Antimalarial Activity and Pharmacokinetic Properties of New Chemical Entities

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DECLARATION

I, Ntokozo Shirley Dambuza, declare that the above thesis, which I hereby submit for the degree *Philosophiae Doctor* at the University of Cape Town, is my own work except where otherwise acknowledge with the guidance of my supervisors.

I declare that the contents of this thesis have not previously submitted by me for a degree for a degree at this or any other institution.

SIGNATURE……

DATE……

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LIST OF ABBREVIATIONS

ACT : Artemesinin-based combinations
ADME/Tox : Absorption, distribution, metabolism, elimination and toxicity
AIDS : Acquired immunodeficiency syndrome
APAD : 3-acetyl pyridine adenine dinucleotide
ATP : Adenine triphosphate
AZT : Azidothymidine
AZT-CQ : Azidothymidine-chloroquine
Caco-2 : Human epithelial colorectal adenocarcinoma cells
CHO : Chinese Hamster Ovarian
DHFR : Dihydrofolate reductase
DHODH : Dehydroorotate dehydrogenase
DHPS : Dihydropteorate synthase
DMEM : Dulbecco's Modified Eagle Media
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
DND*i* : Drugs for Neglected Diseases initiative
DTT : Dichloro-diphenyl-trichloroethane
EMA : European Medicines Agency
FCS : Fetal calf serum
FP-IX : Ferriprotoprophryn IX
GNF : Global Nature Fund
HBSS : Hank’s balanced salt Solution
HEPES : 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
HIV : Human immunodeficiency virus
HLB : Hydrophile-lipophile balance
HPO-CQ : Hydroxypyridone-chloroquine
HRP2 : Histidine-rich protein II
HTS : Highthroughput screening
KFDA : Korean Food & Drugs Administration
LC-MS/MS : Liquid chromatography coupled to mass spectrometry
LOQ : Limit of quantitation

LY : Lucifer yellow
MDR : Multi-drug resistant
MMV : Medicines for Malaria Venture
MR4 : Malaria Research and Reference Reagent Resource Center
MRM : Multiple reaction monitoring
MTT : 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
NAD^+ : Nicotinamide adenine dinucleotide
NADPH : Nicotinamide adenine dinucleotide phosphate
NBT : Nitro Blue Tetrazolium
NCE : New chemical entities
PABA : ρ-Aminobenzoic acid
P_{APP} : Apparent permeability
PBS : Phosphate buffered saline
PD : Pharmacodynamic
PES : Phenazine ethosulfate
PK : Pharmacokinetic
pLDH : Parasite lactacte dehydrogenase
PTFE : Polytetrafluoroethylene
PVDF : Polyvinylidene difluoride
RI : Resistance index
RNA : Ribonucleic acid
Rpm : Revolutions per minute
RPMI : Roswell Park Memorial Institute medium
SAR : Structure – activity relationship
SERCA : Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SI : Selectivity index
Swiss TPH : Swiss Tropical and Public Health Institute
TEER : transepithelial electrical resistance
THF : Tetrahydrofolate
UFLC : Ultra fast liquid chromatography
WHO : World Health Organization

ABSTRACT

Antimalarial Activity and Pharmacokinetic Properties of New Chemical Entities

Ntokozo Shirley Dambuza

Malaria is a disease caused by *Plasmodium falciparum* and is transmitted to a human host when an infected female *Anopheles gambiae* mosquito bites the host. It is estimated that 81% cases and 91% of deaths cause by malaria occurred in the WHO African Region, with children under five years of age and pregnant women being the mostly affected. According to the Centres for Disease Control and Prevention *P. falciparum* has also developed resistance to most antimalarials and this has encouraged the development of new candidates of effective antimalarial drugs.

Twelve antimalaria compounds were tested against *P. falciparum* chloroquine sensitive (D10 and 3D7) and resistant strains (Dd2 and K1). These compounds were tested for cytotoxicity against Chinese Hamster Ovarian (CHO) cell lines. Their pharmacokinetic properties were determined using a mouse model and blood samples were collected at different time intervals and analysed using LC-MS/MS. For *in vivo* efficacy the mice were infected with *P. berghei* strain in a 4-day Peter's test. The parasitaemia was determined from day 3 and the course of the infection was followed for 24 days by microscopic examination of stained blood films every 2 to 3 days. Caco-2 cell lines were used to study permeability properties of these compounds.

IC_{50} values for sensitive and resistant strains were 0.006 to 4 µg/ml (0.014 to 7 µM) and 0.007 to 51µg/ml (0.017 to 105 µM), respectively. Of the twelve compounds only seven were potent against malaria parasites. No significant cytotoxicity was observed. The compounds tested were very insoluble which cause low oral bioavailability, and this affected *in vivo* efficacy. Two potent antimalarials, namely MMV394903 and MMV652120 had high bioavailability at 60 and 69%, respectively

and this resulted in improved *in vivo* efficacy. Permeability studies were performed on Caco-2 cells to investigate the compounds permeability through intestinal lining and the apparent permeability values were less than $3x10^{-5}$cm/s, which shows low to intermediate permeability.

Even though the compounds evaluated in this study were very active antimalarials their insolubility affected their permeability across stomach lining, thus causing poor drug absorption and low oral bioavailability.

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I dedicate this thesis to the memory of my deceased father

<u>Rev. Joseph Grant Themba Dambuza (1934-2011)</u>

In your eyes I've seen God's Love

In your words I've heard His wisdom

Through your life I've found His grace

'by Anonymous'

Chapter 1: Literature Review

Literature Review

1.1. Introduction

Malaria is a disease caused by protozoan organism of the genus *Plasmodium* of Apicomplexan parasites within the suborder Haemosporidiasina, suborder Laveraniine and is transmitted to a human host when a vector, an infected female *Anopheles gambiae* mosquito, bites the host (Clark *et al*., 2004). Many cases of human malaria each year are generally caused by five species, including *Plasmodium falciparum, P. vivax, P. ovale, P. knowlesi* and *P. malariae*. *P. falciparum* causes the most severe symptoms, and infection may become fatal when treatment is delayed for more than 24 hours after presentation of symptoms. Together with *P. vivax*, they are responsible for most cases of malaria and has become one of major public health problem in many regions of Africa, Asia and Latin America (Maltha and Jacobs, 2011; Panichakul *et al*., 2007). The incidence of *P. falciparum* infection in Africa and in Asia is high and in Latin America *P. vivax* malaria is more common to find and this disease is an important factor of morbidity and a problem for economic development worldwide, especially in the countries in which this disease is endemic (Tham *et al.*, 2012; Winzeler, 2008).

1.1.1. The history of malaria

The term 'malaria' is an Italian name derived from *mala aria*, which means the 'bad air', the miasmas evaporating from the stagnant waters of marshes. People from the ancient times believed that the spoiled air was the cause of the malaria fevers (Capanna, 2006; Cox, 2010). The understanding of malaria parasites began in 1880 in Constantine, Algeria, when Alphonse Laveran, a French army surgeon, identified the protozoan for the first time in peripheral human blood (Buonsenso and Cataldi, 2010; Cirillo, 2011). It was reported that he observed several different forms of erythrocytic organisms including crescents, spherical motionless bodies with pigment, spherical moving bodies with pigment and bodies that extruded flagella-like structures all of which he thought were on the outside of the red cells. He also discovered that a drug called quinine

removed these stages from the blood, and then realized that he had found a parasitic protozoan which he called *Oscillaria malariae* (Cox, 2010). In 1885 Italian scientists, Ettore Marchiafava and Angelo Celli proposed the name '*Plasmodium malariae*' because of its resemblance to mycetozoan bodies called plasmodia (Cirillo, 2011).

In 1897 Ronald Ross, a British doctor, discovered that malarial parasites were transmitted by the bite of mosquitoes (Capanna, 2006; Cirillo, 2011). He had no patients to investigate this discovery further; therefore, he studied malaria transmission in birds using female mosquito of *Culex* species. He discovered how the parasites developed on the midgut wall and accumulated in the salivary glands, and at that stage the mosquitoes could infect other birds (Cox, 2010; Verhave, 2010). He concluded that the human malaria parasite went through a similar, if not identical, life cycle, and that it was also transmitted by mosquitoes (Cirillo, 2011). In 1898 Giovanni Battista Grassi, an Italian Zoologist, identified *Anopheles claviger* as a vector responsible for the transmission of human malaria from man to man when he infected a healthy volunteer by exposing him to three mosquitoes of different species, namely, *Anopheles claviger* and two *Culex* species. He also concluded that bird malaria could not be transmitted by the same mosquito responsible for human malaria. (Buonsenso and Cataldi, 2010; Capanna, 2006). The discovery made by Ross and Grassi that malaria is transmitted through the bite of an infected mosquito formed a basis of initial malaria control measures (Petersen *et al*., 2011). Grassi taught people in infected areas not to go out at dusk but to stay in their homes and prevent mosquitoes from entering. Windows and door screens were installed and years later an insecticide called dichlorodiphenyltrichloroethane (DTT) was used, reducing and eliminating disease transmission in more than 10 countries. Unfortunately, the elimination of malaria could not be achieved in underdeveloped countries, such as Sub-Saharan countries, which is proved by the current statistics of malaria infections, with Sub-Saharan countries having the most reported cases (Buonsenso and Cataldi, 2010; Petersen *et al*., 2011).

1.2. A Global View of Malaria Infection

In 2012 the World Health Organization (WHO) developed an annual report on malaria infections occurred in 2011 in the malaria-endemic countries and it highlights the progress made in the fight against malaria. The report shows an estimation of more than 25% decrease in mortality rate since 2000. The largest percentage reduction reported was seen in Europe (99%), America (55%), Western Pacific (42%) and African Regions (33%) (World Health Organization, 2011; World Health Organization, 2012a).

The WHO reported that an estimation of 3.3 billion people were at risk of malaria in 2011 with populations living in sub-Saharan Africa presenting the highest risk of acquiring malaria as indicated in Figure 1.1. It was also estimated that 80% cases and 90% of deaths occurred in the WHO African Region, with children under five years of age and pregnant being the mostly affected.

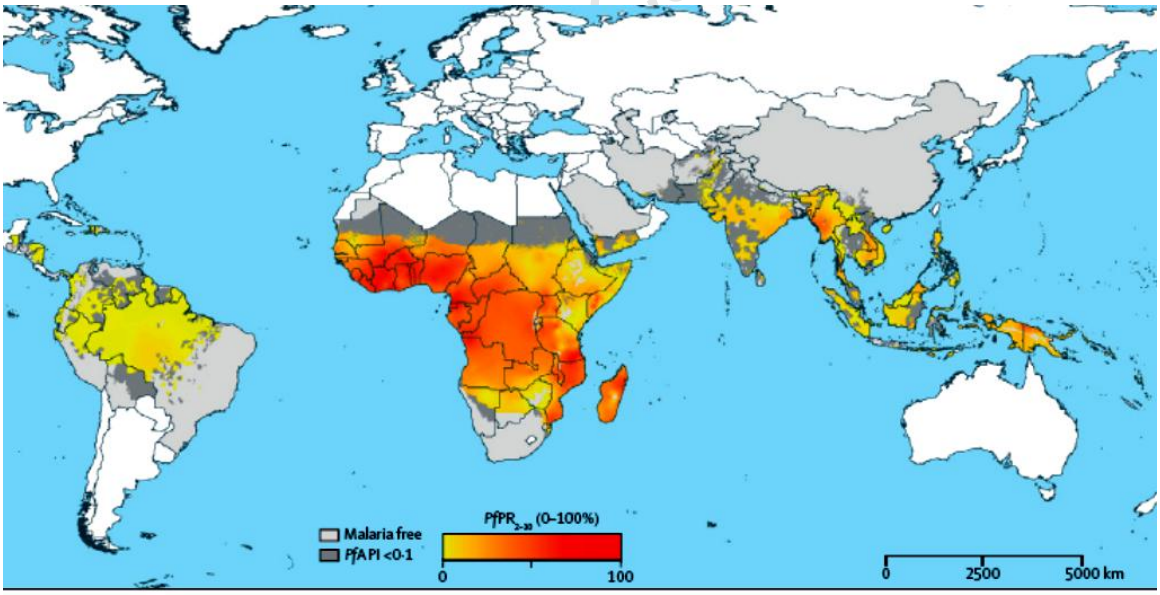


Figure 1.1: Geographic distribution of infection with *Plasmodium falciparum* malaria (Goldenberg *et al*., 2010).

1.2.1. Malaria infection in South Africa

In South Africa, an estimated 10% of the country's population live in malaria-endemic provinces, namely, Limpopo, Mpumalanga and KwaZulu-Natal, along the borders of Zimbabwe and Mozambique. (Moonasar *et al*., 2012; Khosa *et al*., 2013). Recently, an increase in malaria notification has been reported in non-endemic areas, especially in Gauteng because of travellers returning from malaria-endemic areas such as Mozambique (Moonasar *et al*., 2011). Figure 1.2 shows affected regions in South Africa with about 9866 confirmed cases of malaria infection and 54 deaths reported in 2011 (WHO, 2012). About 95% of all malaria cases are caused by the species *P. falciparum* transmitted by a local vector *Anopheles arabiensis* and transmissions occur during the rainy season, which is between September and May (Moonasar *et al*., 2011; Moonasar *et al*., 2012; Khosa *et al*., 2013).

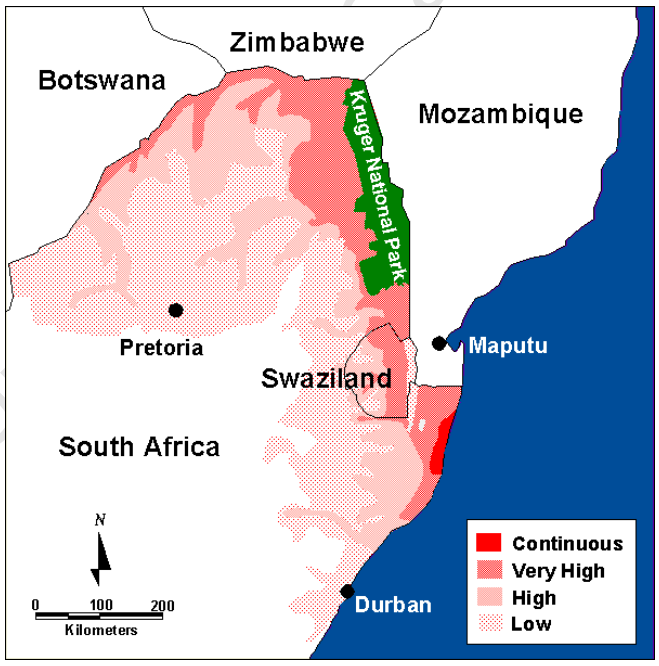


Figure 1.2: Distribution of confirmed malaria cases in South Africa (malaria.org.za).

An official notification of malaria infections in South Africa dates back to 1956 but over the years the Department of Health implemented malaria control and prevention

interventions in the endemic provinces. The use of residual spraying has caused a reduction in malaria transmission in some endemic areas and because malaria is listed as a modifiable disease in South Africa, the disease is diagnosed sooner. If a patient presents signs and symptoms of malaria, blood is collected for confirmation of parasite infection (Khosa *et al*., 2013; Moonasar *et al*., 2011). Implementation of these treatment and prevention policies by the Department of Health has caused a reduction of 85% in malaria cases and the number of deaths has decreased by 81% since 2000 (doh.gov.za, 2012)

1.3. Malaria Pathogenesis

Malaria parasites are unicellular eukaryotes that invade red blood cells of vertebrates. The clinical symptoms and pathology of the malaria disease is caused by blood stage parasites that evade the red blood cells (Bhattacharjee *et al*., 2012; Garcia *et al*., 2008). They have a complex life cycle, which include a sexual cycle in a mosquito vector that transmits the infection during feeding from the host and an asexual cycle inside a human host (figure 1.1). The plasmodia alternate between the host and the mosquito vector, and thus the transmission stages of the parasite had to develop mechanisms for rapid adaptation to the new environment in order to co-exist with the respective host (Kuehn and Pradel, 2010; Tham *et al.*, 2012).

1.3.1. Life cycle in mosquitoes

Plasmodium falciparum resides within the salivary gland of a female *Anopheles* mosquito in its sporozoite stage. When a mosquito ingests human blood during a feed the parasite male and female gametocytes differentiate into ookinetes and migrate out of the blood meal, across the peritrophic matrix to the mid-gut wall where an oocyst is formed (figure 1.1). After a meiotic reduction in chromosome number sporozoites are formed within the oocyst. Eventually the oocyst ruptures and the sporozoites migrate and invade the salivary gland epithelium where they await transfer to the vertebrate host during the next blood meal (Winzeler, 2008).

1.3.2. Life cycle in humans

During biting of an infected female mosquito, *Plasmodium* sporozoites are injected in the blood and these invade hepatocytes where they develop into liver schizonts (Figure 1.3). These schizonts rupture releasing thousands of merozoites into the blood stream where they invade erythrocytes. This commences the asexual blood stage that includes ring stages that develop to trophozoites and these mature into schizonts, a stage supported by the ingestion of host hemoglobin. The rupture of the schizont and release of tens of thousands of merozoites result in invasion of new erythrocytes, which give rise to the cycle of fever and chills in malaria. Some merozoites differentiate into male and female gametocytes which remain in the bloodstream until they are taken up by a feeding mosquito (Maltha and Jacobs, 2011; Tham *et al.*, 2012).

Most deaths occur due to a complication of infections with *P. falciparum* and the disease pathology is produced by the intraerythrocytic stages of the malaria parasite (Foley and Tilley, 1998). In *P. vivax* and *P. ovale* infections, dormant liver stages may cause a relapse weeks to months after adequate prophylaxis or effective blood-stage treatment of a primary infection. *P. malariae* may persist in the blood at undetectable levels without causing symptoms for many years and may later develop into clinical illness in patients under immunosuppressive drugs or after splenectomy (Maltha and Jacobs, 2011).

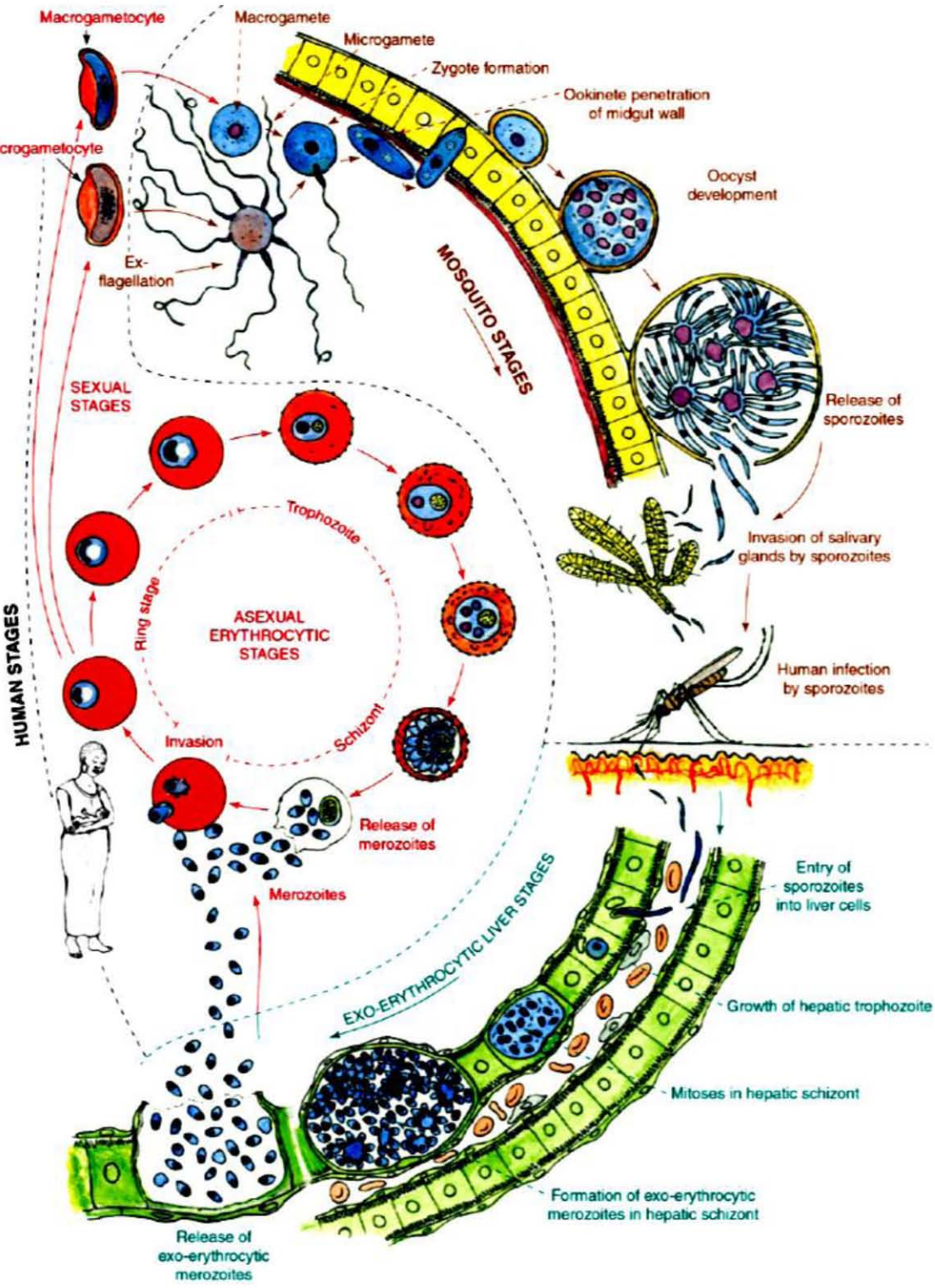


Figure 1.3: <u>Diagram of the life cycle of *Plasmodium falciparum* is described from the sexual cycle inside the mosquito and an asexual cycle inside the host</u> (Bannister and Mitchell, 2003).

1.4. Malaria Treatment

In the review paper of Wernsdorper and Payne, 1991, it is stated that “malaria control is defined as a reduction of malaria to a level at which it does not anymore constitute a major health problem”. This control of malaria rests on the use of antimalarial drugs and the effectiveness of these drugs depends on their ability to interrupt the essential life functions of malaria parasites, thereby inhibiting their multiplication and leading to their removal from the circulation (White, 1998).

Antimalarial drugs target different stages of the malaria life cycle but the majority of these drugs act on the intra-erythrocytic phases of development of the malaria parasite and this is the stage that causes most of the pathology of the disease, giving rise to all of the clinical symptoms of malaria (Foley and Tilley, 1998; Olliaro, 2001). During the intra-erythrocytic phase the parasites evades the host immune system with a mechanism necessary for maintaining its chemical composition and for obtaining from the host cell cytosol all of the nutrients that it requires for its survival (Kirk, 2001). In drug discovery, the goal is to generate inhibitors specific for the parasite proteins and its biochemical pathways. Plasmodium metabolic pathways which are different from those of humans offer excellent opportunities for the development of antimalarials with reduced risks of potential side effects (Olliaro, 2001). Examples of metabolic pathways that can be targeted for the development of novel antimalarials include:

- **The food vacuole**: This is the site for the digestion of the host hemoglobin by the parasite hemoglobinases such as aspartic and cysteine proteases. Inhibiting these enzymes can disrupt the development of parasites in the erythrocyte by preventing the hydrolysis of hemoglobin. Cysteine inhibitors also prevent the erythrocyte rupture, causing an uncompleted erythrocyte cycle (Dahl & Rosenthal, 2005; Olliaro, 2001). Prevention of the polymerization of heme to a non-toxic hemozoin by heme polymerase can cause an accumulation of toxic heme, resulting in the death of the parasite. Antimalarials such as quinoline are known to prevent the formation of hemozoin (Olliaro, 2001).

- **Folate pathway:** This is the pathway that controls DNA synthesis and metabolism of certain amino acids such as methionine and glycine. Folate metabolism offers many possibilities for selective inhibition of biochemical processes that are vital for parasite growth. The reason for that is because enzymes such as dihydropteorate synthase (DHPS) and dihydroneopterin aldolase, which are utilized in the biosynthesis of dihydrofolate, are not present in the mammalian systems (Delfino *et al*., 2002; Warhurst, 2002).
- **Apicoplast:** This organelle participates in lipid biosynthesis and iron metabolism. Studies show that the parasites can survive with no apicoplast or with a chemically damaged apicoplast, but they will be unable to establish a successful new infection. The apicoplast synthesizes a molecule that is needed for the infection process (Vargas Parada, 2010).
- **Pyrimidine biosynthesis:** The malaria parasites require purines and pyrimidines for DNA and RNA synthesis. This is required for their growth and replication during their intraerythrocytic stage of their life cycle. Enzymes involved in the plasmodial *de novo* pyrimidine synthesis are excellent targets for the development of novel antimalarials (Olliaro, 2001).
- **Plasmodial protein kinases:** Kinases utilize adenine triphosphate (ATP) to add phosphate to amino acids of resulting in changes in the activity of proteins, stability, interactions with ligands or localisation of the phosphorylated substrates. Studies show that inhibiting plasmodial protein kinases block the invasion and intraerythrocytic development of *P. falciparum* (Avery *et al.*, 2008; Lim *et al*., 2012; Olliaro, 2001).
- **Glycolysis:** During the erythrocytic stages of the malaria parasites anaerobic glycolysis in the cytoplasm plays an important role in energy production. Targeting enzymes that are involved in the glycolysis could result in the death of the malaria parasites due to limited ATP production. Parasite lactacte dehydrogenase (pLDH), an enzyme responsible for the production of L-lactate as a major product of glycolysis, has kinetic differences from the human LDH isoforms, suggesting that pLDH can be selectively targeted for the development of novel antimalarials (Avery *et al.*, 2008; Mehta *et al*., 2006). Figure 1.4 shows different classes of drugs that have been studied over the years for the treatment of malarial and their mode of action.

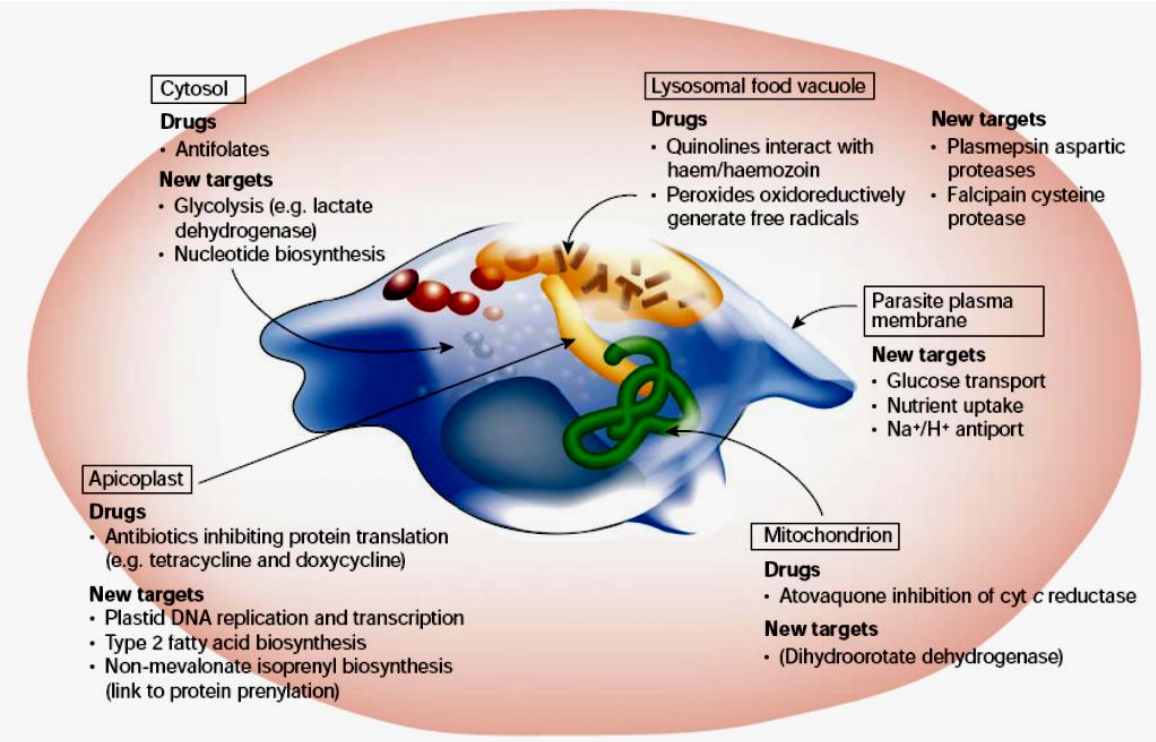


Figure 1.4: Plasmodium life cycle with phases targeted by antimalarial drugs (Ridley, 2002).

1.4.1. Classes of drugs used in the treatment of malaria

Various classes of drugs such as quinolines, artemisinins, antifolates and antibiotics have been shown to exhibit antimalarial activity and have been used for decades for the treatment of malaria (Avery, 2008; Kaur *et al*., 2010)

1.4.1.1. Quinoline and its derivatives

The quinoline drugs share some common structural features with purine nucleotides, such as the heterocyclic quinoline ring that is analogous to the C1-C10 ring of purines (Figure 1.5). Most important use of the quinoline ring is its antimalarial potential (Graves *et al*., 2002). Quinoline-containing antimalarials are among the most important antimalarial drugs ever used. Early studies showed that disubstitution of the quinoline ring is essential for improved antiplasmodial activity and these antimalarials include:

- **Type 1 drugs:** The 4-aminoquinolines chloroquine and Mannich-base amodiaquine, pyronaridine
- **Type 2 drugs:** The aryl-amino alcohols quinine and quinidine, mefloquine, halofantrine (Foley and Tilley, 1998; Olliaro, 2001).

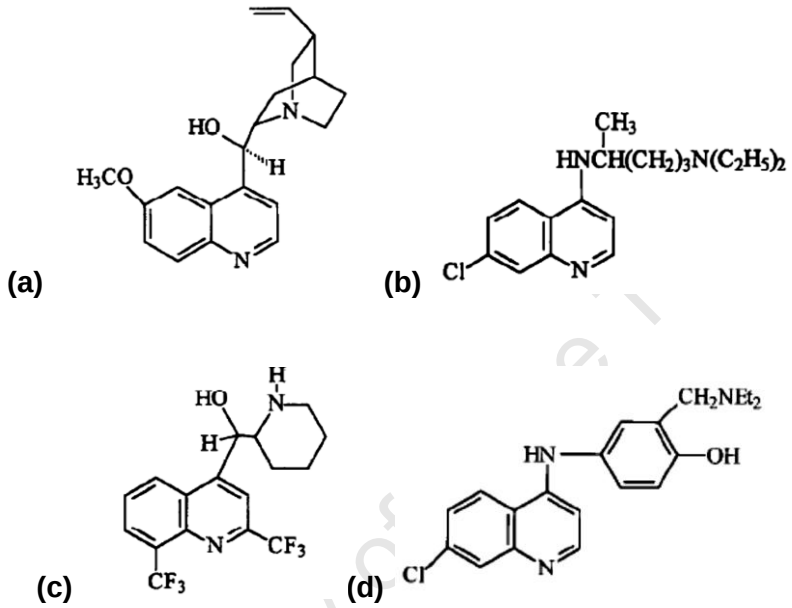


Figure 1.5: Structures of the quinoline-based antimalarial drugs (a) quinine, (b) chloroquine and (c) mefloquine (d) amodiaquine (Foley and Tilley, 1997; Graves *et al*., 2002; Kotecka *et al*., 1997).

Mechanism of action:

These compounds are more effective against the stages of the malaria parasite within the erythrocytes but there are few which are believed to target the hepatic stage (Avery, 2008; Kaur *et al*., 2010). When the parasite infects the erythrocyte, they use their endolysosomal system to digest the hemoglobin in an acidic food vacuole, producing an oxidized form of heme, ferriprotoporphyrin IX (FP-IX), which is the non-protein component of hemoglobin, as a byproduct (Fitch, 2004; Fitch, 1998; Orjih, 2001). Free FP-IX is mainly toxic and can lyse the cell and affect the function of lysozomal enzymes, therefore, the parasite disposes the toxic FP-IX by a polymerization process that crystallizes at least 95% of FP-IX as insoluble brown pigment, hemozoin, and this

allows an uninterrupted growth and proliferation of the parasite (Combrinck *et al*., 2012; Egan *et al*., 1994; Loria *et al*., 1999). Quinoline-based antimalarials are known to inhibit the polymerization of FP-IX by heme polymerase, preventing hemozoin formation. This leads to intraparasitic accumulation of heme, which is highly toxic to the parasites, thereby causing the death of the parasite (Egan *et al*., 1994; Kaur *et al*., 2010).

Quinine:

Quinine is the first line drug for the treatment of severe malaria. It is a cinchona alkaloid that belongs to the aryl amino alcohol group of drugs. It was extracted from the Cinchona Bark, which had been used for the treatment of fever since the 1630s. Quinine has been used in unextracted form by Europeans. In 1820, French researchers Pierre Joseph Pelletier and Joseph Bienaimé Caventou successfully isolated the cinchona alkaloid active ingredient quinine from the crude extracts of cinchona bark (Foley and Tilley, 1998; Solomon and Lee, 2009; Spikes, 1998). Quinine remained the only useful drug for the treatment of malaria until it was displaced in the 19th century by a number of synthetic antimalarials (Spikes, 1998).

Chloroquine:

Chloroquine was introduced in the 1940s and it has been the most widely used therapeutic antimalarial. It was first synthesized in 1934 in a German Pharmaceutical company Bayer AG as a cheaper alternative to quinine (Cooper and Magwere, 2008). Its efficiency, safety, stability and low cost have contributed in making it the most widely used synthetic antimalarial (Slater, 1993). Chloroquine is only active against the intra-erythrocytic stages during which the parasite degrades hemoglobin. It kills malaria parasites by accumulating in the food vacuole of parasites. It has high affinity for FP-IX; therefore it binds tightly and forms a complex with FP-IX, which interferes with the polymerization of FP-IX into hemazoin. Free FP-IX and FP-IX-chloroquine complexes kill parasites by inducing oxidative stress, which may lead to peroxidation of parasite membrane lipids, damage of DNA, oxidation of protein and finally parasite death (Fitch, 1998; Gelb, 2007; Kumar *et al*., 2006).

Other quinolines:

According to Inokuchi, 2013, basic side chains can improve the activity of chloroquine by causing the accumulation into the food vacuole and, hence, for inhibition of hemozoin formation The structure-activity studies showed that substitution in the side chain of chloroquine with an aryl group produced compounds with good activity *in vivo* (O'Neill, 2004). **Amodiaquine** was first introduced as an alternative to chloroquine since it appeared to have activity against chloroquine resistant *Plasmodium falciparum* parasites. Like chloroquine, it is a 4-aminoquinoline, but it is more effective in treating chloroquine-resistant strains of P. vivax. It is also believed that it has the same mode of action as chloroquine (Humphreys *et al*., 2007). **Mefloquine** and **halofantrine** are quinine like aminoalcohols and, like chloroquine; they are both selectively active against the intraerythrocytic stages of parasite development with the parasite food vacuole as its primary target (Bryson and Goa, 1992; Bouchaud *et al*., 2009; Palmer *et al*., 1993). Mefloquine was introduced in the 1970s by the Walter Reed Army Institute of Research as another synthetic analogue of quinine but more hydrophobic than chloroquine (Chevli and Fitch, 1982; Wells *et al*., 2013). Both mefloquine and halofantrine have (Bryson and Goa, 1992) been proven to be a potent antimalarial, especially against chloroquine resistant strains of malaria parasites (Bouchaud *et al*., 2009; Palmer *et al*., 1993). **Piperaquine** is a bisquinoline compound related to chloroquine and other 4-aminoquinolines. It was developed as an antimalarial by Chinese scientists in the 1970s and 1980s and replaced chloroquine as the first-line treatment of falciparum malaria in China in 1978 when chloroquine resistance became a problem (Breman *et al*., 2007; Davis *et al*., 2005).

1.4.1.2. Artemisinin

Artemisinins were discovered in 1971 from an herb Qing Hao, known by its Latin name as *Artemisia annua*, and this herb was known in the past for its activity against intermittent fever for more than 2000 years (Corpolongo, 2012; Wright, 2005). The Chinese discovered that extracts from this plant showed good antimalarial activity in mice and that artemisinin killed the malaria parasites more rapidly with less toxicity than

either chloroquine or quinine and it even killed chloroquine resistant strains (Covello, 2008; Weina, 2008).

Artemisinin is a sesquiterpene lactone peroxide and its poor solubility in water and oil and its low oral bioavailability became two major shortcomings despite possessing good efficacy against malaria (Wang, 2007). In order to overcome these shortcomings natural and semi-synthetic artimisinin derivatives were developed in order to tackle the solubility problems such as water soluble **dihydroartemisinin** (Figure 1.6). Many derivatives that have been synthesized from dihydroartemisinin include **artemether**, **arteether** and **artesitunic acid** (Figure 1.6) and these possessed more activity than artemisinin *in vitro* (Balint, 2001; Avery *et al.*, 2008).

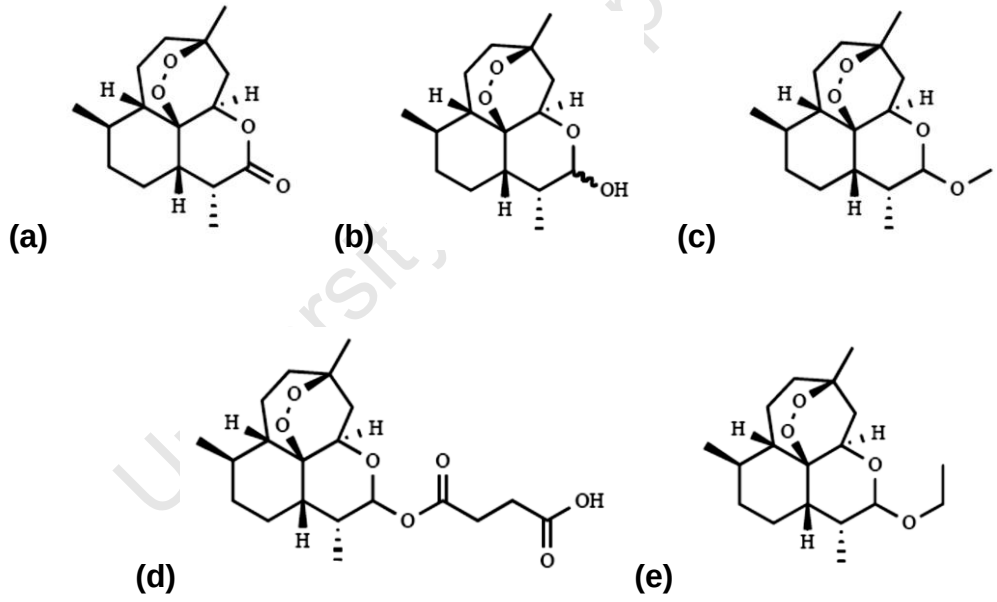


Figure 1.6: Structures of artemisinin (a) and its derivatives; (b) dihydroartemisinin, (c) artemether, (d) artesunic acid and (e) arteether (Avery *et al.*, 2008).

Mechanism of action:

The endoperoxide bridge that is observed in the structures presented in Figure 1.6 was found to be responsible for artemisinin activity against malaria parasites. This was discovered when the metabolites isolated from urine possessed structures that lacked the endoperoxide bridge and had very low antimalarial activity (Hien and White, 1993; Wang *et al*., 2007). Peroxides are highly reactive entities, therefore, studies have shown that the endoperoxide group reacts with ferriprotoporphyrin IX (FP-IX) to produce oxygen centered free radicals which are then converted to carbon centered free radicals within the parasitized erythrocyte (Krishna *et al*., 2004; Woodrow *et al*., 2005). These free radicals react with susceptible groups within the parasite causing alkylation and oxidation of proteins and lipids, damaging the organelles and membranes of the parasites (Balint, 2001; Hien and White, 1993). Studies also show that the artemisinins structure is similar to that of thapsigargin, which is a highly specific inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). This is an ATPase-dependent ionic pump present in *Plasmodium* endoplasmic reticulum membrane related to cation transport and is inhibited by artemisinin and its derivatives (Eckstein-Ludwig *et al*., 2003; Quashie, 2010).

1.4.1.3. Antifolates

Antifolates are antimalarial drugs that inhibit the enzyme dihydrofolate reductase (DHFR), thus inhibiting the metabolic pathway synthesizing tetrahydrofolate (THF), which is a folic acid derivative and a coenzyme in the metabolism of certain amino acids such as methionine and nucleic acids required for cell proliferation (Talisuna *et al*., 2004; Warhurst, 2002). In malaria treatment, inhibiting the function of DHFR deprives the parasite essential folate cofactors because malaria parasites synthesize tetrahydrofolates and precursors *de novo* by salvaging them from the environment (Salcedo Sora *et al*., 2011; Yuthavong, 2002). Antifolates are classified in two classes:

- **Type 1 antifolates:** Sulfonamides and sulfones have structures similar to ρ-aminobenzoic acid (PABA) and they prevent the synthesis of 7,8-dihydropteroate, which is a precursor of dihydrofolate, by competing with PABA for the active site of dihydropteorate synthase (DHPS). Type 1 antifolates include a sulfone dapsone and

sulfonamides sulfadoxine, sulfadazine and sulfalene, whose structures are shown in Figure 1.7 (Delfino *et al*., 2002; Olliaro, 2001)

- **Type 2 antifolates:** Pyrimethamine, biguanides and triazine metabolites and quinazolines inhibit the parasite DHFR, preventing the nicotinamide adenine dinucleotide phosphate (NADPH) dependant reduction of dihydrofolate to tetrahydrofolate

$$\mathbf{DHF + NADPH + H^+} \xrightarrow[\textit{DHFR}]{} \mathbf{THF + NADP^+}$$

These compounds have a similar structure to pteridine ring of dihydrofolate, and therefore compete for the active site of DHFR. Type 2 antifolates are methotrexate, pyrimethamine, cycloguanil, a metabolic product of proguanil and trimethoprim. Their structures are shown in Figure 1.7 (Delfino *et al*., 2002; Olliaro, 2001).

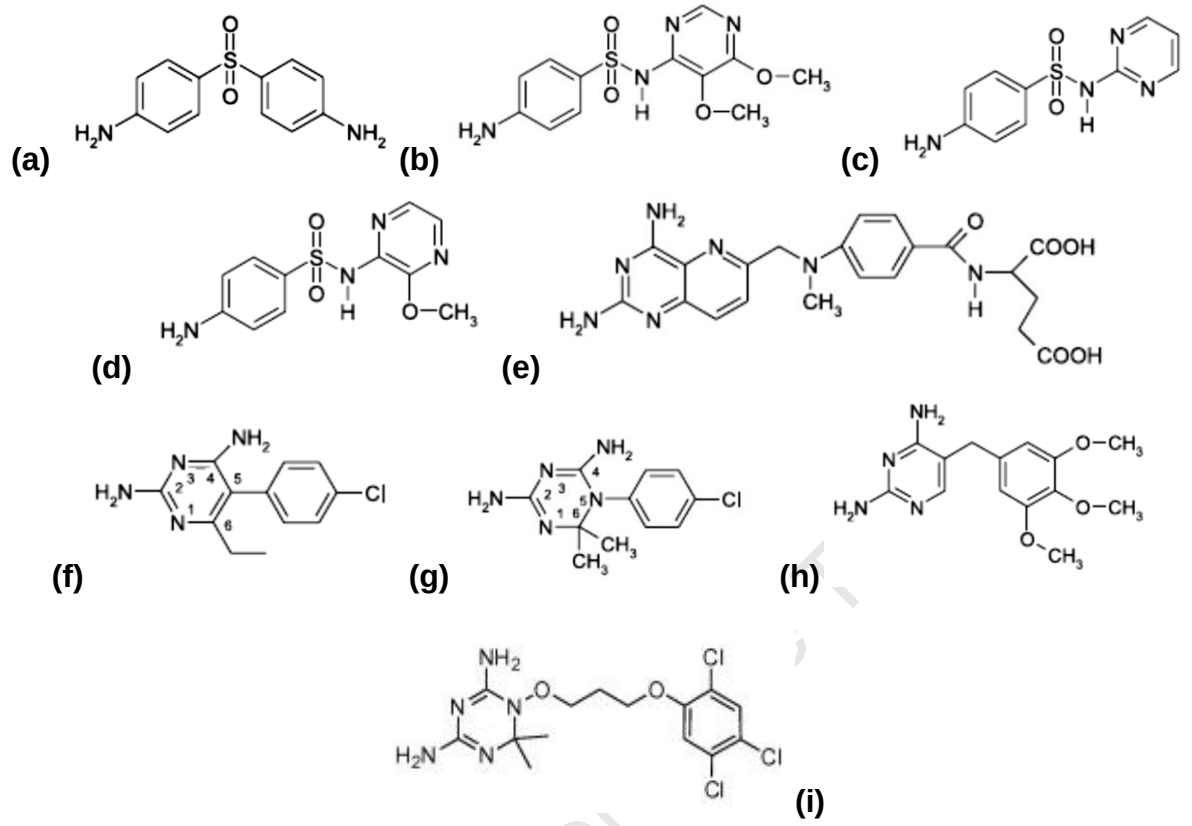


Figure 1.7: Structures of antifolates. Type 1 antifolates are (a) dapsone, (b) sulfadoxine, (c) sulfadiazine and (d) sulfalene and type 2 antifolates are (e) methotrexate, (f) pyrimethamine, (g) cycloguanil, (h) trimethoprim and (i) WR99210 (Delfino *et al*., 2002).

1.4.1.4. Other antimalarials

A number of other chemical classes of drugs have been investigated for the treatment of malaria. Most of these also act on the erythrocytic stage of the parasite.

Antibiotics

Several classes of antibiotics have been used for the treatment of malaria due to their potency against the malaria parasites. Some antibiotics have low antimalarial activity; therefore, they are used in combination with more rapidly acting drugs to treat malaria. Studies show antibiotics such as tetracycline and its derivatives, doxycycline and minocycline, and fosmidomycin act against the plasmodial mitochondrion or an

organelle called the apicoplast, which is related to plant chloroplasts but unique to plasmodia and other apicomplexan parasites. Other antibiotics used in malaria treatment are azithromycin and clindamycin (Avery *et al.*, 2008; Dahl, 2007).

Tetracyclines are broad-spectrum antibiotics with a wide range of potency on a number of bacteria or protozoans such as *Plasmodium* and have been used for treatment of malaria since 1950 (Briolant, 2008). The plasmodial mitochondrion and aplicoplast contain independent genomes encoding prokaryote-like RNA polymerase subunits, 70S ribosomal subunits, tRNAs, and a small number of proteins. Therefore, the tetracyclines bacterial activity affects the function of these organelles by inhibiting prokaryotic translation (Dahl *et al*., 2006; Dahl, 2007). **Doxycyclines** and **minocycline** are derived from naturally occurring tetracyclines produced by *Streptomyces* bacteria. Studies show that they are highly effective against the asexual erythrocytic stages of the malaria parasite and have a similar mechanism as tetracycline (Briolant *et al*., 2010; Tan *et al*., 2011). **Fosmidomycin** is a phosphonic acid derivative that inhibits the biosynthesis of isoprenoids in the apicoplast of *P falciparum*, which are necessary to maintain cellular function in mammalian hosts. This is achieved by inhibiting 1-deoxy-D-xylulose 5-phosphate reductoisomerase of the non-mevalonate pathway, which is absent in humans (Lell *et al*., 2003; Missinou *et al*., 2002; Umeda *et al*., 2011).

Atovaquone

Atovaquone is a lipophilic hydroxynaphthoquinone analog and structurally related to coenzyme Q (ubiquinone). It specifically targets the cytochrome b complex in the inner mitochondrial membrane, thereby inhibiting the respiratory chain of *P. falciparum* which is required for the regeneration of coenzyme Q, which is essential for pyrimidine biosynthesis (Hyde, 2007; Petersen *et al.*, 2011). Atovaquone interferes with the transfer of electrons that are important in maintaining membrane potential of the mitochondrion that allows transport of proteins and small molecules in and out of the organelle (Hyde, 2002).

1.4.2. Drug Toxicity

The World Health Organization defines adverse drug reactions as “harmful, unintended reactions to medicines that occur at doses normally used for treatment” and are among the leading causes of death in many countries (WHO, 2008). Various classes of antimalarials have common adverse effects, which sometimes can be mild, but in some cases fatal adverse reactions have been reported. These include cardiovascular, ocular, neuropsychiatric, gastrointestinal, dermatological and hepatotoxicities (Table 1). Many effective antimalarials are no longer prescribed to patients due to such severe adverse reactions caused by toxicity of the drug (AlKadi, 2007). Sometimes toxicity occurs with long-term usage of antimalarials or drug overdose (Taylor and White, 2004). Studies show that malaria patients of certain groups, such as young, elderly, pregnant and patients suffering from other diseases are at risk of severe adverse reactions (AlKadi, 2007; Nosten *et al*., 2006; Taylor and White, 2004).

Table 1.1: The list of antimalarial drugs and their major adverse effects.

