibited that Nicardipine and Ivermectin showed favourable safely profiles and selectivity indices. Following further validation of these preliminary findings, the two compounds could have potential for further progression and derivatisation as standalone and combinatorial partner drugs for malaria.

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