Class	Drug	Adverse effects	References
Quinolines	Quinine	Titinnus, hypoglycemia, cardiovascular effects, vertigo, visual disorders and hypotension	(AlKadi, 2007; Taylor and White, 2004)
	Chloroquine	Malaise, itching, dizziness, headaches, visual imparement gastrointestinal upset, cardiovascular effects and seizures	(AlKadi, 2007 Taylor and White, 2004)
	Mefloquine	Headache, vertigo, insomnia, gastrointestinal upset, cardiovascular effects, depression, dreams and fatigue.	(Palmer *et al*., 1993)
	Halofantrine	Cardiovascular effects and hepatotoxicity.	(Obi *et al*., 2004)
	Amodiaquine	Hepatatoxicity and agranulocytosis.	(AlKadi, 2007; Taylor and White, 2004)
Artemisinins	Artemisinin Artemether Artesunic Acid	Cardiovascular effects, vertigo, dizziness, abdominal pains diarrhea and anorexia.	(AlKadi, 2007; Salako, 1984)
Antifolates	Pyrimethamine Sulfadoxine Diapsone Proguanil	Cardiovascular effects, vertigo, dizziness, abdominal pains diarrhea and anorexia.	(Salako, 1984; Taylor, 2004)
	Methotrexate	Nausea, vomiting, diarrhoea and oral ulcers	(Roma, 2011)
Antibiotics	**Tetracyclines** Doxycyline Minocycline	Photosensitivities, renal imparement, gastrointestinal upset and oral contraceptive failure.	(AlKadi, 2007)
	Fosmidomycin	Hepatotoxicity and gastrointestinal upset.	(Borrmann *et al*., 2004; Wiesner *et al*., 2003)
Atovaquone		Rash, vomiting, diarrhea, abdominal pain and headache.	(AlKadi, 2007)

1.4.3. Drug Resistance

The use of most antimalarial drugs that have been in the market for decades is not only affected by the development of severe adverse reactions, but also the emergence and spread of drug resistance in many parts of the world, primarily in *Plasmodium falciparum* strains. This has severely limited the use of many effective antimalarials, and has become a major threat to malaria elimination efforts, causing increased morbidity and mortality and a financial burden due to sustenance of replacement therapy (Hastings, 2004). The World Health Organization defines malaria resistance as the "ability of a parasite strain to survive and/or multiply despite the administration absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject" (Bloland, 2001).

1.4.3.1. Mechanism of resistance in *P. falciparum*

Resistance to nearly all antimalarials has been reported in both *P. falciparum and P.vivax*, but the former causes the most virulent form of malaria in humans and it accounts for most of the disease burden (Sanchez *et al*., 2010). The development and spread of resistance in malaria result from interactions between parasite, humans, vector and drugs, enhanced by particular ecological features. Factors that contribute to the emergence of malaria resistance are:

- **The interaction of drug use patterns:** Overuse of antimalaria drugs, incorrect dosing and incomplete therapeutic treatments of active infections can cause treatment failure in the individual and may also cause the development of drug resistance (Hyde, 2007; Wongsrichanalai *et al*., 2002).
- **The characteristics of the drug:** Pharmacokinetic and pharmacodynamic properties of the drug also contribute to the development of resistance. Hydrophobic drugs from earlier treatments with long half-lives tend to remain in circulation and can cause resistance to develop (Rathod *et al*., 1997).
- **Parasite characteristics:** Drug resistance can be caused by different modifications in the malaria parasite cell. Point mutations in the parasite protein-target genes can confer reduced sensitivity to a given drug or a class of drugs (Bloland, 2001).

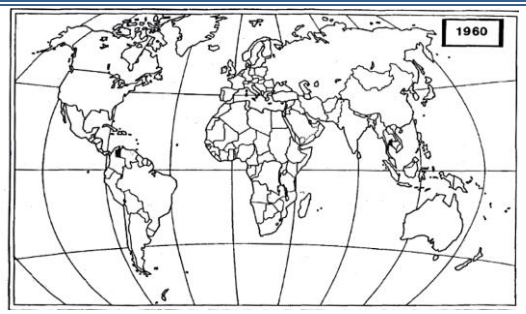
The mutation rate of the parasite has a direct influence on the frequency at which resistance can emerge. While higher mutation rates enable a faster emergence of resistance, they can also lead to an accumulation of harmful mutations (Petersen *et al*., 2011). These mutations can cause a change in the permeability or transport properties across the membranes involved, thus reducing the absorption of the drug (Delfino *et al*., 2002).

- **Vector and environment factors:** Genetic basis of a host's resistance to parasites can be complicated by non-genetic factors, such as environmental quality, which may influence the expression of genetic resistance, thus altering the patterns of disease and the host's response to selection (Lambrechts *et al*., 2006). Studies show that temperature and nutrients greatly affect host biochemical, physiological and behavioral processes and can enhance parasite development and host exploitation and, therefore, virulence (Wolinska and King, 2009). Level of transmission influences the rate of development and spread of drug resistance. Increased risk of resistance development has been reported in areas of low transmission where infections are acquired infrequently and the majority of these infections are symptomatic, which means that treatment will be received. (White, 1999; Wongsrichanalai *et al*., 2002). In higher-transmission areas the majority of infections are asymptomatic and usually only a small fraction of infections are exposed to antimalarial drugs (White, 2004).

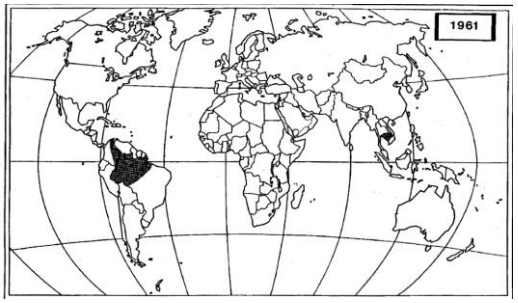
Examples of first line malaria drugs that have been compromised by the evolution of parasites resistance include chloroquine, atovaquone and pyrimethamine.

Chloroquine and related compounds

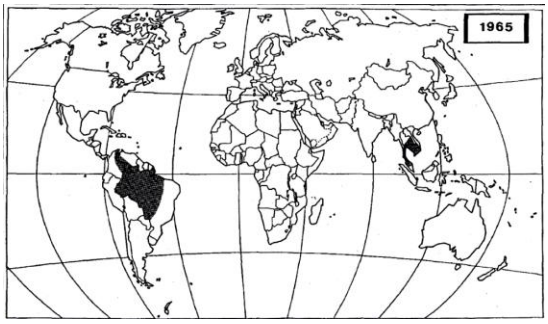
Resistance of *P. falciparum* to chloroquine emerged in the late 1950s in Colombia and at the Cambodia-Thailand and by the 1960s and 1970s resistance had spread through South America, South East Asia, and India. In Africa resistance was only detected in 1970s and, currently, it has been reported that chloroquine-resistant *P falciparum* is widespread across all malaria-endemic areas (Guerin *et al*., 2002; Wellems and Plowe, 2001). The review paper by Wernsdorfer and Payne gave an illustration of how chloroquine resistance spread throughout the malaria-endemic areas (Figure 1.8).



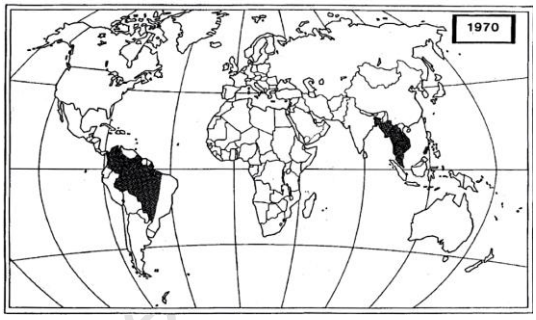
1960



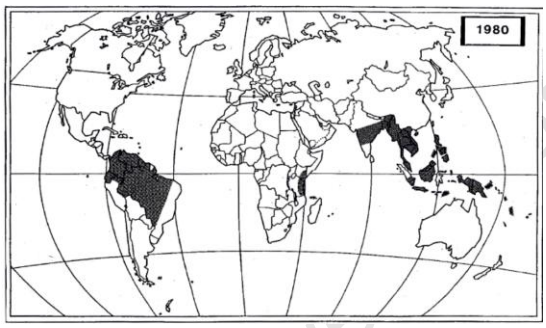
1961



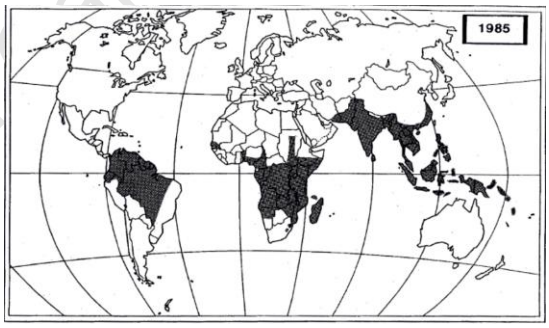
1965



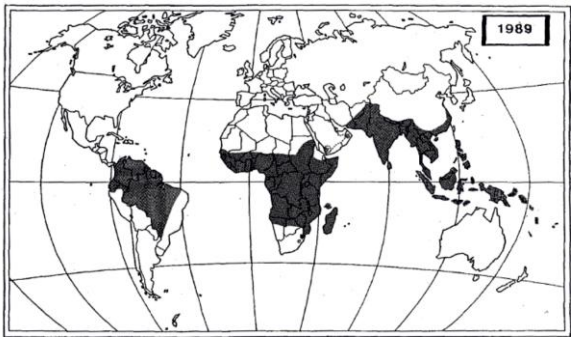
1970



1980



1985



1989

Figure 1.8: <u>Chloroquine resistance in *Plasmidium falciparum* from 1960 to 1989</u> (Wernsdorfer and Payne, 1991).

In chloroquine-resistant parasites, the drug is excluded from the site of action, therefore, this cause the parasites to accumulate less chloroquine in their acid food vacuoles than chloroquine-sensitive parasites (Foley and Tilley, 1998). The cause of this resistance has a genetic basis; which involves an amino acid mutation at the 76th position, Lys-76 to Thr (K76T), of the *P. falciparum* chloroquine resistance transporter (*Pfcrt*), a membrane transporter protein localized to the acid food vacuole (Figure 1.9). Mutations at Cys-72 to Ser (C72S), Met-74 to Iso (M74I), Asn-75 to Glu (N75E), and Asn-75 to Lys (N75K) are also associated with resistance and all these mutations cause the pH across the vacuolar membrane to increase, thus affecting the uptake of chloroquine which is dependent on the maintenance of the pH gradient (Awasthi *et al*., 2011; Foote and Cowman, 1994; Griffing *et al*., 2010; Wongsrichanalai *et al*., 2002).

Mefloquine resistance was first observed near the Thai-Cambodian border in the late 1980s. The development of mefloquine resistance in Thailand may have been influenced by the heavy use of quinine and its derivatives just before the introduction of mefloquine (Wongsrichanalai *et al*., 2002). Mefloquine resistance correlates well with resistance to halofantrine and quinine and is often associated with mutations in or amplification of the ATP-dependent P-glycoprotein pump homologue, encoded by the MDR gene family (White, 1999). *P. falciparum* multidrug resistance transporter 1 (*pfmdr1*) (Figure 1.9) is located in the parasite vacuolar membrane with its ATP-binding domain facing the cytoplasm and it directs drug movement into the vacuole (O'Neill, 2012). Mutations Asn-86 to Tyr (N86Y), Tyr-184 to Phe (Y184F), Ser-1034 to Cys (S1034C), Asn-1042 to Asp (N1042D) and Asp-1246 to Try (D1246Y) in the *pfmdr1* gene are associated with drug resistance (Griffing *et al*., 2010).

Studies show that antimalarial drugs with similar drug structure and drug binding site structure can cause cross-resistance between antimalarial drugs. Chloroquine, mefloquine, quinine and halofantrine are structurally related drugs and previous studies conducted in animal models of malaria demonstrated cross resistance between these drugs. The use of chloroquine in some malaria endemic countries prior to the use of mefloquine is suspected to be the cause of cross-resistance, induced by previous

chloroquine drug pressure (Palmer *et al*., 1993). Petersen and colleagues also reported cross-resistance between chloroquine and amodiaquine with mutations in *PfCRT* and *PfMDR1*, which are associated with decreased susceptibility to both drugs but the cross-resistance is incomplete because some chloroquine resistant parasites remain susceptible to amodiaquine.

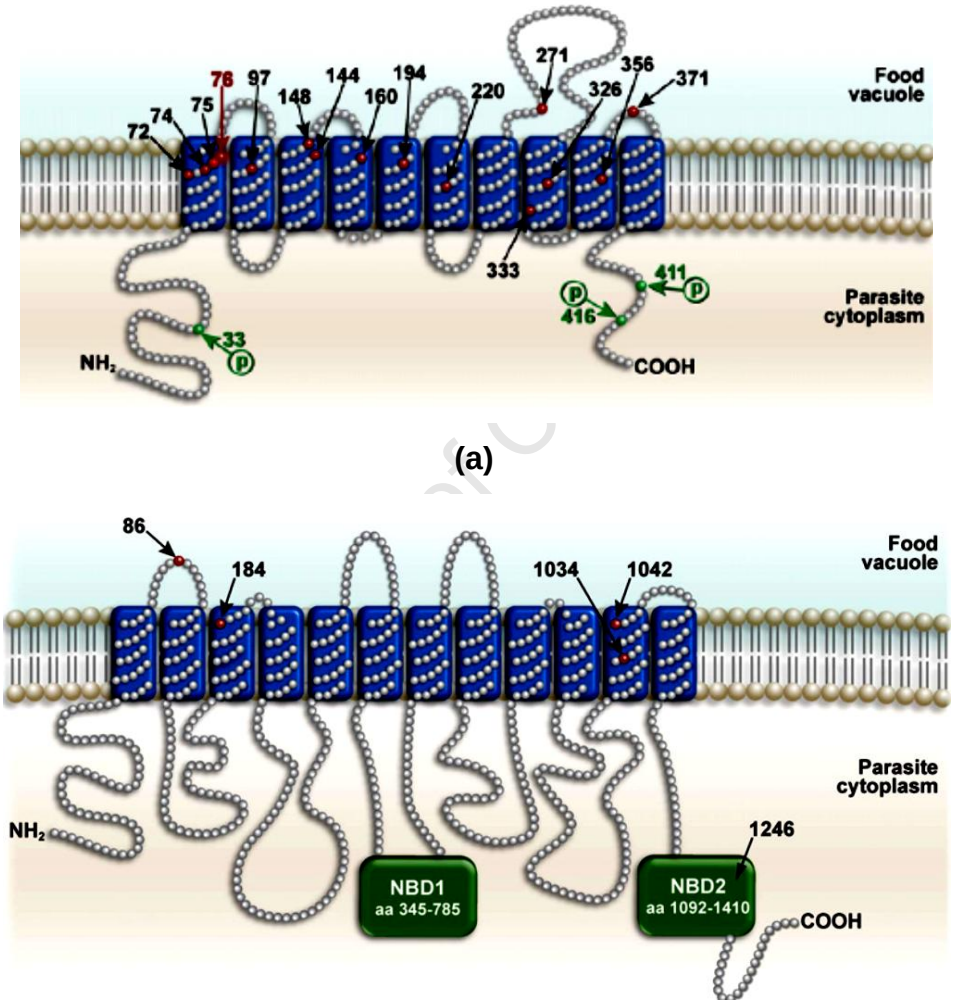
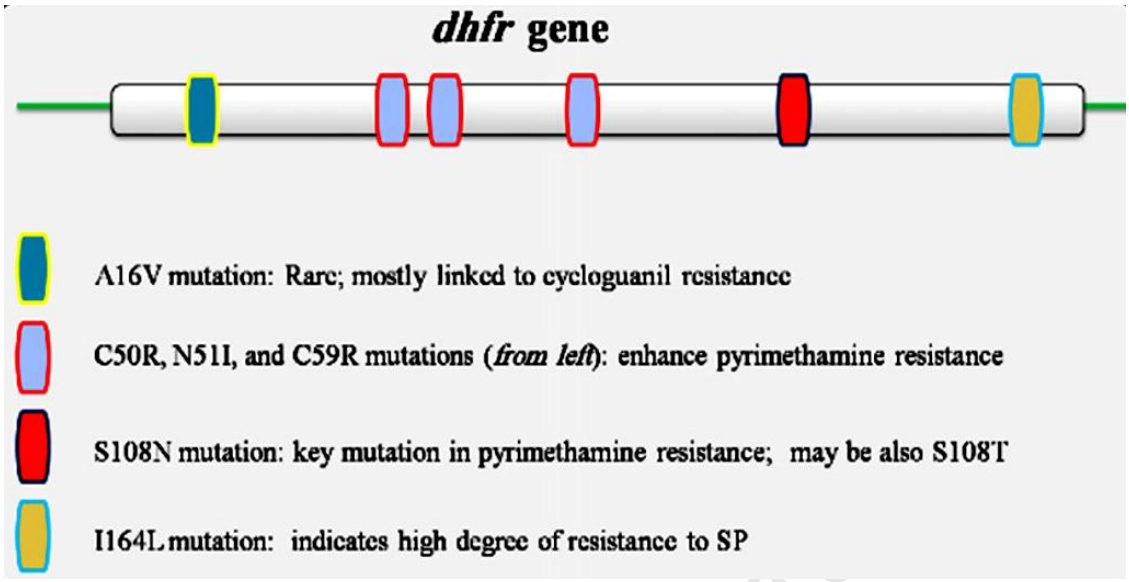


Figure 1.9: A model of *P. falciparum* chloroquine resistance transporter (*Pfcrt*) with lysine to threonine substitution at position 76 indicated by a red arrow and P *falciparum* multi-drug resistance transporter 1 (*PfMDR1*) indicated by a black arrows found in chloroquine-resistant parasites (Sanchez *et al.*, 2010).

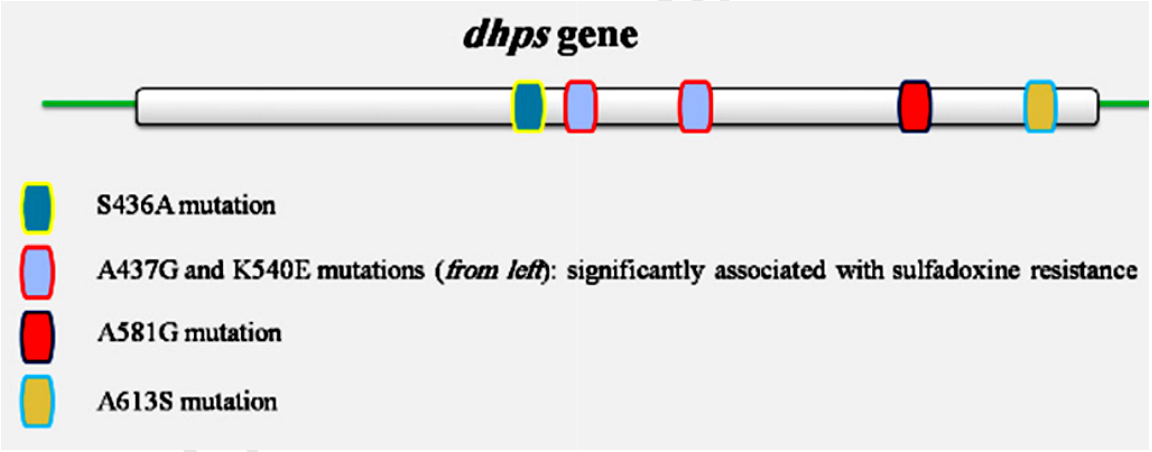
Antifolates

Antifolate resistance was first reported in the 1950s when pyrimethamine and proguanil were introduced as single antimalarial treatments. Studies reported that resistance to antifolate drugs in *P. falciparum* is caused by point mutations in the genes encoding for dihydrofolate reductase (*pfdhfr*) and dihydropteroate synthase (*pfdhps*), reducing the binding affinity of the enzyme for the drug (Durand *et al.*, 2000; Sibley *et al.*, 2001). Resistant mutants of DHFR appear to have arisen in Southeast Asia and then spread to Africa. Studies show that a small number of amino acid replacements in the parasite *pfdhfr* are the genetic basis of pyrimethamine resistance in *P. falciparum*. Resistant mutants in *pfdhfr* are associated with a combination of 4 amino acid changes, in particular, Asn-51 to Ile (N51I), Cys-59 to Arg (C59R), Ser-108 to Asn (S108N), and Ile-164 to Leu (I164L) (Figure 1.10). These 4 replacements affect the enzyme-binding pocket and reduce the binding affinity for pyrimethamine (Lozovsky *et al.*, 2009). Parasites with double mutations at Ala-16 to Val and Ser-108 to Thr in the *pfdhfr* gene are resistant to cycloguanil but susceptible to pyrimethamine. The two drugs are very closely similar; therefore, parasites with cross-resistance to both drugs have either single or multiple mutations at amino acid residues 51, 59, 108 and 164 (Rastelli *et al.*, 2000).

P. falciparum dihydropteroate synthase (*pfdhps*) has five point mutations that cause resistance to dihydropteroate synthase inhibitors. Ala-437 to Gly (A437G) and Lys-540 to Glu (K540E) are the main cause for resistance evolution against type 1 antifolates. Resistance is enhanced with the addition of the following mutations: Ser-436 to Ala or Phe (S436A/F), Ala-581 to Gly (A581G) and Ala-613 to Ser or Thr (A613S/T) (Figure 1.10). These mutations are associated with *P. falciparum resistance to* sulfonamides *sulfadoxine and* sulfone diapsone (Abdul Ghani *et al.*, 2012; Nzila, 2006).



(a)



(b)

Figure 1.10: Genetic mutations associated with antifolate resistance. (a) Pyrimenthamine resistance *dhfr* mutations and (b) sulfadoxine resistance *dhps* mutations. (Abdul Ghani *et al.*, 2012).

Artemisinins

Studies show that artemisinin resistance is brought about by mutational changes in the artemisinin binding site of *PfATP6* on *P*. *falciparum.* A mutation at Leu-263 to Glu (L263E) results in reduced susceptibility of the parasites to artemisinins (Tanabe *et al.*, 2011). Another gene that has been associated with artemisinin resistance is *P*. *falciparum* multidrug resistance protein 1 (*pfmdr1)* with three mutations, S1034C, N1042D, and D1246Y, which are similar to mutations responsible for quinoline resistance, also contribute to increased sensitivity to artemisinin (Chavchich *et al.*, 2010).

Atovaquone

Resistance to atovaquone arose because of mutations in the parasite mitochondrial genome cytochrome *bc1* (*PfCYTbc*) at position Tyr-268 to Ser (Y268S) (Fisher *et al.*, 2012; Krishna *et al.*, 2004). Studies show that amino acid mutations associated with atovaquone resistance occur around atovaquone-binding site. Mutations in these positions are consistent with a reduced binding affinity of atovaquone for malaria parasite *PfCYTbc* (Korsinczky *et al.*, 2000).

1.4.4. Combination therapy

The incidence of antimalarial drug resistance can be reduced by the use of combination therapy. It has been found that if a drug, for which the parasite has developed resistance and is withdrawn from use for some time; if it is combined with another effective drug the sensitivity to that drug may return, delaying the development of resistance (Bloland, 2001; Mehlotra *et al.*, 2006). This is due to the fact that drug resistance is caused by mutations in genes which control the structure of the biochemical target in the parasite and this causes the synergistic effect of the combined drugs with a different mechanisms of action and different resistance mechanisms to be effective even in the presence of resistance to the individual components (Bloland, 2001; Wilairatana *et al.*, 2002).

The combinations of drugs currently prescribed can be divided into 2 categories: non-artemesinin and quinine based combinations and artemesinin based combinations (Kremsner *et al.*, 2004).

1.4.4.1. Non-Artemesinin based combinations

Sulfadoxine-Pyrimethamine

Sulfadoxine/pyrimethamine, also known as Fansidar, is a combination of antifolates (dihydrofolate reductase (*DHFR*) and dihydropteroate synthetase (*DHPS*) inhibitors) and was used in many African countries as treatment for *P. falciparum* between the 1960s and the 1980s since the development and spread of chloroquine resistance (Kremsner *et al.*, 2004; Wang *et al.*, 1997). The advantages of sulfadoxine/pyimethamine include low cost, simple dosing, and a few side effects effects. However, the emergence of resistance to sulfadoxine-pyrimethamine, which results from mutations in the DHFR and to a lesser degree in DHPS, presented reduction in drug susceptibility (Staedke *et al*., 2001). The quadruple DHFR mutant genotype containing I164L confers almost complete sulfadoxine/pyrimethamine resistance DHPS gene of the parasite as described previously and this was replaced with chloroquine, amodiaquine or mefloquine combined with sulfadoxine-pyrimethamine (Peters *et al*., 2007; ter Kuile *et al*., 2007).

- **Sulfadoxine-Pyrimethamine *plus* Chloroquine or Amodiaquine:** Combination therapy of amodiaquine or chloroquine plus sulfadoxine-pyrimethamine was introduced as first-line treatment against uncomplicated malaria in 2000 (Marfurt *et al*., 2007). Studies show that administering chloroquine or amodiaquine combined with sulfadoxine-pyrimethamine, presented better clearance of parasites from the blood compared to chloroquine or amodiaquine, or sulfadoxine-pyrimethamine alone (McIntosh and Greenwood, 1998). Amodiaquine is a 4-aminoquinoline like chloroquine but remains more efficacious than chloroquine, in many areas with chloroquine resistance (Dorsey *et al.*, 2002). When combined with sulfadoxine-pyrimethamine, it is more potent than the chloroquine combination but studies show that it causes agranulocytosis and liver damage (Kremsner *et al.*, 2004).

- **Sulfadoxine-Pyrimethamine *plus* quinine:** Studies show that when sulfadoxine-pyrimethamine is combined with quinine its efficacy increases and it also shortens the long treatment duration of seven days of quinine to three days, thus enhancing compliance and reducing side effects (Matsiegui *et al.*, 2006).
- **Sulfadoxine-Pyrimethamine *plus* Mefloquine:** Treatment with the combination of sulfadoxine-pyrimethamine and mefloquine (also known as Fansimef) has been successful in many parts of the world. Side-effects such as mild dizziness, nausea, and vomiting have been reported with use of this combination and are no longer recommended for use due to emergence of resistance (De Souza, 1985).

1.4.4.2. Artemisinin-based combinations (ACT)

The artemisinins are the most potent antimalarials available and the WHO specifically recommends the use of artemisinin-based combination therapy (ACT) as the standard policy in the treatment of uncomplicated falciparum malaria (Obonyo *et al*., 2007). Artemisinin and its derivatives have a very short half-life, which means the patient must take the drug for at least seven days and therefore, the partner compound needs to be a relatively longer acting drug with a different mode of action. This is to ensure that it is able to persist at a parasiticidal concentration until all the infecting parasites have been killed (Grueninger and Hamed, 2012; Sinclair *et al.*, 2009). This combination also reduces the seven day treatment of artemisinin to three days when given in combination with slowly eliminated antimalarials and this provides complete protection for the artemisinin derivative from resistance. However, this leaves the slowly eliminated compound partially protected by the artemisinin derivative (Reyburn, 2010; White, 2004).There are five ACTs that are recommended for the first-line treatment of uncomplicated falciparum malaria in adults and children:

- **Artemether–lumefantrine:** Artemether-lumefantrine, also known as Coartem®, Riamet® and Lonart®, is the first fixed-dose artemisinin-based combination therapy recommended and pre-qualified by WHO for the treatment of uncomplicated malaria caused by *P. falciparum*. It has been shown to be effective both in sub-Saharan Africa and in areas with multi-drug resistant *P. falciparum* in Southeast Asia (Falade *et al.*, 2004; Olliaro, 2004). Artemether is rapidly eliminated and reinfection occurs frequently

when given as monotherapy for less than seven days, but when combined with lumefantrine, which has a much longer half-life, there is a low recrudescence rate (Falade *et al.*, 2004).

- **Artesunate–amodiaquine**: A fixed-dose combination of artesunate-amodiaquine, also known as Coarsucam®, was launched in February 2007 and in some African countries it is considered as first-line treatment for children with uncomplicated malaria. It is highly effective against multidrug resistant *P. falciparum* malaria and is well tolerated (Kremsner *et al.*, 2004).
- **Artesunate–mefloquine:** Artesunate-mefloquine combination therapy, also known as Artequin®, was highly effective in reducing the incident of malaria in the Northwestern border of Thailand where mefloquine resistance had developed (White, 2004). This combination also reduces the occurrence of nausea, dysphoria, and dizziness, which are induced when mefloquine is used as monotherapy (Breman *et al.*, 2007).
- **Artesunate plus sulfadoxine–pyrimethamine:** Artesunate and sulfadoxine-pyrimethamine combination, also known as Ariplus®, is used in areas where sulfadoxine-pyrimethamine susceptibility is high such as South America, the Middle East, and South Asia (Breman *et al.*, 2007).
- **Dihydroartemisinin–piperaquine:** Dihydroartemisinin – piperaquine combination, also known as Artekin®, was developed in China and registered in China and Cambodia. It has proved tolerable and efficacious in treating multi-drug resistant strains in China, Cambodia, Vietnam and Thailand (Mutabingwa, 2005).

1.4.4.3. Other combinations

- **Antibiotics *plus* Quinine:** Combinations of quinine with antibiotics such as tetracycline, doxycyline, clindamycin and azithromycin significantly improved the treatment efficacy compared to quinine alone in the treatment of drug-resistant malaria. In combination with clindamycin, the treatment course for quinine can be shortened to three days, which may improve adherence and, unlike with doxycycline or tetracycline combinations, the quinine and clindamycin combination can be administered to both children and pregnant women (Kremsner *et al.*, 2004; Obonyo and Juma, 2012).

- **Atovaquone plus proguanil:** Previous studies proved that when atovaquone is combined with proguanil, a dihydrofolate antagonist used for antimalarial treatment and prophylaxis, act synergistically against the parasites and lead to high cure rates in patients with malaria (Lell *et al.*, 1998). This combination shows good safety and tolerability in children and adults, with high efficacy against *P falciparum* malaria when used in a three day regimen (Kremsner *et al.*, 2004).

1.4.5. New antimalarial drugs under development

There are a number of antimalarial compounds in different stages of preclinical and clinical development. These include combinations of known drugs or new substances from drug classes already used in antimalarial therapy, some of which are described in the previous section, and compounds with novel or even unknown mechanism of action.

1.4.5.1. Recently approved antimalarials

Two major artemisinin combination therapies (ACTs) include **Artemether-lumefantrine** (Coartem® and Coartem® Dispersible from Novartis) and artesunate-**amodiaquine** (Coarsucam™/Artesunate Amodiaquine-Winthrop® from Sanofi) (Anthony *et al*., 2012). Other types of ACT that are recently approved for the treatment of uncomplicated malaria are:

- **Dihydroartemisinin-piperaquine** (Eurartesim® from Sigma-tau) was approved by the European Medicines Agency (EMA) in October 2011. The two drugs are pharmacokinetically mismatched due to vastly different half-lives of 1 h and 20 days (Anthony *et al*., 2012; Schrader *et al*., 2012).
- **Pyronaridine-artesunate** (Pyramax® from Shin Poong Pharmaceuticals) was approved by the Korean Food & Drugs Administration (KFDA) in August 2011 and was approved by the EMA in February 2012 (Anthony *et al*., 2012).
- **Artesunate-mefloquine** (ASMQ) is a fixed-dose combination produced by Cephalon/Mepha as a paediatric formulation in Africa, and by the Drugs for Neglected Diseases initiative (DND*i*) in partnership with Farmanguinhos for use in Brazil. WHO prequalification was obtained in September 2012 (Anthony *et al*., 2012).

1.4.5.2. Antimalarials in clinical development

OZ439 was developed in a Medicines for Malaria Venture (MMV) project in collaboration with the University of Nebraska, the Swiss Tropical and Public Health Institute, and Monash University. It is a peroxide based antimalarial with superior pharmacokinetics to the artemisinins and clinical efficacy as a single agent in phase IIa studies, which have now been completed. The next stage is to investigate drug interactions in volunteers with potential partner drugs (Anthony *et al*., 2012; Burrows *et al*., 2013).

NITD609 is a spiroindolone developed by the Novartis Institute for Tropical Diseases in Singapore as part of collaboration with the Swiss Tropical and Public Health Institute and the Dutch Biomedical Primate Research Center. It started human proof of concept in 2012; just under 5 years after the initial screen began. NITD609 inhibits the P-type sodium transporter ATPase 4 (*PfATP4*), causing an increase in the concentration of sodium ions in the parasite, which is toxic to the cell. It has now passed proof of concept, and has been shown to clear parasites in patients infected with either *P. vivax* or *P. falciparum* (Anthony *et al*., 2012; Burrows *et al*., 2013).

GNF156 is an imidazolopiperazine identified by the GNF (Global Nature Fund) compound library collection conducted by Genome Foundation of Novartis in San Diego as part of the same collaborative network as NITD609. GNF156 acts *via* a novel mechanism, involving a gene called *P. falciparum* Cyclic Amine Resistance Locus (*Pfcarl*) and is currently in Phase IIa clinical trials (Anthony *et al*., 2012; Burrows *et al*., 2013; Schrader *et al*., 2012).

1.4.5.3. Antimalarials in pre-clinical development

DSM265 is a triazolopyrimidine that selectively inhibits the dehydroorotate dehydrogenase (DHODH) of *P. falciparum, an enzyme that* catalyzes the oxidation of dihydroorotate to orotic acid, the fourth step in the de novo pyrimidine biosynthesis. This compound was identified by University of Texas Southwestern, in collaboration with the University of Washington, Monash University and MMV (Anthony *et al*., 2012; Burrows *et al*., 2013; Schrader *et al*., 2012).

P218 is a DHFR inhibitor developed by BIOTEC, a research center created by the Thai government with in collaboration with Monash University, the London School of Hygiene and Tropical Medicine and MMV. This compound combines the pyrimidine ring of pyrimethamine (Figure 1.7f and i) which brings potency, and the linker of the DHFR inhibitor WR99210 which tolerates mutations due to its flexibility. P218 finished preclinical development (Anthony *et al*., 2012; Biamonte *et al*., 2013; Burrows *et al*., 2013; Schrader *et al*., 2012).

MMV390048 is an aminopyridine compound which has the potential to become a part of a single-dose cure for malaria. This compound was developed through collaboration between a chemistry team at the University of Cape Town, biology assay teams at the Swiss Tropical and Public Health Institute, and pharmacokinetics at the Center for Drug Candidate Optimization at Monash University in Australia. MMV390048 was initially identified by Griffith University scientists in Australia as part of MMV's extensive malaria screening campaign of around 6 million compounds and is due to enter human trials next year (Leonard and Leonard, 2012; MMV, 2012; Nordling, 2013).

1.5. Drug Discovery

The development and spread of resistance has prompted the search for the identification of effective alternative novel antimalarial drugs with minimal side effects for the treatment of the malaria disease. In drug discovery, the development of lead drug candidates from combinatorial synthesis and high throughput screening for drug sensitivity has enabled synthesis and purification of hundreds of pharmacologically active antimalarial compounds. These methods also provide data on biological parameters such as potency, absorption, distribution, metabolism, excretion, and toxicity, thereby facilitating a rapid identification and selection of lead drug candidates (Kalyan Kumar *et al.*, 2010; van Breemen and Li, 2005). Studies show that only a small fraction of drug candidates that were selected for clinical development eventually became marketed drugs and this may be due to factors such as lack of sensitivity towards the parasites *in vivo*, toxicity or poor pharmacokinetics (Kaplita *et al.*, 2005).

1.5.1. Antimalarial drug sensitivity screening

In the discovery of antimalarial drugs high-throughput screening is employed in order to identify new chemical entities that might lead to drug candidates at a much higher rate. *In vitro* assays have been developed to determine the sensitivity of malaria parasites to both existing and new antimalarials (Smilkstein *et al.*, 2004; Kaplita *et al.*, 2005; Baniecki *et al.*, 2007; World Health Organization, 2011; World Health Organization, 2012b);. These assays are based on measuring the efficacy of antimalarials against parasite growth and development in culture (Noedl *et al.*, 2003b). Not all these assays are suitable for high-throughput screening because of factors such as cost, safety, assay stability and robust performance, speed, equipment availability, and quality of data produced (Duffy and Avery, 2012; Smilkstein *et al.,* 2004). Examples of these *in vitro* assays that are used to measure the inhibition of parasite multiplication include:

- **Microscopic examination of stained blood smears:** Microscopic examination of Giemsa-stained thick and thin blood film is essential in the diagnosis of malaria and in quantifying parasitized erythrocytes. This method has been used for over a century in malaria diagnosis because it provided all the parameters needed to guide treatment, such as identifying the presence of parasites, the infecting species, and the level of parasitemia. Although this method is sensitive and inexpensive, reading of test results by visual counting of parasites remains laborious and can be subject to error because of ocular fatigue, and therefore, making it unsuitable for high-throughput use (Erdman and Kain, 2007; Planche *et al.*, 2001)).
- **Flow cytometry:** Flow cytometry makes use of DNA dyes which cause parasitized red blood cells to fluoresce, thus differentiating parasitized red blood cells from uninfected red blood cells because of the fact that normal red blood cells lack DNA. In drug-susceptibility measurements, parasitemia in blood samples is measured by counting and comparing the ratio of red blood cells which stain positive for DNA to the total number of red blood cells analyzed. The advantage of using this method is that it can use an asynchronous malaria parasite culture, thus simplifying the assay and allowing the screenings to be performed fast (Grimberg *et al.*, 2008; Grimberg *et al.*, 2009; Grimberg, 2011)

- **Fluorescent-based assays:** Fluorescent-based assays use nucleic acid intercalating dyes such as SYBR green I, PicoGreen and YOYO-1. These dyes bind malaria DNA in any erythrocytic stage of *P. falciparum* growth; therefore, they are used to measure malaria growth inhibition in drug-susceptibility assays (Johnson *et al.*, 2007). This method has an advantage of being simple and a one-step procedure (Rason *et al.*, 2008).
- **Isotopic assays:** In this method antimalarial activity of test compounds is measured based on the inhibition of uptake radiolabeled hypoxanthine, a nucleic acid precursor, by the parasites (Desjardins *et al.*, 1979). This method is accurate and reliable, but requires radioactive materials that pose safety and disposal problems. It is also relies on expensive radioisotopes and involve multiple processing steps that are technically demanding (Baniecki *et al.*, 2007).
- **Histidine-rich protein II (HRP2) assay:** HRP2 is a water-soluble antigen localized in several cell compartments and is also expressed by erythrocytes infected by *P. falciparum* and immature gametocytes (Mayxay *et al.*, 2001; Noedl *et al.*, 2002; Van den Ende *et al.*, 1998). The HRP2 assay is based on the measurement of HRP2 in the course of the parasite growth and multiplication using an enzyme-linked immunosorbent assay (ELISA) test kit. It is more sensitive than isotopic assay, with little technical equipment required and because it is ELISA based, it is very easy and faster, even when testing a large number of samples. However, it requires 72 hours of culture time, which is longer than other assays (Noedl *et al.*, 2003).
- **Parasite lactate dehydrogenase assay:** Lactate dehydrogenase (LDH) is the last enzyme in the glycolytic pathway and is used as an indicator for the presence of malaria. Drug-susceptibility assays measure the synthesis of pLDH by growing parasites and the enzyme levels correspond to the parasite density after exposure to drugs. This method can detect multiple species of malaria but requires an initial parasitemia of greater than 1% in order to receive interpretable results, affecting its sensitivity (Noedl *et al.*, 2003; Druilhe *et al.*, 2007; Kaddouri *et al*., 2006) .

Potent antimalarial drug candidates identified through these drug sensitivity assays may exhibit phenomenal efficacy *in vitro* but that is only a small part of what ultimately makes

a lead compound into a viable drug. A drug candidate must also have appropriate physical and chemical properties to be readily absorbed, to pass through multiple cell or tissue layers, to get to the right organs, to perform its activity safely, to be deactivated and ultimately to be eliminated. In other words, poor drug properties (ADME/Tox results) will cause the termination of its development (Wishart, 2007).

1.5.2. ADME/Tox Drug Properties

Drug properties such as absorption, distribution, metabolism, elimination and toxicity (ADME/Tox) are major parts of pharmacokinetics and play a crucial role in the final clinical success of a drug candidate (Li, 2001). *In vitro* ADME–Tox characterization is used to provide a preliminary prediction of the *in vivo* behavior of a compound to assess its potential to become a drug (Yu and Adeyoni, 2003). ADME/Tox data, whether predicted or experimentally measured, can provide key insights into whether a drug lead has the right properties to ultimately be a marketable drug (Wishart, 2007). Determining this data and identifying candidates with undesirable ADME/Tox properties in early stages of drug discovery will save both time and expense as the cost of drug development is much greater than the cost of drug discovery (Liu *et al.*, 2001; Palm *et al.*, 1997).

There are a variety of experimental assays that have been developed, which characterize each aspect of the drug discovery processes. These include physicochemical methods and biological assays using subcellular fractions, primary cell culture, cell lines, tissues and whole organs (Yu and Adeyoni, 2003).

Absorption

In drug discovery, oral administration of therapeutics is the route of choice for drug delivery because oral drug absorption is a crucial criterion in screening of new drug candidates as it may restrict oral bioavailability of many potential drug candidates (Delie & Rubas, 1997; Shah, 2006). Factors that affect drug absorption can be physicochemical and biological.

- **The physicochemical factors:** These include dosage form, size and shape, rates of disintegration, disaggregation and dissolution, and rates of release from polymeric dosage forms. Drug factors include solubility, chemical and enzymatic stability, lipophilicity, hydrogen-bonging potential, conformation, pK_a, molecule size and shape and affinity for transporters (Meunier *et al.*, 1995).
- **Biological factors:** These include stomach emptying, intestinal motility, the pH and enzymes of the intestinal lumen, the intestinal mucosa barrier, and liver function (Meunier, 1995). Permeability studies make use of cell culture based models by studying the mechanism of drug absorption, to screen intestinal permeability and to study passive and active diffusion of drug molecules (Kaylan Kumar *et al.*, 2010). In these studies, several human colon carcinoma cell lines, namely; Caco-2, T-84 and HT-29, have been widely used because of their ability to express morphological and biochemical features of mature differentiated enterocytes and goblet cells (Meunier *et al.*, 1995; Wilson *et al.*, 1990).

Distribution

Once the drugs have been absorbed into the circulation, they need to be distributed to individual tissues to exert their clinical effects and this depends on blood flow to the tissues, and solubility and uptake into those tissues (Chillistone and Hardman, 2008). The drugs pass through four body fluid compartments, namely; plasma, interstitial fluid, transcellular fluid and transcellular fluid compartments and their distribution depends on their physicochemical properties such as ionization, lipid solubility, and molecular size and shape (Satoskar and Bhandarkar, 1969).

In pharmacokinetics, the distribution is described by the parameter V_D, the apparent volume of distribution, which measures the relative distribution of drugs between tissue and plasma and depends on the plasma and tissue binding and the lipophilicity of the drug. Drugs that are mostly confined to the plasma due to high plasma binding have a small volume of distribution, while non-ionized lipid soluble drugs which readily cross epithelial barriers distribute rapidly and bind to tissues will have a large volume of distribution (Chillistone and Hardman, 2008; Oie, 1986).

Metabolism

Absorbed drugs are transported via the portal circulation to the liver, where they are subjected to hepatic metabolism followed by elimination as bile or via the kidneys. The purpose of drug metabolism is to transform lipophilic drugs, which are desirable for absorption, to more water-soluble molecules so they can be more easily excreted from the body (Li, 2001; Tingle and Helsby, 2005). Drug metabolizing enzymes can be classified into two major groups: Phase I drug metabolizing enzymes, cytochrome p450 enzymes, are responsible for either introduction of a polar functional group or the unmasking of a polar functionality and Phase II drug metabolizing enzymes are generally transferases (uridine diphosphoglucuronosyl transferase, glutathione S-transferase, N-acetyl transferase, and sulfotransferase) and are responsible for conjugating a xenobiotic or its metabolite (Asha and Vindyavathi, 2009; Iyer, 1999 and Sinz).

Several animal and human *in vitro* liver models have been developed such as isolated enzymes, liver microsomes, isolated hepatocytes, and organ slices. Data obtained from these models are used for the interpretation of structure-activity relationships and prediction of pharmacokinetic properties in humans. Human liver microsomes account for the most popular *in vitro* model because of the presence of cytochrome *P*450 monooxygenases in the liver microsomes (Asha and Vindyavathi, 2009; Gunaratna & Kissinger, 1997).

Elimination

Drug elimination is the irreversible removal of drugs from the body. The kidney serves as the major organ responsible for the removal of most drugs, especially hydrophilic drugs. Other organs of drug elimination are the liver, which secretes about a liter of bile, and the lungs, where drugs are exhaled. Occasionally, other routes may include saliva, sweat, tears, hair and breast milk (Alavijeh *et al.*, 2005; Wilkinson *et al.*, 2008).

The rate at which drugs are removed from the body is calculated as drug elimination rate constant, *Ke* and it depends upon its plasma concentration. Another important

parameter in pharmacokinetics is clearance, which is a measure of the removal of drug from the body per unit time (Wilkinson *et al.*, 2008).

Toxicity

It has been estimated that over 25% of drug candidates would fail in clinical trials due to unexpected adverse drug properties. A desirable drug has a high therapeutic index, which means, the plasma level required to exert a toxic effect would be significantly higher than that required for therapeutic efficacy (Li, 2001).

A number of toxicity predictive approaches have been implemented in many drug discovery and development laboratories such as cytotoxicity assays for the evaluation of toxic mechanisms. These assays are performed using tumor cell lines, which contain little drug-metabolizing capacity and yield little information about the metabolic activation of the parent compound as a result of the formation of toxic metabolites or deactivation of parent toxicity by metabolic detoxification (Li, 2001). Therefore, intact human hepatocytes have been used for evaluation of metabolic fate of toxic and non-toxic drugs (Li, 2007).

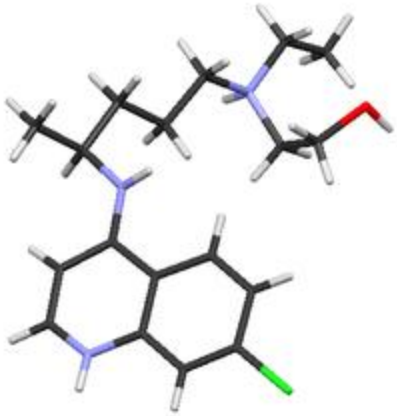
1.6. Aims and Objectives

The search for potential antimalarial compounds have motivated chemists from the medicinal chemistry programme of the University of Cape Town Department of Organic Chemistry to synthesize new chemical entities that are efficacious against drug-resistant strains and provide cure within a reasonable time with minimum side effects. Therefore, this study was developed as part of a collaborative drug discovery project with the medicinal chemistry group with the aim of:

- Performing *in vitro* antiplasmodial testing of new chemical entities by using a dose-response test against chloroquine sensitive and resistant *Plasmodium falciparum* strains by employing a high-throughput screening in order to identify potential animalarial compounds. Using a high-throughput screening in dose response tests in this study allows assaying of large number of potential compounds at a higher rate.

- Determining the selectivity of active compounds by performing *in vitro* cytotoxic assays using cancer cell lines. Selectivity testing evaluates the ability of the compound to target a particular cell population and this property is desirable because and ideal antimalarial must be able to kill the malarial parasites but not to affect host cells (Mencher and Wang, 2005).
- Investigating the pharmacokinetic properties of the each active compound in a mouse model. Section 1.5.2. described drug properties which can affect pharmacokinetics of a particular compound which ultimately affects its absorption, distribution, metabolism, and excretion in an animal model. These properties can be estimated *in vitro* in order to determine the 'drug-likeness' of a compound according to Lipinski's 'rule of five' but determining these properties *in vivo* is better suited because it offers conclusive insights about the nature of the compound and it observes the overall effects of an experiment on a living subject.
- Performing efficacy studies on selected active compounds using a mouse model. Determining the efficacy of the compounds *in vivo* can also show that the therapeutic effect exerted by the compounds correlates with the pharmacokinetic properties.
- Performing permeability studies in order to study their drug absorption properties using colon carcinoma cell lines. Estimation of drug absorption in permeability studies can give insights on the pharmacokinetic properties of a compound because studies have shown that poor drug absorption can result in poor pharmacokinetic properties (van De Waterbeemed *et al*., 2001).

Chapter 2: Selection of New Chemical Entities for Antimalarial Testing



Selection of New Chemical Entities for Antimalarial Testing

2.1. Introduction

The emergence and spread of drug-resistant malaria parasites is the major threat to effective malaria control. Therefore, this has stimulated interest in new chemical entities (NCEs) as potential antimalarials.

Two of the many strategies to discover NCEs is molecular hybridization in which two drugs are covalently fused together by combining their pharmacophoric moieties in order to produce a new hybrid compound with potentially improved efficacy when compared to the parent drugs (Andayi *et al*., 2013; Viegas-Júnior *et al*., 2007) and phenotypic whole cell high throughput screening (Guiguemde *et al*., 2012; Paquet *et al*., 2012). Antiplasmodial *in vitro* screening of these hybrid and phenotypic HTS compounds led to the identification of potential antimalarial lead candidates. Among these active hybrid compounds, two structural classes were selected as part of this collaborative study in order to determine their *in vivo* efficacy and pharmacokinetic properties. These are azidothymidine-chloroquine (AZT-CQ) and hydroxypyridone-chloroquine (HPO-CQ) hybrids. Another class of orally active antimalarial compounds, namely 2-aminopyridines, was also included for pharmacokinetic evaluation.

2.1.1. Azidothymidine-chloroquine (AZT-CQ) hybrids

AZT-CQ hybrids are molecules generated by covalent linkage of chloroquine-based 4-aminoquinoline diamines and azidothymidine (AZT) (Aminake *et al*., 2012). These hybrid molecules were designed in order to target the human immunodeficiency virus (HIV) and *P. falciparum* simultaneously. Malaria and HIV/AIDS are the causative agents of the most devastating diseases that result in most deaths in the Sub-Saharan Africa and in many areas both diseases are endemic (Aminake *et al*., 2012). Malaria patients that are infected with HIV have a higher viral load than non-malaria patients because

infection with *P. falciparum* stimulate HIV-1 replication through the production of interleukin 6 and tumour necrosis factor α, thus causing a faster progression of HIV-1 disease (Whitworth *et al*., 2000). Previous studies by Boelaert, Sperber & Piette, 2001 reported an improved anti-HIV activity when AZT is combined to chloroquine *in vitro.* These findings inspired the design and synthesis of the AZT-CQ hybrid molecules, namely VAK 31DS, VAK 37DS, VAK 59DS, AKAZTQ and AKAZTBQ and their chemical structures are presented in Figure 2.11.

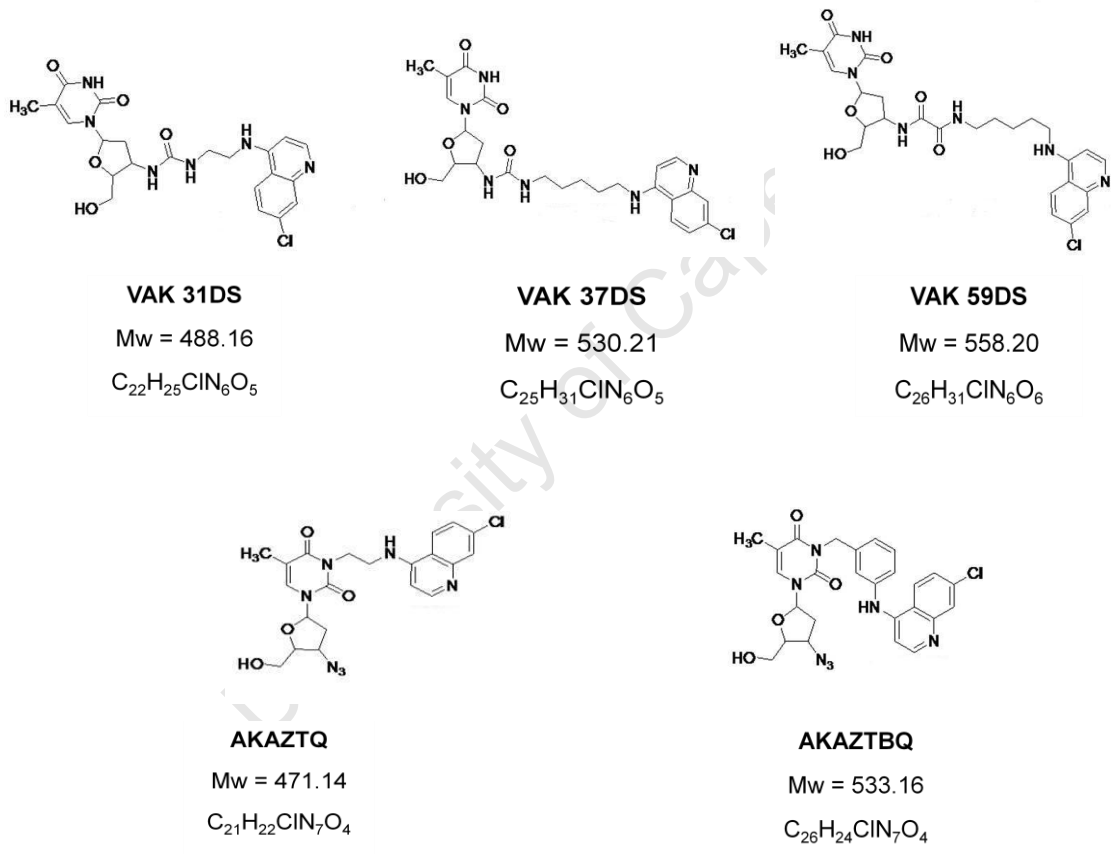


Figure 2.11: <u>The structures of synthesized AZT-CQ hybrids.</u>

2.1.1. Hydroxypyridone-chloroquine (HPO-CQ) hybrids

Hydroxypyridones are iron-chelating agents known to suppress malaria growth *in vivo* and *in vitro* (Andayi *et al*., 2013*;* Hershko *et al*., 1991). Iron chelators have been studied as alternative malaria drugs for many years because of their ability to interact with available iron in the nucleus and parasite cytosol, thereby interfering with the iron-dependent metabolism of malaria parasites and inhibit their development (Ferrer *et al*., 2012; Lytton *et al*., 1994). Both hydroxypyridones and chloroquine target the erythrocytic stage of malaria life cycle, which is highly dependent on iron; therefore, for the purpose this study, *N*-alkyl-3-hydroxypyridin-4-ones were combined with chloroquine in order to achieve a synergistic antiplasmodial effect against chloroquine resistant strains of *P. falciparum* by enhancing hemozoin inhibition when compared to inhibition by chloroquine alone (Andayi *et al*., 2013).

Two compounds, namely D13 (from kojic acid derived double drugs series) and 13AB.2HCl (from ethylmaltol derived double drug series) were selected among a series HPO-CQ hybrid compounds in order to assess them for *in vivo* efficacy and pharmacokinetic properties (Figure 2.12).

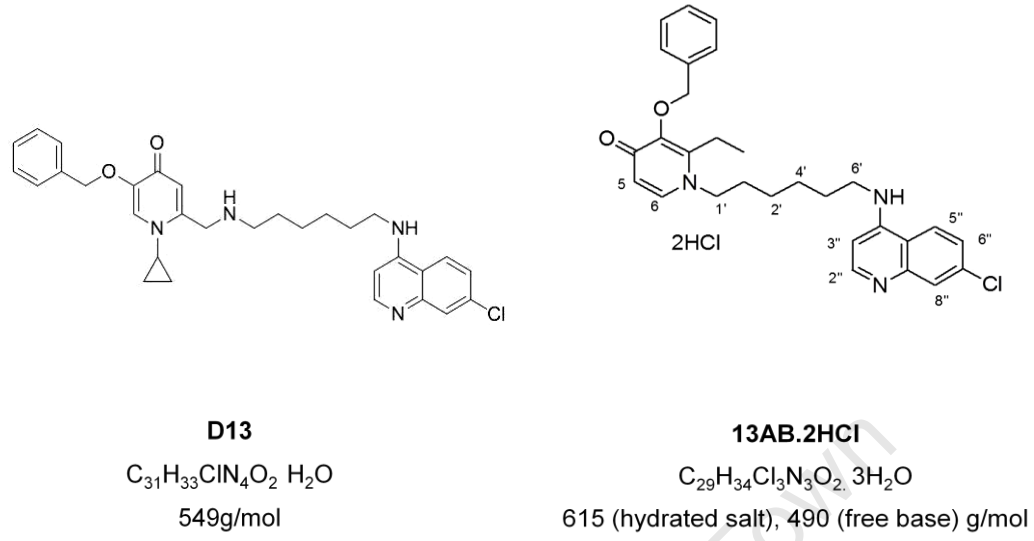
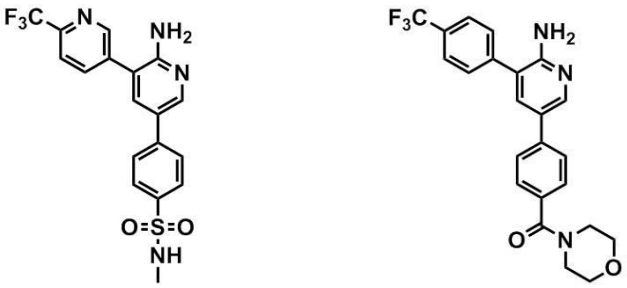


Figure 2.12: The structures of synthesized HPO-CQ hybrids.

2.1.3. 2-Aminopyridines

3,5-Diaryl-2-aminopyridines were identified from an imaged based HTS of a BioFocus DPI SoftFocus kinase library as promising selective *in vitro* antiplasmodial hits. A total of 36608 compounds were screened for antiplasmodial activity of which 442 demonstrated high antiplasmodial activity against chloroquine sensitive and resistant strains of *P. falciparum*. A number of active compounds with varying aryl groups at positions 3 and 5 of the aminopyridine core were identified. Compounds MMV394903 and MMV652120 (Figure 2.13) were among the active analogues synthesized in order to explore the structure – activity relationship (SAR) around aryl substitution (Younis *et al*., 2012).



MMV652120

$C_{18}H_{15}F_3N_4O_2S$

408.4g/mol

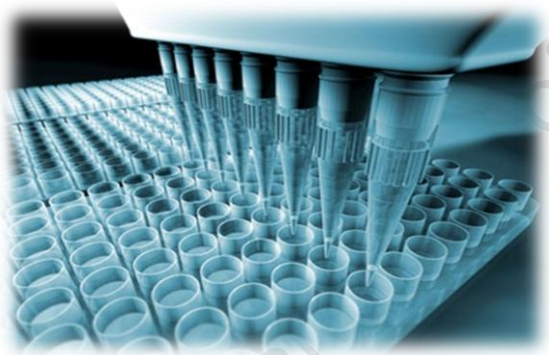
MMV394903

$C_{23}H_{20}F_3N_3O_2$

427.43g/mol

Figure 2.13: <u>The structures of synthesized 3,5-Diaryl-2-aminopyridines.</u>

Chapter 3: *In vitro* Antiplasmodial Activity and Cytotoxicity of Antimalarial Compounds



In vitro Antiplasmodial Activity and Cytotoxicity of Antimalarial Compounds

3.1. Introduction

In vitro assays provide valuable insights into the mechanism of action of drugs by providing data on catalytic action of an isolated enzyme or binding of an antibody to a defined antigen in the presence of the drugs. They have been used for many years to assess newly synthesized compounds that specifically interfere with the parasite biochemistry. They also provide information on the toxicity of the antimalarial compounds by monitoring the growth of an engineered cell line when exposed to the compound (Eckstein, 2010; Shelby *et al*., 1996). Section 1.5.1 discusses various examples of *in vitro* assays that are used to measure the inhibition of parasite multiplication with advantages that result in an economical and rapid assay for primary drug screening. The principles are the same for all these assays but the technique used to measure the parasite growth is different. In this study, the parasite lactate dehydrogenase (pLDH) assay was applied in order to determine the sensitivity of various *Plasmodium* strains to the newly synthesized compounds discussed in chapter 2. This assay is fast and easy to perform and has the advantage of being quantitative, making it a practical method for screening a large number of compounds. The absence of genetic variation in the pLDH gene also provides an advantage over tests such as HRP-2 based tests and therefore it can be used for detecting all four species of human malaria parasites (Goodyer & Taraschi, 1997; Houzé *et al*., 2011).

3.2. Materials and Methods

3.2.1. Antiplasmodial assay

The human parasite *Plasmodium falciparum* strains used in this project were chloroquine sensitive strains (3D7 and D10) and chloroquine resistant strains (Dd2 and K1) and these were obtained from the malaria reagent depository, The Malaria Research and Reference Reagent Resource Center (MR4) (ATCC, Manassas, VA).

3.2.1.1. Cultivation of parasites

The parasites were continuously cultured *in vitro* according to the method described by Trager and Jensen but with modifications (Trager & Jensen, 1976). The cultures were maintained at 37^{o}C in O-positive (O^{+}) human erythrocytes supplied by Blood Transfusion Services Laboratory at Groote Schuur Hospital in Cape Town. The complete culture medium used contained RPMI 1640 (Sigma-Aldrich Chemical Company) growth medium supplemented with 25mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (Sigma), 22mM glucose (Sigma), 5g/l albumax (Gibco-BRL Laboratories), 25mM sodium carbonate (Sigma) and 0.3mM hypoxanthine. In order to control microbial contamination 50µg/l gentamicin (Sigma) was added to the culture medium. The medium was pre-filtered through a millipore 0.45 µm durapore filter (Merck & Co.) and was further sterilized using a sterile 0.22µm hydrophilic durapore cartridge filters (Merck).

3.2.1.2. Preparation of erythrocytes

In the malaria lifecycle the erythrocytic stages are most often associated with the pathogenesis of the malaria parasite and a major target of drug development (Schuster, 2002). Therefore, preparation of human erythrocytes is essential because they are the primary reagent in the culture of malaria parasites as they provide nutrients and a location for asexual reproduction, thereby essential for their development. Human erythrocytes of all groups were used for the growth of parasites but type O^{+} erythrocytes was preferred because they are compatible with serum or plasma from any blood group (Read and Hyde, 1993; Schuster, 2002). The erythrocytes were washed before they

were used to culture parasites to remove leukocytes and the preservatives added on donation as described in Table 3.2.

Table 3.2: The procedure for washing erythrocytes.

Reagent	Volume (ml)
Transfer erythrocytes to a sterile centrifuge tube	30
Mix with culture medium	20
Spin at 1200 x g for 5 minutes	
Remove the pale layer which is formed by the leukocytes by suction using a sterile Pasteur pipette	
Repeat the procedure keep the washed erythrocytes at 4°C for approximately 4 weeks	

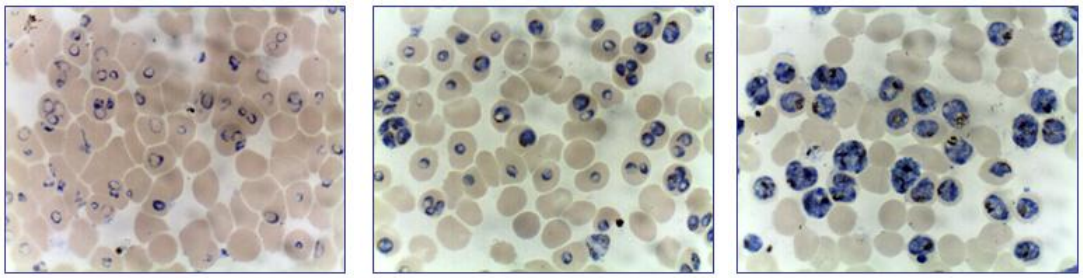
3.2.1.3. The parasite culture

The parasites were cultured in sterile 250ml Greiner Bio-One culture flasks (Lasec, SA). Forty milliliters of parasite culture was prepared with 2% hematocrit[1] and 2% parasitemia[2] and the culture medium was replaced daily. The culture was transferred to a sterile centrifuge tube and spun at 750 x g for 4 minutes and the supernatant was removed by suction. In order to monitor the parasitemia or to determine the life-cycle stages a blood smear was prepared on a microscope slide and stained with 10% Giemsa stain (Merck) in PBS following methanol fixation. The slides were allowed to stand for 5 minutes and washed with water before observing microscopically under an oil-immersion objective (100x). The Giemsa stain was used because of its high-quality staining of chromatin and nuclear membrane of cells. It binds strongly to the DNA at the regions with high concentration of adenine-thymine rich bonds (Barcia, 2007; O'Connor, 2008). Erythrocytes don't have any nucleus; therefore uninfected erythrocytes appear normal when stained with Giemsa stain, but malarial DNA in infected cells shows up blue, thus making it possible to visualize malaria. Figure 3.14 shows different stages of parasite growth as view under the microscope during a continuous culture.

[1] **Hematocrit:** The percentage volume of erythrocytes in blood.
[2] **Parasitemia:** The percentage of parasitized erythrocytes.

Parasite synchronization

Malaria parasites grow in synchrony with each other *in vivo* as they undergo repeated cycles in the erythrocytes (Kwiatkowski, 1989). However, as they grow in culture they tend to lose the normal synchronicity in their life cycle and this may affect *in vitro* antiplasmodial tests because these tests evaluate the effect of drug on the maturation of parasite from ring to schizont stage. Therefore, synchronized *P. falciparum* cultures are required before antiplasmodial assays are performed (Srinivas and Puri, 2002). In order to obtain a loosely synchronous ring stage 5% sterile aqueous D-sorbitol (Sigma) was used. Sorbitol causes lysis of infected erythrocytes containing the late stages of the malaria parasites (Schuster, 2002). Twenty milliliters of sorbitol was used to resuspend the pellet obtained by centrifuge, as described earlier. The suspension was allowed to stand at 37^{o}C for 10 minutes, preferably in a waterbath, and then centrifuged again to pellet the parasitized erythrocytes. The pellet was resuspended in complete medium and transferred to a new culture flask, where it was distributed evenly on the surface of the flask. The culture was gassed with a mixture of 5% CO_2, 3% O_2 and 92% N_2 before incubating at 37^{o}C and this was due to the fact that the parasites are microaerophilic, therefore, low oxygen levels have a beneficial effect on the growth of malaria parasites when cultured *in vitro* (Wong *et al.*, 2012).



(a) (b) (c)

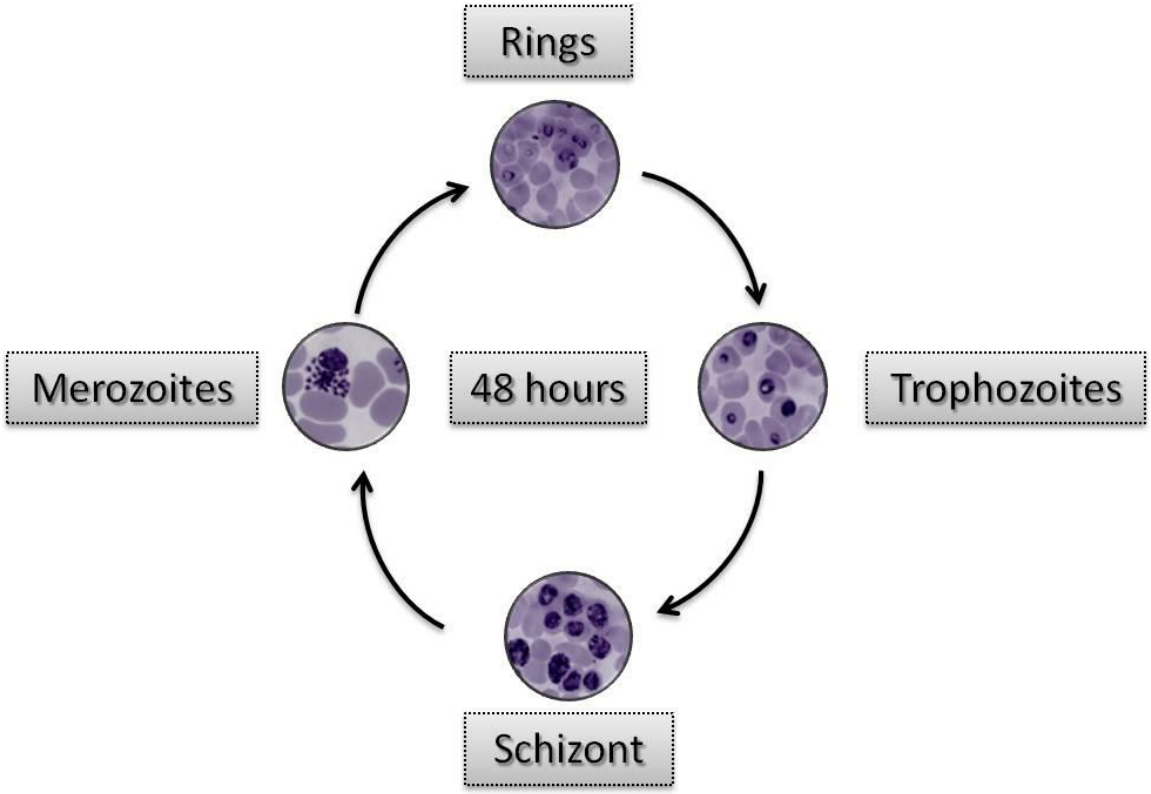


Figure 3.14: The intraerythrocytic developmental stages of malaria parasites observed after invasion during a continous culture. (a) The ring stage observed after 6 to 22 hours, (b) the trophozoite stage between 22 and 38 hours and (c) the schizont stage between 38 and 48 hours. The merozoites are observed at 48 hours just before erythrocytic invasion (Radfar *et al*., 2009).

3.2.1.4. Preparation of parasites for antiplasmodial assay

The *in vitro* antiplasmodial activities of the compounds were evaluated against D10, 3D7, Dd2 and K1 strains of *P. falciparum*. The antiplasmodial assay was initiated with the parasites in the trophozoite stage and with a desired parasitemia and hematocrit of 2%. The parasitemia was calculated according to the formula described below:

$$\frac{\text{No of parasitized erythrocytes}}{\text{Parasitized erythrocytes} + \text{Non-parasitized erythrocytes}} \times 100$$

3.2.1.5. Preparation of test compounds

The test compounds were prepared to a 20 mg/ml stock solution in 100% DMSO. The dissolved compounds were sonicated for 30 minutes at 37°C to enhance solubility and were stored at -20ºC. The compounds were tested as a suspension if not completely dissolved. Further dilutions were prepared on the day of the experiment with complete medium as a diluent and chloroquine was used as the reference drug in all experiments. The final concentration of DMSO that the parasites were exposed to did not exceed 0.5% in culture medium, which has no measurable effect on parasite viability.

The compounds were tested at a starting concentration of 100 – 0.1 μg/ml and chloroquine was tested at a starting concentration of 1 and 0.1 μg/ml for resistant strain and sensitive strains, respectively. The compounds were then serially diluted 2-fold in complete medium in a 96-well microtiter plate (Lasec) to give 10 concentrations. The same dilution technique was used for all samples, including chloroquine. Each concentration was measured in triplicate. Nonparasitized erythrocytes were used as a negative control and parasitized erythrocytes without any test compound were used as positive control. A full dose-response was performed for all compounds to determine the concentration inhibiting 50% of parasite growth (IC_{50} value). The plates were incubated for 48 hours at 37°C in a gassing chamber containing a mixture of 5% CO_2, 3% O_2 and 92% N_3. In order to quantify parasite viability and to determine the effect of the compounds on the parasite a parasite lactate dehydrogenase (pLDH) assay was used

as described by Makler *et al.*, 1993 and Makler *et al.*, 1998 but with the volumes of the the parasitized suspension and Nitro Blue Tetrazolium (NBT) modified.

3.2.1.6. Parasite lactate dehydrogenase assay

pLDH is a glycolytic enzyme essential for energy production and parasite development and is expressed at high levels in the anaerobic, asexual blood stage of the malaria lifecycle (Piper, 1999; Turgut Balik *et al.*, 2001). It catalyzes the dehydrogenation of L-lactate and converts it to pyruvate and requires the regeneration of nicotinamide adenine dinucleotide (NAD^+) for continued use in the glycolytic pathway (Brown, 2004; Xu, 2007). pLDH utilizes a co-enzyme, 3-acetyl pyridine adenine dinucleotide (APAD), which is an analog of NAD but with a higher oxidation potential, and metabolizes about 200-fold faster than human erythrocyte LDH (Grimberg *et al.*, 2008; Noedl *et al.*, 2003).

In a pLDH assay, pyruvate is formed when pLDH from a blood lysate infected with parasites is mixed with a buffered solution of APAD and L-lactate called MalStat. APAD is reduced to APADH. The quantification of the product was facilitated by adding Nitro Blue Tetrazolium (NBT) and phenazine ethosulfate (PES). APADH in turn reduces blue tetrazolium, forming a blue formazan product (Figure 3.15) which can be measured by spectrophotometry.

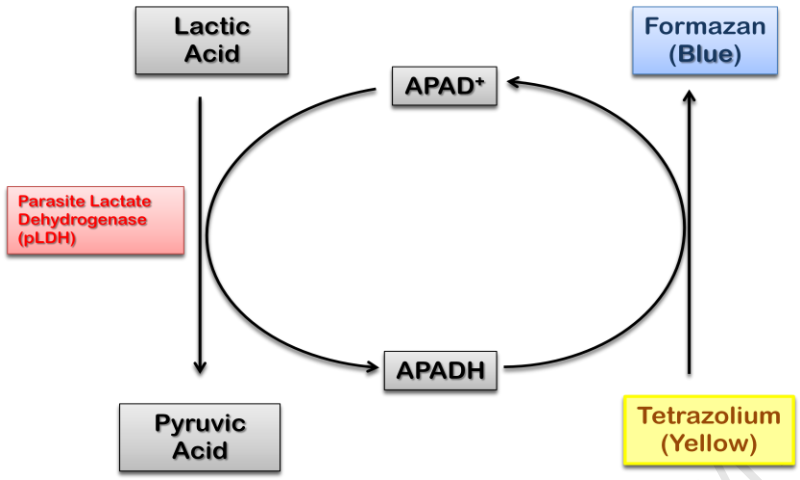


Figure 3.15: The MalStat reaction for detecting pLDH.

After completing a 48 hour cycle at 37 °C the plates were transferred to a −20 °C freezer to lyse the red blood cells. The plates were allowed to thaw at room temperature and pLDH levels in the blood suspension were measured in a new 96-well microtitre plate according to the procedure described below in Table 3.3.

Table 3.3: The procedure for the pLDH assay using MalStat reagent.

Reagent	Volume (µl)
Add MalStat reagent in a 96 well polystyrene plate	100
Transfer parasitized suspension (from the 48 hour incubation)	15
Add NBT	25
Leave in the dark at room temperature until a blue color develops	
Measure absorbance at 600nm using a Modulus™ Microplate Multimode Reader by Turner Biosystems	

The amount of formazan (blue product) produced is directly proportional to parasite viability, therefore, the parasite percentage survival was calculated in each well using the formula described below:

$$\frac{A_{\gamma 600nm}\ \text{Test well (Parasitized erythrocytes + Test compound)}}{A_{\gamma 600nm}\ \text{Control well (Parasitized erythrocytes + No test compound)}} \times 100$$

The IC_{50}-values were obtained by regression analysis, using a non-linear dose-response curve-fitting equation in GraphPad Prism v4.0 software.

3.2.2. Cytotoxicity Assays

The cell line used in this study was Chinese Hamster Ovarian (CHO).

3.2.2.1. Harvesting cells

The cells were grown in a 50:50 (v/v) mixture of Dulbecco's Modified Eagle Media (DMEM) (Highveld Biologicals, SA) and Ham's Nutrient Mixture F-12 (Sigma). DMEM consists of amino acids, vitamins, glucose and salts, and Ham's Nutrient Mixture F-12 contains L-proline because CHO cells lack the active gene form needed for proline synthesis and therefore have a requirement for proline for growth (Kao & Puck, 1967; Kao & Puck, 1968). The medium was supplemented with 10% heat inactivated fetal calf serum (FCS) (Highveld Biologicals) and 1% gentamycin (Sigma). Cells produce CO_2 during growth but it affects the pH of the medium, making it more acidic by reacting with water to form carbonic acid according to the equilibrium:

$$H_2O + CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

Therefore, both DMEM and Ham's Nutrient Mixture F-12 uses a sodium bicarbonate (Sigma) buffer system (3.7 g/L) in order to mimic the buffering system of blood, maintaining a pH of 7.1 in the medium (Barngrover *et al.*, 1985; Kraig *et al.*, 1986; Lee, 2003). Sodium bicarbonate dissociates into sodium and bicarbonate ions:

$$H_2O + CO_2 + NaCO_2 \rightleftharpoons Na^+ + H^+ + 2HCO_3^-$$

After adding sodium bicarbonate to the medium the pH was adjusted to 7.1, which is the optimum pH required for cell growth and the medium was pre-filtered through a millipore 0.45 µm durapore filter (Merck & Co.) and was further sterilized using a sterile 0.22µm

Sterivex-GV PVDF Durapore (Merck & Co.) filter. The fetal calf serum was only added after the medium was filter-sterilized.

3.2.2.2. The cell culture

All cell lines were cultured in Greiner Bio-One filter cap cell culture flasks with a growth area of 75 cm² (Lasec). These flasks are made of high-grade polystyrene required for adherent cell growth and are also pre-treated, to improve cell adhesion and proliferation. The cells were grown at 37°C and 5% CO_2 and were fed with medium every 2 days until they covered about 80% of the surface area available (80% confluency[3]). They were subcultured before 100% confluence was reached, as described by Freshney, 2005, in order to avoid the culture dying.

Subculturing the cells

For subculture the cells need to be brought into the suspension. Firstly, the medium was aspirated from the culture flask and the remaining traces of medium on the monolayer were washed off gently with 5 ml pre-warmed sterile PBS as the medium acts as an inhibitor of trypsin (Freshney, 2005). The cells were then covered with 5 ml trypsin (Highveld Biologicals), which is used to release the cells from the flask. The detaching process took about 1 to 5 minutes at 37°C, as the degree of adhesion changes from cell line to cell line, and the detached cells were suspended in 5 ml growth medium. The cells were diluted and reseeded in another culture flask.

3.2.2.2. Preparation of cells for cytotoxicity assay

When the cells reached 70-80% confluence they were subcultured and the cell suspension was centrifuged in a sterile 15 ml tube at 750 rpm for 4 minutes and the supernatant was aspirated completely. The pellet was resuspended in 5ml medium and 20 μl was removed and transferred to a 1.5 ml eppendorf tube containing 20 μl crystal violet, which was used to stain the cells for counting in a hemocytometer. The cell concentration in the suspension was calculated using this formula (Freshney, 2005):

[3] **Confluency:** When a monolayer of cells in which all cells are in contact with other cells all around their periphery, and no available substrate is left uncovered.

$$[\text{Cells}]\ \text{cells/ml} = \text{dilution factor} \times \frac{\text{Average of cells counted per small square}}{\text{Volume of a small square (0.0001ml)}}$$

The cells were seeded into 96-well plates at a density of 10000 cells/well for cytotoxicity tests. They were allowed to attach overnight and were then exposed to the test compounds for 48 hours in an incubator set 37^{o}C and 5% CO_2.

3.2.2.3. Preparation of test compounds

The compounds were prepared in a similar manner as in the antiplasmodial assay. The compounds were tested at a starting concentration of 100 – 3.125 μg/ml and emetine (Sigma), a very toxic alkaloid known as protein synthesis inhibitor in eukaryotic cells, was tested at a starting concentration of 100 μg/ml – 0.001 μg/ml and it was used as a reference drug (Grollman, 1966). The compounds were then serially diluted 2-fold in complete to give 6 concentrations. The same dilution technique was used for all samples, including emetine and each concentration was measured in triplicate. The 96 well plates that were incubated with cells overnight were removed from the incubator and the medium in the wells was aspirated. The test compounds were added to the wells with cells. The wells without any cells were used as a negative control and the positive controls were the wells with cells but no test compounds. A full dose-response was performed for all compounds to determine the concentration inhibiting 50% of cell growth (IC_{50}–value). The plates were incubated for 48 hours at 37^{o}C and 5% CO_2. In order to quantify parasite viability and to determine the toxicity of the compounds on the cells, an MTT assay was used as described by Denizot & Lang, 1986 and Twentyman & Luscombe, 1987 but with modifications where the incubation period was between 3 and 4 hours.

3.2.2.4. Procedure for the cytotoxicity assay

An MTT assay is a colorimetric assay that is used for measuring cell proliferation. In functionally intact mitochondria of actively growing cells an enzyme, succinate dehydrogenase, catalyzes the reduction of yellow water-soluble tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) to a blue water-

insoluble formazan product (Figure 3.16), using succinate, NADH, and NADPH as substrates (Dreiem *et al.*, 2005; Twentyman & Luscombe, 1987; van Meerloo *et al.*, 2011).

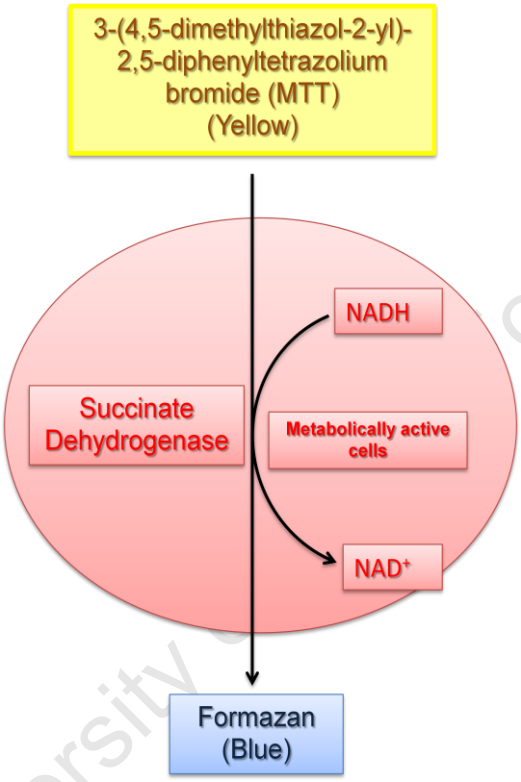


Figure 3.16: The conversion of MTT by metabolically active cells to a blue formazan product.

After completing a 48 hour cycle at 37 °C the cells were further incubated with MTT according to the procedure described below in Table 3.4.

Table 3.4: The procedure for the MTT assay.

Reagent	Volume (µl)
Add MTT reagent to the cells, with the medium still in the wells	25
Incubate for 3 to 4 hours at 37°C and 5% CO_2	
Centrifuge the plates at 200 rpm for 10 minutes	
Aspirate the supernatant	
Add DMSO	100
Shake the plate for 2 minutes to allow the crystals to dissolve completely in DMSO	
Measure absorbance at 540nm using a Modulus™ Microplate Multimode Reader by Turner Biosystems	

In most cell populations the total mitochondrial activity is related to the number of viable cells, therefore, the amount of formazan (blue product) produced is directly proportional to cell viability. The percentage survival of the cells was calculated in each well using the formula described below:

$$\frac{A_{\gamma 540nm}\ \text{Test well (Cells + Test compounds)}}{A_{\gamma 540nm}\ \text{Control well (Cells + No test compounds)}} \times 100$$

The IC_{50}-values were obtained by regression analysis, using a non-linear dose-response curve-fitting equation in GraphPad Prism v4.0 software.

3.3. Results and Discussion

A total of twelve antimalarial compounds were designed with the ultimate aim of overcoming the burden of drug resistant *P. falciparum* strains. These compounds consist of two hybrid classes, namely AZT-CQ hybrids and HPO-CQ hybrid, and 3,5-diaryl-2-aminopyridines. In addition to malaria resistance as the reason for the synthesis of these compounds, AZT-CQ hybrids were also designed to target the human immunodeficiency virus (HIV), as described in chapter 2. However, this study only focused on the investigation of the AZT-CQ hybrid compound's ability to suppress the growth of *P. falciparum* sensitive and resistant strains *in vitro*.

To determine the potential of these compounds as effective antimalarials, resistant strains K1 and Dd2 and sensitive strains 3D7 and D10 were used in order to determine the sensitivity of these compounds in comparison to chloroquine, a known antimalarial drug with reduced potency against drug-resistant strains.

3.3.1. *In vitro* antiplasmodial activity and cytotoxicity of the AZT-CQ hybrids

3.3.1.1. The AZT-CQ hybrids

The AZT-CQ hybrid compounds, VAK 31DS, VAK 37DS, VAK 59DS, AKAZTQ and AKAZTBQ, were tested for antiplasmodial activity against sensitive strains 3D7 and D10 and the dose response curves obtained were compared to those of chloroquine and are presented in Figure 3.17 and Figure 3.18.

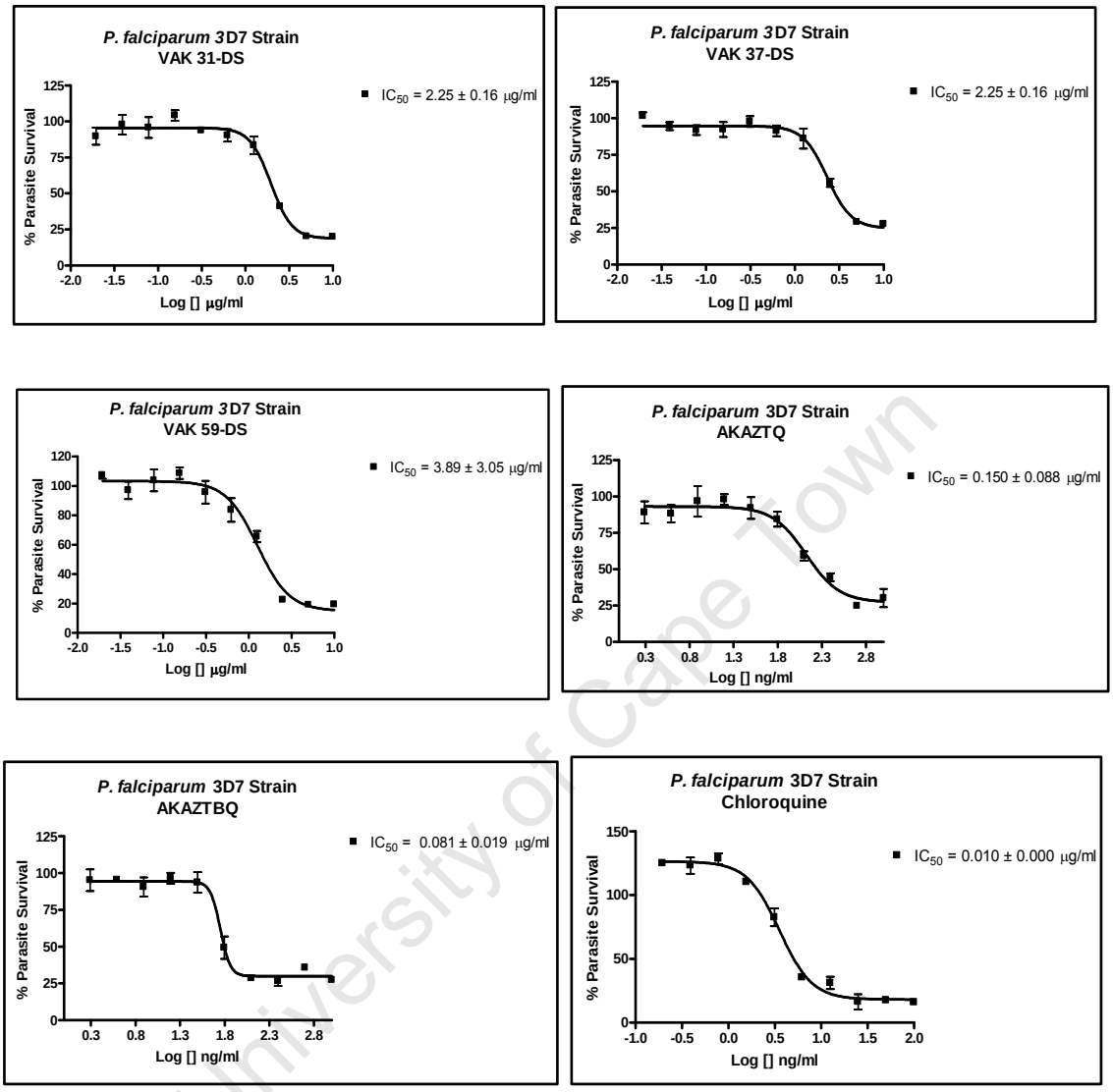


Figure 3.17: Dose response curves of the AZT-CQ hybrids tested against chloroquine sensitive strain 3D7. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.

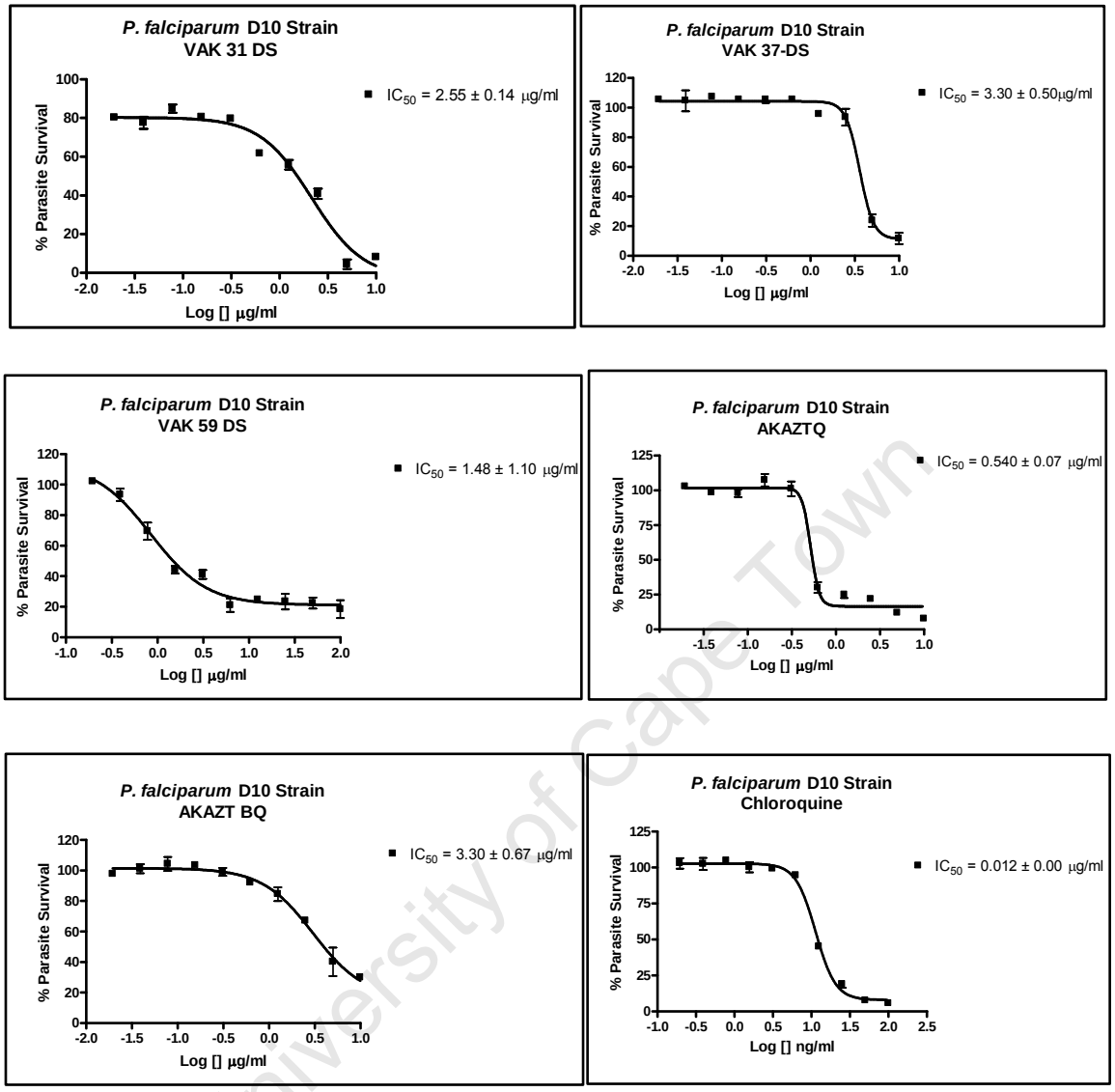


Figure 3.18: Dose response curves of the AZT-CQ hybrids tested against chloroquine sensitive strain D10. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.

The IC_{50} values calculated from the dose response curves were in the range of 0.081 to 3.9 µg/ml against 3D7 and 0.5 to 3.3 µg/ml against D10, both significantly higher than those of chloroquine. The experiment was repeated using resistant strains K1 and Dd2. The dose response curves for these compounds are presented in Figure 3.19 and Figure 3.20.

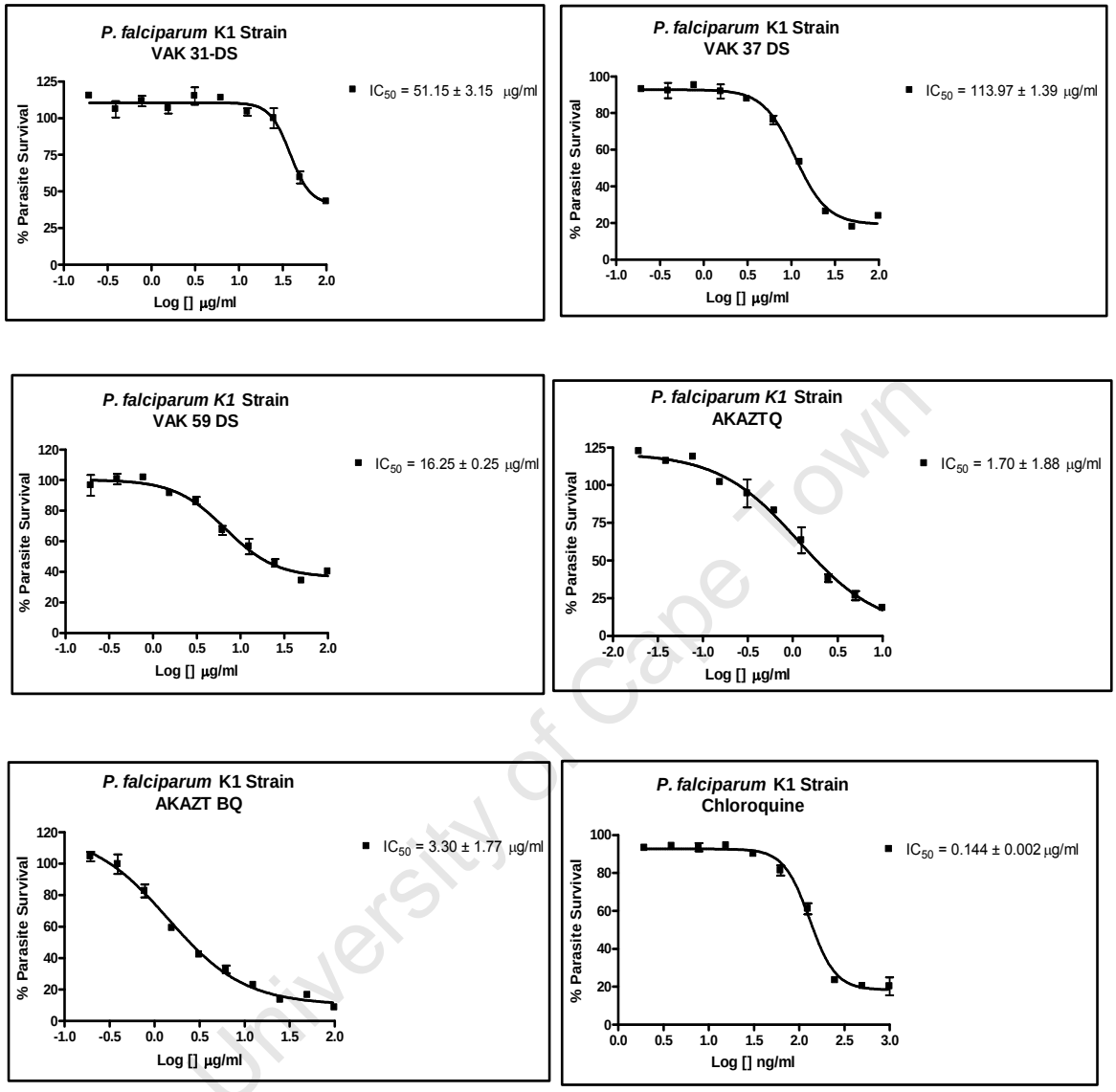


Figure 3.19: Dose response curves of the AZT-CQ hybrids tested against chloroquine resistant strain K1. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.

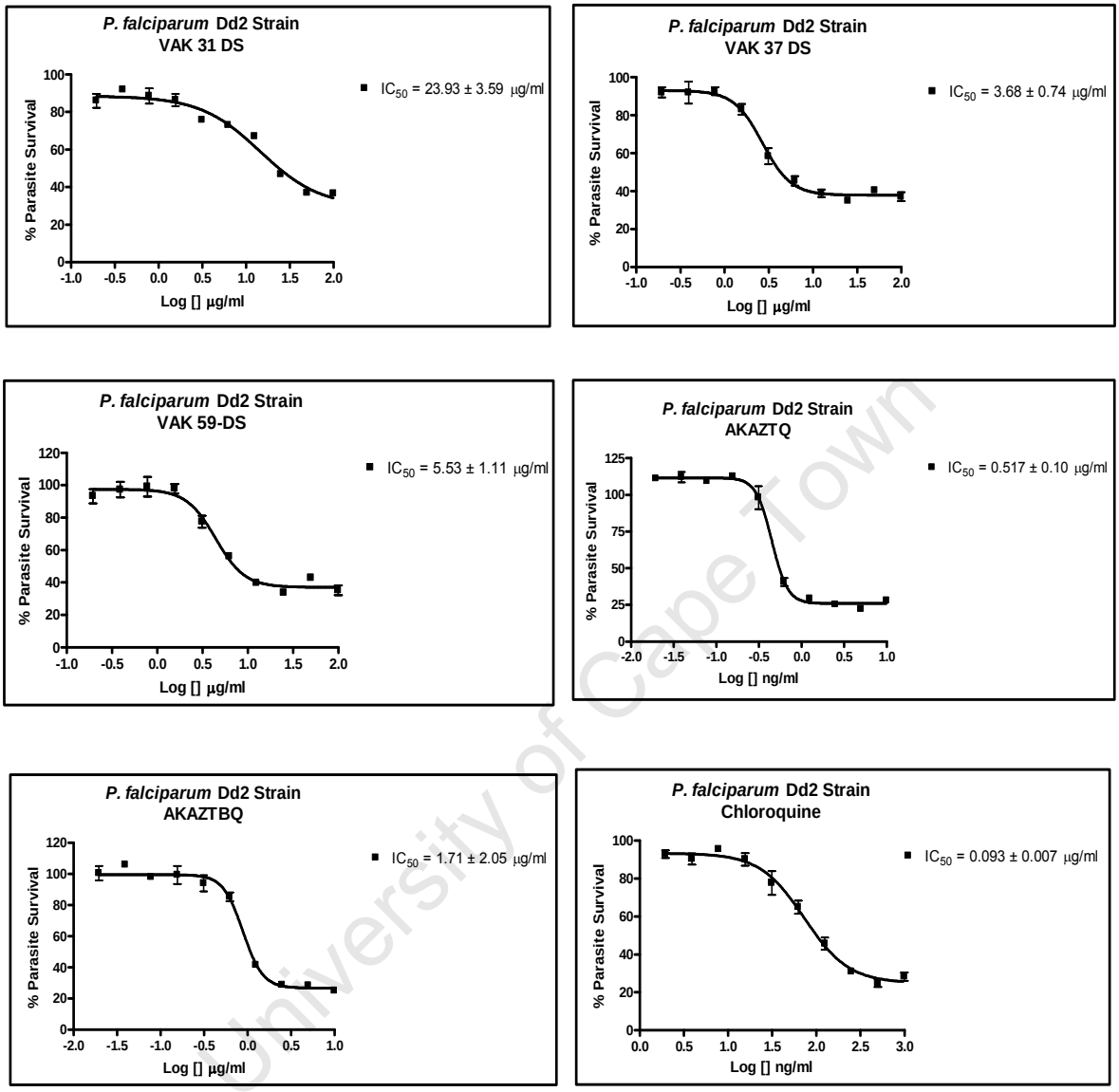


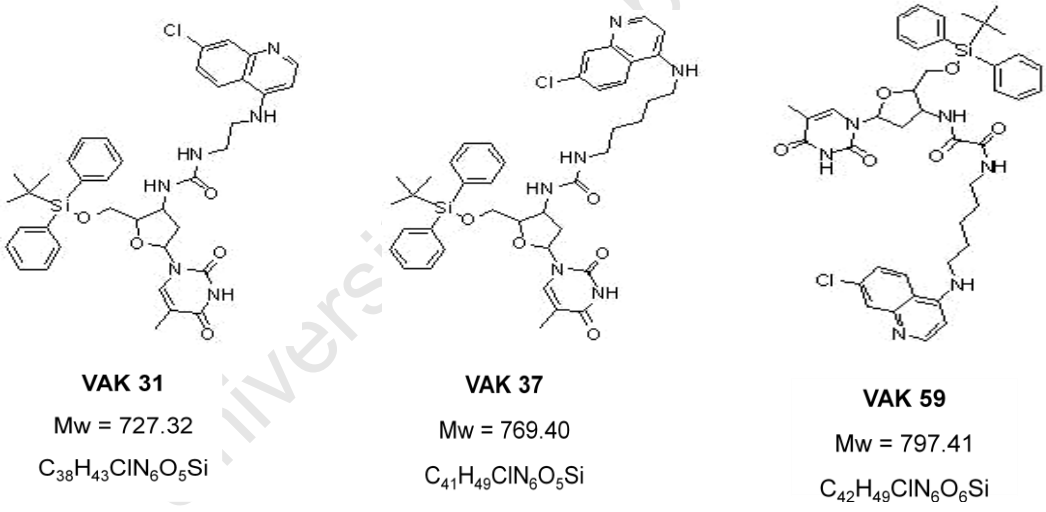
Figure 3.20: Dose response curves of the AZT-CQ hybrids tested against chloroquine resistant strain Dd2. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.

The IC_{50} values of the compounds tested against K1 and Dd2 were in the range of 1.7 to 114 µg/ml and 0.5 to 24.0 µg/ml, respectively. The antiplasmodial activity of the hybrid compounds was much lower when compared to chloroquine (IC_{50} values which were 0.144 and 0.093 µg/ml for K1 and Dd2, respectively). No further tests were

performed on VAK 31DS, VAK 37DS and VAK 59DS. The antiplasmodial activity of AKAZTQ and AKAZTBQ against the resistant strains was greater than the other three compounds but still significantly lower than chloroquine.

3.3.1.2. The silylated AZT-CQ hybrids

In a routine testing of intermediate compounds, *in vitro* antiplasmodial activity was performed on a batch of AZT-CQ hybrids which had, not undergone desilylation. The core structure of these compounds is similar to that of desilylated AZT-CQ but with the *tert*-butyldiphenylsilyl group still present (Figure 3.21). The presence of this bulky and lipophilic *tert*-butyldiphenylsilyl protecting group caused the compounds to be more insoluble than the AZT-CQ hybrids. The rather surprising results are presented in Figure 3.22 and Figure 3.23.



VAK 31

Mw = 727.32

$C_{38}H_{43}ClN_6O_5Si$

VAK 37

Mw = 769.40

$C_{41}H_{49}ClN_6O_5Si$

VAK 59

Mw = 797.41

$C_{42}H_{49}ClN_6O_6Si$

Figure 3.21: The structures of synthesized silylated AZT-CQ hybrids.

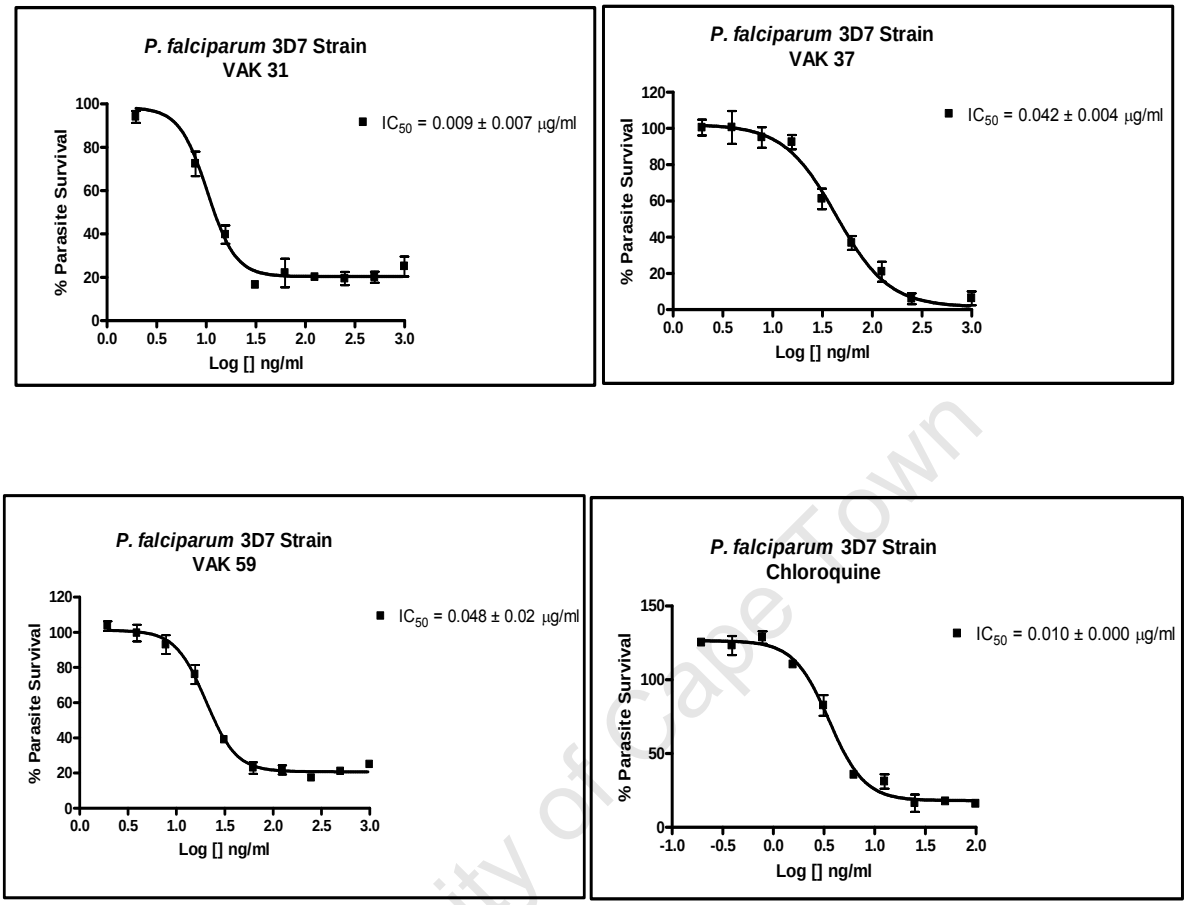


Figure 3.22: Dose response curves of the silylated AZT-CQ hybrids tested against chloroquine sensitive strain 3D7. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.

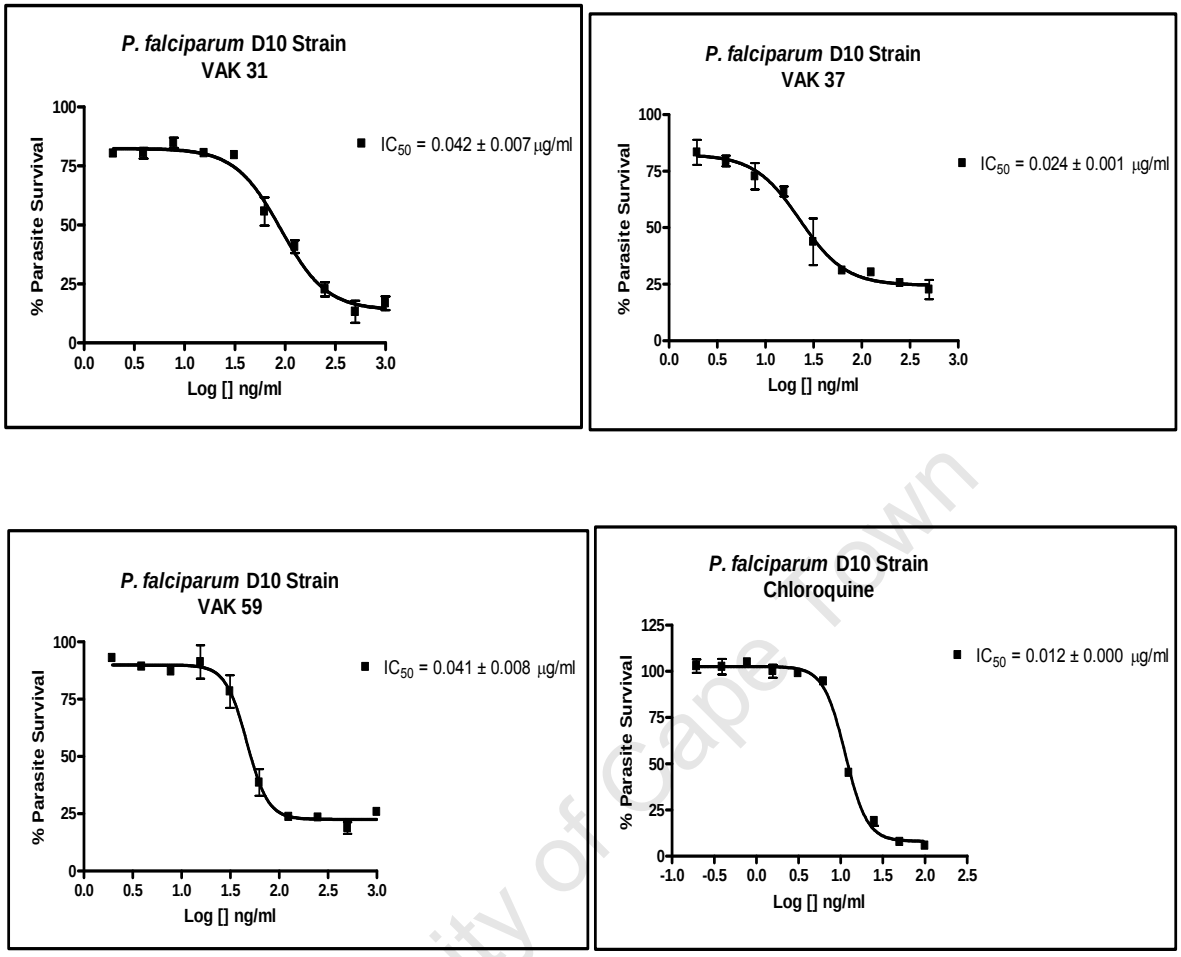


Figure 3.23: Dose response curves of the silylated AZT-CQ hybrids tested against chloroquine sensitive strain D10. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12

The IC_{50} values were significantly lower than the desilylated AZT-CQ hybrids. The activity was therefore tested against the resistant strains K1 and Dd2 and the dose response curves are presented in Figure 3.24 and Figure 3.25.

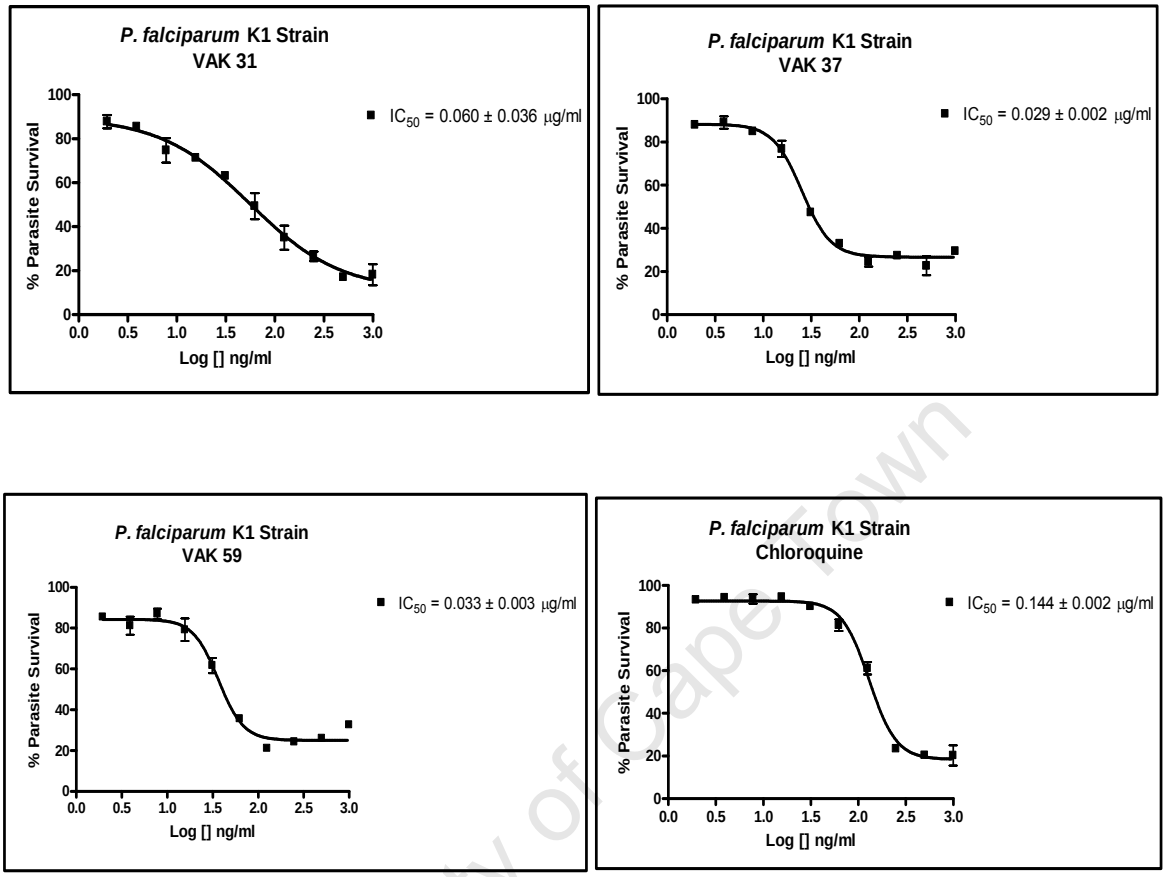


Figure 3.24: Dose response curves of the silylated AZT-CQ hybrids tested against chloroquine resistant strain K1. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.

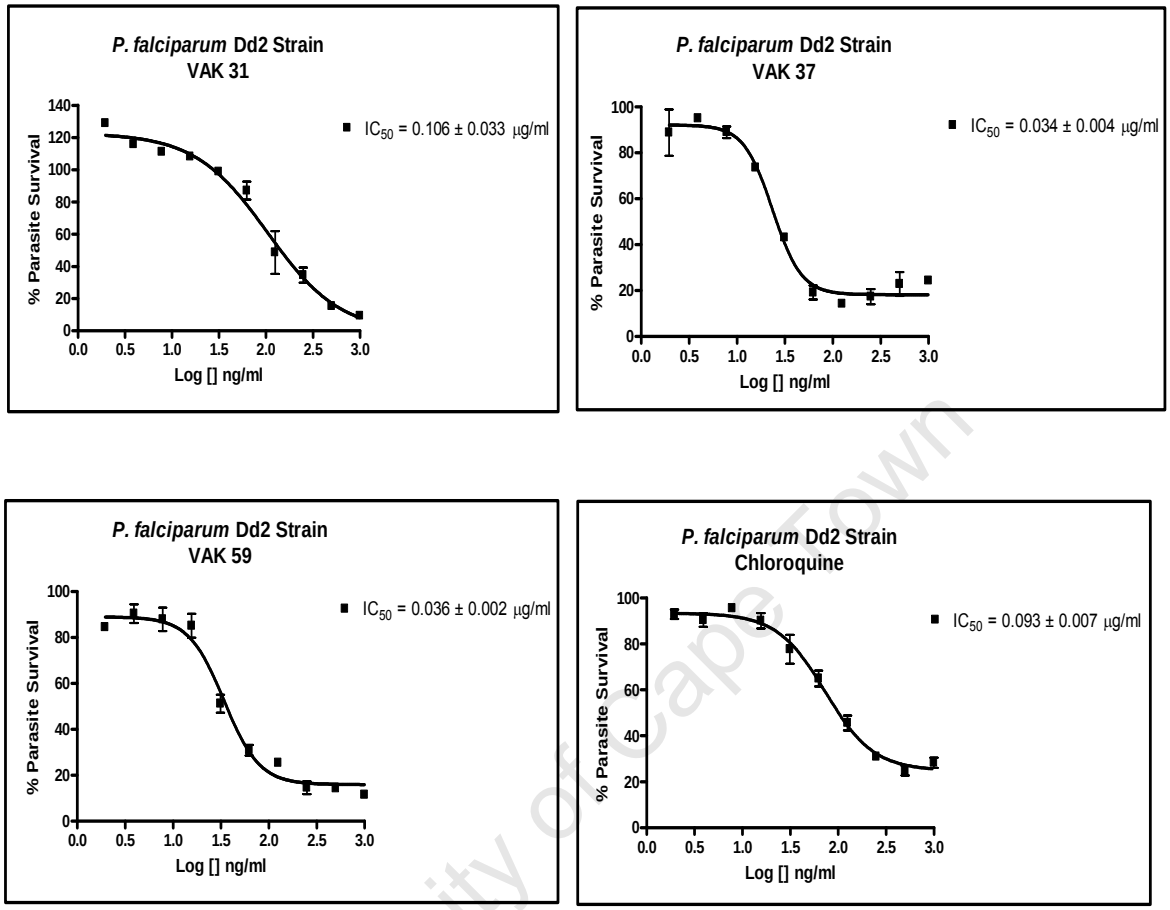


Figure 3.25: Dose response curves of the silylated AZT-CQ hybrids tested against chloroquine resistant strain Dd2. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.

The IC_{50} values of the compounds tested against the resistant strain K1 and Dd2 were in the range of 0.029 to 0.060 µg/ml and 0.034 to 0.105 µg/ml, respectively. This activity was not only significantly lower than the desilylated hybrids but also lower than chloroquine (IC_{50} values which were 0.144 and 0.093 µg/ml for K1 and Dd2, respectively), except for VAK 31 which presented the highest IC_{50} value of 0.106 µg/ml against Dd2. In order to determine the specificity of the antiplasmodial effect of the compounds, they were subjected to cytotoxicity testing against CHO cells and emetine

was used as a reference compound. The dose response curves are presented in Figure 3.26

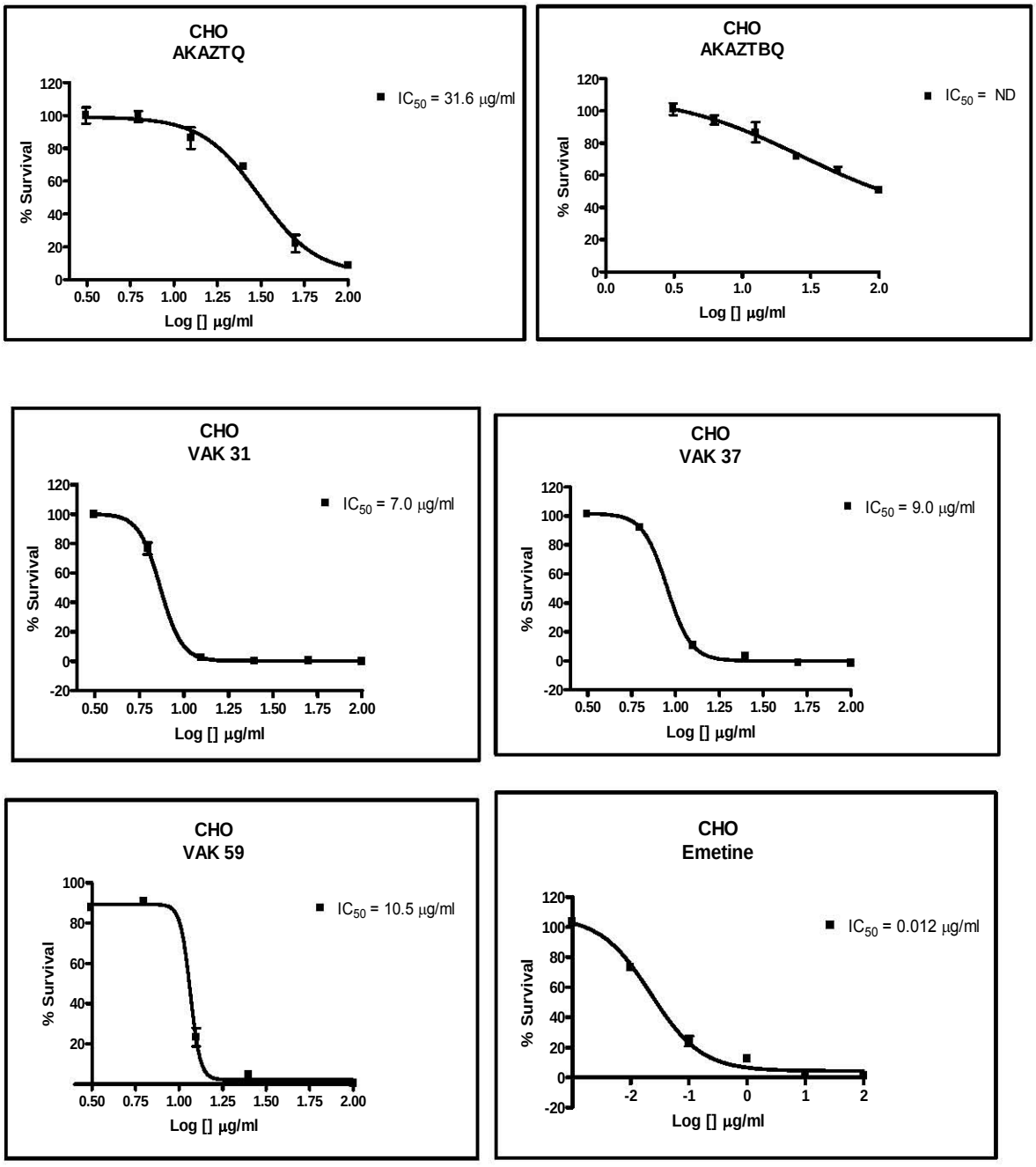


Figure 3.26: Dose response curves of the AZT-CQ hybrids tested against CHO cell lines for cytotoxicity evaluation. Each point represents the mean of two independent (n=2) experiments each performed in triplicate. For emetine, n=8.

The IC_{50} values calculated were 1000 times higher than that of emetine, which is known for its toxic properties. In order to demonstrate the differential activity of these compounds, selectivity index (SI) values were calculated. For each compound the SI value was calculated using the highest IC_{50} value, which is more likely to give the lowest SI value. This is because the greater the SI value is, the more selective the compound. An SI value less than 2 indicates general toxicity of that particular compound. The SI values were calculated according to this formula (Badisa *et al*., 2009):

$$\text{Selectivity Index (SI)} = \frac{IC_{50}\ \text{mammalian cell}}{IC_{50}\ \text{parasite}}$$

The IC_{50} value calculated for AKAZTQ was 31.6 µg/ml and the SI value was greater than 61. This value was calculated using the IC_{50} values obtained against K1 which was the highest value for AKAZTQ. The $IC_{50,}$ and hence the SI, values for AKAZTBQ could not be determined because the cell viability in the presence of this compound exceeded 50%. These results suggest that the cytotoxicity of AKAZTQ and AKAZTBQ was insignificant.

The calculated SI value for VAK 31 was greater than 66 and the IC_{50} in Dd2, which was the highest for VAK 31, and was used to calculate the SI values. For VAK 37 and VAK 59 the calculated SI values were greater than 214 and 219, respectively. All SI values were much greater than 2; therefore, the toxicity presented by these compounds was insignificant, indicating the specificity of the antiplasmodial activity.

Table 3.5 summarizes the IC_{50} values (in µg/ml) of all eight AZT-CQ hybrid compounds that were screened for antiplasmodial activity and cytotoxicity. The choroquine and emetine IC_{50} values listed in the table is the mean value of IC_{50}'s calculated from multiple experiments and has been used as a reference for all the compounds that were tested.

Table 3.5: The summary of IC_{50} values (µg/ml) and selectivity index (SI) values of the AZT-CQ hybrid compounds. The values obtained from antiplasmodial tests represent the mean of three independent experiments each performed in triplicate and the cytotoxicity values represent the mean of two independent experiments each performed in triplicate.

Test Compounds	Sensitive Strains		Resistant Strains		Cells	SI**	P-value	RI##
AZT-CQ hybrids	**3D7**	**D10**	**K1**	**Dd2**	**CHO**			
VAK 31DS	2.25	2.55	51.15	23.93	NA*	ND#	0.029	ND
VAK 37DS	2.61	3.30	13.97	3.68	NA	ND	0.029	ND
VAK 59DS	3.89	1.48	16.25	5.53	NA	ND	0.029	ND
AKAZTQ	0.150	0.54	1.70	0.517	31.6	>19	0.029	ND
AKAZTBQ	0.081	3.30	3.30	1.71	>100	ND	0.114	ND
Silylated AZT-CQ hybrids	**3D7**	**D10**	**K1**	**Dd2**	**CHO**			
VAK 31	0.009	0.042	0.060	0.106	7.0	>66	0.886	<11.8
VAK 37	0.042	0.024	0.029	0.034	9.0	>214	1.0	<1.4
VAK 59	0.048	0.041	0.033	0.036	10.5	>219	1.0	<0.9
Reference Compounds	**3D7**	**D10**	**K1**	**Dd2**	**CHO**			
Chloroquine (n=12)	0.010	0.012	0.144	0.093				
Emetine (n=8)					0.012			

***NA – Not assayed, #ND – Not determined, >100 – Highest concentration tested**
****SI value - Values calculated using the highest IC_{50} value.**
##RI value – Values calculated using the highest CQR IC_{50} value and lowest CQS IC_{50} value.

In order to compare the efficacy of these compounds to that of chloroquine for significant differences, a nonparametric Mann-Whitney U test was performed using the statistical functions of GraphPad Prism 4. P-values obtained by the two-tailed test for the desilylated AZT-CQ hybrid compounds were considered significant for at $P<0.05$ and insignificant for the silylated AZT-CQ hybrids at $P>0.05$. AKAZTBQ was the only compound from the desilylated AZT-CQ hybrid compounds with insignificant difference at $P=0.114$ ($P>0.05$). This means that the efficacy of silylated AZT-CQ hybrid compounds is similar to that of chloroquine but enhanced when tested against the resistant strains. Table 3.4 also shows similar IC_{50} values of silylated AZT-CQ hybrid compounds for sensitive and resistant strains. The resistance index (RI) was calculated for these compounds using the lowest IC_{50} value of the sensitive and the highest IC_{50} of

the resistant strain for each compound which should give you the highest RI value for that compound. This value provides a quantitative measurement of the antiplasmodial activity against chloroquine-resistant strains relative to that against chloroquine-sensitive strains (Ekoue-Kovi *et al*., 2009). The RI value is calculated according to this formula (Kaushik *et al*., 2012):

$$\text{Resistance Index (RI)} = \frac{\text{Chloroquine Resistant Strain } IC_{50}}{\text{Chloroquine Sensitive Strain } IC_{50}}$$

The RI values calculated for silylated AZT-CQ hybrid compounds were in the range of 11.8 and 0.7 and these values were less than that of chloroquine which was 14. Desirable RI values were observed for VAK37 and VAK59 at 1.4 and 0.9, respectively, and these were 10 times less that the RI value for chloroquine which shows less resistance.

The silylation of AZT using *tert*-butyldiphenylsilylchloride as a protective group in the synthesis of the AZT-CQ hybrids was done in order to achieve chemoselectivity, a process that is required in multistep organic synthesis (Corey *et al*., 1985). Desilylated AZT-CQ hybrids were the intended final products after a successful desilylation process by the removal of the *tert*-butyldiphenylsilyl group on the AZT part of the compound (Figure 2.11 and Figure 3.21). The poor activity of all the hybrids against both sensitive and resistant strains of *P. falciparum* indicates that the nature of the coupling of chloroquine to AZT results in loss of antiplasmodial activity. An unanticipated finding was that retention of the silyl group on the AZT moiety of the hybrid molecule not only preserved activity against the sensitive strain but significantly enhanced activity against the chloroquine resistant strain. Retention of the group would likely inhibit activity of AZT against HIV replication but its ability to overcome chloroquine resistance is noteworthy.

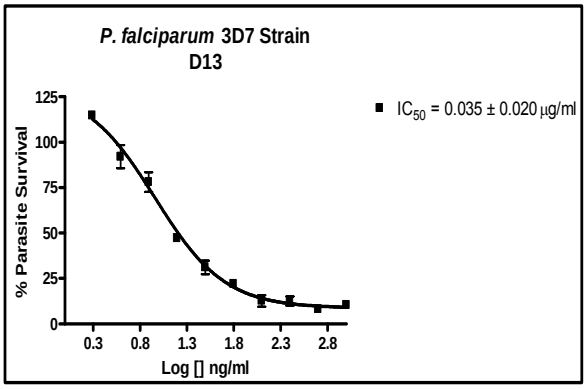
The efficacy of the desilylated AZT-CQ hybrid compounds was 100-fold less than that of the silylated AZT-CQ hybrids and the P-value indicated a significant difference when compared to the efficacy of chloroquine. This means that the efficacy of the AZT-CQ hybrid compound against *P. falciparum* strains may be enhanced further by chemical

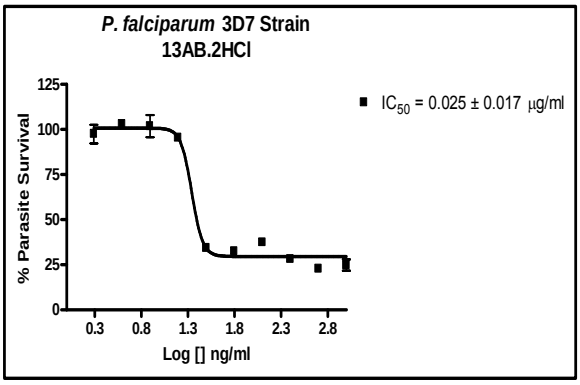
modification of their functional groups, while retaining both antimalarial and anti-HIV activity of the compound. However, the presence of lipophilic groups such as *tert*-butyldiphenylsilyl reduces the solubility of the compounds as was observed in this study. In drug discovery, properties such as solubility play a very crucial role as they can affect ADMET properties of the drugs because poor solubility can reduce drug absorption following oral administration and this may cause low oral bioavailability, making the drug a less desirable candidate for further development (Bose *et al*., 2010).

The next section will discuss the results obtained when another group of hybrid compounds was assessed for antiplasmodial activity.

3.3.2. *In vitro* antiplasmodial activity and cytotoxicity of the HPO-CQ hybrids

Compounds D13 and 13AB.2HCl are HPO-CQ hybrid compounds that were synthesized in order to enhance the activity of chloroquine by combining it with an iron chelator. These compounds were tested for antiplasmodial activity against sensitive strains 3D7 and D10 and the dose response curves obtained were compared to those of chloroquine, and these are presented in Figure 3.27 and Figure 3.28.





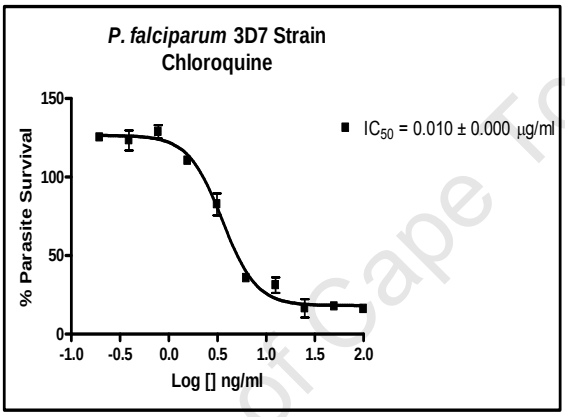


Figure 3.27: Dose response curves of the HPO-CQ hybrids tested against chloroquine sensitive strain 3D7. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.

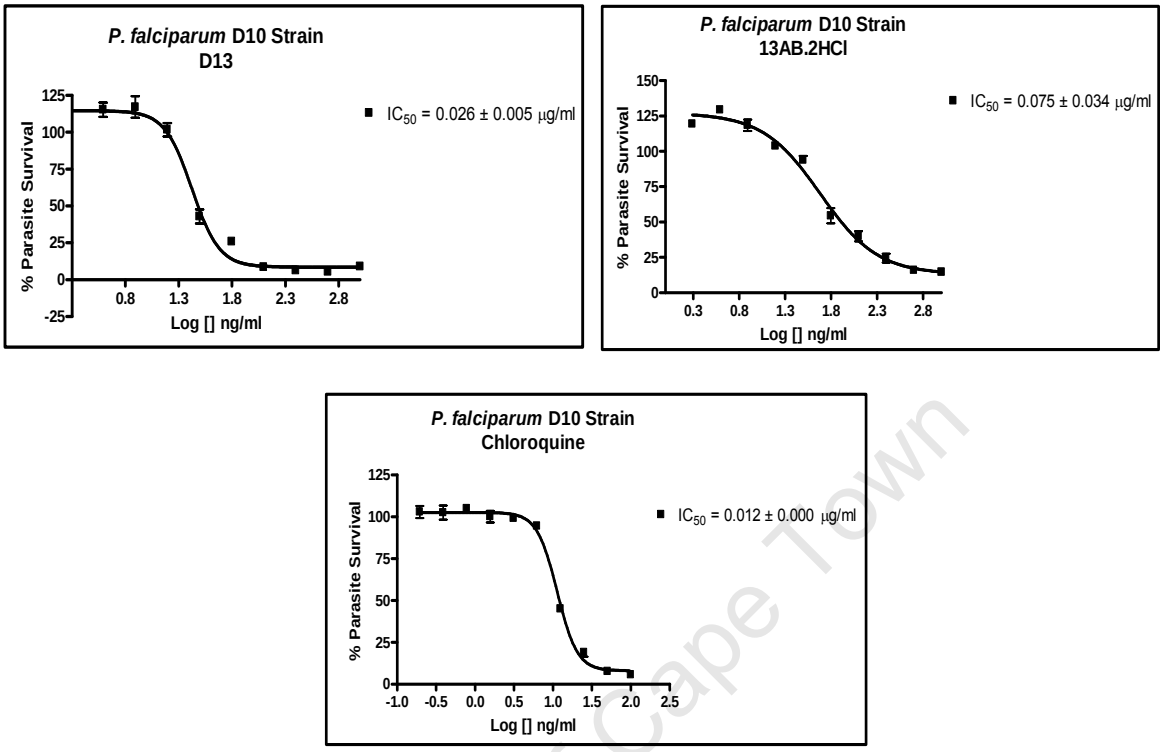
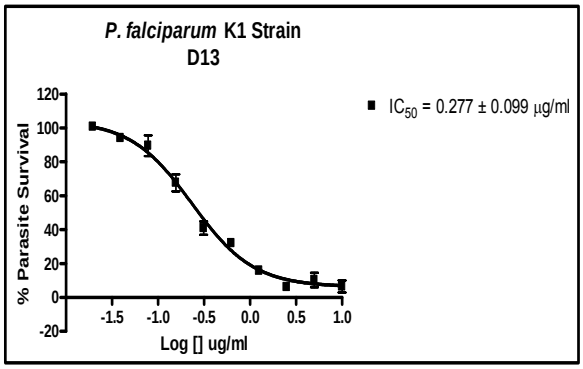
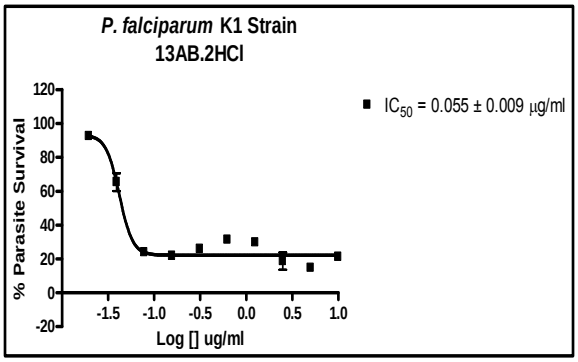


Figure 3.28: Dose response curves of the HPO-CQ hybrids tested against chloroquine sensitive strain D10. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.

The IC_{50} values calculated from the dose response curves were in the range of 0.025 to 0.035 µg/ml against 3D7 and 0.026 to 0.075 µg/ml against D10. These values were higher than that of chloroquine, with IC_{50} values of 0.010 and 0.012 µg/ml against 3D7 and D10, respectively. In order to compare their activity with that of chloroquine against chloroquine–resistant strains activity was tested against K1 and Dd2 strains. The dose response curves for these compounds are presented in Figure 3.29 and Figure 3.30.





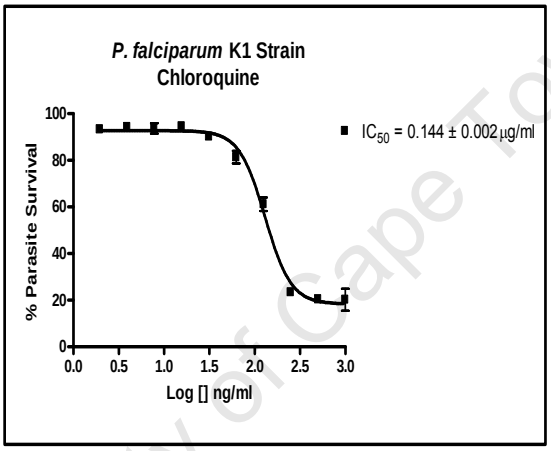


Figure 3.29: Dose response curves of the HPO-CQ hybrids tested against chloroquine resistant strain K1. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.

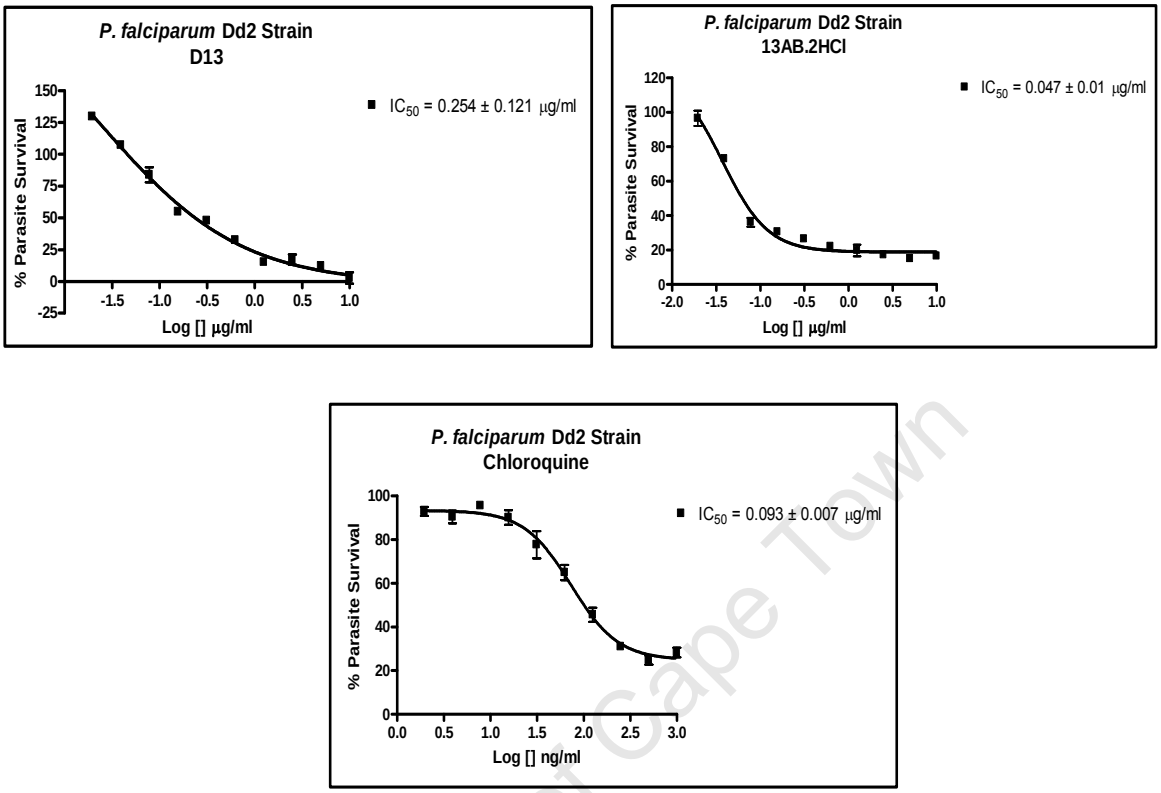


Figure 3.30: Dose response curves of the HPO-CQ hybrids tested against chloroquine resistant strain Dd2. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.

The IC_{50} values of the compounds tested against the resistant strain K1 and Dd2 were compared to those of chloroquine (IC_{50} values of 0.144 and 0.093 µg/ml for K1 and Dd2, respectively). 13AB.2HCl was more active than chloroquine with IC_{50} values of 0.055 and 0.047 µg/ml for K1 and Dd2, respectively. D13 on the other hand was less active than chloroquine with IC_{50} values of 0.277 and 0.254 µg/ml for K1 and Dd2, respectively. Both compounds were tested for cytotoxicity using a CHO cell line in order to establish specificity of activity. Emetine was used as a reference compound. The dose response curves are presented in Figure 3.31.

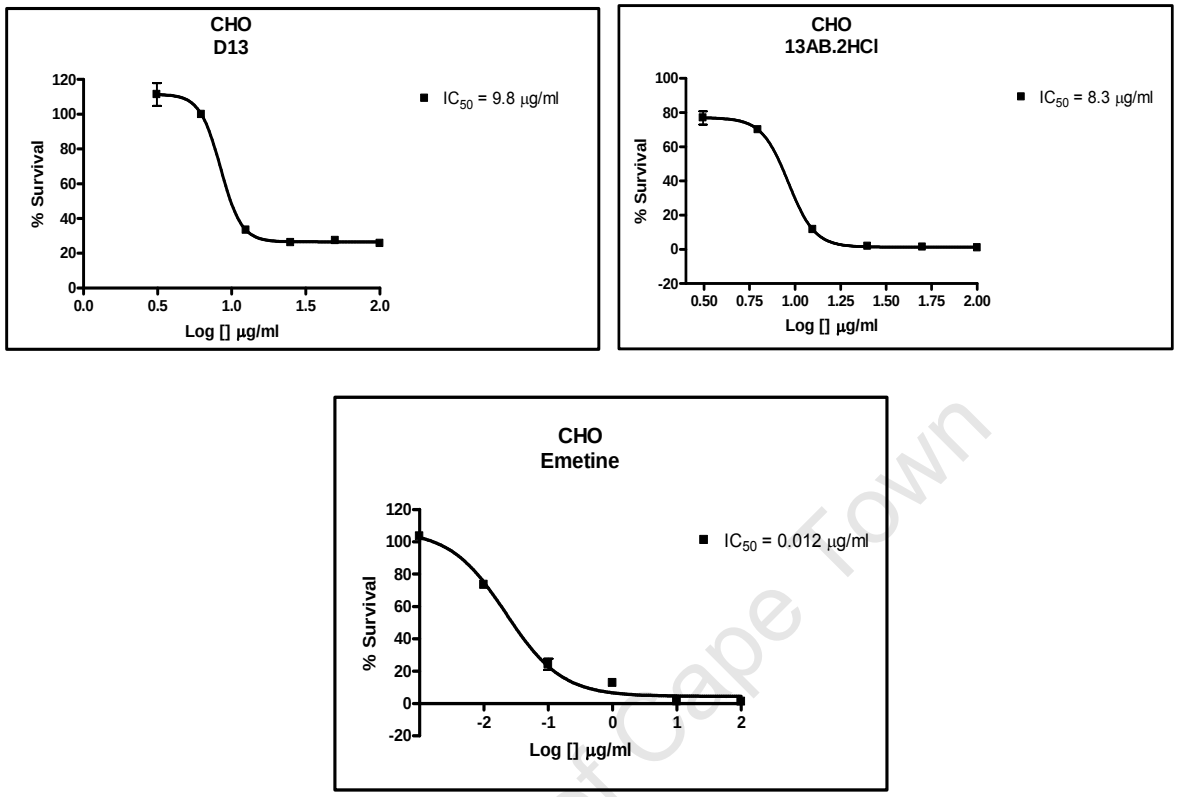


Figure 3.31: Dose response curves of the HPO-CQ hybrids tested against CHO cell lines for cytotoxicity evaluation. Each point represents the mean of two independent (n=2) experiments each performed in triplicate. For emetine, n=8.

The sensitivity of the CHO cell lines towards D13 and 13AB.2HCl was about 1000 times less than that of emetine, with IC_{50} values of 9.8 and 8.3 µg/ml. The calculated SI value for D13 was greater than 39 and for 13AB.2HCl the SI value was greater than 111, indicating a specific antiplasmodial effect.

The IC_{50} values (in µg/ml) of HPO-CQ hybrid compounds that were screened for antiplasmodial activity and cytotoxicity are summarized in Table 3.6. The P-values were calculated using the nonparametric Mann-Whitney U test in order to compare the efficacy of chloroquine with that of the HPO-CQ hybrids.

Table 3.6: The summary of IC_{50} values (µg/ml) and selectivity index (SI) values of the HPO-CQ hybrid compounds. The values obtained from antiplasmodial tests represent the mean of three independent experiments each performed in triplicate and the cytotoxicity values represent the mean of two independent experiments each performed in triplicate.

Test Compounds	Sensitive Strains		Resistant Strains		Cells	SI	P-value	RI
HPO-CQ hybrids	**3D7**	**D10**	**K1**	**Dd2**	**CHO**			
D13	0.035	0.026	0.277	0.254	9.8	>39	0.343	<10.7
13AB.2HCl	0.025	0.075	0.055	0.047	8.3	>111	1.0	<2.2
Reference Compounds	**3D7**	**D10**	**K1**	**Dd2**	**CHO**			
Chloroquine (n=12)	**0.010**	**0.012**	**0.144**	**0.093**				
Emetine (n=8)					**0.012**			

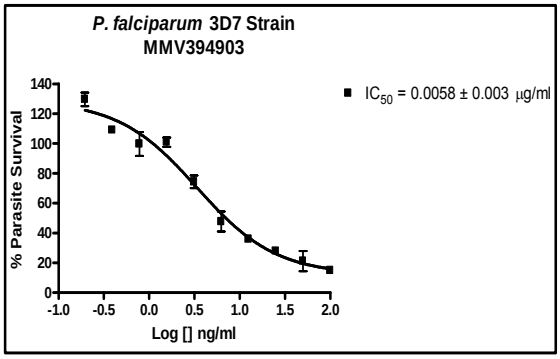
The data presented in Table 3.6 shows that the difference in efficacy of the HPO-CQ hybrids when compared to chloroquine is insignificant at P>0.05 in all chloroquine sensitive strains. However, activity of the two compounds against the resistant strains differed significantly. 13AB.2HCl had an RI value of 2.2 and the RI value of D13 was 10.7. The ethyl maltol derived HPO-CQ hybrid (13AB.2HCl) possessed greater antiplasmodial activity against chloroquine–resistant strains when comparing to chloroquine and D13, the kojic acid derived HPO-CQ hybrid. In a study performed by Andayi and coworkers, a group ethyl maltol derived HPO-CQ hybrids, which included 13AB.2HCl, were reported with enhanced antiplasmodial activity against resistant strains K1 and W2 (Andayi *et al*., 2013). This study tested 13AB.2HCl against another resistant strain, Dd2, and the results also show high activity and a very low RI value of 2.2. Therefore, the hydroxypyridone structure on the hybrid compounds tested in this study apparently influenced their efficacy against the resistant strains.

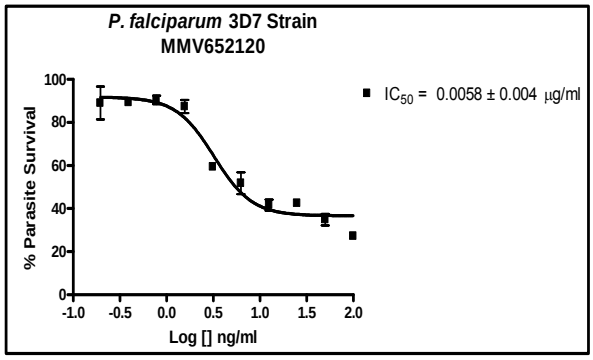
The data obtained in this study showed the chemical modification of chloroquine by combining it with other rationally selected and designed chemical entities resulted in compounds with antiplasmodial activity that is comparable to that of chloroquine alone. No assays were performed to determine the mode of action of the hybrid compounds. Andayi and colleagues performed β-hematin inhibition assays in order to determine

whether combining chloroquine with an iron chelator will enhance β-hematin inhibition as proposed mode of action of chloroquine. No β-hematin inhibition was observed for 13AB.2HCl in their study even though some of the HPO-CQ hybrid compounds they tested showed reasonable β-hematin inhibition (Andayi *et al*., 2013). Therefore, it is possible that the efficacy of 13AB.2HCl is via a different mechanism other than β-hematin inhibition.

3.3.3. *In vitro* antiplasmodial activity and cytotoxicity of the 2-aminopyridines

3,5-diaryl-2-aminopyridines, which are not hybrid compounds are described in Chapter 2, were also assessed for antiplasmodial activity. The activity of 3,5-diaryl-2-aminopyridines, namely MMV394903 and MMV652120, was determined by incubating sensitive strains 3D7 and D10 and the dose response curves obtained were compared to that of chloroquine, and these are presented in Figure 3.32 and Figure 3.33





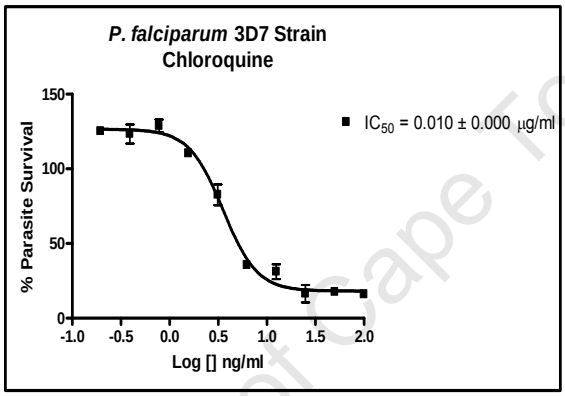


Figure 3.32: Dose response curves of the 2-aminopyridones tested against chloroquine sensitive strain 3D7. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.

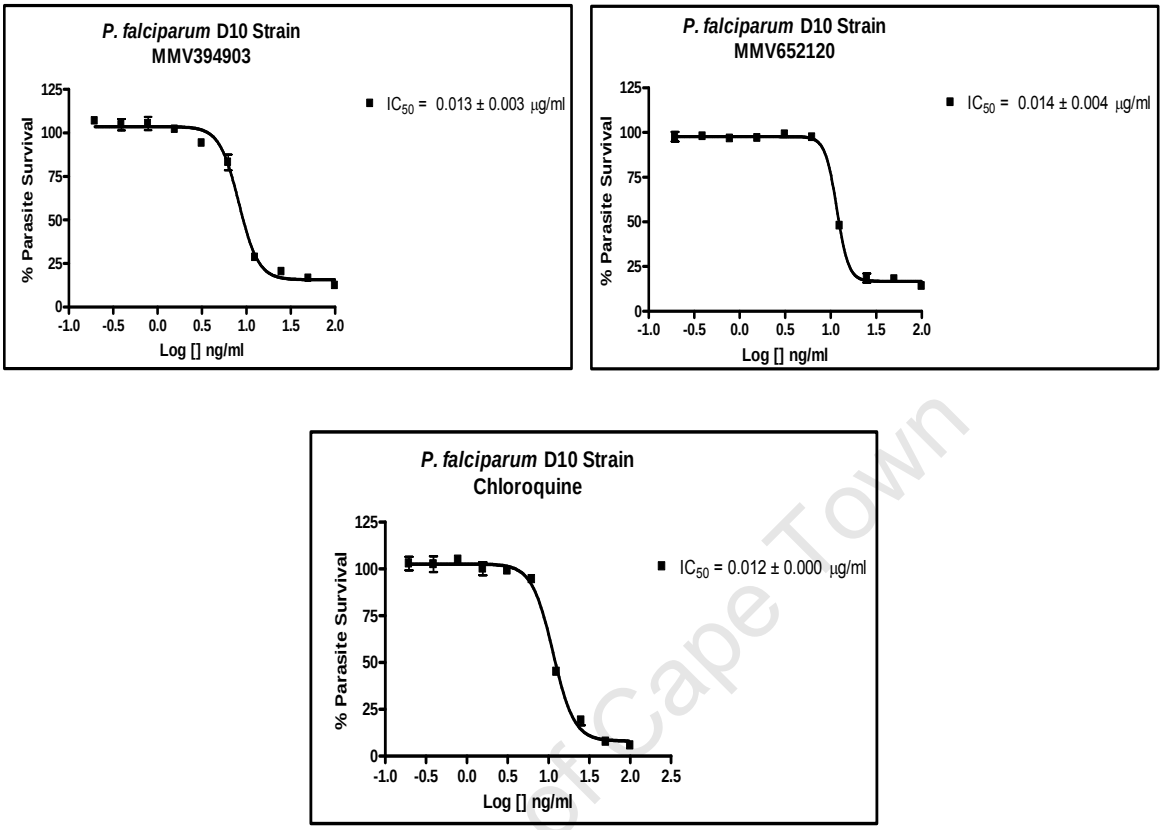


Figure 3.33: Dose response curves of the 2-aminopyridones tested against chloroquine sensitive strain D10. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.

MMV394903 and MMV652120 showed significant antiplasmodial activity with IC_{50} values of 0.0058 µg/ml for both compounds against 3D7 and 0.013 to 0.014 µg/ml against D10. These values were in the same range as those of chloroquine against sensitive strains. Therefore in order to compare their activity with that of chloroquine against chloroquine–resistant strains K1 and Dd2, the experiment was repeated and the dose response curves for these compounds against the resistant strains are presented in Figure 3.34 and Figure 3.35.

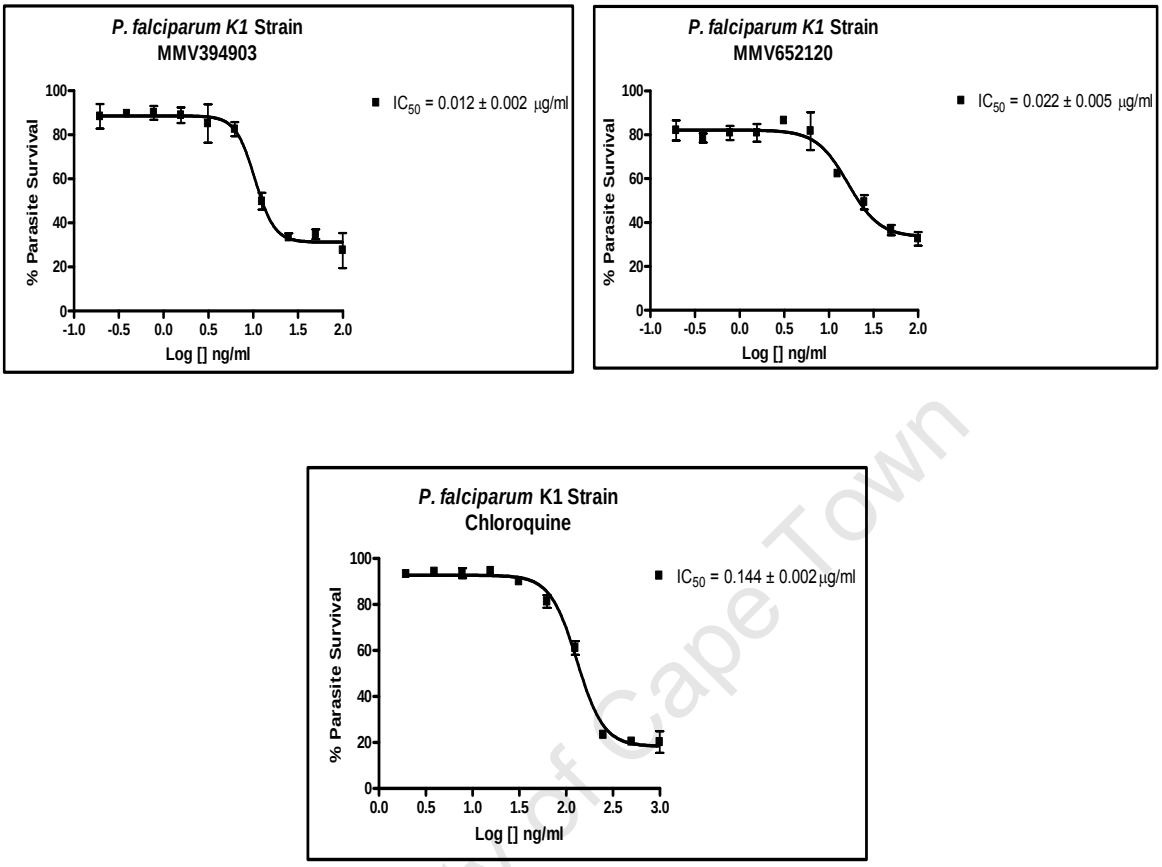
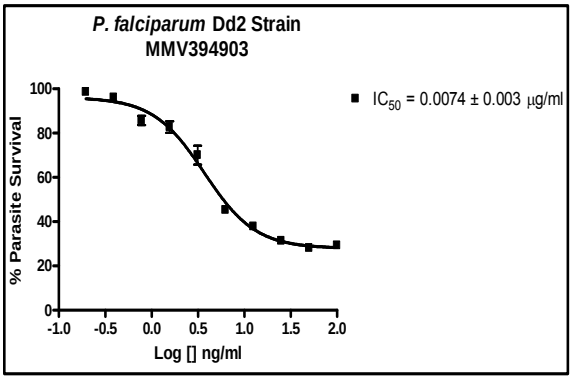
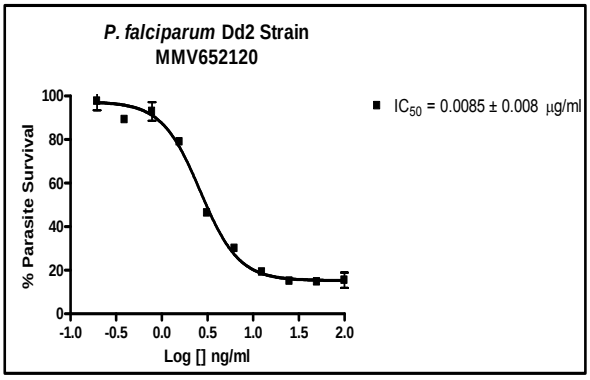


Figure 3.34: Dose response curves of the 2-aminopyridones tested against chloroquine resistant strain K1. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.





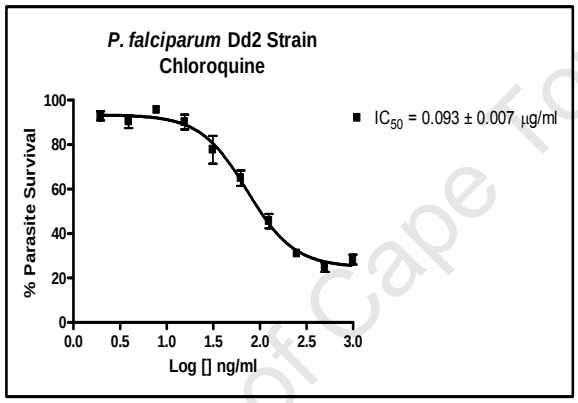


Figure 3.35: Dose response curves of the 2-aminopyridones tested against chloroquine resistant strain Dd2. Each point represents the mean ± standard deviation of three independent (n=3) experiments each performed in triplicate. For chloroquine, n=12.

MMV394903 and MMV652120 possessed antiplasmodial activity that was greater than that of chloroquine against the resistant strains with IC_{50} values of 0.012 and 0.022 µg/ml against K1 and 0.0074 and 0.0085 µg/ml against Dd2, respectively. Since these compounds were potent against chloroquine–resistant strains, cytotoxicity evaluation was performed against CHO cells as previously described. The dose response curves are presented in Figure 3.36.

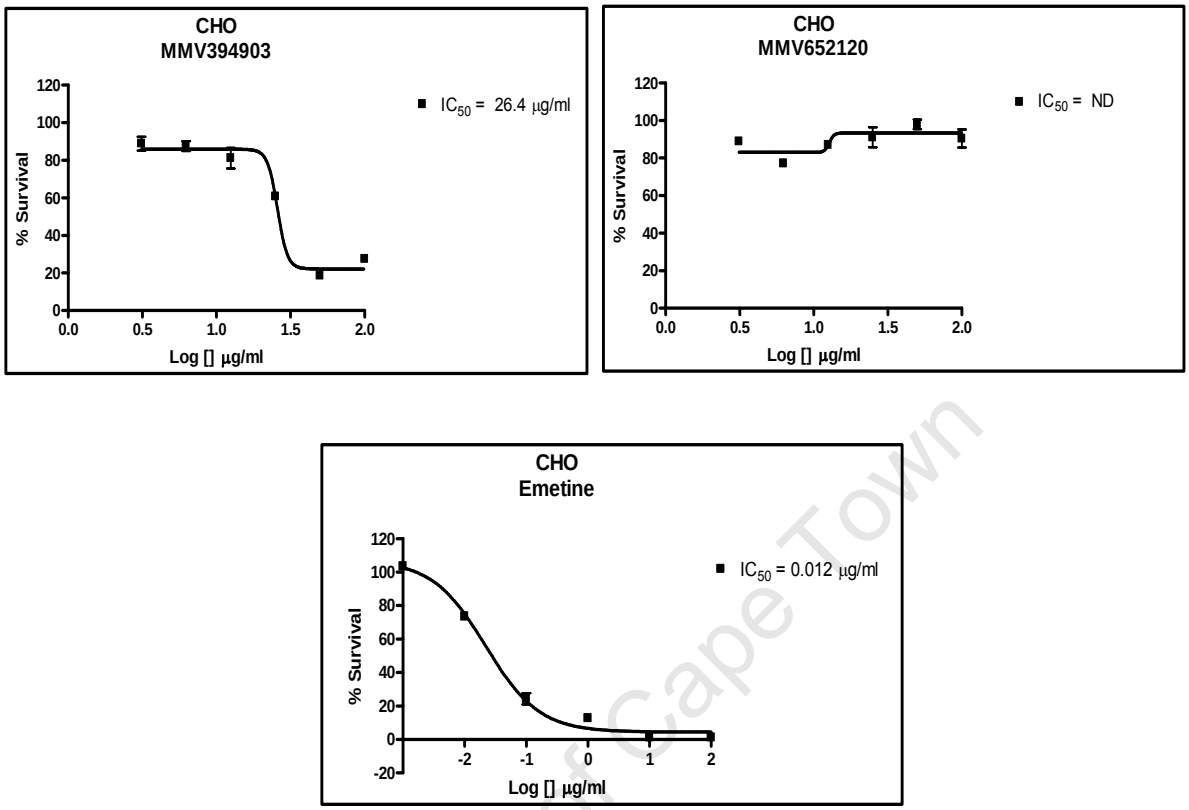


Figure 3.36: Dose response curves of the 2-aminopyridones tested against CHO cell lines for cytotoxicity evaluation. Each point represents the mean of two independent (n=2) experiments each performed in triplicate. For emetine, n=8.

The *in vitro* cytotoxicity of MMV394903 was lower than that of the reference drug emetine and the selectivity index was greater than 2031. The IC_{50} and therefore the SI, value for MMV652120 could not be determined because the cell viability in the presence of this compound exceeded 50%, even at the highest concentrations. The toxicity of these compounds was insignificant.

Table 3.7 summarizes the IC_{50} values (in µg/ml) of 2-aminopyridines that were screened for antiplasmodial activity and cytotoxicity. The P-values were calculated using the nonparametric Mann-Whitney U test in order to compare the efficacy of chloroquine with that of MMV394903 and MMV652120. The difference in efficacy of 2-aminopyridines

when compared with chloroquine is insignificant at P>0.05 in all strains as indicated in Table 3.7.

Table 3.7: The summary of IC_{50} values (µg/ml) and selectivity index (SI) values of the 2-aminopyridones compounds. The values obtained from antiplasmodial tests represent the mean of three independent experiments each performed in triplicate and the cytotoxicity values represent the mean of two independent experiments each performed in triplicate.

Test Compounds	Sensitive Strains		Resistant Strains		Cells	SI	P-Value	RI
2-Aminopyridines	**3D7**	**D10**	**K1**	**Dd2**	**CHO**			
MMV394903	0.0058	0.013	0.012	0.0074	26.4	>2031	0.2	<2.1
MMV652120	0.0058	0.014	0.022	0.0085	>100	ND	0.34	<3.4
Reference Compounds	**3D7**	**D10**	**K1**	**Dd2**	**CHO**			
Chloroquine (n=12)	**0.010**	**0.012**	**0.144**	**0.093**				
Emetine (n=8)					**0.012**			

[#]ND – Not determined, >100 – Highest concentration tested

The 3,5-diaryl-2-aminopyridines are a novel class of antimalarial compounds found to be potent against chloroquine–resistant strains, as indicated by the data presented in Table 3.7. RI values of 2.1 and 3.4 imply that the activity is caused by a different mechanism of action from that of chloroquine. Structure-activity relationship analysis of 3,5-diaryl-2-aminopyridines conducted by Younis *et al* and Cabrera *et al* also reported IC_{50} values that were comparable to that of chloroquine in the sensitive strain, but much less active when compared to artesunate, one of the most active antimalarial compounds. Amongst other things, it was established that the presence of the carboxamide and methylsulfonylphenyl groups, within the chemical structures of MMV394903 and MMV652120 in Figure 2.13, is associated with their potency against *P. falciparum* strains (González Cabrera *et al*., 2012; Younis *et al*., 2012).

Table 3.8 summarizes the IC_{50} values of all the compounds that were tested in this study.

Table 3.8: The summary of IC_{50} values (µg/ml) and selectivity index (SI) values of all compounds tested. The values obtained from antiplasmodial tests represent the mean of three independent experiments each performed in triplicate and the cytotoxicity values represent the mean of two independent experiments each performed in triplicate.

Test Compounds	Sensitive Strains		Resistant Strains		Cells	SI	P-value
AZT-CQ hybrids	**3D7**	**D10**	**K1**	**Dd2**	**CHO**		
VAK 31DS	2.25	2.55	51.15	23.93	NA*	ND#	0.029
VAK 37DS	2.61	3.30	13.97	3.68	NA	ND	0.029
VAK 59DS	3.89	1.48	16.25	5.53	NA	ND	0.029
AKAZTQ	0.150	0.54	1.70	0.517	31.6	>19	0.029
AKAZTBQ	0.081	3.30	3.30	1.71	>100	ND	0.114
Silylated AZT-CQ hybrids	**3D7**	**D10**	**K1**	**Dd2**	**CHO**		
VAK 31	0.009	0.042	0.060	0.106	7.0	>66	0.886
VAK 37	0.042	0.024	0.029	0.034	9.0	>214	1.0
VAK 59	0.048	0.041	0.033	0.036	10.5	>219	1.0
HPO-CQ hybrids	**3D7**	**D10**	**K1**	**Dd2**	**CHO**		
D13	0.035	0.026	0.277	0.254	9.8	>39	0.343
13AB.2HCl	0.025	0.075	0.055	0.047	8.3	>111	1.0
2-Aminopyridines	**3D7**	**D10**	**K1**	**Dd2**	**CHO**		
MMV394903	0.0058	0.013	0.012	0.0074	26.4	>2031	0.2
MMV652120	0.0058	0.014	0.022	0.0085	>100	ND	0.34
Reference Compounds	**3D7**	**D10**	**K1**	**Dd2**	**CHO**		
Chloroquine (n=12)	**0.010**	**0.012**	**0.144**	**0.093**			
Emetine (n=8)					**0.012**		

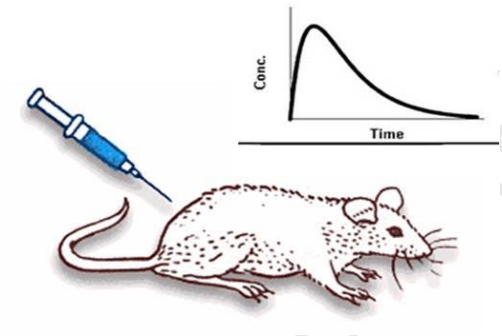
***NA – Not assayed, #ND – Not determined, >100 – Highest concentration tested**

Table 3.8 summarizes the IC_{50} values of all compounds tested in this study. The origin of the strains that were used is geographically diverse and the genetic studies of these parasites DNA also showed that they are all genetically diverse (Mu *et al*., 2002). The exact origin of 3D7 is unknown, but assumed to be of African origin. It is a cloned line from the isolate NF54 obtained from an airport worker in Amsterdam (Cowman *et al*.,

1991). D10 was cloned from the Papua New Guinean isolate FCQ-27; K1 is a clone originated from Thailand and Dd2 originated from South East Asia (Indo-China) (Elandalloussi & Smith, 2002; Mehlotra *et al*., 2008; Mu *et al*., 2002). The data presented in table 3.7 shows similarities in the efficacy of the newly synthesized antiplasmodial agents against sensitive strains (3D7 and D10). The majority of the IC_{50} values were in the same range for both strains. This may imply that the genetic diversity between the two strains does not affect the susceptibility towards the studied compounds. AKAZTQ and AKAZTBQ are the only compounds with IC_{50} values that are significantly different in 3D7 and D10. This is presumably due to the instability of these compounds as indicated by the standard deviation in Figure 3.17 and Figure 3.18. Since storage may have affected the activity of these compounds, the working solutions were prepared on the day of the experiment. The resistant strains on the other hand varied in their sensitivity towards the test compounds. Dd2 is classified as moderately resistant to chloroquine while K1 is known to be a multidrug resistant strain with reported resistance against chloroquine, cycloguanil and pyrimethamine (Daher *et al*., 2006; Vivas *et al*., 2007). K1 was more sensitive to the silylated AZT-CQ hybrids and the 2-aminopyridines, thus making these compounds potential candidates for further assessment.

The prerequisite for testing potent compounds for *in vivo* efficacy is their selective antiplasmodial activity. It is important to know that the effect of the drug is only due to parasite death in its presence and not an indirect effect resulting from toxicity towards mammalian cells. Therefore determining cytotoxicity is important to avoid false positive and/or misleading results (Ayisi *et al*., 2011). Selective index values for these compounds were determined in order to demonstrate their selectivity. All values were greater than 2 and the lowest value was observed for D13 with SI of 39. This was an indication that the cytotoxicity of all the compounds was very low and that the antiplasmodial activity is not due to a general cytotoxic effect. Pharmacokinetic properties of active compounds were evaluated and this is discussed in the following chapter.

Chapter 4: Pharmacokinetic Evaluation of Promising Antimalarial Compounds in a Mouse Model



Pharmacokinetic Evaluation of Promising Antimalarial Compounds in a Mouse Model

4.1. Introduction

The previous chapter discussed *in vitro* antiplasmodial and cytotoxic activity of synthetic compounds. It is noteworthy that *in vitro* tests do not represent the real physiological environment and are restricted in their capacity to mimic whole animal metabolism and distribution. Most hit molecules identified from *in vitro* tests fail in the preclinical stage due to lack of adequate *in vivo* efficacy in animal models because of poor pharmacokinetic (PK) properties. Studies have shown that compounds with favorable PK properties are more likely to be efficacious (Lee, 2003). Since *in vivo* PK studies provide insight into complex biological systems, evaluation of drug candidates in animal models during pre-clinical studies generates information on the suitability of the test compound as a drug candidate with respect to bioavailability and clearance after dosage. PK studies also facilitate correlation of drug concentration at the site of action with pharmacological response (Ingelse *et al*., 2008; Li *et al*., 2013).

In pre-clinical drug discovery, animal PK studies are often performed in mice. Mice are an excellent animal model for studying human biology and behavior because of their close phylogenetic relation to humans (Sibal & Samson, 2001). For this reason the mouse became the animal model of choice in this study to perform initial pharmacokinetic studies of compounds discussed in the previous chapters in order to demonstrate exposure (or lack thereof) prior to conducting efficacy studies. Compounds VAK31, VAK37, VAK59, AKAZTQ, AKAZTBQ, D13, 13AB.2HCl, MMV394903 and MMV652120 had *in vitro* antimalarial activity that was comparable to that of chloroquine, as described in chapter 3, therefore, were selected for pharmacokinetic evaluation.

4.2. Materials and Methods

Male and female C57BL/6 mice (20-30g) were obtained from the University of Cape Town Medical School Animal Unit. The mice were housed in well ventilated cages at room temperature (approximately 22^{o}C) and 60% relative humidity with constant supply of food and water and were monitored twice daily. The study was authorized by the Faculty of Health Science Animal Research Ethics Committee before its commencement; Reference No. 012/020. All the work was performed according to the guidelines established by Austin and colleagues, 2004.

4.2.1. Pharmacokinetic studies

4.2.1.1. Test compound formulations

Preparation of a reference formulation

The compounds were dissolved in DMSO and diluted further with phosphate buffered saline (PBS) to give a final DMSO concentration of 1%.

Preparation of water-in-oil (o/w) emulsions

Compounds with low solubility were also prepared in oil-in-water (o/w) microemulsions which consist of oil droplets dispersed in water with the aid of surfactant and a cosurfactant for improved oral bioavailability (Chern & Liu, 2000). This type of oral delivery system is designed to enhance oral bioavailability of poorly water-soluble drugs with potential therapeutic effects by improving their solubility. Surfactants are used as absorption enhancers because they can increase wetting and aqueous dissolution rates of drug compounds (Kim and Park, 2004). Addition of oil and water can lead to the formation of a clear and stable oil-in-water microemulsion system, which can be combined with a co-surfactant (Lawrence & Rees, 2000).

In this study Tween 80 (Sigma) and Span 80 (Sigma), with hydrophile-lipophile balance (HLB) of 15 and 4.3, respectively, were used as surfactants for o/w emulsions. HLB is value that is based on whether a substance is water soluble (high HLB value greater than 10) or oil soluble (low HLB less than 10). Span 80 was used with another

surfactant, pluronic F68 (Sigma) because of its low HLB ratio. Oils are used because of their ability to solubilize water-insoluble compounds and in this study oleic acid (Sigma) and ethyl linoleate (Sigma) were used as oils (Georges *et al.*, 1987). Co-surfactants are used to increase the solubilizing power of the surfactant system and these are mainly alcohols, amines and ether-alcohols. Ethanol (Merck) and isopropyl alcohol (Sigma) were used as co-surfactants. Alcohols are small molecules (containing less than 8 carbons) and therefore have a greater affinity for oil/water interface. For the purpose of this study ethanol and isopropyl alcohol were used because of their short chain in order to form a mixture that is less viscous, which is easier to administer to mice. Longer chain alcohols lead to a higher viscosity (Kaur *et al*., 2012). Two microemulsion formulations were prepared according to the composition described in publications by Gondogdu and co-workers and Wu and co-workers with modifications in the composition of the formulations (Gundogdu *et al.*, 2011; Wu, Wang & Que, 2006). The compositions used in this study have a varying aqueous content and are described in Table 4.9.

Table 4.9: The composition of the optimal microemulsion formulations.

Formulation	Oil (%)	Surfactant 1 (%)	Surfactant 2 (%)	Cosurfactant (%)	Aqueous
Microemulsion A	**Ethyl linoleate**	**Tween 80**	**N/A**	**Ethanol**	**Water**
	5	11		4	80
Microemulsion B	**Oleic Acid**	**Span 80**	**Pluronic F68 (20% in PBS)**	**Isopropyl alcohol**	**PBS**
	20	20	12.5	10	37.5

N/A: Not applicable

Emulsion characterization studies were not performed in this study. These include particle size analysis to measure droplet size and particle size distribution, ξ potential to measure surface droplet to assess stability of emulsion, and pH which are usually performed when preparing microemulsions.

4.2.1.2. Test drug administration

The PK study involved the oral dosing (200 µl) of 10 week old C57BL/6 male mice at 20 mg/kg and intravenous dosing (100 µl) via the dorsal penile vein at 2 to 4 mg/kg under anesthesia. For oral dosage a gavage needle was used for the administration of test compounds directly into the lower esophagus or stomach. This type of needle has a ball tip that makes them less traumatic on delicate oral and esophageal tissues and reduces the chance of introducing the needle into the larynx. It also minimizes spillage of the low volume of test compounds which was easily introduced to the mouth and throat (Ryan & Vandenbergh, 2006).

4.2.1.3. Blood sampling

Initial PK studies were performed on groups of three animals to estimate the pharmacokinetic properties of the test compounds. Blood samples were collected at 5 to 20 µl serially by needle prick on the tail vein near to the tip of the tail at 0.5, 1, 2, 4 and 6 hours post-dosing. For comprehensive PK studies groups of 5 animals were used and blood samples were collected 0.17, 0.5, 1, 2, 3, 5, 7 and 9 hours post-dosing. Serial sampling technique was used in which 20-30µl of blood was drawn from the tail vein. The disadvantage of using serial blood sampling is that mice are small in size and that causes difficulties when collecting blood over time from a single mouse. This could also compromise animal physiology and result in unreliable pharmacokinetic data. Alternatively, parallel blood sampling can be used which can result in dosing large number of animals for a full pharmacokinetic profile because each animal is subject to only one blood draw. It can also produce suboptimal quality of pharmacokinetic data caused by animal variability and dosing error because of having one animal per time point. Therefore, serial blood sampling was a preferred method of blood sampling in this study because it provides quality data and allows comparison of inter-animal differences in PK profiles (Jun Tang & Redmond, 2008; Kurawattimath *et al*., 2012; Peng *et al*., 2009; Zhu *et al*., 2005). Lithium heparin coated MiniCollect® Plasma Tubes (Lasec SA) were used to collect the blood samples. Heparin is an anticoagulant that prevents blood from clotting by binding and activating antithrombin, thus inhibiting the coagulation

cascade. The collected blood samples were placed on ice immediately after sampling, were briefly vortexed and then stored at -80^{o}C until analysis.

4.2.1.4. LC-MS/MS Assay

A sensitive and specific liquid chromatography coupled to mass spectrometry (LC-MS/MS) method was developed for the analysis of nine antimalarial compounds that were evaluated for pharmacokinetic properties. The combination of liquid chromatography and mass spectrometry offers the advantages of both liquid chromatography, as a powerful and versatile separation technique and mass spectrometry, as a powerful and sensitive detection and identification technique. Another advantage of LC-MS/MS is that it can be automated, which allows high throughput (Li *et al*., 2013; Koren *et al*., 2012; Sosa-Ferrera, Mahugo-Santana & Santana-Rodríguez, 2012). For method development the compounds were prepared at 1mg/ml stock in methanol and were further diluted with 50% acetonitrile: 0.1% formic acid to make a final concentration of 1µg/ml.

Sample preparation

Prior to analysis, the dosed blood samples were thawed and brought to room temperature and then mixed by vortex to ensure homogeneity. The samples were precipitated according to the procedure described in table 4.4.

Table 4.10: Procedure for sample precipitation

Reagent	Volume (µl)
Thawed whole blood collected from the mice	20
Milli-Q water	50
Acetonitrile	150
Vortex for 15 seconds and sonicate for 10 minutes	
Vortex for 15 seconds and centrifuge for 5 minutes @ 13000 x g	
Transfer supernatant to a flat bottom glass insert in glass vial and place it in the LC autosampler for analysis	

Analyst software version 1.5.2 (AB Sciex) was employed for data acquisition, peak-area integration and quantitation of compounds in blood samples. Calibration curves were

derived in each analytical run in duplicate and were employed to extrapolate blood concentration of the compounds tested. The calibration curves are presented in Appendix A. LC-MS/MS analysis was done in the Division of Clinical Pharmacology by Miss Alicia Evans.

Chromatography

The LC system employed was an ultra fast liquid chromatography (UFLC) system (Shimadzu) and the separation of the compounds was performed on a Phenomenex, Luna 5 µm PFP(2), 100 Å, 50 mm × 2 mm analytical column. The mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of acetonitrile. The flow rate was set at 500 µl/min and the temperature of the column was maintained at 40^{o}C. Two gradient systems were used:

A. For the separation of VAK31, VAK37, VAK59, AKAZTQ, AKAZTBQ, D13 and 13AB.2HCl the mobile phase B was increased from 5% to 95% B over 4 min, after that, phase B was returned to 5% within 0.1 minutes, then equilibrated for 3 minutes.

B. For the separation of MMV394903 and MMV652120 the mobile phase was increased from 5% to 95% B over 2 minutes and returned to 5% B within 0.1 minutes, then equilibrated for 3 minutes. The chromatograms are presented in Appendix A.

Detection

The MS system consisted of an API 3200 Q-Trap hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex) which was operated at unit resolution in the multiple reaction monitoring (MRM) mode. The calibration range for all the compounds was between 7.8 ng/ml and 1000 ng/ml and the accuracy (%Nom) for the calibration curves was between 90.31% and 104.0%. Table 4.11 and Table 4.12 give an overview of the MS parameters and the instrument settings and the MS/MS spectra is presented in Appendix A.

Table 4.11. Mass spectrometer settings

Parameter	Setting
Source temperature	500°C
Curtain gas	25 psi
Gas 1	50 psi
Gas 2	70 psi
CAD gas	Medium
Ion spray voltage	5500 kVolts
Ionization mode	Positive

Table 4.12: Important MS parameters used for the detection of all the test compounds on an API 3200 Q-Trap.

Compounds analyzed	Q1 (Da)	Q3 (Da)	Dwell time (msec)	DP	CE (volts)	EP (volts)	CXP (volts)
VAK31	727.3	222.2	40	101	53	10	4
VAK37	769.4	307.2	40	56	55	10	4
VAK59	797.3	335.3	40	111	63	10	4
AKAZTQ	472.2	331.2	40	56	27	10	4
AKAZTBQ	534.2	393.2	40	66	31	10	4
D13	531.2	91.1	40	91	89	10	2
13AB.2HCl	490.2	261.2	40	61	73	10	2
MMV394903	428.1	227.2	40	81	77	10	4
MMV652120	408.9	315.2	40	101	55	10	2

Q1 = protonated mass of compound; Q3 = mass of fragment ion; DP = declustering potential; CE = collision energy; EP = entrance potential; CXP = collision cell exit potential

4.3. Results and discussion

4.3.1. AZT-CQ hybrid compounds

AZT-CQ hybrid compounds and intermediate compounds were evaluated *in vitro* for antiplasmodial activity, as discussed in the previous chapter. Of the eight compounds that were evaluated, only intermediates with silyl group possessed potent activity, presenting most activity against chloroquine resistant strains K1 and Dd2. These are VAK31, VAK37 and VAK59 and the calculated IC_{50} values were in the range of 33 to 60 ng/ml for K1 and 34 to 106 ng/ml for Dd2. However, the activity of AZT-CQ hybrids (desilylated) was 10 to 100 fold less than the silylated hybrids, therefore, of the five desilylated AZT-CQ, only two compounds were selected for pharmacokinetic evaluation, namely AKAZTQ and AKAZTBQ, with IC_{50} values in the range of 1.7 to 3.3 µg/ml and 0.5 to 1.7 µg/ml against chloroquine resistant strains K1 and Dd2, respectively. Even though the activity of these two compounds was much lower than that of chloroquine against chloroquine resistant strains, they were included in this study in order to compare their pharmacokinetic properties with those of the silylated AZT-CQ hybrids.

Silylated AZT-CQ hybrid compounds

VAK31, VAK37 and VAK59 prepared with the reference formulation were administered to mice in a snapshot PK study. The mice were dosed at 20 mg/kg orally and the blood samples collected were submitted for bioanalysis by LC-MS/MS. The compounds could not be detected in the blood because the concentrations were below the limit of quantitation (LOQ). In order to potentially improve the bioavailability of these compounds, formulations A and B, which consist of different aqueous contents as described in Table 4.9, were prepared. There was no improvement observed in the bioavailability of VAK 31 and VAK 37. The concentrations in the blood remained below LOQ and therefore could not be detected. VAK59 was the only compound detected and the concentrations were slightly above the LOQ and the concentration levels are presented in Table 4.13. Figure 4.37 shows the mean concentration vs time profiles of VAK59 in formulations A and B obtained after the mice were dosed orally in a PK study.

Table 4.13: Blood concentration levels of VAK59 in o/w microemulsion formulation A and B in PK study. Data represent mean ± standard deviation data points obtained from three mice.

Time (hours)	[VAK59] ng/ml	
	Formulation A	Formulation B
0	0	0
0.5	15.87 ± 5.2	12.45 ± 0.5
1	19.10 ± 0.6	21.25 ±1.1
2	16.67 ± 2	17.90 ± 1.8
4	14.80 ± 4.7	14.49 ± 5.2
6	8.05 ± 3	11.09 ± 4
8	*5.78 ± 2	7.45 ± 2.5

***Concentration levels were extrapolated from the calibration curves in Appendix A**

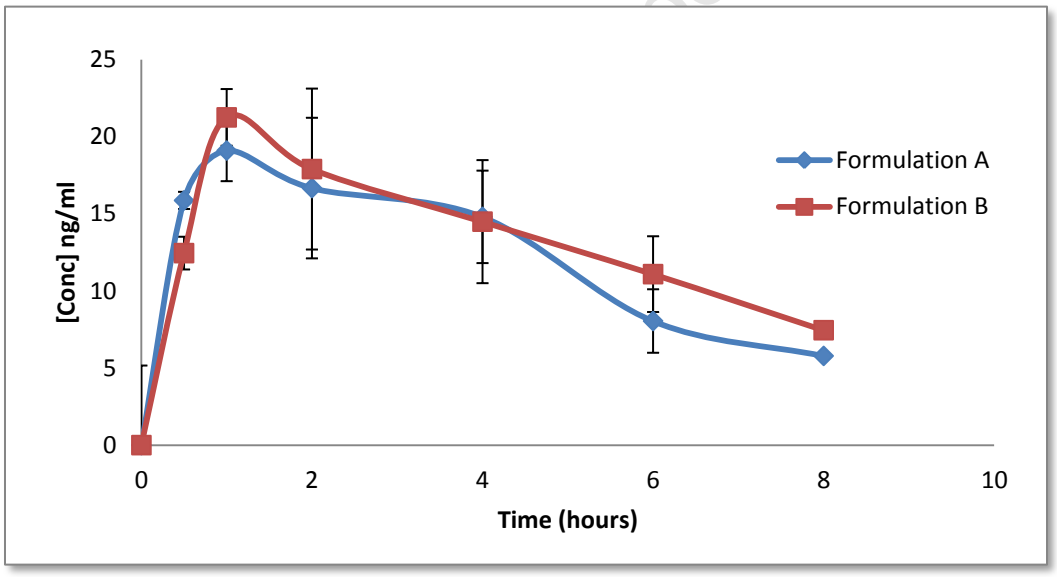


Figure 4.37: Blood concentrations of VAK 59 in C57BL/6 mice blood after oral administration of 20 mg/kg in microemulsion formulation A and B. Data represent mean ± standard deviation of data points obtained from three mice.

The preparation of VAK59 in formulations A and B caused a slight increase in the blood concentrations. This procedure was repeated using an intravenous dosage at 4 mg/kg. Formulation B contained less aqueous content, which resulted in animal toxicity when

dosed intravenously. For this reason only formulation A was used for intravenous administration. Table 4.14 and Figure 4.38 shows the mean concentrations in mice blood after intravenous administration of 4 mg/kg VAK59 in formulation A.

Table 4.14: Blood concentrations of VAK59 following an IV dosage in a comprehensive PK study. Data represent mean ± standard deviation of data points obtained from five single mice.

Time (hours)	[VAK59] ng/ml
0.17	1183.33 ± 102
0.50	695.67 ± 186
1	377.00 ± 62.6
2	226.67 ± 28.7
3	114.70 ± 67.7
5	97.17 ± 57.7
7	63.97 ± 30.6
9	60.17 ± 24

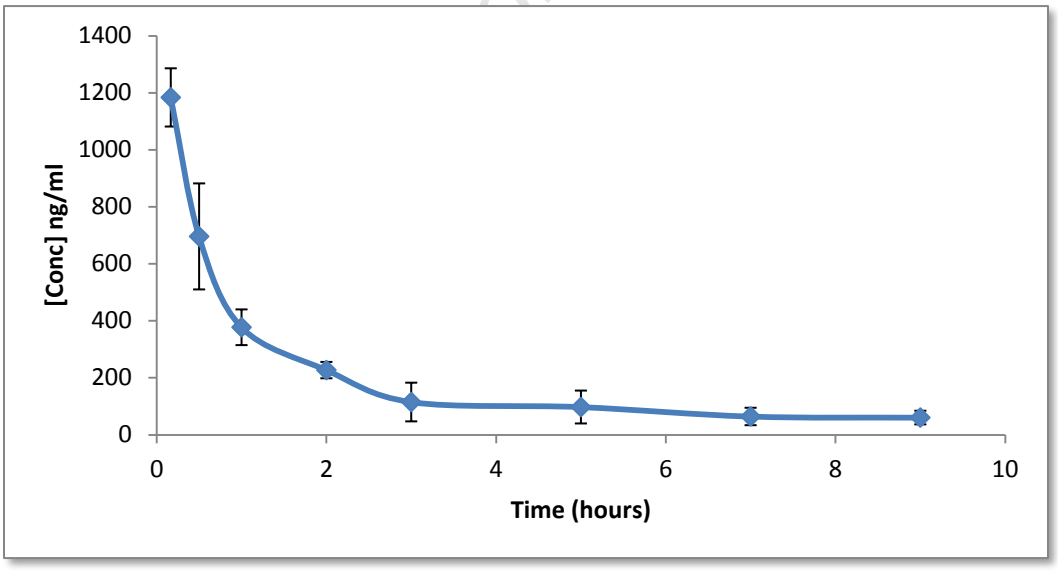


Figure 4.38: Blood concentrations of VAK 59 in C57BL/6 mice blood after intravenous administration of 4 mg/kg. Data represent mean ± standard deviation of data points obtained from five mice.

The mean concentration-time data was used to calculate the PK parameters by non-compartmental analysis using PK Solutions 2.0 Pharmacokinetic Analysis Software (Summit Research Services, Montrose, USA). The PK parameters are given in Table 4.15.

Table 4.15: Pharmacokinetic parameters of VAK59 compounds after administration in formulations A and B. Data represent mean ± standard deviation of data points obtained from five single mice.

Parameters		$T_{1/2}$ (hours)	T_{Max} (hours)	C_{Max} (ng/ml)	Vd (L/kg)	Vss (L/kg)	CL (ml/min/kg)	$AUC_{0-\infty}$ hr.ug/L	BA (%)
VAK59	Ref	No Data							
	Oral A	3.0 ± 0.7	1	19 ± 0.6	N/A			124.3 ± 14	1.3
	Oral B	4.8 ± 1.8	1	21.3 ±1.1	N/A			144.9 ± 30	3.3
	IV	0.6 ± 0.1	N/A		24.6 ± 11.2	51.6 ± 34	32.08 ± 8	2183 ± 475	N/A

N/A: Not applicable; No Data: Blood concentrations were below LOQ; $T_{1/2}$, *half-life;* T_{max}: Time to reach C_{max}; C_{max}: Peak plasma concentration $AUC_{0-\infty}$: Area under the curve; CL: Plasma clearance; V_d: Apparent volume of distribution V_{ss}: Volume of distribution at steady state; BA: Bioavailability

The data presented in Table 4.15 shows that even though the preparation of VAK59 in formulations A and B cause an increase in the oral bioavailability in mice, the PK properties still remain poor. The C_{max} values were between 19 and 21 ng/ml and AUC values of 124 to 145 hr.µg/L for formulations A and B, respectively. These values were very low; hence the oral bioavailability was also low, with values between 1.3 and 3.3. To compare the difference in the influence of the two formulations used, a nonparametric Mann-Whitney U test was performed using the statistical functions of GraphPad Prism 4. A P-value obtained by the two-tailed test for VAK59 in formulations A and B was considered insignificant at P=0.49 (P>0.05).

Following IV dosage, high mean concentration of VAK59 in mice blood (1200 ng/ml) was observed (Figure 4.38). The data in Table 4.15 also shows a very short half-life of 0.6 and it is possible that the high clearance rate of 32.1 ml/min/kg and high volume of distribution of 24.6 L/kg influenced the short half-life of VAK59. However, VAK59 possessed high *in vitro* antiplasmodial activity against chloroquine sensitive and resistant strains with IC_{50} values in the range of 33 to 48 ng/ml. Therefore, VAK59 was selected for *in vivo* efficacy investigation. Even though the oral bioavailability was very

low it would be interesting to investigate whether the high blood concentration of this potent compound will reduce parasitemia and to investigate how the parameters presented in Table 4.15 will influence the efficacy of VAK59 in mice following IV administration.

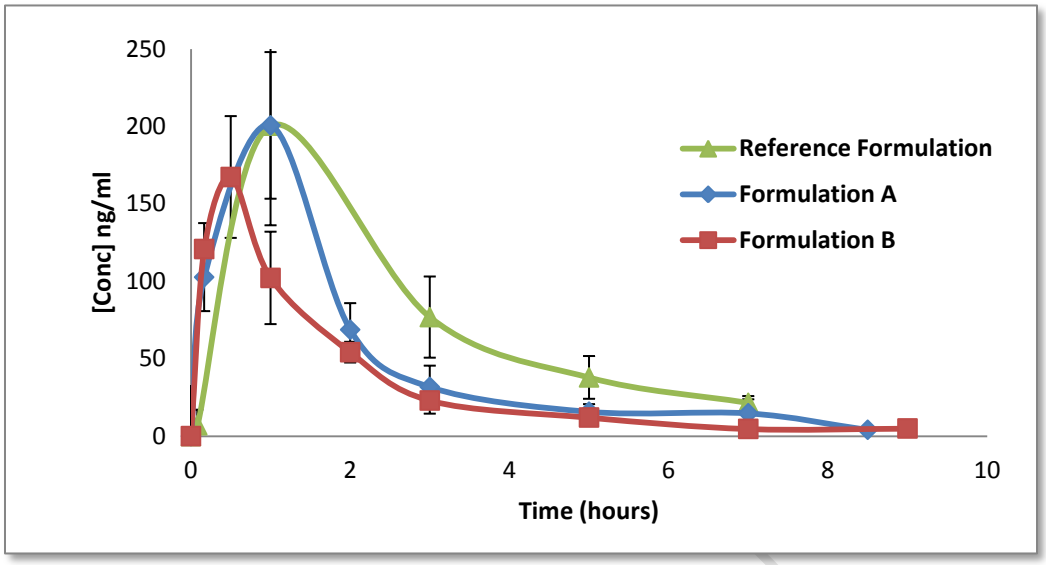
Desilylated AZT-CQ hybrid compounds

Chapter 3 discussed the chemical structures of silylated AZT-CQ hybrid compounds and how the presence of *tert*-butyldiphenylsilyl, a bulky protective group, affected their solubility. In the absence of this group, antiplasmodial activity was less, as discussed in the previous chapter albeit there was enhanced solubility. In order to establish whether or not solubility of the desilylated AZT-CQ hybrid compounds will translate into improved bioavailability, a PK study was performed on AKAZTQ and AKAZTBQ. The test compounds were prepared with the reference formulation were administered orally at 20 mg/kg in PK study. The mice were also dosed at 20 mg/kg orally with compounds prepared in formulations A and B to further enhance their solubility and at 4 mg/kg intravenously in formulation A. The blood concentrations measured are listed in Table 4.16 for AKAZTQ and Table 4.17 for AKAZTBQ and their mean concentration vs time profiles after oral and intravenous administration are presented in Figure 4.39 and Figure 4.40.

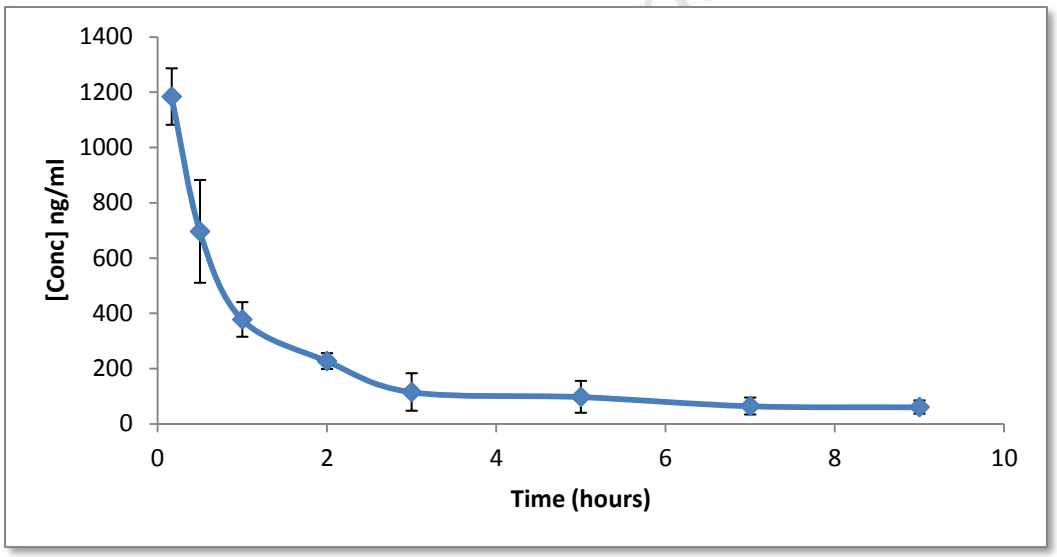
Table 4.16: Blood concentrations (ng/ml) of AKAZTQ following an oral and IV dosage in a PK study. Data represent mean ± standard deviation of data points obtained from five mice. For the reference formulation n=3.

Time (hours)	AKAZTQ (Oral @ 20 mg/kg)			AKAZTQ (IV @ 4 mg/kg)
	Reference	Formulation A	Formulation B	Formulation A
0	0	0	0	**N/A***
0.17	7.03 ± 10	102.67 ± 22.0	120.83 ± 32.3	537.67 ± 136.9
0.50	ND	ND**	167.33 ± 16.7	340.00 ± 86.8
1	200.50 ± 64.4	200.67 ± 47.4	102.17 ± 39.3	214.00 ± 27.4
2	ND	68.73 ± 17.1	54.20 ± 29.8	114.47 ± 23.5
3	76.87 ± 26.2	31.77 ± 13.7	23.00 ± 6.7	73.77 ± 28.1
5	37.90 ± 13.8	15.80 ± 4.9	12.02 ± 8.5	72.13 ± 32.3
7	21.53 ± 4.3	14.78 ± 9.0	#4.67 ± 2.9	45.07 ± 6.8
9	ND	#4.10 ± 1.4	#4.93 ± 1.6	36.77 ± 18.3

***N/A: Not Applicable; **ND: Not Determined**
#Concentration levels were extrapolated from the calibration curves in Appendix A



(a)



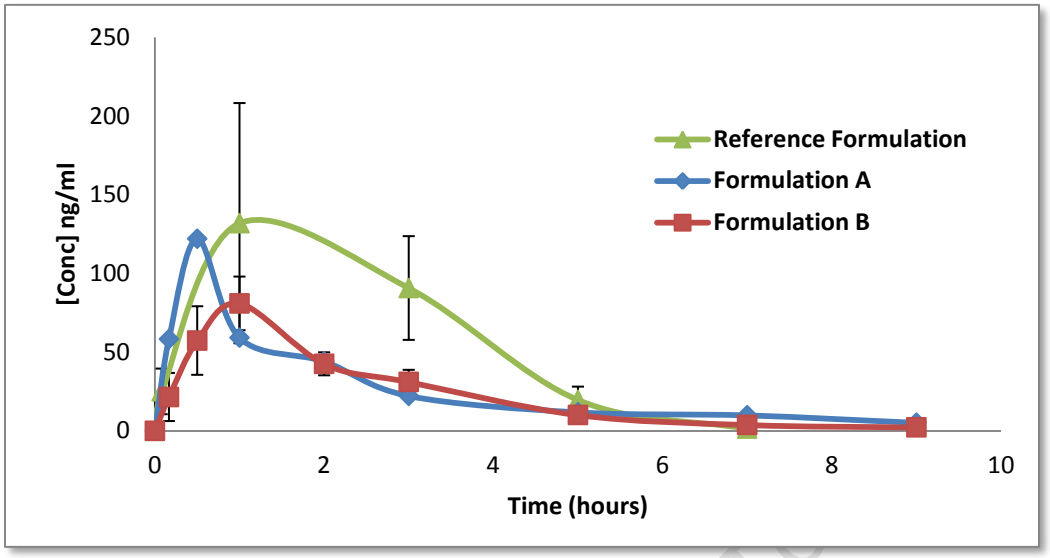
(b)

Figure 4.39: Blood concentration of AKAZTQ in C57BL/6 mice blood after oral administration of 20 mg/kg and intravenous administration of 4 mg/kg compound. Data represent mean ± standard deviation of data points obtained from five mice. For the reference formulation n=3

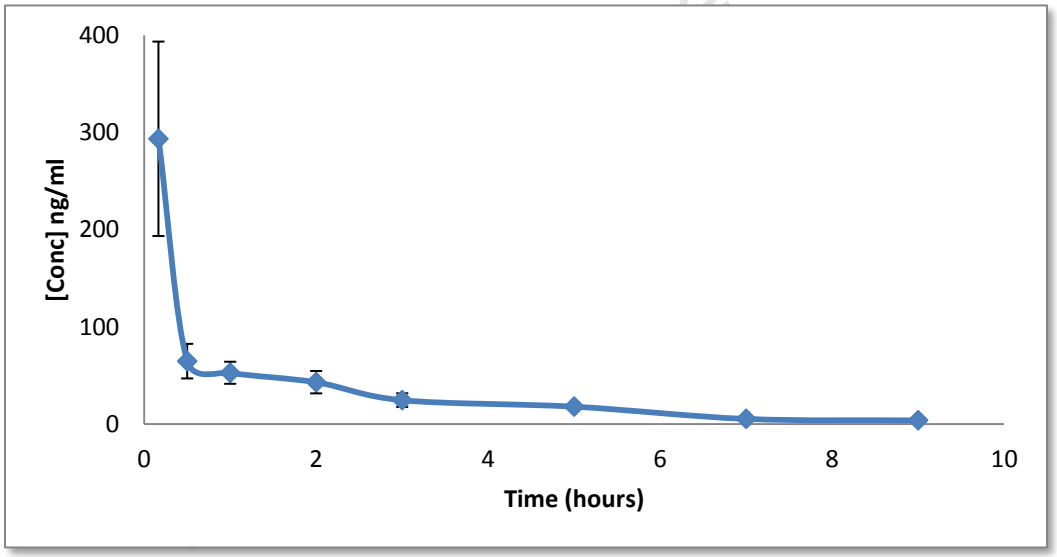
Table 4.17: Blood concentration levels of AKAZTBQ following an oral and IV dosage in a comprehensive PK study. Data represent mean ± standard deviation of data points obtained from five single mice. n=1 for formulation A and n=3 for the reference formulation.

Time (hours)	AKAZTBQ (Oral @ 20 mg/kg)			AKAZTBQ (IV @ 4 mg/kg)
	Reference	Formulation A	Formulation B	Formulation A
0	0	0	0	**N/A***
0.17	25.03 ± 14.5	58.30	21.47 ± 18.9	293.33 ± 100.0
0.50	ND**	122.00	57.33 ± 15.3	64.73 ± 17.8
1	131.90 ± 76.3	59.20	80.93 ± 21.8	52.80 ± 11.3
2	ND	44.10	42.55 ± 17.0	43.10 ±11.5
3	90.70 ± 33	22.20	31.07 ± 7.3	24.67 ± 7.0
5	19.67 ± 8.4	11.80	10.07 ± 7.7	18.03 ± 2.5
7	#1.42 ± 1.3	9.90	#3.76 ± 2.1	#5.46 ± 2.6
9	ND	#5.16	#2.22 ± 1.0	#3.97 ± 3.0

***N/A: Not Applicable; **ND: Not Determined**
#Concentration levels were extrapolated from the calibration curves in Appendix A



(a)



(b)

Figure 4.40: Blood concentrations of AKAZTBQ in C57BL/6 mice blood after oral administration of 20 mg/kg and intravenous administration of 4 mg/kg compound. Data represent mean ± standard deviation of data points obtained from five mice. For the reference formulation n=3.

The mean concentration-time points were used to calculate the PK parameters and these are listed in Table 4.18.

Table 4.18: Pharmacokinetic parameters of desilylated hybrid compounds after administration in formulations A and B. Data represent mean ± standard deviation of data points obtained from five mice.

Parameters		$T_{1/2}$ (hours)	T_{Max} (hours)	C_{Max} (ng/ml)	Vd (L/kg)	Vss (L/kg)	CL (ml/min/kg)	$AUC_{0-\infty}$ hr.ug/L	BA (%)
AKAZTQ	**Ref**	2.6 ± 1.3	1	200.5±64	**N/A**			633.1 ±59	12.4
	Oral A	3.6 ± 2.3	1	200.7±47	**N/A**			255.1 ± 6	10.0
	Oral B	1.86 ± 0.5	0.5	167.3±17	**N/A**			191 ± 51	9.5
	IV	0.8 ± 4	**N/A**	**N/A**	21 ± 14.6	26.3 ± 20	44.9 ± 5	192 ± 23	**N/A**
AKAZTBQ	**Ref**	0.7 ± 0.1	1.7 ± 1.2	138 ± 70	**N/A**			429.4 ±144	30.1
	Oral A	2.34	0.5	122	**N/A**			240.1	16.85
	Oral B	1.5 ± 1.8	1	80.9 ± 22	**N/A**			215 ± 9.1	17
	IV	0.5 ± 0.4	**N/A**	**N/A**	45 ± 11.3	55.3 ± 13	254 ± 75	40 ± 11	**N/A**

N/A: Not applicable; $T_{1/2}$, half-life; T_{max}: Time to reach C_{max}; C_{max}: Peak plasma concentration $AUC_{0-\infty}$: Area under the curve; CL: Plasma clearance; Vd: Apparent volume of distribution V_{ss}: Volume of distribution at steady state; BA: Bioavailability

The data presented in Table 4.18 compares the PK parameters of AKAZTQ and AKAZTBQ prepared in the reference formulation and in formulations A and B. For AKAZTQ, a shift in T_{max} values is observed, with formulation B presenting a T_{max} of 0.5 hours, which indicates that this formulation facilitated a rapid absorption of AKAZTQ when compared to the reference formulation and formulation A which had a T_{max} values of 1 hour. Formulation B also caused a decrease in C_{max} values when comparing with those of the compound prepared in the reference formulation and formulation A, with formulation B presenting a C_{max} value of 167 ng/ml and the reference formulation and formulation A had a C_{max} value of 201 ng/ml. The $AUC_{0-\infty}$ value of the reference formulation was higher than that of formulation A and B, which were 255 and 191 hr.ug/L for formulation A and B, respectively. Therefore, this caused the oral bioavailability of AKAZTQ to decrease when prepared in formulation A and B, as observed with the values presented in Table 4.18. The half-life of formulation A was 3.6 hours which was longer than that of the reference formulation and formulation B at 2.6 and 1.9 hours, respectively. To compare the difference in the influence of the two formulations used, a nonparametric Mann-Whitney U test was performed using the statistical functions of GraphPad Prism 4. A P-value obtained by the two-tailed test for AKAZTQ prepared in the reference formulation and formulations A and B was considered insignificant at P=0.84 (P>0.05).

For AKAZTBQ a similar trend was observed wherein the reference formulation has a higher (30%) bioavailability as indicated in Table 4.18. However, the oral bioavailabilities of AKAZTBQ prepared in formulations A and B were similar with a %BA value of 17%. C_{max} value of the reference formulation was 138 ng/ml, which was higher than those of formulations A and B at 122 and 81 ng/ml, respectively. The $AUC_{0-\infty}$ value was also higher for the reference formulation at 429.4 hr.ug/L, hence a greater oral bioavailability was observed. The T_{max} values show a slower absorption of AKAZTBQ in the reference formulation than formulation A and formulation B with T_{max} values of 1.7 hours for the reference formulation. The preparation of AKAZTBQ in microemulstion formulations caused faster absorption of the compound, hence the T_{max} were earlier at 1 and 0.5 hours. The half-life of formulation A was 2.3 and was longer than that of the reference formulation and formulation B with values of 0.7 and 1.5 hours, respectively. The P value for AKAZTBQ prepared in reference formulation and microemulsion formulations A and B was also insignificant at P=0.55 (P>0.05).

In the IV data, both AKAZTQ and AKAZTBQ had short half-lives of 0.8 and 0.5 hours, respectively. This is presumably caused by the high clearance rates and volumes of distribution. The clearance rates were 45 and 254 ml/min/kg and the apparent volumes of distribution were 21 and 45 L/kg for AKAZTQ and AKAZTBQ, respectively.

The increase in the bioavailabilty of AKAZTQ and AKAZTBQ suggests that solubility-limited absorption may have contributed to the poor bioavailability of poorly soluble silylated compounds when comparing the data presented in Table 4.15 and Table 4.18. The PK parameters of the desilylated AZT-CQ hybrid compounds suggest that the *tert*-butyldiphenylsilyl group in the silylated AZT-CQ hybrid compounds affected the oral bioavailability of this class of compounds. The efficacy of AKAZTQ and AKAZTBQ was 50 to 100 fold less than that of silylated AZT-CQ hybrid compounds when evaluated *in vitro*. Therefore, with low efficacy, low C_{max} values and a short half life, the compounds were deemed poor candidates for *in vivo* efficacy.

4.3.2. HPO-CQ hybrid compounds

In the previous chapter, D13 and 13AB.2HCl were assessed for antiplasmodial activity and the IC_{50} values were in the range of 25 to 277 ng/ml. Since compounds were active against chloroquine sensitive and resistant strains *in vitro*, their PK properties were evaluated.

D13 and 13AB.2HCl were administered to mice orally and intravenously at 20 mg/kg and 2 mg/kg prepared with the reference formulation, respectively, in an initial PK study to determine their oral bioavailability. Table 4.19 shows blood concentrations of D13 and 13AB.2HCl following oral and intravenous administration and the mean concentration vs time profiles are presented in Figure 4.41.

Table 4.19: Blood concentrations (ng/ml) of D13 and 13AB.2HCl following an oral and IV dosage in an initial PK study. Data represent mean ± standard deviation of data points obtained from three single mice. For 13AB.2HCl n=1.

Time (hours)	D13		13AB.2HCl
	Oral @ 20 mg/kg	IV @ 2 mg/kg	IV @ 2 mg/kg
0	0	**N/A***	**N/A**
0.08	ND**	88.30 ± 8.8	259.50 ± 96.4
1	14.60 ± 11.7	56.83 ± 9.9	25.66 ± 23.0
3	8.83 ± 6.4	55.97 ± 7.4	7.68 ± 3.7
5	#5.67 ± 9.2	31.33 ± 7.5	6.63 ± 1.7
7	0	12.19 ± 3.5	7.42 ± 0

***N/A: Not Applicable; ****ND: Not Determined**
#Concentration levels were extrapolated from the calibration curves in Appendix A

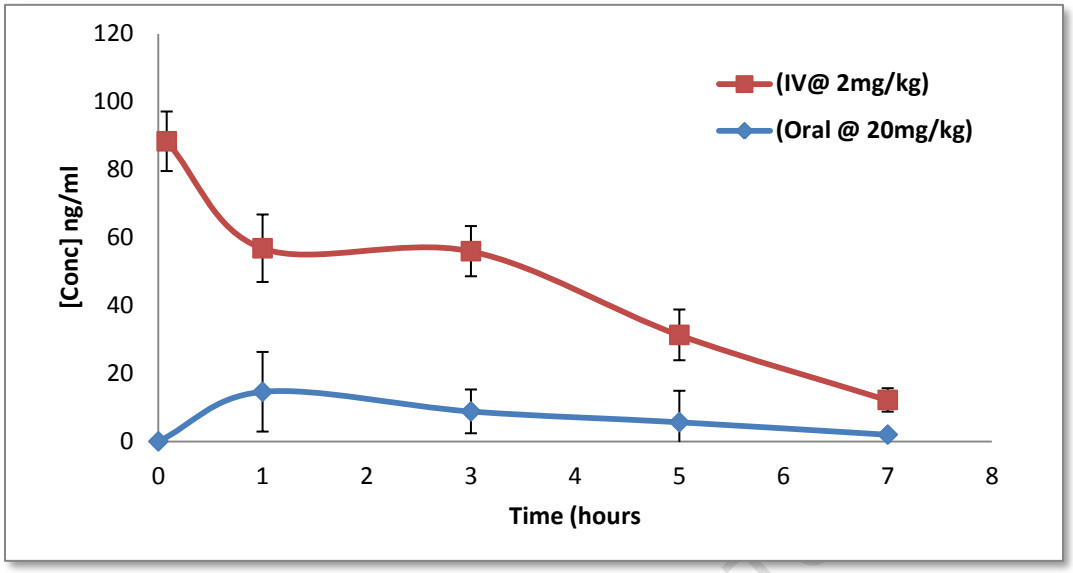


Figure 4.41: Blood concentrations of D13 in C57BL/6 mice blood after oral and intravenous administration of 20 and 2 mg/kg compound, respectively. Data represent mean ± standard deviation of data points obtained from three mice.

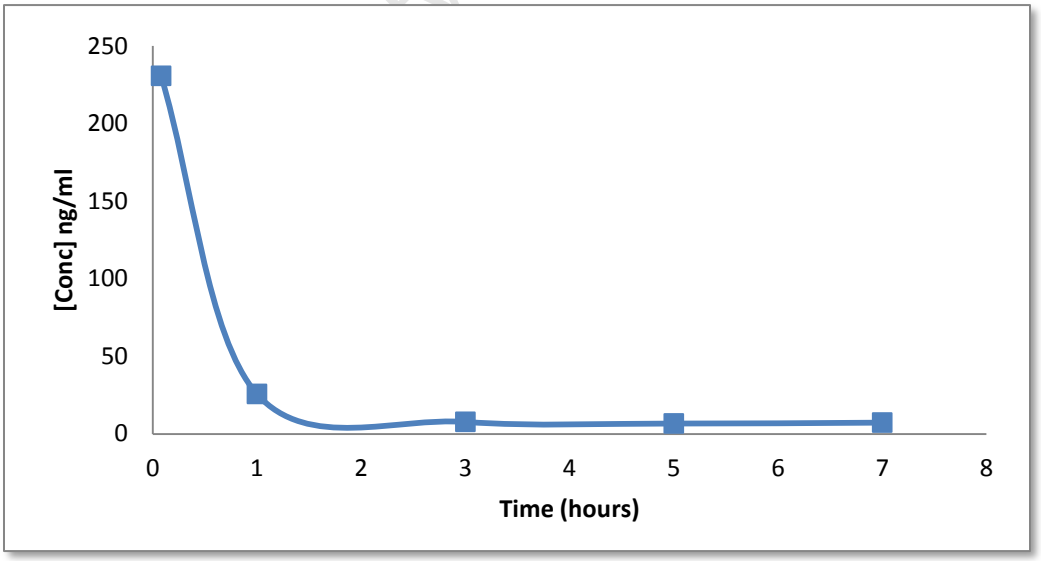


Figure 4.42: Blood concentrations of 13AB.2HCl in C57BL/6 mice blood after intravenous administration of 20 2 mg/kg compound. Data represent mean ± standard deviation of data points obtained from three mice.

The initial PK data for D13 and 13AB.2HCl is shown in Figure 4.41 and Figure 4.42. The blood levels for D13 compounds were very low in the mice dosed orally and 13AB.2HCl could not be detected because the values were below LOQ. In order to enhance bioavailability the compounds were prepared in formulations A and B. The mice were dosed at 20 mg/kg and 4 mg/kg orally and intravenously, respectively. Intravenous administration was done using compounds prepared in formulation A. The concentrations for D13 and 13AB.2HCl 'oral groups' were obtained from one mouse because the concentration levels in other mice were below LOQ. As such this data cannot be considered reliable because if only one out of 5 mice had detectable levels in the blood, it could imply that an error occurred during administration of the compounds. One mouse could have been dosed more in that particular group. All mice dosed orally with 13AB.2HCl prepared in formulation B had blood levels that were below LOQ. Table 4.20 shows concentrations of D13 and 13AB.2HCl following an intravenous dosage and the mean concentration vs time profiles are presented in Figure 4.43 and Figure 4.44.

Table 4.20: Blood concentrations (ng/ml) of D13 and 13AB.2HCl following an IV dosage in a comprehensive PK study. Data represent mean ± standard deviation of data points obtained from five single mice. For oral data n=1.

Time (hours)	D13 (IV @ 4 mg/kg)	13AB.2HCl (IV @ 4 mg/kg)
	Formulation A	Formulation A
0	N/A*	N/A*
0.17	314.67 ± 52.4	159.67 ± 57.3
0.50	237.33 ± 28.3	91.17 ± 24.1
1	203.67 ± 37.4	53.20 ± 9.8
2	187.00 ± 34.7	20.05 ± 12.3
3	170.00 ± 39.1	15.94 ± 9.0
5	131.93 ± 32.2	9.64 ± 8.4
7	92.53 ± 32.2	**4.82 ± 2.9
9	85.30 ± 30.3	**1.88 ± 1.3

*N/A: Not Applicable **Concentration levels were extrapolated from the calibration curves in Appendix A

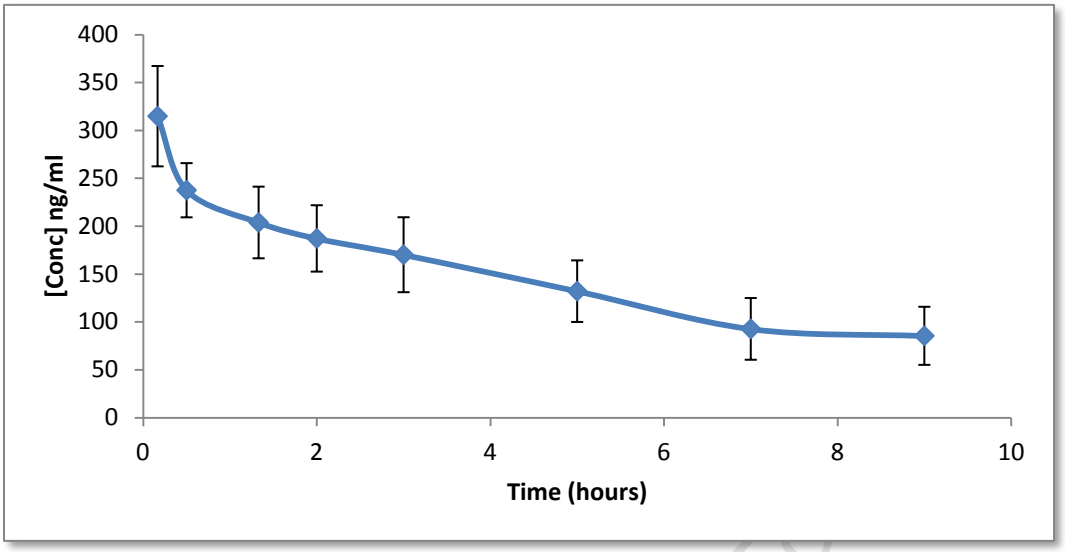


Figure 4.43: Blood levels of D13 in C57BL/6 mice blood after intravenous administration of 4 mg/kg compound. Data represent mean ± standard deviation of data points obtained from five single mice.

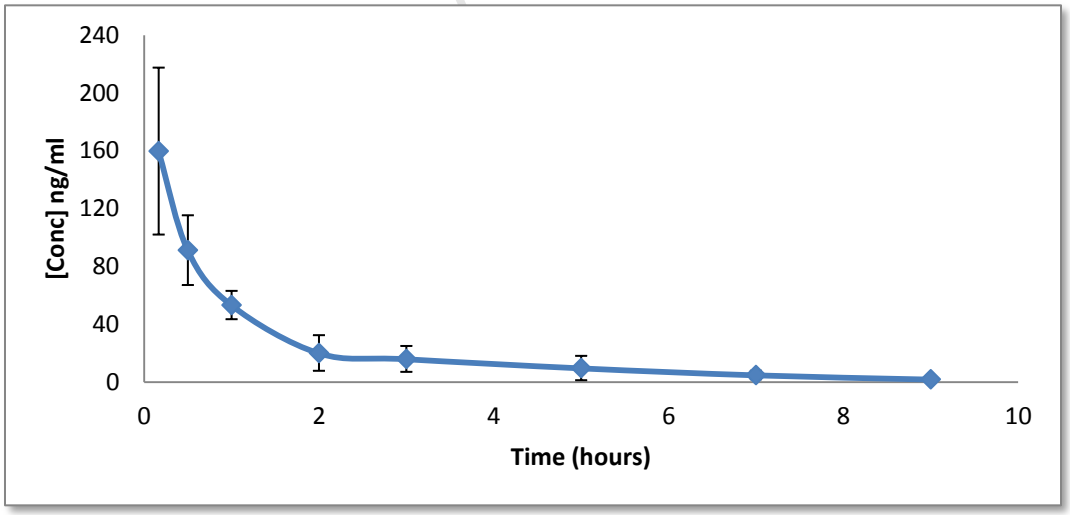


Figure 4.44: Blood levels of 13AB.2HCl in C57BL/6 mice blood after oral administration of 20 mg/kg of compound in formulation A and intravenous administration of 4 mg/kg compound. Data represent mean ± standard deviation of data points obtained from five single mice. For oral data n-1.

The PK parameters for D13 and 13AB.2HCl are listed in Table 4.21.

Table 4.21: Pharmacokinetic parameters of HPO-CQ hybrid compounds after administration in formulations A and B. Data represent mean ± standard data points obtained from five single mice. For oral data n-1.

Parameters		$T_{1/2}$ (hours)	T_{Max} (hours)	C_{Max} (ng/ml)	Vd (L/kg)	Vss (L/kg)	CL (ml/min/kg)	$AUC_{0-\infty}$ hr.ug/L	BA (%)
D13	Ref	No Data							
	Oral A	No Data							
	Oral B	No Data							
	IV	4.3 ± 1.02	N/A		19.7 ± 6	20.1 ± 6	27.6 ± 8	1793 ± 315	N/A
13AB. 2HCl	Ref	No Data							
	Oral A	No Data							
	Oral B	No Data							
	IV	0.7 ± 0.2	N/A		92.2 ± 17	154.1 ± 1	366 ± 46.6	450 ± 34	N/A

N/A: Not applicable; No Data: Blood concentrations were below LOQ; $T_{1/2}$, half-life; T_{max}: Time to reach C_{max}; C_{max}: Peak plasma concentration; $AUC_{0-\infty}$: Area under the curve; CL: Plasma clearance; Vd: Apparent volume of distribution V_{ss}: Volume of distribution at steady state; BA: Bioavailability

The IV data presented in Table 4.21 for D13 shows a clearance rate of 28 ml/min/kg, a high apparent volume of distribution of 20 L/kg and a half-life of 4.3 hours. The AUC value was also at 1793 hr.ug/L. Even with the half-life of 4.3 hours, the compound remained at detectable levels of 80 ng/ml after 9 hours at the end of the dosing interval. This means that the elimination of the compound from circulation was slower when compared to other compounds discussed earlier. When considering these properties and low IC_{50} values of 26 to 277 ng/ml against sensitive and resistant strains, D13 was selected for *in vivo* efficacy studies. Even though oral bioavailability was low, investigating the efficacy of D13 following an intravenous dosage will be worthwhile.

Compound 13AB.2HCl on the other had a very short half life of 0.7 hours and after 2 hours the blood levels dropped from 160 to 20 ng/ml because of a high clearance rate of 366 ml/min/kg. The volume of distribution was also high at 92 L/kg. Even though compound 13AB.2HCl displayed potent activity *in vitro*, the PK properties presented in Table 4.21 suggested that it was a poor candidate for *in vivo* efficacy studies. Therefore, only compound D13 from this group was evaluated for *in vivo* efficacy.

4.3.3. 2-Aminopyridine compounds

MMV394903 and MMV652120 are 3,5-diaryl-2-aminopyridine compounds that possess high efficacy towards chloroquine sensitive and resistant strains as discussed in the previous chapter. The IC_{50} values of MMV394903 and MMV652120 were in the range of 6 to 22 ng/ml and more potent than chloroquine by 2 to 10 fold. The *in vivo* PK study was thus investigated. A snapshot PK study was performed on MMV394903 and MMV652120 by administering them 20 mg/kg orally to mice to determine their oral bioavailability. Blood samples were collected at different time intervals and the blood concentrations are presented in Table 4.22. The mean concentration vs time profiles of MMV394903 and MMV652120 after oral administration in a snapshot PK study are presented in Figure 4.45.

Table 4.22: Blood concentration levels of MMV394903 and MMV652120 following an oral dosage in a snapshot PK study. Data represent mean ± standard deviation of data points obtained from three single mice.

Time (hours)	MMV394903	MMV652120
0	0	0
0.5	889 ± 229.8	2123.33 ± 899.5
1	2494 ± 248.9	1082 ± 556.3
2	979.67 ± 147.7	338.67 ± 231.4
4	554.33 ± 377.5	61.1 ± 33.6
6	152.3 ± 54.6	21.87 ± 10.4
8	55.6 ± 38.5	10.55 ± 6.4

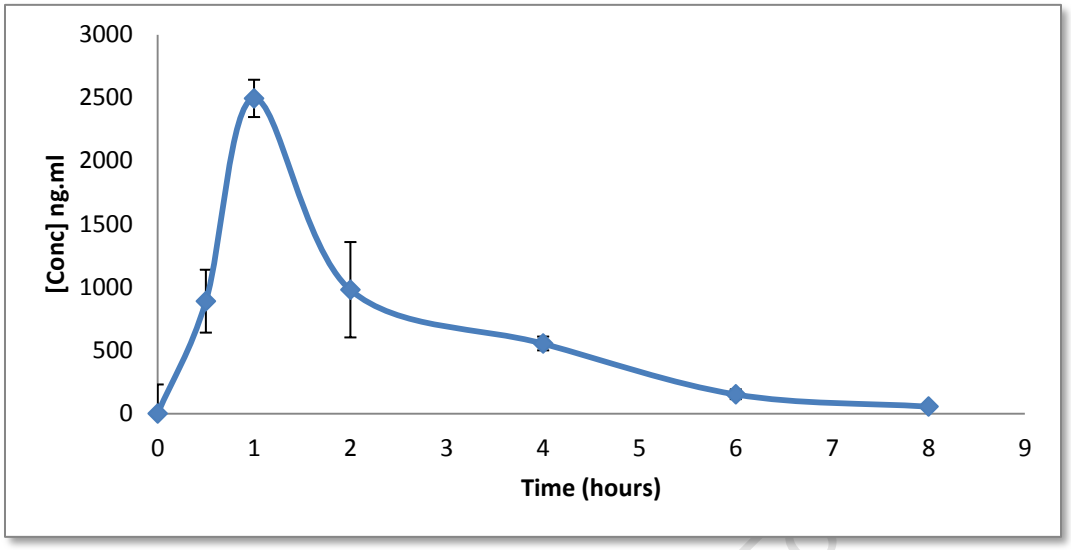


Figure 4.45: Blood concentrations of MMV394903 in C57BL/6 mice blood after oral administration of 20 mg/kg. Data represent mean ± standard deviation of data points obtained from three single mice.

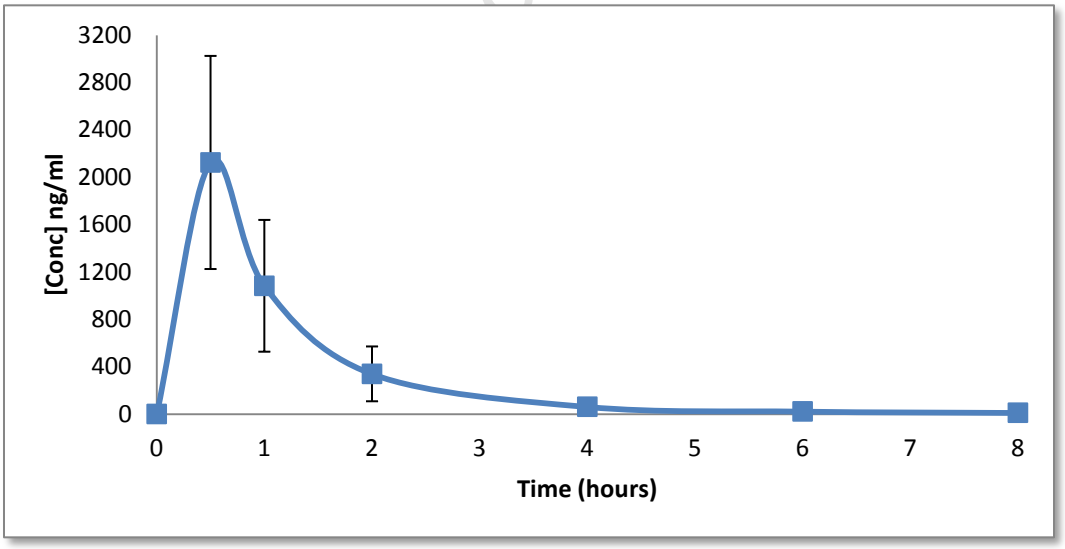


Figure 4.46: Blood concentrations of MMV652120 in C57BL/6 mice blood after oral administration of 20 mg/kg. Data represent mean ± standard deviation of data points obtained from three mice.

The blood levels of both compounds were very high with C_{max} values of 2500 ng/ml for MMV394903 and 2120 ng/ml for MMV652120. The T_{max} value for MMV652120 was 0.5 hours which shows that the absorption was much faster than that of MMV394903, with a T_{max} value of 1 hour. A comprehensive study was then performed and this included IV dosage. The compounds were dosed orally at 20 mg/kg and intravenously at 4 mg/kg. MMV394903 and MMV652120 suspensions were prepared without the formulations. Table 4.23 shows blood levels of MMV394903 and MMV652120 following oral and intravenous administration in mice and the concentration vs time profiles are presented in Figure 4.47.

Table 4.23: Blood concentration levels of MMV394903 and MMV652120 following an oral and IV dosage in a comprehensive PK study. Data represent mean ± standard deviation of data points obtained from five single mice.

Time (hours)	MMV394903		MMV652120	
	Oral	IV	Oral	IV
0	0	**N/A***	0	**N/A**
0.17	390 ± 67.1	900.33 ± 26.3	1176.67 ± 188.2	392.33 ± 24.3
0.5	724 ± 47.5	464 ± 45.7	1363.33 ± 211.3	164.33 ± 15.8
1	818.33 ± 34.1	188 ± 40.0	1084 ± 124.1	107.2 ± 18.4
2	564 ± 72.8	74.3 ± 19.4	374.33 ± 175.3	91.1 ± 13.9
4	117.37 ± 62.5	16.5 ± 4.3	1.29 ± 1.2	56.13 ± 20.6
6	48.9 ± 23.5	**2.85 ± 0.5	0	39.9 ± 6.7
8	31.12 ± 1.9	**0.39 ± 0.3	**0.14 ± 0.24	22.89 ± 11.3

***N/A: Not Applicable; ****Concentration levels were extrapolated from the calibration curves in Appendix A**

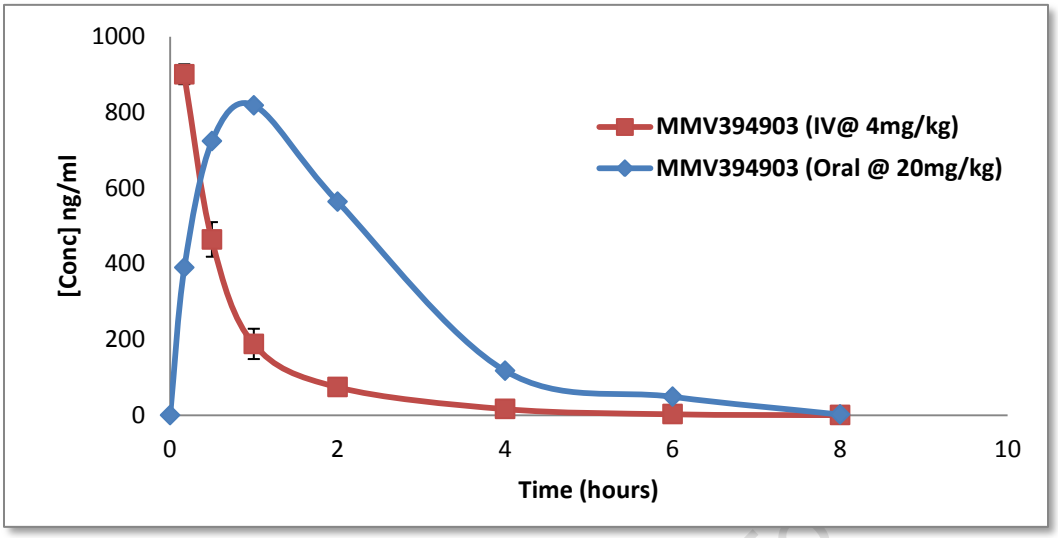


Figure 4.47: Blood concentrations of MMV394903 in C57BL/6 mice blood after oral and intravenous administration. Data represent mean ± standard deviation of data points obtained from five single mice.

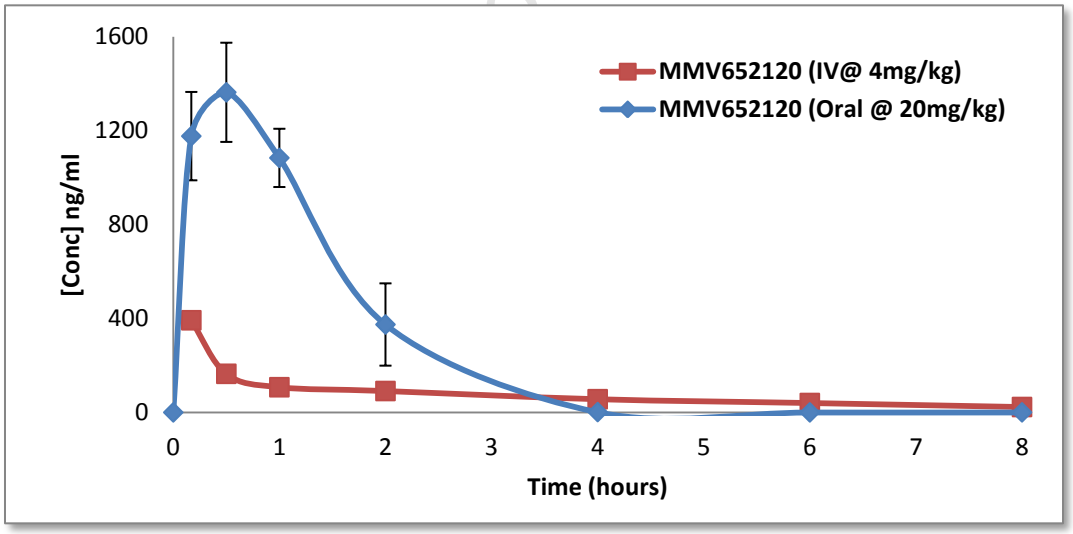


Figure 4.48: Blood concentrations of MMV652120 in C57BL/6 mice blood after oral and intravenous administration. Data represent mean ± standard deviation of data points obtained from five single mice.

The data points presented in Figure 4.47 were used to estimate the PK parameters of MMV394903 and MMV652120 and all the values are listed in Table 4.24.

Table 4.24: Pharmacokinetic parameters of 3,5-Diaryl-2-aminopyridine compounds after oral and intravenous administration. Data represent mean ± standard deviation of data points obtained from five mice.

Parameters		$T_{1/2}$ (hours)	T_{Max} (hours)	C_{Max} (ng/ml)	Vd (L/kg)	Vss (L/kg)	CL (ml/min/kg)	$AUC_{0-\infty}$ hr.ug/L/l	BA (%)
MMV 394903	Oral	2.53 ± 0.1	1	818.3 ± 34	N/A			2560 ± 691	69
	IV	0.87 ± 0.03	N/A		31.7 ± 6.5	9.51 ± 1	83.6 ± 12	739 ± 89	N/A
MMV 652120	Oral	2.96 ± 0.2	0.5	1363 ± 211	N/A			2235 ± 211	59.7
	IV	0.68 ± 0.6	N/A		22.9 ± 0.6	27 ± 1.3	92.6 ± 19	715 ± 156	N/A

N/A: Not applicable; $T_{1/2}$, half-life; T_{max}: Time to reach C_{max}; C_{max}: Peak plasma concentration $AUC_{0-\infty}$: Area under the curve; CL: Plasma clearance; V_d: Apparent volume of distribution V_{ss}: Volume of distribution at steady state; BA: Bioavailability

MMV394903 and MMV652120 had high oral bioavailability at 69 and 60% and high AUC values of 2560 and 2235 hr.ug/L, respectively as shown in Table 4.24. The T_{max} values show that MMV652120 has a more rapid absorption than MMV394903 with T_{max} values of 0.5 and 1 hour, respectively. Both compounds had short half-lives in the range of 2.5 to 3.0 hours. The IV data, on the other hand, shows a shorter half-life for both compounds at 0.87 and 0.68 hours for MMV394903 and MMV652120, respectively. The clearance rate was very high at 84 and 93 ml/min/kg and a high volume of distribution at 32 and 23 L/kg for MMV394903 and MMV652120, respectively.

When considering the fact that these compounds were found to be potent against chloroquine sensitive and resistant strains, having high AUC values and high bioavailability could be an advantage for these compounds when evaluated for *in vivo* efficacy.

Table 4.25: Summary of pharmacokinetic parameters for all the compounds evaluated. Data represent mean ± standard deviation of data points obtained from five mice. For reference formulations n=3.

Parameters		$T_{1/2}$ (hours)	T_{Max} (hours)	C_{Max} (ng/ml)	Vd (L/kg)	Vss (L/kg)	CL (ml/min/kg)	$AUC_{0-\infty}$ hr.µg/L	%BA
AZT-CQ Hybrid Compounds									
VAK59	Ref	No Data							
	Oral A	3.0 ± 0.7	1	19 ± 0.6	N/A			124.3 ± 14	1.3
	Oral B	4.8 ± 1.8	1	21.3 ±1.1	N/A			144.9 ± 30	3.3
	IV	0.6 ± 0.1	N/A		25 ± 11.2	51.6 ± 34	32.08 ± 8	2183 ± 475	N/A
AKAZTQ	Ref	2.6 ± 1.3	1	200.5±64	N/A			633.1 ±59	12.4
	Oral A	3.6 ± 2.3	1	200.7±47	N/A			255.1 ± 6	10.0
	Oral B	1.86 ± 0.5	0.5	167.3±17	N/A			191 ± 51	9.5
	IV	0.8 ± 4	N/A		21 ± 14.6	26.3 ± 20	44.9 ± 5	192 ± 23	N/A
AKAZTBQ	Ref	0.7 ± 0.1	1.7 ± 1.2	138 ± 70	N/A			429.4 ±144	30.1
	Oral A	2.34	0.5	122	N/A			240.1	16.85
	Oral B	1.5 ± 1.8	1	80.9 ± 22	N/A			215 ± 9.1	17
	IV	0.5 ± 0.4	N/A		45 ± 11.3	55.3 ± 13	254 ± 75	40 ± 11	N/A
HPO-CQ Hybrid Compounds									
D13	Ref	No Data							
	Oral A	No Data							
	Oral B	No Data							
	IV	4.3 ± 1.02	N/A		19.7 ± 6	20.1 ± 6	27.6 ± 8	1793 ± 315	N/A
13AB.2HCl	Ref	No Data							
	Oral A	No Data							
	Oral B	No Data							
	IV	0.7 ± 0.2	N/A		92.2 ± 17	154.1 ± 1	366 ± 46.6	450 ± 34	N/A
2-Aminopyridone Compounds									
MMV 394903	Oral	2.53 ± 0.1	1	818 ± 34	N/A			2560 ± 691	69
	IV	0.87 ± 0.03	N/A		31.7± 6.5	9.51 ± 1	83.6 ± 12	739 ± 89	N/A
MMV 652120	Oral	2.96 ± 0.2	0.5	1363 ± 211	N/A			2235 ± 211	59.7
	IV	0.68 ± 0.6	N/A		22.9± 0.6	27 ± 1.3	92.6 ± 19	715 ± 156	N/A

N/A: Not applicable; No Data: Blood concentrations were below LOQ; $T_{1/2}$, half-life; T_{max}: Time to reach C_{max}; C_{max}: Peak plasma concentration; $AUC_{0-\infty}$: Area under the curve; CL: Plasma clearance; Vd: Apparent volume of distribution V_{ss}: Volume of distribution at steady state; BA: Bioavailability

Table 4.25 summarizes the PK parameters of all the compounds that were evaluated in this study. Estimation of these parameters is important in pharmacokinetic studies because they represent the exposure of the body to the drug by summarizing the drug kinetics in the whole population (Urso *et al.*, 2002). Clearance is a very important

parameter because it explains how well a drug is removed irreversibly from the circulation. It is defined as the volume of fluid that is completely cleared of drug per unit time. It is used for constructing dosage recommendations in clinical situations (Thomson, 2000). High clearance values were reported in this study, as indicated in Table 4.25. These high estimates of blood clearance could be a consequence of the high volumes of distribution observed. The volume of distribution describes the relationship between the measured systemic concentrations and amount of drug in the body and the values reported in this study were high for all the compounds, as shown in Table 4.25. This parameter is dependent on a variety of drug properties such as hydrophobicity, plasma protein binding, and tissue components, including proteins (Grover & Benet, 2009). Of the nine compounds that were evaluated in this study, eight of them exhibited poor solubility. Not surprisingly, only the hydrochloride salt 13AB.2HCl had good solubility. Generally, the higher the lipophilicity of a drug, the stronger its binding to protein and the greater its distribution. Lipophilic compounds dissolve in fat and interact with cell membrane phospholipids, resulting in strong tissue binding reduced plasma concentrations and high volume of distribution (Dörwald, 2012; Lin & Lu, 1997; van de Waterbeemd *et al.*, 2001). Drug clearance and the volume of distribution can influence the half-life of a compound and can be altered by changes in either parameter. The half-life is defined as the time taken for 50% of the drug to be eliminated, and similarly to the clearance parameter (Toutain & Bousquet-Mélou, 2004). In this study the half lives of the compounds were in the range of 0.5 to 4.3 hours for both orally and intravenously dosed compounds. These values mean that the compounds are eliminated faster from circulation resulting in doses that were not effective in inhibiting the growth of the parasites in the blood. Highly soluble compounds such as 13AB.2HCl are expected to have a low volume of distribution because they do not diffuse to muscle and adipose tissues and therefore, the blood levels can be high. However, hydrophilic compounds with low molecular weight can move through the endothelial slit junctions of the capillaries into the interstitial fluid (Clark *et al*., 2011). 13AB.2HCl, on the other hand, has a molecular weight of 490 g/mol as a free base and 615 as a hydrated salt. Because of a larger molecular weight of this compound it is

possible that it can cross the endothelial cells with the help of transporters into the interstitial fluid.

Oral bioavailability defines how much drug gets into systemic circulation following oral administration (Veber *et al*., 2002). Low bioavailability becomes a major hindrance to the development of new generation drugs. In this study only two out of nine compounds had relatively high oral bioavailability, namely MMV394903 and MMV652120 with values of 69 and 60%, respectively. AKAZTQ and AKAZTBQ had moderate oral bioavailability and the rest of the compounds had very low bioavailability, even after preparing them in o/w microemulsions to enhance their oral bioavailability. One of the most important factors that affect oral bioavailability is drug absorption from the gastrointestinal tract. Drug dissolution is a determinant for absorption and is influenced by the solubility of the drug (Kim & Park, 2004). Wasan and co-workers reported that when hydrophobic drugs are co-administered with high-fat meals they get mixed micelles within the intestinal tract due to a combination of increased bile flow and the presence of the fatty components introduced as part of the meal. This causes the solubility of the drug to improve and thus increase its ability to enter enterocytes, leading to an enhanced drug bioavailability (Wasan *et al*., 2008). In this study o/w microemulsions were used to enhance the solubility of the compounds. No significant increase in oral bioavailability was observed. In some cases, oral bioavailability was reduced. An example of this was observed with AKAZTQ and AKAZTBQ. A reduction in oral bioavailability is reported in Table 4.25 for both compounds. AKAZTBQ bioavailability was reduced by 43% when prepared in o/w microemulsion formulations. This may suggest that the presence of the surfactants and co-surfactants had minimal influence on the absorption albeit the rate at which the compounds were absorbed was influenced, as noted in the T_{max} values in Table 4.18 where the T_{max} values shifted by 0.5 hours.

There are methods that have been developed to assist with the interpretation of the PK parameters. These methods include *in vitro* ADME (Absorption, Distribution, Metabolism, Excretion) studies, including metabolic stability in human or mouse liver

microsomes, permeability in Caco2 cells as well as direct and indirect transporter uptake studies to determine whether the compounds are substrates for human uptake transporters (Beconi *et al*., 2010). These studies can give you an idea on what properties of the drug contribute to the PK profile. In order to establish which ADME processes contributed to the poor oral biovailability of the compounds assessed in this study, absorption studies were performed on Caco-2 cells. Results from these studies will be presented and discussed in chapter 6.

All the parameters discussed above can influence the success of new chemical entities as potential drug candidates. Some of the compounds (AKAZTQ, AKAZTBQ and D13) did not display high antiplasmodial activity, when compared to chloroquine (albeit they were evaluated for PK properties). This is because it is possible that less potent molecules *in vitro* can turn out to be more effective *in vivo* because they may possess favourable kinetics such as greater absorption, better distribution, etc. On the other hand the other six compounds that were assessed in this study, namely VAK31, VAK37, VAK9, 13AB.2HCl, MMV394903 and MMV652120, are potent antiplasmodial compounds with activity greater than that of chloroquine against chloroquine-resistant strains. VAK31, VAK37, VAK59 and 13AB.2HCl are hybrid compounds and *in vitro* studies discussed in chapter 3 proved that the presence of another compound attached to chloroquine can enhance its activity against chloroquine resistant strains. However, this was not the case with *in vivo* PK properties. Unfavorable pharmacokinetics was observed with these hybrid compounds. Chloroquine alone has a an oral bioavailability of 79 to 90% and a half life of 7 hours in mice but in this case when combined with another molecule, AZT and hydroxypyridones, the oral bioavailability was reduced significantly (Salako, 1985; Zakaria *et al*., 2010). Even though these hybrid compounds were potent *in vitro*, they had unfavorable pharmacokinetics properties *in vivo*, therefore, making them poor candidates for further development.

As discussed earlier, the concentration- time profiles of VAK59 and D13 following an intravenous administration in mice (Figure 4.38 and Figure 4.43) showed very high blood concentration of VAK59 but with a very short half life and a longer half life of D13.

MMV394903 and MMV652120 in Figure 4.47 also showed high blood concentrations following oral administration. Therefore, these compounds were selected for *in vivo* efficacy and results will be presented and discussed in the next chapter.

Chapter 5: Evaluation of the Efficacy of Antimalarial compounds in mice in Mice

Evaluation of the Efficacy of Antimalarial Compounds in Mice

5.1. Introduction

In the previous chapter, the pharmacokinetic (PK) properties of 9 antimalarial compounds, namely VAK31, VAK37, VAK59, AKAZTQ, AKAZTBQ, D13, 13AB.2HCl, MMV394903 and MMV652120, were discussed. Investigating the PK properties of these compounds was performed in this study because PK principles predict optimal therapeutic effect, or pharmacodynamic response of the compounds tested (Schentag, 2001). Modeling of the relationship between drug concentration in the blood and efficacy also allow one to determine which pharmacokinetic/pharmacodynamic (PK/PD) dosing parameter best correlates with treatment outcomes (Andes, 2002). The PK parameters of the above mentioned compounds were determined in the previous chapter in order to identifying candidates suitable for evaluation of *in vivo* antiplasmodial activity. A majority of these compounds had low bioavailability, which means that the blood concentration levels were very low, and therefore were unlikely to exert any therapeutic effect on a mouse infected with plasmodium parasites. The compounds VAK59 and D13 were selected for *in vivo* antiplasmodial activity. VAK59, MMV394903 and MMV652120 showed high blood concentration levels following oral dosage and the D13 concentration vs time profile showed a slower clearance rate than other compounds after an intravenous dosage in a mouse model. This chapter therefore, investigates the correlation of the measurements of PK parameters of these compounds to their pharmacological action at the site of infection. This chapter will also discuss *in vivo* efficacy results of MMV394903 and MMV652120 which were obtained in a study conducted by the Swiss Tropical and Public Health Institute (Swiss TPH).

5.2. Materials and Method

5.2.1. Test compound preparation

All samples were prepared according to the method described in section 4.2.1.1. of the previous chapter.

5.2.2. The parasite

The chloroquine sensitive *Plasmodium berghei* (ANKA strain), which originated from the Congo River basin in Central Africa, was used to assess *in vivo* antiplasmodial activity of the test compounds. It belongs to a group of *Plasmodium* species that infect rodents and is used extensively in preclinical antiplasmodial studies.

5.2.3. Parasite inoculation

The parasites were maintained *in vivo* in a healthy C57BL/6 mouse by inoculation with 250 µl of a 1:1 (v/v) suspension of erythrocytes infected with *P. berghei* in phosphate buffered saline (PBS). The parasites in the host mouse were monitored under microscope from the third day post-inoculation until 20% parasitemia was reached. On the day of the experiment the host mouse was anaesthetized intraperitoneally with a mixture of ketamine (120 mg/kg) and xylizine (16 mg/kg). Whole blood from the host mouse was drawn by cardiac puncture into a Vacuette® Heparin tube (Lasec SA) and the mouse was killed in a sealed chamber by inhalation of halothane. A suspension of *P. berghei* parasitized erythrocytes (1×10^7) in PBS was prepared and the test mice were infected with 200 µl of this suspension intraperitoneally.

5.2.4. Evaluation of schizontocidal activity (Peters 4-day test)

Evaluation of the curative potential of the test compounds was done using the Peters 4-day test as described by Peters, 1975. The mice were dosed orally at 20 and 40 mg/kg and intravenously at 2 and 5 mg/kg 2 hours post-infection for 4 consecutive days. On day 0, blood samples were collected serially from each mouse at 0.5, 1, 3 and 7 hours post-dosing in order to provide quantitative measurements of drug exposure which is

needed for the sound interpretation of the efficacy of the antimalarials (Jun Tang & Redmond, 2008). Chloroquine was used as a reference drug and was dosed orally at 10 mg/kg. The mice were also dosed orally with PBS as a blank control. The parasitemia was determined from the third day by preparing thin blood films from the tail of each mouse and the smears were fixed with methanol and stained with Giemsa stain. This was repeated every two to three days to monitor the efficacy of the test compounds. The weights of the mice were recorded daily and the mice were monitored twice daily for discomfort or distress.

5.3. Results and discussion

Of the nine compounds that were evaluated for PK properties in the previous chapter only four were selected for *in vivo* efficacy studies. VAK59, D13, MMV394903 and MMV652120 are potent antimalarials with IC_{50} values in the range of 22 to 277 ng/ml and 7.5 to 254 ng/ml against chloroquine resistant strains K1 and Dd2, respectively (Table 3.8). The concentration vs time profiles of these compounds in Figure 4.38, Figure 4.43, Figure 4.47 and Figure 4.48 of the previous chapter showed high blood concentration levels after they were administered to the mice orally and intravenously. Therefore, *in vivo* efficacy studies were performed to investigate whether the high blood concentration of these potent compounds will reduce parasitemia and to investigate how the parameters in Table 4.25 of chapter 4 will influence their efficacy in mice.

5.3.1. *In vivo* efficacy of VAK59

VAK59 is a silylated AZT-CQ hybrid compounds and *in vitro* studies showed high efficacy against chloroquine sensitive and resistant strains. The IC_{50} values of VAK59 against resistant strains K1 and Dd2 were 33 and 36 ng/ml, respectively. Pharmacokinetic evaluation of this compound showed low oral bioavailability with blood concentration levels below LOQ. The half life was promising, and other methods like nano-formulation could improve oral bioavailability. The mice were dosed orally at 20 and 40 mg/kg and intravenously at 2 and 5 mg/kg following a successful inoculation of the mice with *P. berghei*. A snapshot PK study was performed on day 0 on the ’40 mg/kg’ oral and ‘5 mg/kg’ IV groups to measure the exposure of the malaria-infected

mice to the drug. The compound could not be detected in the '40 mg/kg' oral group because the concentration values were below the LOQ. This shows consistency with the previous PK study in chapter 4 on VAK59 where low oral bioavailbility was observed with concentration values below LOQ. Table 4.14 shows the concentration levels in mice blood and the concentration vs time profile of VAK59 following an intravenous administration in mice at 5 mg/kg is shown in Figure 5.49.

Table 5.26: Blood concentrations of VAK59 C57BL/6 mice blood infected with *P. berghei* following an IV dosage. Data represent mean ± standard deviation of data points obtained from five mice.

Time (hours)	[VAK59] ng/ml
0.17	1792.5 ± 492.2
1	229.4 ± 33.3
3	113.62 ± 22.9
7	58.4 ± 10.8

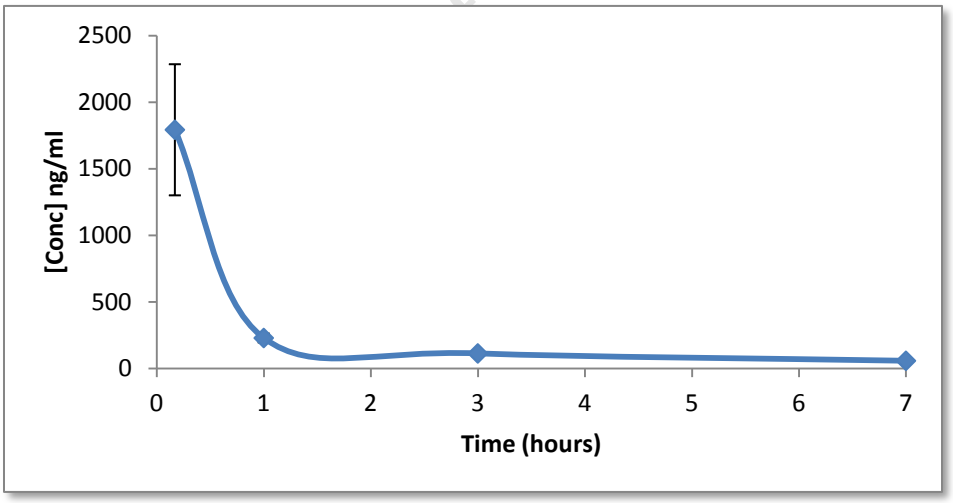
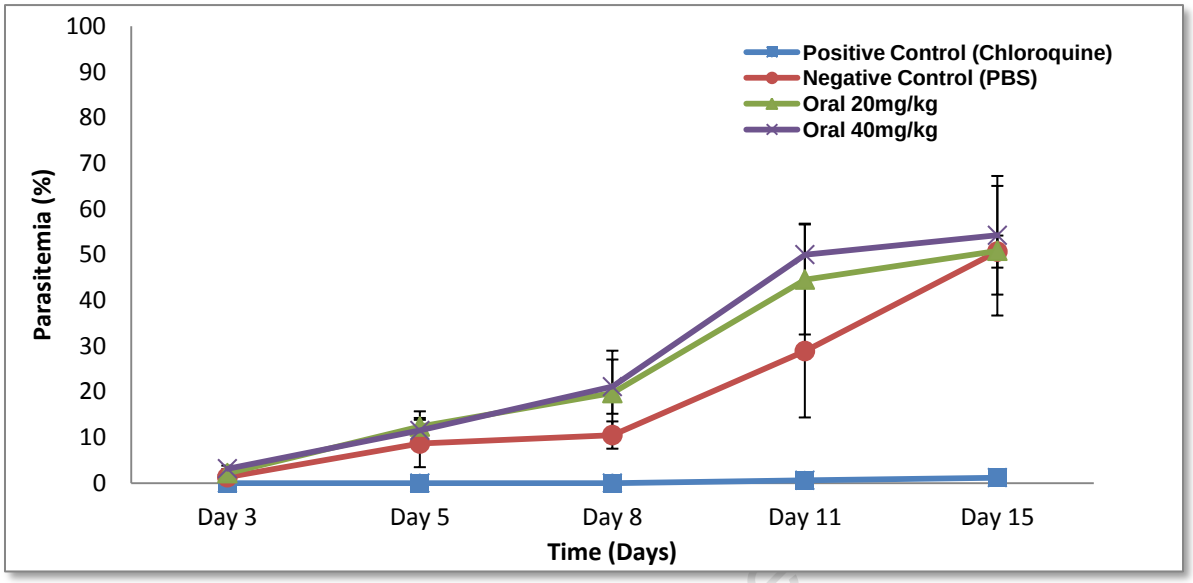


Figure 5.49: Blood levels of VAK59 in C57BL/6 mice blood infected with *P. berghei* after intravenous administration of 5mg/kg of compound. Data represent mean ± standard deviation of data points obtained from five single mice.

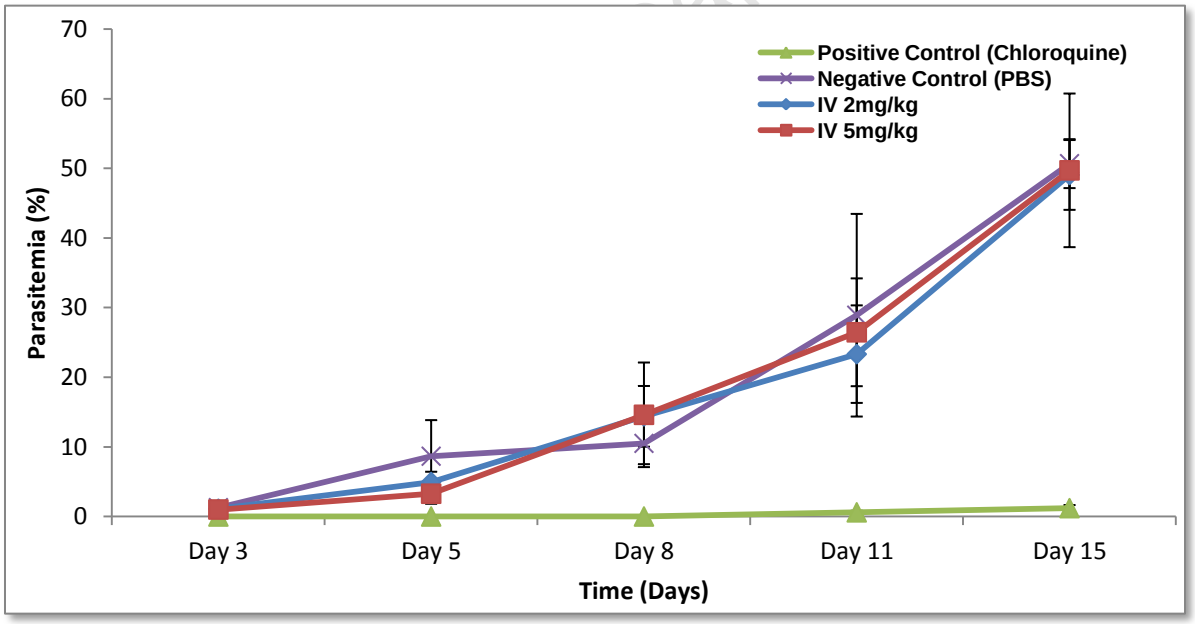
Figure 5.49 shows blood levels of VAK59 following a 5 mg/kg intravenous administration. The concentration vs time profile of VAK59 shows that after 1 hour post

dosage the blood levels had decreased from 1800 ng/ml to 230 ng/ml which correlates well with high clearance rate that is presented in Table 4.25. The mice were dosed once every day on a four day course to ensure constant exposure to the drug. Chloroquine was dosed at 10 mg/kg orally as a positive control and PBS was dosed orally as a negative control. The course of the infection was followed by microscopic examination of stained blood films every 2 to 3 days from day 3 for to day 15, when the mice showed signs of distress due to high parasitemia. The parasitemia profile in mice blood following an oral and intravenous dosage of VAK59 is shown in Figure 5.50.

The data shows that on day 3, the parasitemia was already ±1% in all the mice, including the mice dosed intravenously. Even though blood levels were high following the intravenous administration the compound was cleared much faster, as indicated by the high clearance rate of 32 ml/min/kg and a short half life of 0.6 hours, as presented in Table 4.25. This could imply that the length of time the compound remained in circulation was not sufficient for the compound to be effective against all the parasites in the blood. Figure 5.50 also shows that the high blood levels of VAK59 after each of the four doses, as indicated by the concentration vs time profile in Figure 5.49, may have caused a reduction in the parasitemia because on day 5 the parasitemia was much lower compared to that of the mice dosed orally. The parasitemia in the mice that were dosed orally increased at a much higher rate even when comparing with the 'negative control group' (PBS dosed mice). The PK data shows that VAK 59 has a very low bioavailability with blood levels that were below LOQ following oral dosage of 20 and 40 mg/kg. Consequently, an increase in parasitemia is expected. In the chloroquine group the parasitemia was only detected on day 15 post infection, as indicated in Figure 5.50.



(a)



(b)

Figure 5.50: Parasitaemia profiles of the effective doses of VAK59 in C57BL/6 mice blood infected with *P. berghei* after (a) oral and (b) intravenous administration of compound. Data represent mean ± standard data points obtained from five mice.

5.3.2. *In vivo* efficacy of D13

D13 is a HPO-CQ hybrid compound with antimalarial activity against chloroquine sensitive and resistant strains. The IC_{50} of D13 against resistance strains K1 and Dd2 were 277 and 254 ng/ml and the PK parameters showed that oral bioavailability of this compound was very low as indicated in chapter 4. However, a slower clearance rate and a longer half life of 4 hours following an intravenous dosage of 4 mg/kg of D13 were observed. The concentration vs time profile of D13 shows that after 9 hours post IV dosage the blood concentration was 85 ng/ml, as indicated in Table 4.20 of chapter 4. With *in vitro* IC_{50} values of 35 and 26 ng/ml against sensitive strains 3D7 and D10, respectively, it is possible that D13 can be effective in suppressing the growth of the *P. berghei* strain if the compound can remain at such levels even after 9 hours. Therefore, in order to determine the efficacy of D13 the mice were dosed orally at 20 and 40mg/kg and intravenously at 4 and 8 mg/kg following a successful inoculation of the mice with *P. berghei*. A snapshot PK study was performed on day 0 on all the test groups to measure the exposure of the malaria-infected mice to the drug. The blood concentration levels of the mice that were dosed orally were very low and could not be detected in both 20 and 40 mg/kg groups. Table 5.27 shows blood concentration levels of D13 following IV administration in mice infected with *P. berghei* and the concentration vs time profiles are shown Figure 5.51.

Table 5.27: Blood concentrations of D13 C57BL/6 mice blood infected with *P. berghei* following an IV dosage PK study. Data represent mean ± standard deviation of data points obtained from five mice.

Time (hours)	D13 (ng/ml)	
	IV @ 4mg/kg	IV @ 8mg/kg
0.25	714.6 ± 186.0	886.7 ± 314.9
1	645.8 ± 133.4	493.4 ± 183.0
3	287.2 ± 47.4	393 ± 76.6
7	96.3 ± 14.4	182.6 ± 47.7

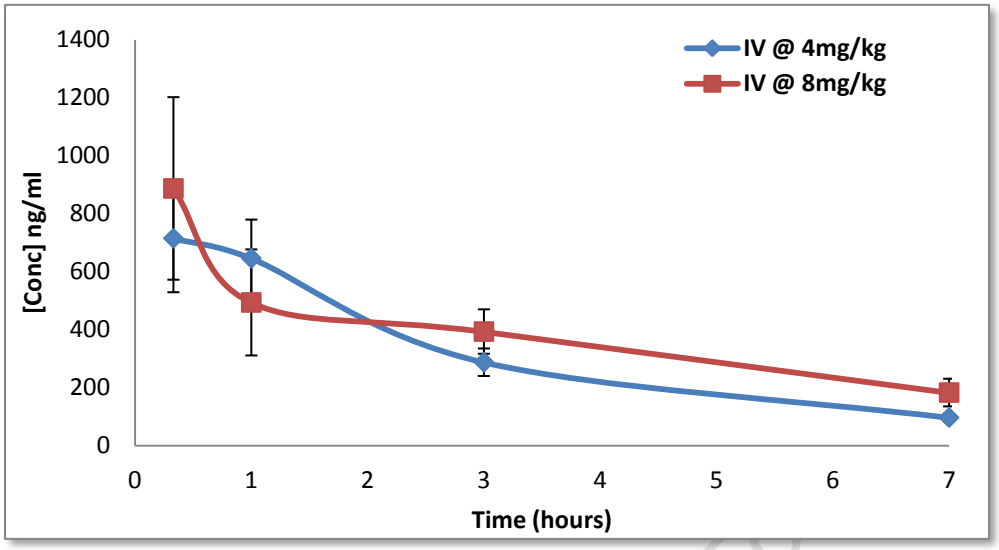
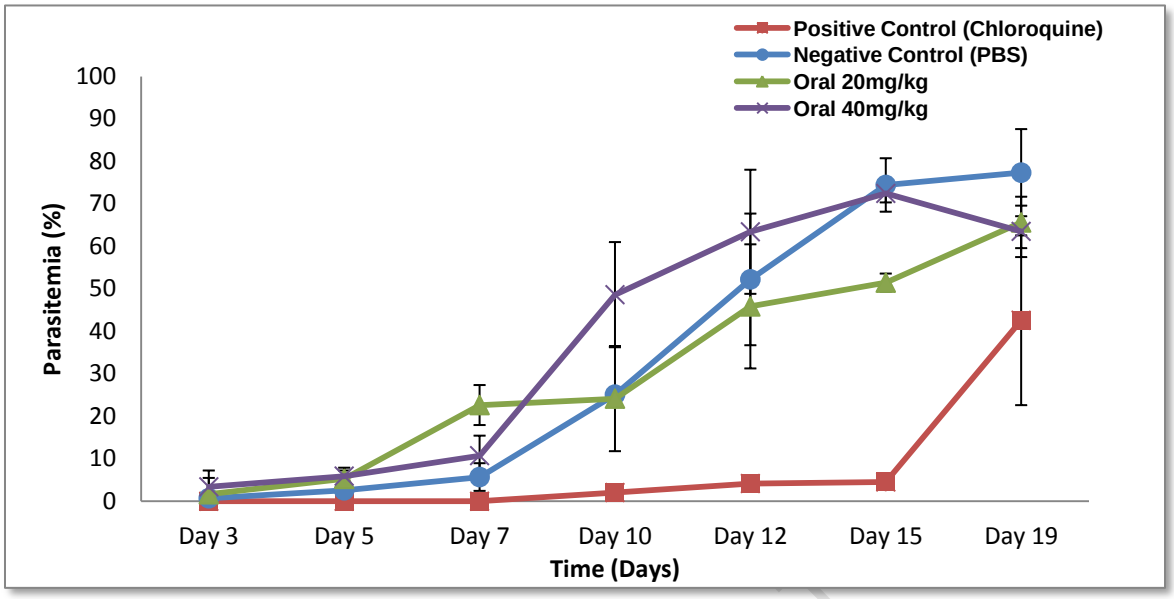
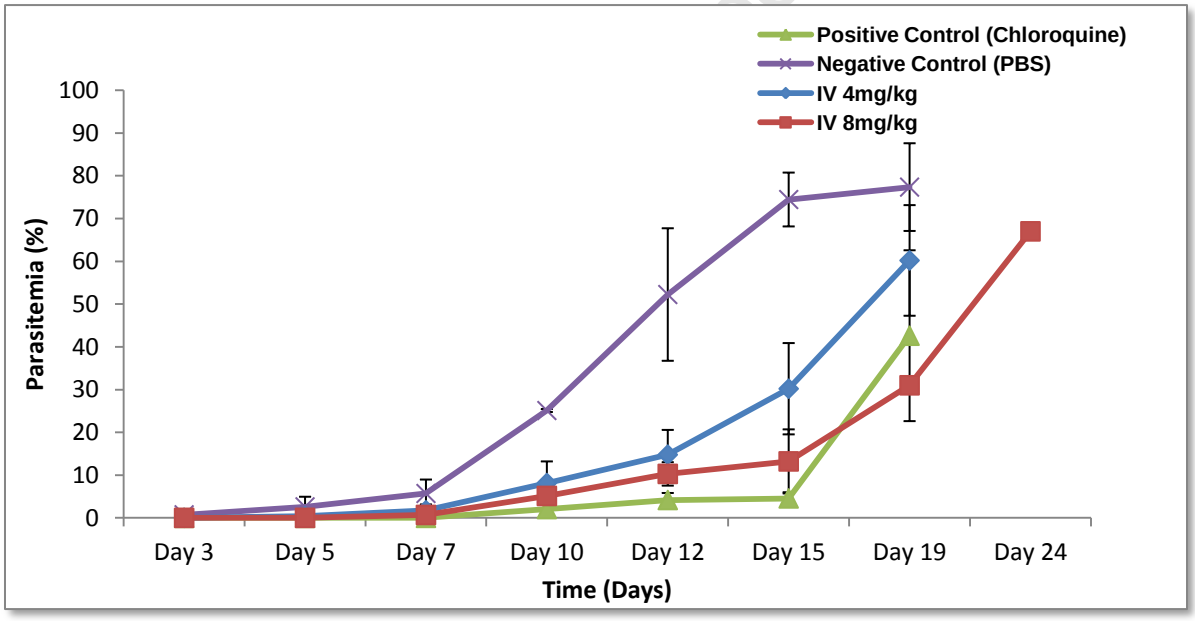


Figure 5.51: Blood levels of D13 in C57BL/6 mice blood infected with *P. berghei* after intravenous administration of 4mg/kg and 8mg/kg of compound. Data represent mean ± standard deviation of data points obtained from five mice.

The mice were dosed once every day on a four day course to ensure constant exposure to the drug in order to inhibit the multiplication of the parasites in the mice blood. Chloroquine was dosed at 10 mg/kg orally as a positive control and PBS was dosed orally as a negative control. The course of the infection was followed from day 3 for 24 days by microscopic examination of stained blood films every 2 to 3 days. The parasitemia profiles of mice blood following an oral and intravenous dosage of D13 are presented in Figure 5.52.



(a)



(b)

Figure 5.52: Parasitaemia profiles of the effective doses of D13 in C57BL/6 mice blood infected with *P. berghei* after (a) oral and (b) intravenous administration of compound. Data represent mean ± standard data points obtained from five mice.

In the mice that were dosed orally, the parasites were visible at a parasitemia of ± 3% on day 3. This data correlates well with the PK data in chapter 4 which shows low bioavailability of D13. In a PK study following oral dosage of D13 in infected mice the blood levels were too low to have any effect on reducing parasitemia and thus allowing the disease to progress as indicated by mice weight loss and a rise in the parasitemia levels, as shown in Figure 5.52a. As expected, the mice that were dosed intravenously showed high blood levels of D13 and the PK study in Figure 5.51 shows that the compound remained at high levels in both 4 and 8 mg/kg dosed mice. The PK data presented in Table 4.25 of the previous chapter shows that D13 had a half life of 4.3 hours following intravenous dosage and a clearance rate of D13 was 28 ml/min/kg. Table 5.27 shows that after 7 hours following intravenous dosage the blood concentration levels were relatively high at 96 and 183 ng/ml for 4 and 8 mg/kg, respectively. This resulted in a reduced parasite multiplication rate as indicated by undetected parasitemia on days 3 and 5. However, the levels were not high enough to clear the parasites in the blood because after the four-day course of treatment the parasites that remained in the mice blood began to multiply and were only detected on day 7 with a parasitemia of 1.8 and 0.7% for mice dosed at 4 and 8 mg/kg, respectively, as shown in Figure 5.52b. Nevertheless, D13 proved to be an effective antimalarial compound *in vivo* when dosed intravenously and further modifications on the chemical structure could improve its PK properties such as oral bioavailability, thus improving its efficacy.

5.3.3. *In vivo* efficacy of MMV394903 and MMV652120

MMV394903 and MMV652120 are 2-aminopyridines that are very potent against chloroquine resistant strains K1 and Dd2 with IC_{50} values of 12 and 22 ng/ml against K1 and 7.7 and 8.5 ng/ml against Dd2, respectively. The PK properties of these compounds were evaluated and the PK parameters are listed in Table 4.25 of chapter 4. This data shows that MMV394903 and MMV652120 have bioavailability of 69 and 60% and figure 4.10 shows high blood concentration levels for both compounds following oral administration. In a mouse infected with a sensitive strain, *P. beghei*, the presence of potent compounds such as these at such high concentrations could

interfere with the parasite multiplication, causing these compounds to be effective antimalarials *in vivo.* High oral bioavailability is another property that made these compounds better candidates for *in vivo* efficacy studies. The Swiss TPH conducted *in vivo* efficacy studies on MMV394903 and MMV652120 in mice infected with *P. berghei* (ANKA) to prove this hypothesis. The mice were dosed orally at 50 mg/kg and 3 mg/kg with MMV394903 and MMV652120, respectively, and were assessed using the 4-day Peter's test. On day 5 a 99% inhibition was observed for MMV394903 and 97% inhibition for MMV652120 (parasitemia profiles not provided by the Swiss TPH) (González Cabrera *et al*., 2012; Younis *et al*., 2012). Chloroquine was more active with 100% inhibition on day 5. MMV394903 and MMV652120 possessed greater *in vitro* activity towards plasmodial parasites when comparing with chloroquine but *in vivo* the compounds were less active. In the publication by Younis *et al*., 2012 series of 2-aminopyridines were subjected in an extensive SAR study. Clinical candidate MMV390048 was among these compounds and the presence of pyridine and sulfonyl groups was found to be the major contributor in the efficacy of these compounds. Figure 5.53 compares the structures of MMV394903 and MMV652120 to that of MMV390048.

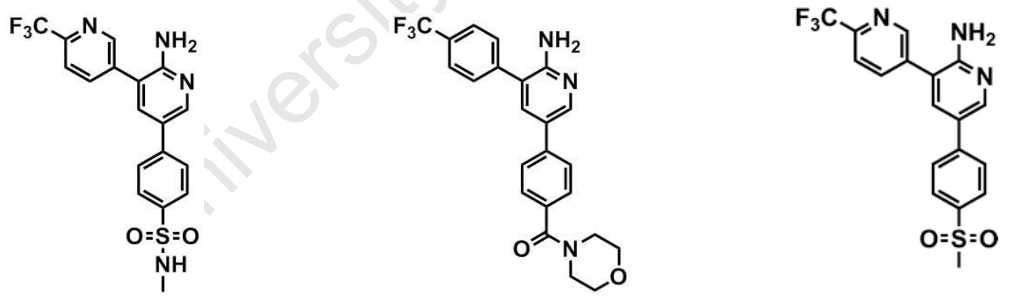


Figure 5.53: The structures of 3,5-Diaryl-2-aminopyridines.

A study conducted by the Swiss TPH on MMV390048 shows that a single dose of 30 mg/kg was found to be curative in a *Plasmodium berghei* mouse model. They performed a similar study on MMV394903 and a 95% inhibition of a single dose was observed on day 5. Even four doses of 50 mg/kg, as described above, were not curable

towards *P. berghei* infected mouse. Therefore, the difference in the efficacy of these compounds can be attributed to the substitution in their chemical structures. In the previous chapter it was discussed that the presence of carboxamide and methylsulfonylphenyl contributed to the activity of MMV394903 and MMV652120 but when comparing the structure of these compounds to that of MMV390048 it can be concluded that the sulfonyl group in MMV390048 is a major contributor to *in vivo* efficacy of this compound. This is confirmed by the similarities observed in the structures of MMV652120 and MMV390048 where the methylsulfamoyl of MMV652120 is substituted with the methanesulfonyl group, thus enhancing its efficacy.

The data presented in this study shows a correlation between PK parameters with an *in vivo* pharmacological effect of the antimalarial compounds evaluated in this study. It can be seen that compounds with higher oral bioavailability and a longer half life are likely to be more effective *in vivo* than compounds with low bioavailability and shorter half life even if they are less active *in vitro.* The bioavailability of chloroquine in mice is 79 to 90% with a half life of about 7 hours (Salako, 1985; Zakaria *et al*., 2010). Therefore, even if VAK59, MMV394903 and MMV652120 were more active than chloroquine *in vitro,* the fact that chloroquine has a longer half life and a higher oral bioavailability than these compounds a more effective pharmacological action is expected. D13 was also more effective than VAK59 following an intravenous administration even though it was less active *in vitro.* This is because D13 was cleared slower than VAK59 from the circulation, causing its half life to be longer as seen in Table 4.25 of chapter 4 where the half life of D13 was 4.3 hours and 0.6 hours for VAK59 following an intravenous administration. One can argue that these compounds were tested on different species of malaria parasites which have different sensitivity profiles and life cycles (*P. falciparum* has a 48 hour life cycle and *P. berghei* has a 24 hour life cycle) (Orjuela-Sánchez *et al*., 2012; Patel *et al*., 2013). Even though the aim of synthesizing chloroquine hybrids was to develop better antimalarial candidates, this study showed that combining chloroquine with another compound can cause a change in the physicochemical properties of the hybrid, thus affecting its pharmacokinetic properties. For future studies on these compounds, such as further structural modification, *in silico*

screening is required in order to predict the properties of their derivatives as well as their pharmacokinetic events before chemical synthesis.

Chapter 6: Determination of the Permeability of active compounds to Predict Absorption

Determination of the Permeability of Active Compounds to Predict Absorption

6.1. Introduction

The previous chapter discussed the pharmacokinetic properties of nine antiplasmodial compounds. Seven of these had very low oral bioavailability as indicated by very low blood levels following oral dosing and very low AUC values. Studies have shown that low oral bioavailability is one of the factors contributing to only a small fraction of drug candidates being selected for clinical development (Kaplita *et al*., 2005).. Therefore, it is important to understand the behavior of a drug molecule during its administration phases.

Drug absorption is one of the important factors affecting oral bioavailability because the drug must first be absorbed for it to be in systemic circulation in order to exert its pharmacological effect. Drug absorption can also be a factor contributing to variability in drug response. The small intestine represents the main site of drug absorption (Conrado *et al.*, 2010). During intestinal drug absorption, therapeutic compounds first enter intestinal epithelial cells from their apical side and then pass through the epithelia to the basal side before they finally appear in the blood stream. Drug absorption involves a complex transfer process across the intestinal lining, which includes passive diffusion through the cell membrane of enterocytes (transcellular transport) or via the tight junction between the enterocytes (paracellular transport) (Figure 6.54). Lipophilic compounds can rapidly partition from the luminal fluid into the cell membranes of the intestinal epithelium and are mainly transported via a transcellular pathway. Hydrophilic drugs and peptides partition poorly into the cell membranes and are subsequently transported via the paracellular pathway (Meunier *et al.*, 1995; Shah *et al.*, 2006).

The intestinal epithelium controls the entry of drug molecules but bioavailability studies do not give a clear insight on drug absorption across the intestinal epithelial. Thus a

highly standardized *in vitro* model that mimics the intestinal mucosa is required in order to assess the permeability of potential new drugs as part of the drug development process (Le Ferrec *et al*., 2001; van Breemen and Li, 2005). The Caco-2 cell line displays the most highly differentiated properties under standard culture conditions and is used extensively in drug discovery to screen for the oral absorption of potential drug candidates (Kamath *et al*., 2007; Wilson *et al*., 1990). They are derived from a human colorectal adenicarcinoma and were used for the first time to model human intestinal absorption in the 1980s. They undergo a spontaneous enterocytic differentiation in culture until they reach confluence where polarised intestinal cells are formed, possessing an apical brush border and tight junctions between adjacent cells (Figure 6.54). They express small intestine microvillous hydrolases (sucrose iso-maltase, lactase, aminopeptidase N, dipeptidylpeptidase IV) and nutrient transporters. This is unlike other human colon carcinoma cell lines such as T-84 cells and HT-29. HT-29 only differentiates when they are induced by the addition or the substitution of various chemicals from the culture media (Meunier *et al.*, 1995; Sambuy *et al*., 2005). Full expression is achieved between, approximately, 15 to 20 days in culture (Le Ferrec *et al*., 2001; van Breemen and Li, 2005).

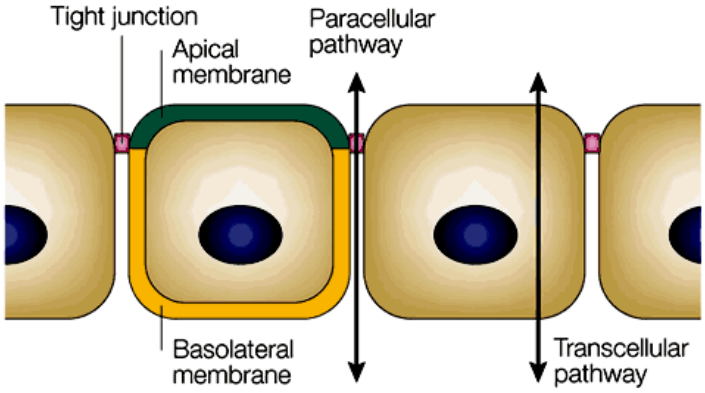


Figure 6.54: Schematic drawing of intestinal epithelial cells. The junctional complex is located at the most apical region of lateral membranes (Tsukita *et al.,* 2001).

This study describes the use of Caco-2 cell culture model for the characterization of intestinal permeability properties of antiplasmodial compounds that were assessed in the pharmacokinetic study discussed in the previous chapter.

6.2. Materials and method

The human colon carcinoma cell lines used in this project were Caco-2 cells obtained from Highveld Biological, South Africa.

6.2.1. Harvesting cells

The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Highveld Biological, South Africa) which was supplemented with 10% heat inactivated fetal calf serum (FCS) (Highveld Biologicals) and 1% gentamycin (Sigma). The cells growth conditions are similar to those described in section 3.2.2 of chapter 3.

6.2.2. Cultivation of cells on permeable supports

The inserts that were used were Corning 6.5 mm Transwell with 0.4μm pore polycarbonate membrane insert attached to the wells of a 24 well polystyrene plates (Sigma). 0.4 μm pore size is the smallest and is primarily used in transport studies. The membranes are also available in 3 and 5 μm pore size but these are used for cell invasion, chemotaxis and motility studies. There are three types of transwell membranes that can be used for transport studies, namely collagen-coated PTFE, polyester and polycarbonate membranes. In this study the polycarbonate membrane was preferred because of a high pore density of $1x10^8$ pores/cm^3 which has an influence in the osmosis process (Corning Booklet; Zhang *et al*., 2013). The permeability study was done according to the methods described by Alhamoruni and co-workers, Hubatsch and co-workers and Wilson (Alhamoruni *et al*., 2010; Hubatsch, 2007; Wilson *et al*., 1990).

When the cells reached 70-80% confluence at passage 42 they were subcultured as described in section 3.2.2. The cell suspension was centrifuged in a sterile 15 ml tube at 750 rpm for 4 minutes and the supernatant was aspirated completely. The pellet was

resuspended in 5 ml medium and 20 μl was removed and transferred to a 1.5 ml eppendorf tube containing 20 μl crystal violet, which was used to stain the cells for counting in a hemocytometer. The filters were pre-wet with 50 μl of medium for 2 minutes and the cells were seeded at 20000 cells per insert by dispensing 200 μl of the resuspended cell solution. The plate was incubated at 37^{o}C overnight for a maximum of 16 hours and non-adherent cells were removed from the apical medium and replaced with 200 μl fresh medium to reduce the risk of multilayer formation. Aspiration was avoided in the first 9 days post seeding. The medium was changed on alternate days, first basolaterally and then apically. The basolateral compartment contained 600 μl and the apical compartment contained 200 μl.

6.2.3. Monolayer integrity measurement

6.2.3.1. Transepithelial electrical resistance (TEER)

On day 14 the confluency or integrity of the cells was determined by measuring transepithelial electrical resistance (TEER) using an Ohmmeter. TEER measures the resistance to pass current across a monolayer. Electrical resistance across a monolayer represents the paracellular resistance, which consist of the resistance of the junction and the intercellular space, and the transcellular resistance, which consists of the resistances of the apical and basolateral cell membranes. TEER values higher than 300 Ω cm^{-2} indicate adequate monolayer integrity (van Breemen & Li, 2005; Harhaj & Antonetti, 2004). At the end of the growth period the plates were allowed to equilibrate at room temperature for 1 hour. The electrical resistance across the monolayer was measured using an Ohm Meter equipped with probes by placing one probe inside the filter well and one into the medium in the growth well. The electrical resistance was measured for each well and the resistance of a blank insert (no cells) was determined to subtract the background value according to the formula:

$$TEER = (R_m - R_b) \times A$$

Where $\mathbf{R_m}$ was the reading obtained from the monolayer, $\mathbf{R_b}$ was obtained from the blank insert and **A** was the surface area of the insert filter membrane which was 0.33cm^3. Another method was required to confirm the confluency of the cells because cells grown on polycarbonate membranes cannot be viewed under the microscope.

6.2.3.2. Lucifer yellow method

Lucifer yellow (LY) (Sigma) is a low molecular weight paracellular marker used to measure the integrity of the cell line. Other low and high molecular weight markers used to measure cell confluency are [^{14}C] inulin, [^{14}C] polyethylene glycol, [^{3}H] mannitol and [^{3}H] dextran. These markers travel through paracellular diffusion and have low permeability when the monolayers reach confluence because of the tight junctions (Hidalgo, 1989). Apparent permeability (P_{APP}) value of LY obtained was used to determine whether the cells have reached confluency before the permeability of the test compounds was determined.

6.2.4. Procedure for the permeability assay

6.2.4.1. Sample preparation

The donor compounds were antimalarials, low permeability control LY and high permeability controls atenolol, propranolol and cimetidine. All these were dissolved in DMSO and were diluted to 100µg/ml with transport buffer, a Hank's balanced salt Solution (HBSS) from Sigma, to give a final DMSO concentration of 1%. The transport buffer consist of HBSS + 10mM Hepes + 0.35g/l NaHCO3, pH 7.4 (1:100 1M Hepes in HBSS). VAK59, D13 and 13AB.2HCl were also prepared in formulations A and B, according to the method described in Table 4.9 of the previous chapter.

6.2.4.2. Drug transport assay

The monolayers were rinsed twice with HBSS. 200µl of the test compounds were added into the apical chambers of the monolayers inserted in a 24 well plate containing 600 µl HBSS. The plate was placed at 37^{o}C with orbital shaking at 60 rpm for 2 hrs and 50 µl was removed from the apical and the basolateral wells and diluted 100 times for

analysis by LC-MS/MS using the method describe in the previous chapter. LY analysis was performed by constructing a standard curve in a range of 1 to 0.05 µg.ml. A blank with HBSS in 0.01% DMSO as a final concentration was prepared. 200 µl of the diluted LY aliquots was transferred to a solid black 96 well plate together with the standards. The plates were read in a fluorescent reader at an excitation/emission wavelength of 490/530nm using a Modulus^{TM} Microplate Multimode Reader by Turner Biosystems.

6.3. Results and Discussion

The permeability of nine antiplasmodial compounds was measured using a Caco-2 cell line grown on a polycarbonate membrane. In order to determine the confluence of the cells, resistance was measured in the inserts and the measured TEER value was 2475 Ω cm^{-2} which was very high and its reliability was questioned. In order to determine whether high TEER values were caused by cell confluence a low permeability marker, LY, was used. The confluence of the cells is measured by determining the leakage of the cells by measuring the amount of LY in the receiver well. LY has low permeability; therefore low concentrations are expected in the receiver well (Volpe, 2010). A standard curve was prepared in order to determine the concentration of LY in the donor and the receiver well. Figure 6.55 shows a typical LY standard curve.

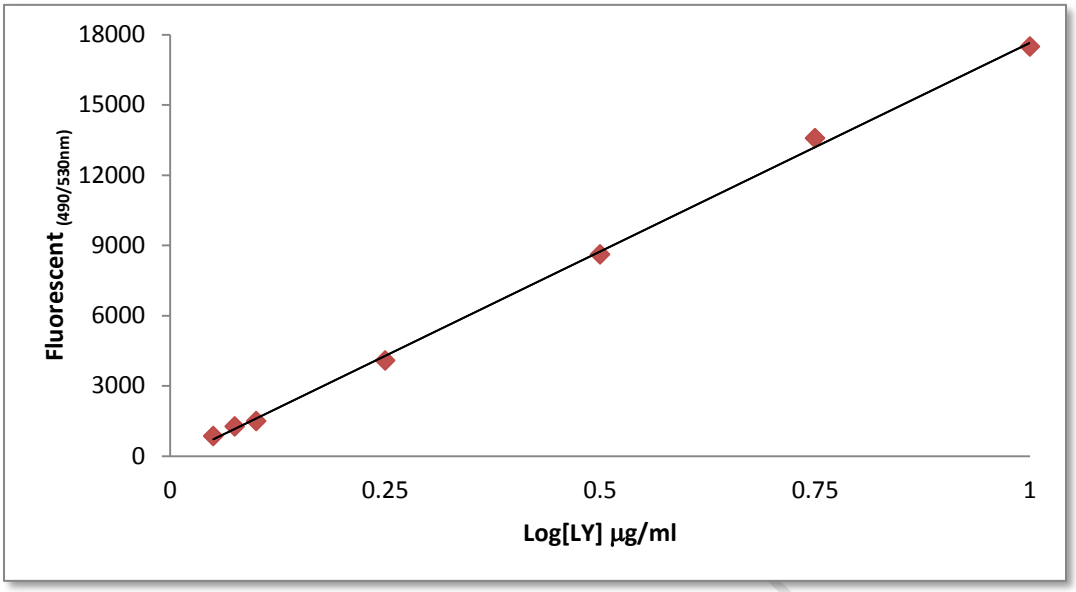


Figure 6.55: A typical lucifer yellow standard curve. (r^2 = 0.999). Data represents the mean of data points performed in duplicate.

The values obtained from the standard curve were used to calculate the permeability parameters. These parameters give values that interpret the outcome of the permeability process. They also indicate whether there were any leakages due to non-confluent cells. Apparent permeability (P_{APP}) is a parameter used to predict human intestinal absorption by documenting compound appearance in the receiver side and is calculated using the following formula:

$$P_{APP} = \frac{V_{receiver}}{Area \ X \ Time} \ X \ \frac{[Compound_{receiver}]}{[Compound_{initial}]}$$

Where $V_{receiver}$ is the volume (in ml) in the acceptor well, Area is the surface area of the membrane (0.33cm^3 for Caco-2 plate), and time is the total transport time in seconds (Corning Booklet). Another parameter that is calculated is flux percentage which measures the % permeability of the compounds. The formula used to calculate % flux was:

$$\% \text{ Flux} = 100 \text{ X } \frac{[\text{Compound}_{\text{receiver}}] \text{ X } V_{\text{receiver}}}{[\text{Compound}_{\text{donor}}] \text{ X } V_{\text{donor}}}$$

Mass balance is a parameter used to define the sum of the amounts of compound recovered in the apical and basal compartments at the end of the transport experiment as a percentage of the initial apical donor amount (Farrell *et al*., 2012). The mass balance was calculated according to this formula:

$$\text{Mass Balance} = 100 \text{ X } \frac{([\text{Final Compound}_{\text{receiver}}] \text{ X } V_{\text{receiver}}) + ([\text{Final Compound}_{\text{donor}}] \text{ X } V_{\text{donor}})}{[\text{Initial Compound}] \text{ X } V_{\text{donor}}}$$

Calculated parameters for LY are presented in Table 6.28.

Table 6.28: Permeability parameters for lucifer yellow. Data represents the mean of data points performed in duplicate.

Permeability parameters	Lucifer Yellow
P_{APP} (cm/s)	5.3×10^{-6}
Theoretical P_{APP} (cm/s)	**$0.4\text{-}1 \times 10^{-6}$**
% Flux	0.7
Theoretical % Flux	**0.3 - 2**
Mass Balance (%)	102

Completely absorbed drugs have high P_{APP} values ($P_{APP} > 1 \times 10^{-6}$ cm/s) whereas incompletely absorbed drugs have low P_{APP} values ($P_{APP} < 1 \times 10^{-7}$ cm/s) in the Caco-2 monolayer (Artursson, Palm & Luthman, 2012). LY P_{APP} value was 5.3×10^{-6} cm/s which is between low and high permeability range and 5 times higher than the theoretical value. When looking at the % flux of 0.7%, this value shows that LY permeability was low and that means the cell monolayer was intact. Flux values greater that 2% suggest the possibility of leakage during the assay due to a non confluent monolayer (Zhang *et al*., 2013). Mass balance values that are between 65 and 135% in the cell transport system show that compounds have permeability measurements that are usually reliable so that using permeability for absorption projection and efflux substrate identification purposes will be straight forward (Liu *et al*., 2010). The mass

balance calculated for LY was 102% which shows the accuracy of the data presented and it can be concluded that with a low % flux of 0.7% the monolayer was intact and no leakage was observed. Therefore, the monolayer was ready for transport studies.

The permeability of nine antiplasmodial compounds was evaluated using the transport assay used to determine the permeability of LY. Inter-laboratory variation in observed P_{APP} values exists; therefore, the values are usually linked to known low and high permeability control compounds, which are included in experiments. Examples of typical low permeation controls are mannitol, lucifer yellow and atenolol, whereas cimetidine and propranolol are often used as high permeation markers. Atenolol, propranolol and cimetidine were included in this study to overcome the typical biological variation in cell based permeation measurements. Table 6.29 show permeability parameters of high and low permeability controls.

Table 6.29: Permeability parameters of high and low permeability controls evaluated. Data represents the mean of data points performed in duplicate.

Permeability parameters	Atenolol	Propranolol	Cimetidine
P_{APP} (cm/s)	$9.4x10^{-5}$	$2.0x10^{-4}$	$2.0x10^{-4}$
Theoretical P_{APP} (cm/s)	**$5x10^{-5}$**	**$2\text{-}3x10^{-5}$**	**$9\text{-}2x10^{-4}$**
% Flux	37.21	78.4	70.1
Theoretical % Flux	**50**	**90**	**95**
Mass Balance (%)	45.6	92.3	82

The values obtained in this study were compared to theoretical values described by Wilson and co-workers. The P_{APP} values were in the same range as theoretical values for all the controls. The %flux values were lower than the theoretical values by 10 to 20% and the mass balance for atenolol was very low which suggested a low absorption rate. Since the parameters of the controls were close to the theoretical values, the values obtained for the antiplasmodial compounds can be assumed to be reliable. Table 6.30 shows the permeability parameters of all the compounds evaluated in this study.

Table 6.30: Permeability parameters of all antimalarials evaluated. Data represents the mean of data points performed in duplicate.

Permeability parameters		P_{APP} (cm/s)	% Flux	Mass Balance (%)
AZT-CQ Hybrid Compounds				
VAK 31	**1% DMSO**	$7.1x10^{-6}$	1.8	10
VAK 37	**1% DMSO**	$5.7x10^{-6}$	1.3	10.4
VAK59	**1% DMSO**	$3.0x10^{-6}$	1.2	25.3
	Formulation A	$2.8x10^{-6}$	1.2	70.7
	Formulation B	$1.7x10^{-5}$	6.4	70.8
AKAZTQ	**1% DMSO**	$2.5x10^{-6}$	1	13.5
AKAZTBQ	**1% DMSO**	$2.5x10^{-6}$	1	14
HPO-CQ Hybrid Compounds				
D13	**1% DMSO**	$3.5x10^{-6}$	1.4	38.6
	Formulation A	$6.7x10^{-6}$	2.8	73.2
	Formulation B	$4.4x10^{-5}$	17.3	85.6
13AB.2HCl	**1% DMSO**	$1.6x10^{-5}$	6.5	160
	Formulation A	$2.1x10^{-5}$	8.3	76.4
	Formulation B	$2.9x10^{-5}$	11.5	67.2
2-Aminopyridines				
MMV394903	**1% DMSO**	$2.5x10^{-6}$	1	37
MMV652120	**1% DMSO**	$2.7x10^{-6}$	1.1	13.1

As described earlier, compounds with high permeability have P_{APP} values higher than 1×10^{-6} cm/s (Corning Booklet). The data presented above shows P_{APP} values in a range of $2.9x10^{-5}$ to $2.5x10^{-6}$, which suggests that they have intermediate permeability in Caco-2 cells. However, the % flux calculated for each compound shows that the majority of the compounds have low permeability with values in the range of 1 to 17.3%. VAK59 and D13 in formulation B and 13AB.2HCl show a % flux of 6.5 to 17.3%. All the compounds prepared in formulation B showed enhanced permeability when compared to the compounds prepared in DMSO only. Even though DMSO has been used for many years to enhance solubility of water insoluble compounds, the compounds remain suspended or precipitate when diluted with an aqueous substance such as water or

culture medium. On the other hand, compounds prepared in o/w microemulsions become more soluble even when an aqueous substance is added because when an insoluble compound is dissolved in oil, surfactants and co-surfactants and an aqueous substance is added micelles are formed instead of a precipitate. For this reason enhanced permeability with compounds prepared in o/w microemulsions is expected. Formulation A has a high aqueous content when compared to formulation B. As such formulation B can be expected to be more effective than A as indicated in Table 6.30 AKAZTQ and AKAZTBQ were only prepared in DMSO because there was not enough material for o/w emulsion preparations.

Table 4.25 shows that MMV394903 and MMV652120 had relatively high bioavailabilities of 69 and 60% even when prepared with reference formulation only. The permeability of these compounds, therefore, is expected to be high across the intestinal epithelium because the rate and extent of drug release and absorption in the gastrointestinal tract contribute to the bioavailability of that drug. The data presented in Table 6.30 show a flux % of 1% for both compounds, which indicate low permeability. This was also observed with AKAZTQ and AKAZTBQ, which have bioavailabilities of 12 and 30%, respectively. Their flux % was also 1%. The improper correlation between Caco-2 permeability and oral bioavailability of these compounds can be explained by a low mass balance. This is usually the case when the concentration of the compounds in the donor and receiver wells is very low, which implies that very little compound diffused through the membrane. All the compounds prepared in DMSO had very low permeability with % flux below 2%. Because these compounds did not dissolve completely, transportation of the drug to the basal side of the monolayer was not effective as indicated by low mass balance value presented in Table 6.30 (Liu *et al*., 2010). 13AB.2HCl is water soluble and was the only compound prepared in DMSO that had a higher % flux than other compounds.

The data presented for compounds prepared in 1% DMSO show that estimating permeability of hydrophobic compounds in the Caco-2 monolayers can lead to unreliable data because of their poor solubility and high lipophilicity, which may result in

considerable retention of compounds by the cell monolayer as well as non-specific binding or adsorption to membrane surface (Krishna *et al*., 2001). For this reason a test was performed to determine whether low mass balance was caused by binding of compounds that were not completely dissolved to the polycarbonate membrane. The permeability assay was repeated but no cells were grown on the polycarbonate membranes. The compounds were prepared in 1% DMSO in order to compare their %flux to the one obtained from the Caco-2 permeability assay. There was very little compound detected on the receiver side on all the compounds, giving a %flux that is less than 0.5% and a mass balance of ± 10% for all the compounds. Low mass balance was caused by the reduction of compound concentration on both the donor and receiver wells in the absence of Caco-2 monolayers, and these results show a contribution of compound adsorption on the membrane surface. Atenolol and propranolol were used as controls and both gave a %flux of 71.5 and 67% with a mass balance of 85 and 80.3%, respectively. There were no solubility issues with the test controls observed. Thus based on these findings it was concluded that the permeability results of all the compounds prepared in 1% DMSO were not reliable due to the fact that these compounds did not dissolve completely in the medium. This may have resulted in these compounds binding to the polycarbonate membrane, which would have meant that very little compound was detected on the receiver side.

The compounds that were prepared in o/w microemulsions had high mass balance values because the presence of micellar aggregates caused by the surfactants facilitated the diffusion of the compounds through the membrane. In this case no binding was expected because the compounds were completely dissolved in the micelles (Kobayashi *et al*., 2002; Rosslee *et al*., 2004).

Numerous studies have shown a good correlation between permeability in the Caco-2 monolayers and the oral absorption in humans but this method is not always reliable for determining the bioavailability of compounds. The binding of insoluble compounds to the membrane surface is one of the reasons for unreliable data and in some cases overrprediction of oral absorption of compounds with a high intestinal first-pass effect

can be expected because Caco-2 cells lacks the phase I metabolic enzymes, such as CYP3A4 (Lau *et al*., 2004). Nevertheless, the results discussed in this study show a possibility that poor oral absorption can be one of the factors that affected oral bioavailability of antiplasmodial compounds assessed, resulting in poor PK properties.

Chapter 7: Summary and Conclusion

Research Summary and Conclusion

The data presented in this thesis addresses a few of the many factors that cause potential antimalarials to be discarded in the early drug development stages. Many potential drug candidates with very high *in vitro* potency show poor efficacy and PK properties when assessed *in vivo*, due to a range of factors which include solubility, permeability and metabolic stability. In pharmaceutical drug design, aqueous solubility is considered to be a fundamental and important physicochemical property because water-insoluble compounds can result in poor drug absorption and ultimately poor oral bioavailability.

This thesis discussed twelve compounds that were evaluated for antiplasmodial activity against chloroquine sensitive strains 3D7 and D10 and resistant strains K1 and Dd2. AZT-CQ hybrids were designed in order to inhibit the growth of malaria parasites and HIV, simultaneously because in areas such as Sub-Saharan Africa these diseases are endemic and coexist. The activity of the AZT-CQ hybrid compounds against both chloroquine sensitive and resistant strains was reduced by 10 to 100 fold when compared to chloroquine. When the intermediates of these compounds were tested for antiplasmodial activity their IC_{50} values were comparable to that of chloroquine against sensitive strain but superior IC_{50} values were observed for resistant strains. The intermediates of the AZT-CQ hybrids were silylated with a *tert*-butyldiphenylsilyl group and its presence resulted in activity greater than that of chloroquine. AZT was the only part of the molecule that was silylated and this bulky group would likely affect the anti-HIV activity of the compound. Despite good *in vitro* activity the solubility of the silylated hybrids was reduced and this affected their PK properties. The bioavailability was very low even when administered in o/w microemulsions. Poor PK was also observed when VAK59 was administered intravenously with a very high elimination rate. This collectively accounted for the poor in vivo efficacy in the *P. berghei* mouse model.

AKAZTQ and AKAZTBQ on the other hand were desilylated AZT-CQ hybrids that were evaluated for PK properties. These compounds were more soluble than the silylated AZT-CQ hybrid and this resulted in an improved bioavailability of 12 and

30% for AKAZTQ and AKAZTBQ, respectively. Studies have shown that structural modifications can improve pharmacokinetic properties but might also negatively impact on other desirable properties such as activity. This is observed by the desilylation of silylated AZT-CQ hybrids to give rise to AZT-CQ hybrids. *In vitro* antiplasmodial activity of VAK 31DS, VAK 37DS and VAK 59DS was reduced by up to 1000 fold when the *tert*-butyldiphenylsilyl group was removed. This further supports the fact that substructures that have minimal effect on structure-activity relationship (SAR) must be identified first during structural modifications (Kerns, 2003).

D13 and 13AB.2HCl are HPO-CQ hybrid compounds with *in vitro* activity comparable to that of chloroquine. D13 had had poor solubility, and together with 13AB.2HCl had very low oral bioavailability, even when administered in o/w microemulsions. This is presumably due to solubility-limited absorption. When D13 was administered intravenously a longer half life was observed and this property caused the compound to show activity against *P. berghei in vivo.* On day 7 post infection 2% inhibition was observed when the mice were dosed intravenously with 4 and 8 mg/kg D13. A study performed on Caco-2 cell line grown on polycarbonate membrane to investigated drug absorption of all active compounds confirmed that the compounds had very low permeability across the intestinal epithelium, which may have contributed to low oral bioavailability observed with the compounds. This is likely due to poor solubility of these compounds which negatively impacted permeability as indicated by the Caco2 studies.

Another group of compounds evaluated were 2-aminopyridines, MMV394903 and MMV652120, which were very potent antimalarial compounds with *in vitro* IC_{50} values 10 times superior to those of chloroquine against resistant strains. The oral bioavailability of these compounds was relatively high with BA% values of 69 and 60% for MMV394903 and MMV652120, respectively. Efficacy evaluation performed at the Swiss Tropical and Public Health Institute (Swiss TPH) showed that the compounds were active *in vivo* but did not achieve a 100% suppression of *P. berghei* and infection progressed faster when comparing with the activity of chloroquine

against *P. berghei in vivo*. MMV394903 and MMV652120 also had poor solubility and therefore this led to undesirable PK properties as seen in Table 4.25.

In most cases poor PK properties of compounds identified as potent antiplasmodial *in vitro* agents are due to poor solubility, For example, the silylated AZT-CQ hybrid compounds VAK31, VAK37 and VAK59 were very potent *in vitro* against resistant strains but poor solubility may also have contributed to poor oral bioavailability and permeability. Attempts were made to improve their pharmacokinetic profiles by preparing o/w microemulsion formulations but no significant improvement was observed. Compounds with poor solubility apparently diffuse to the interstitial fluid by binding on to the phospholipids on the capillary membrane, resulting in rapid elimination of the compound from the blood and high distribution and this explains the PK data in Table 4.25. The clearance rate of the compounds reported in this table was greater than 27 ml/min/kg and the volume of distribution was greater than 19 L/kg. Even though MMV394903 and MMV652120 had %BA values of 69 and 60 % the fact that they had poor aqueous solubility caused high volume of distribution and clearance rate values and these were responsible for short half lives, thus affecting their *in vivo* efficacy.

In silico approaches have also been used by chemists to obtain information to help identify lead compounds with potential low developability liabilities, thereby enabling the selection of candidates for lead optimization and potential drug development (Valerio, 2009). *In silico* tools provide guidance regarding structural modifications that may improve desirable properties before compound synthesis can be undertaken (Butina *et al.*, 2002). *In silico* tools can also be used to predict the solubility of the compounds. As the spread of malaria parasite drug resistance increases in malaria endemic countries, there remains an urgent need to develop new effective antimalarial candidate drugs and using *in silico* procedures can provide early prediction of ADME properties. This may facilitate early elimination of compounds with poor pharmacokinetic properties to and allow those designed with optimal properties to proceed.

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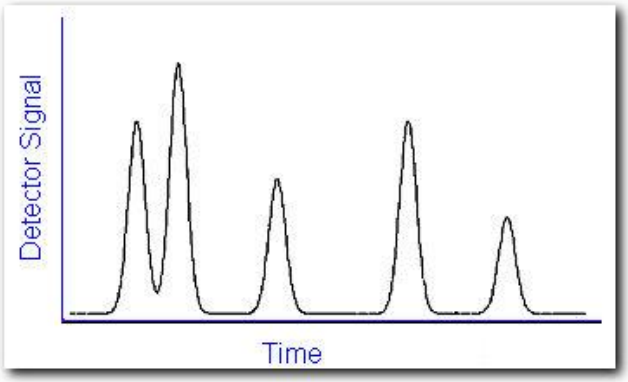
Appendix A: Calibration Curves, Mass Spectra and Chromatograms



Calibration Curves, Mass Spectra and Chromatograms

A.1. VAK31 Detection by LC-MS/MS

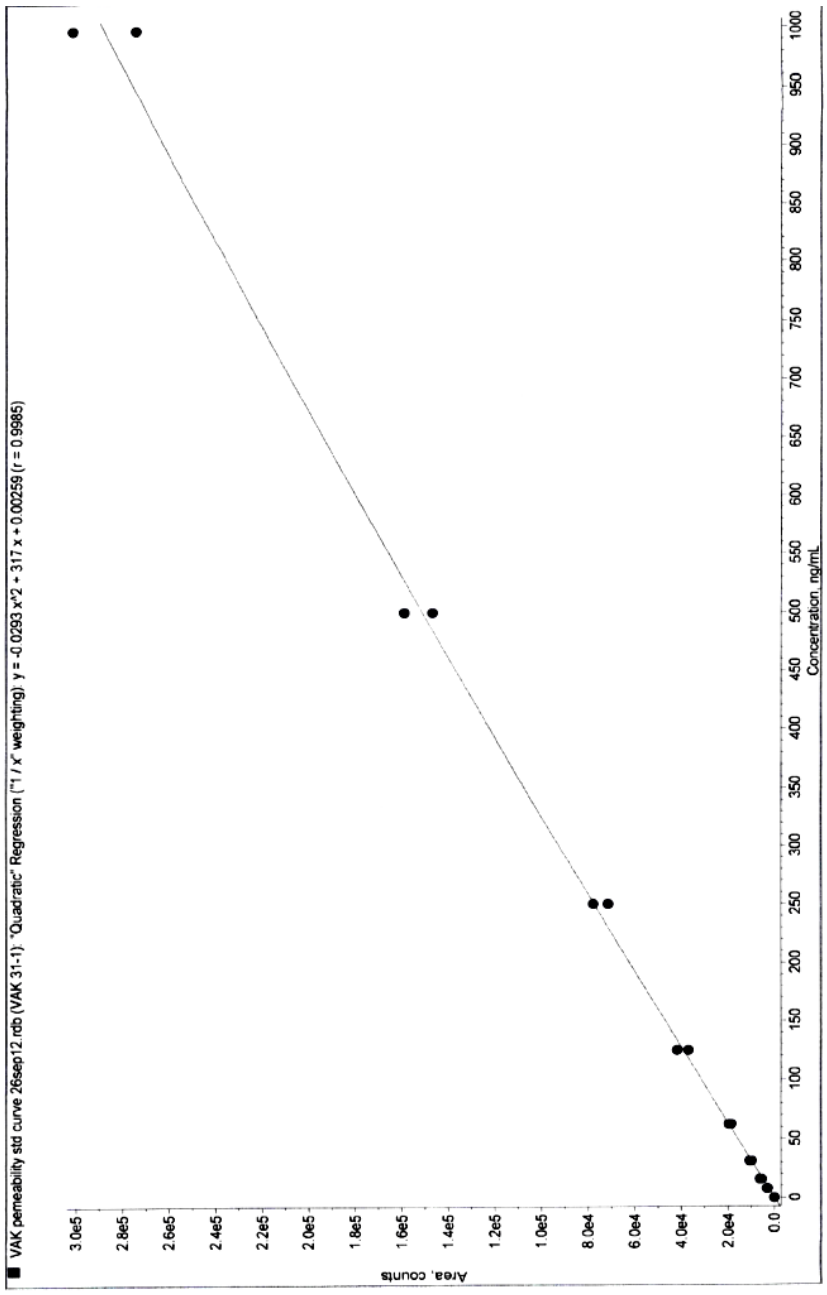


Figure A.56: Typical calibration curve for VAK31 detection by LC-MS/MS.

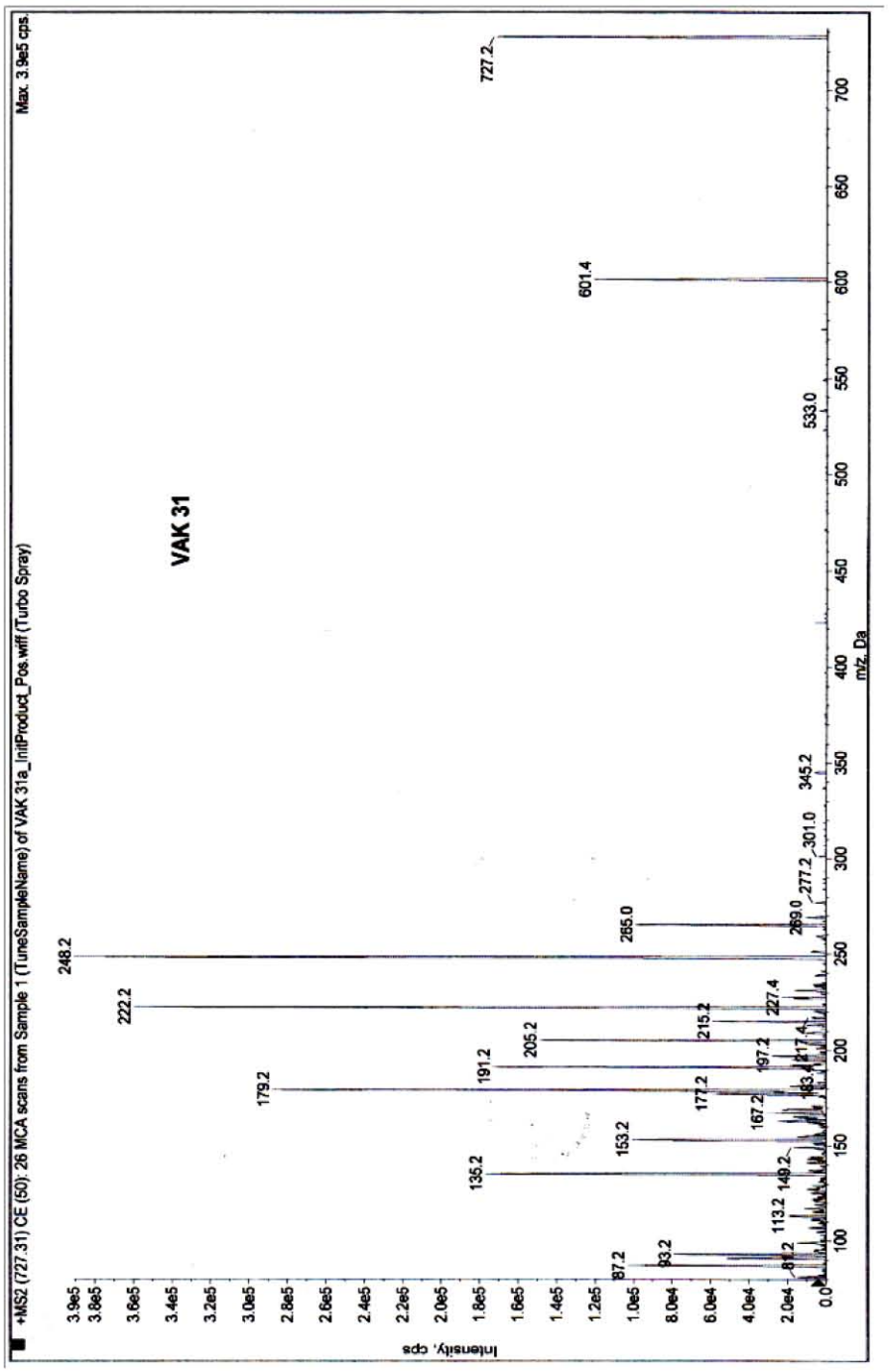


Figure A.57: Typical mass spectrum of VAK31 detection by LC-MS/MS.

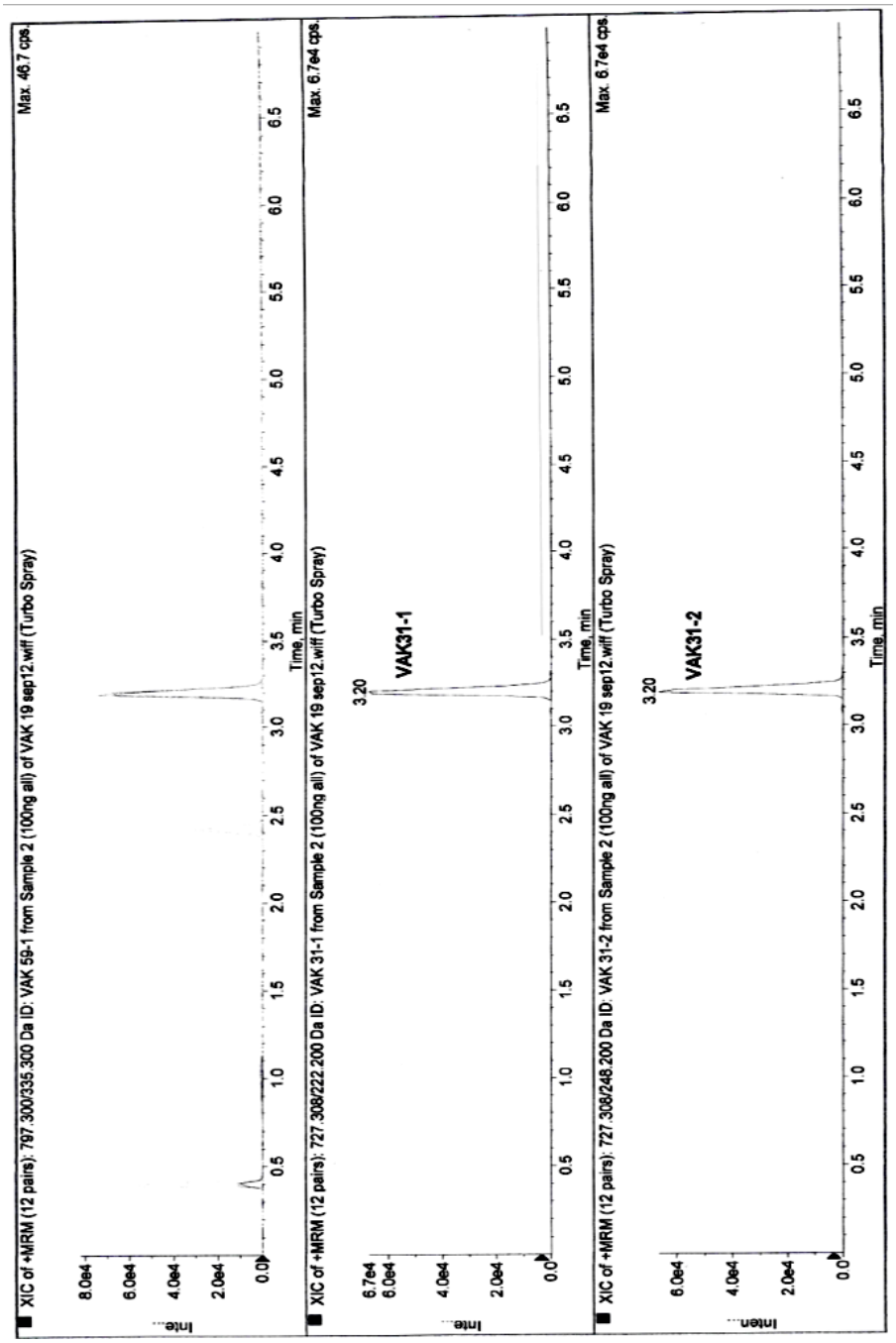


Figure A.58: Typical LC/MS/MS chromatograms in MRM mode for the analysis of VAK 31.

A.2. VAK37 Detection by LC-MS/MS

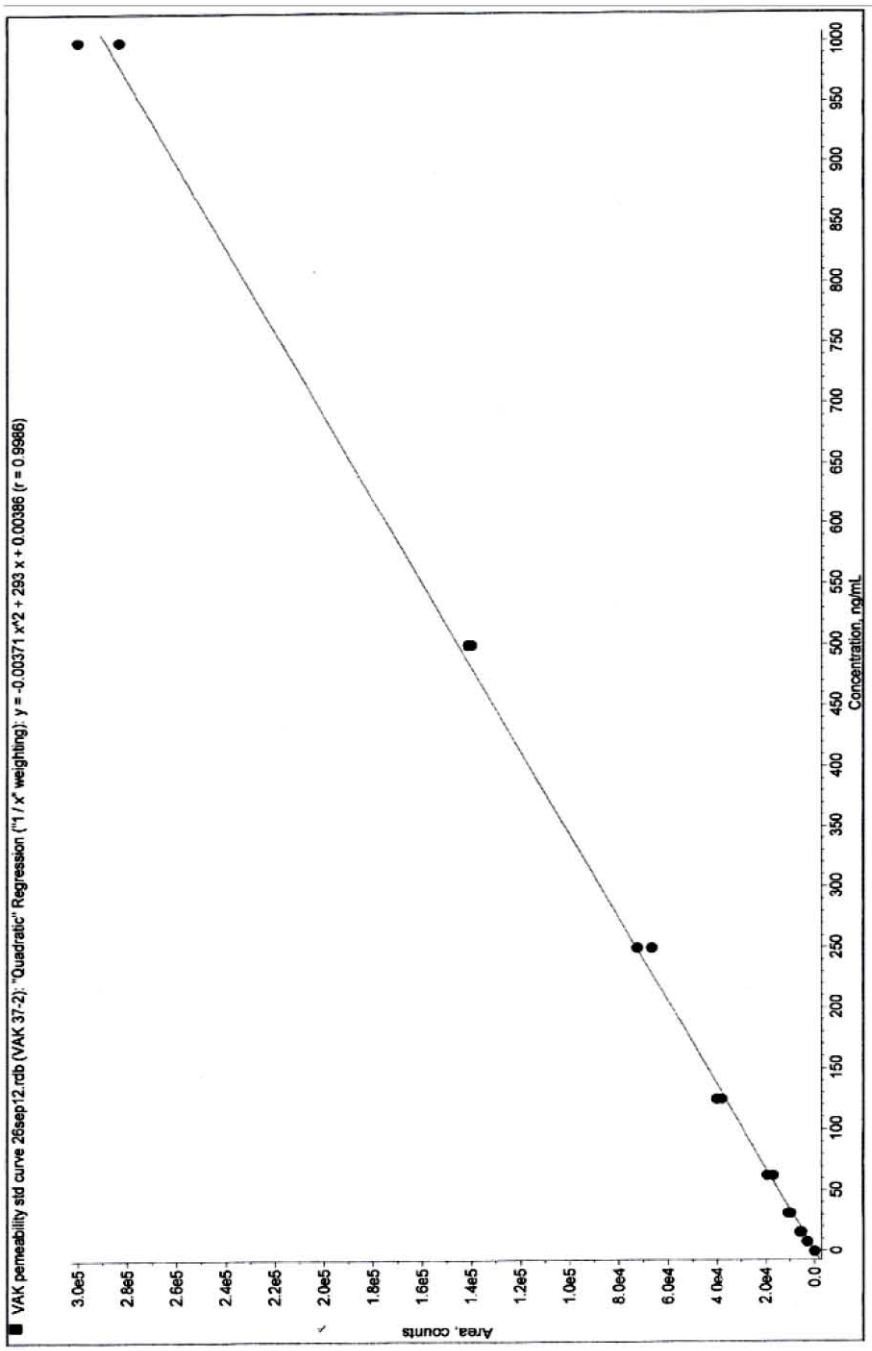


Figure A.59: Typical calibration curve for VAK37 detection by LC-MS/MS.

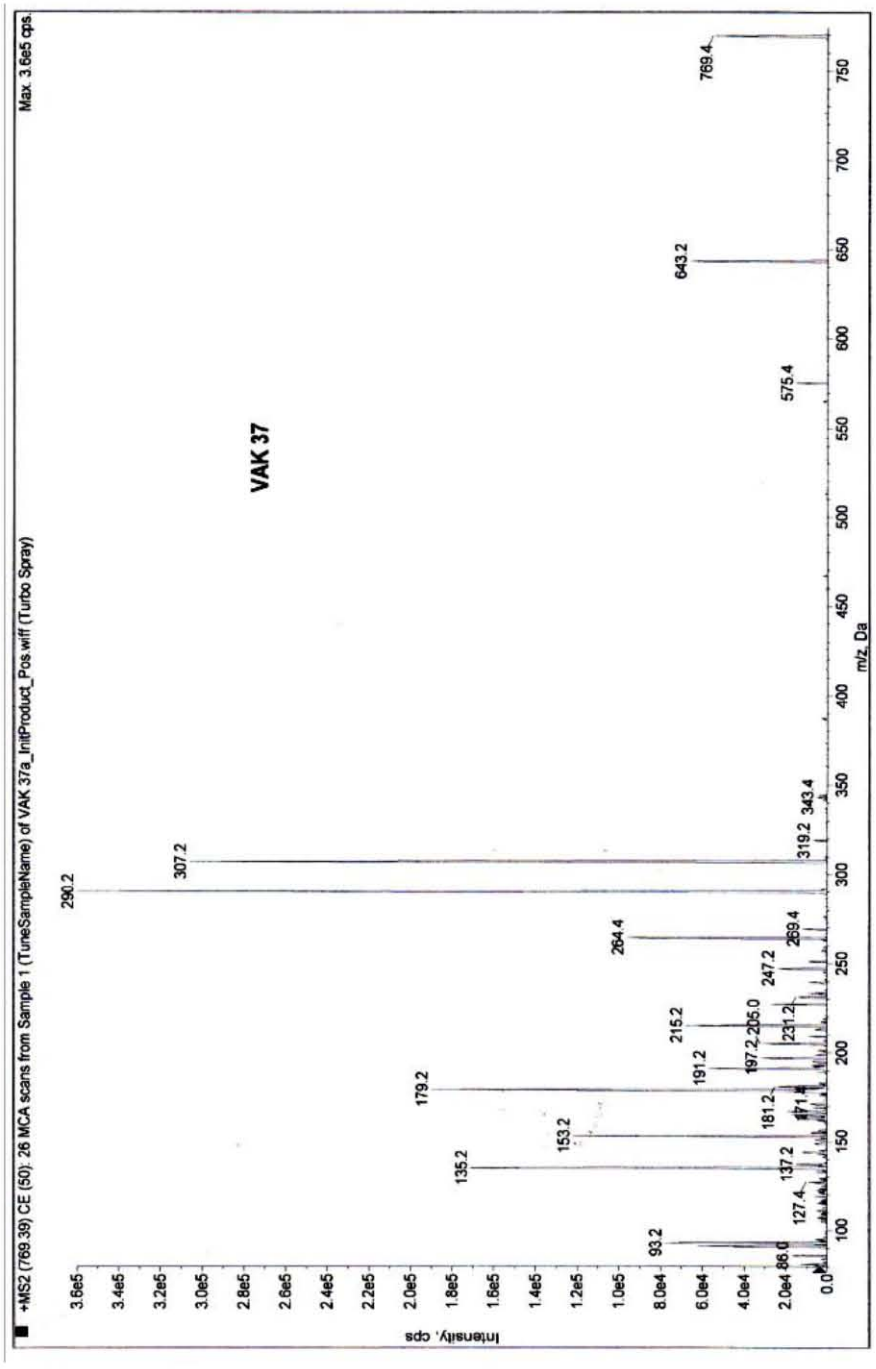


Figure A.60: Typical mass spectrum of VAK37 detection by LC-MS/MS.

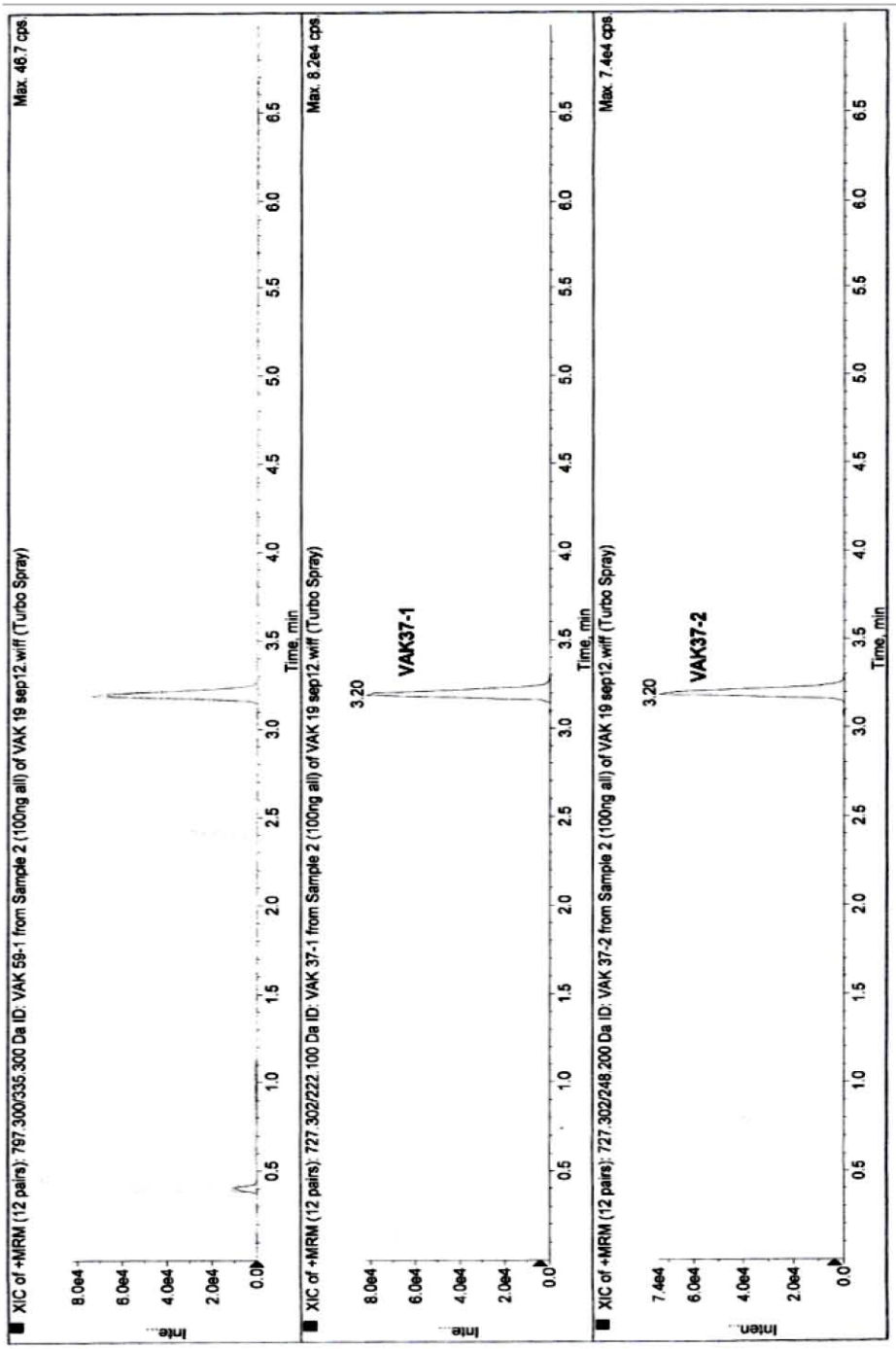


Figure A.61: Typical LC/MS/MS chromatograms in MRM mode for the analysis of VAK37.

A.3. VAK59 Detection by LC-MS/MS

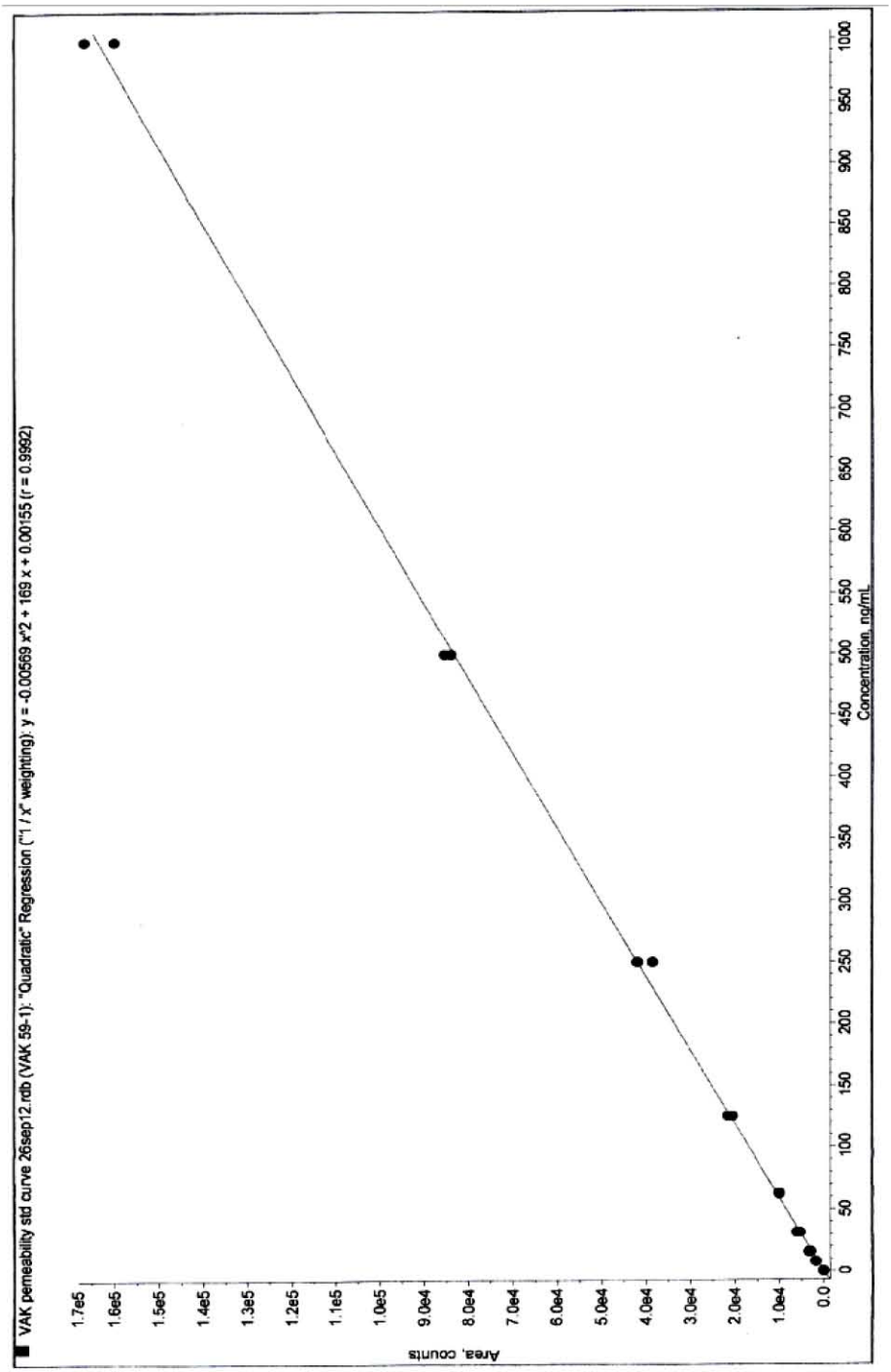


Figure A.62: Typical calibration curve for VAK59 detection by LC-MS/MS.

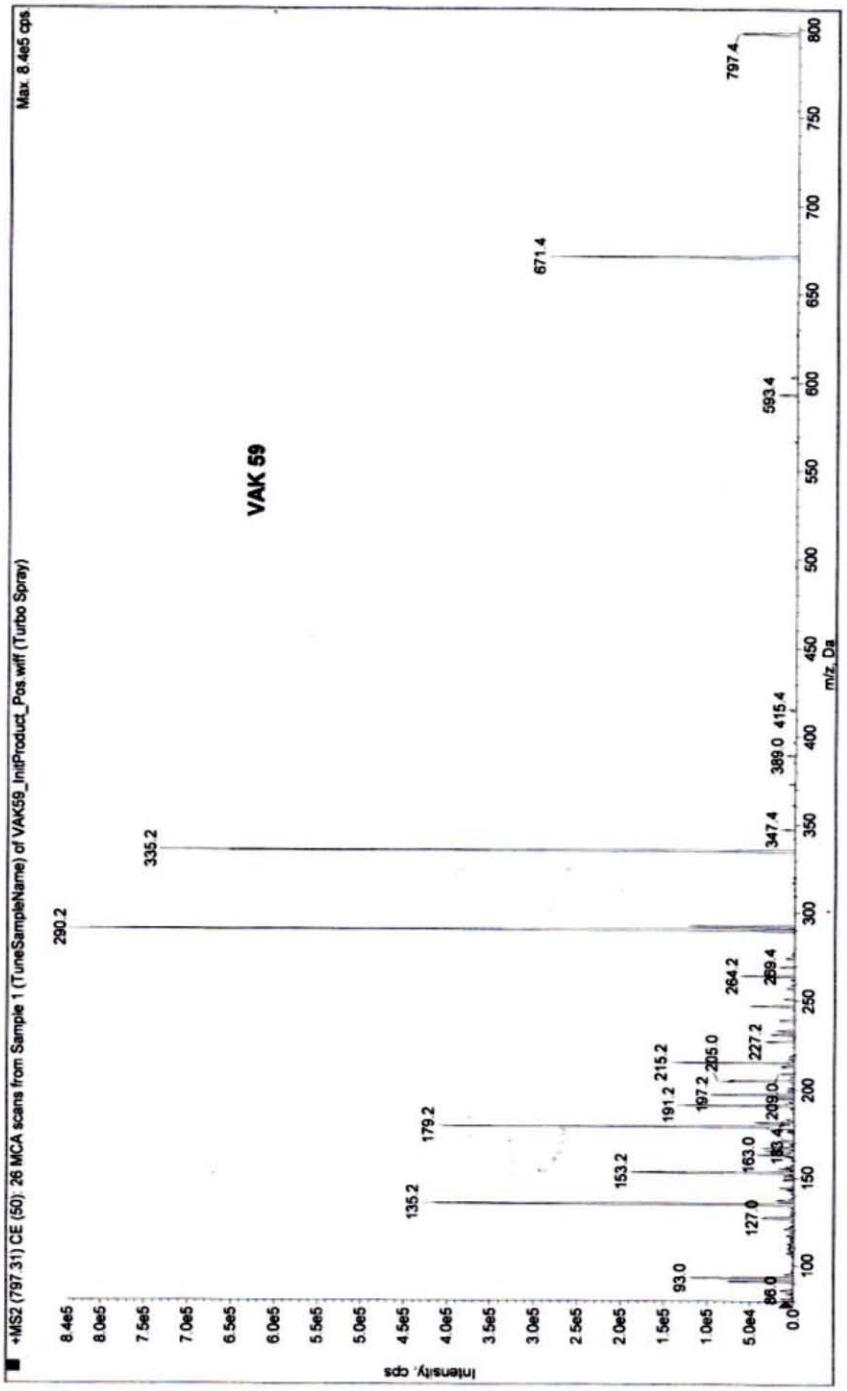


Figure A.63: Typical mass spectrum of VAK59 detection by LC-MS/MS.

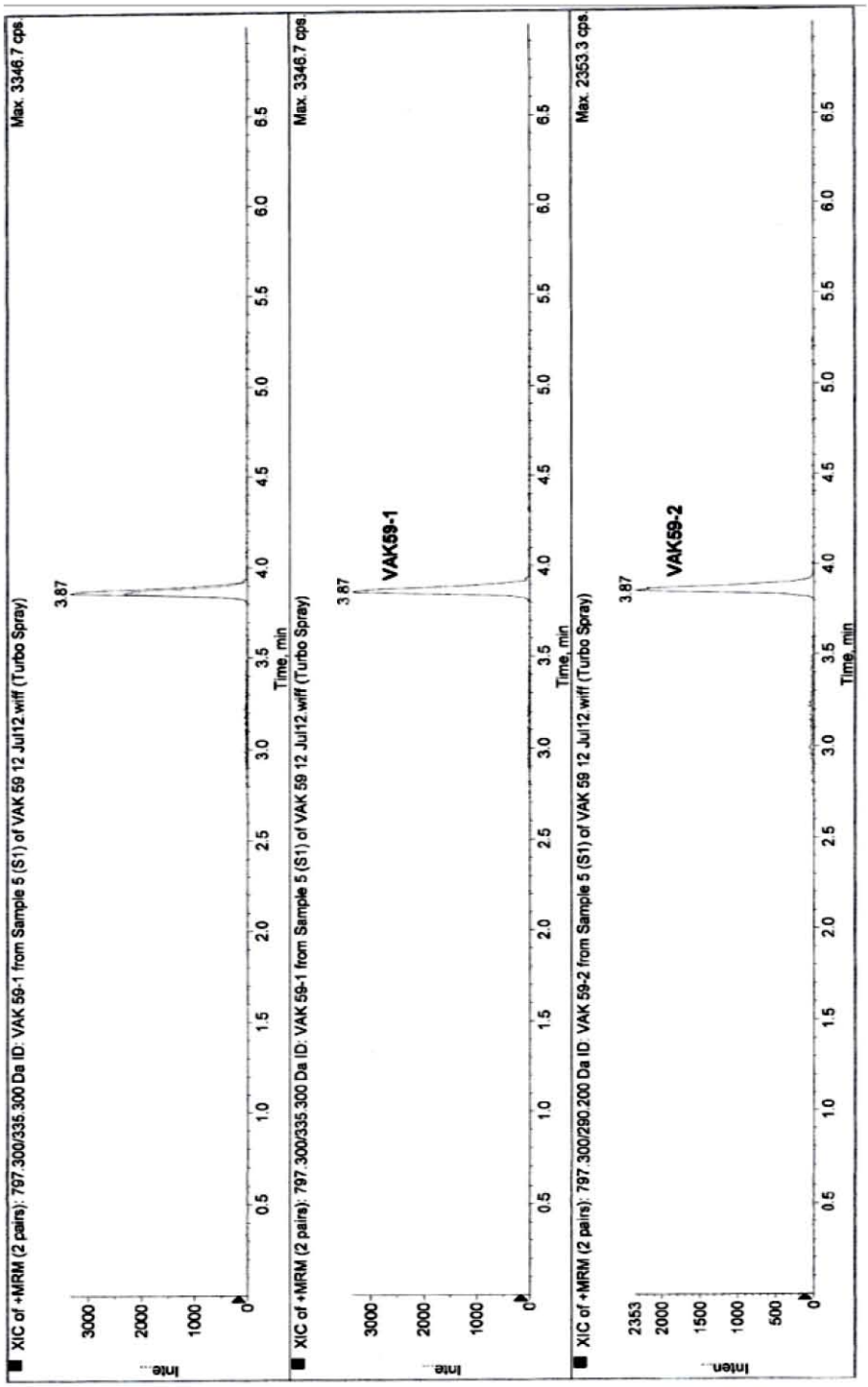


Figure A.64: Typical LC/MS/MS chromatograms in MRM mode for the analysis of VAK 59.

A.4. AKAZTQ Detection by LC-MS/MS

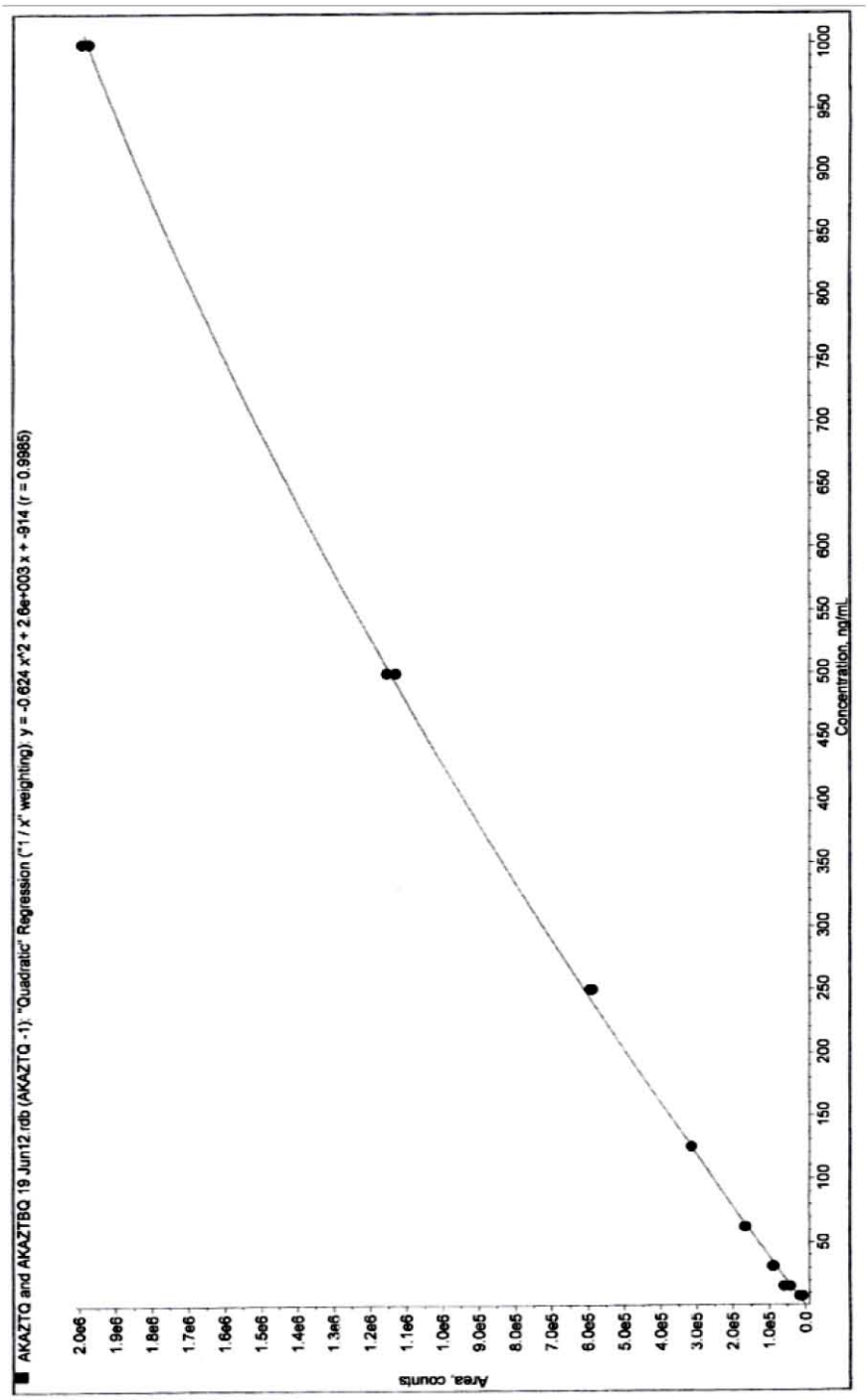


Figure A.65: Typical calibration curve for AKAZTQ detection by LC-MS/MS.

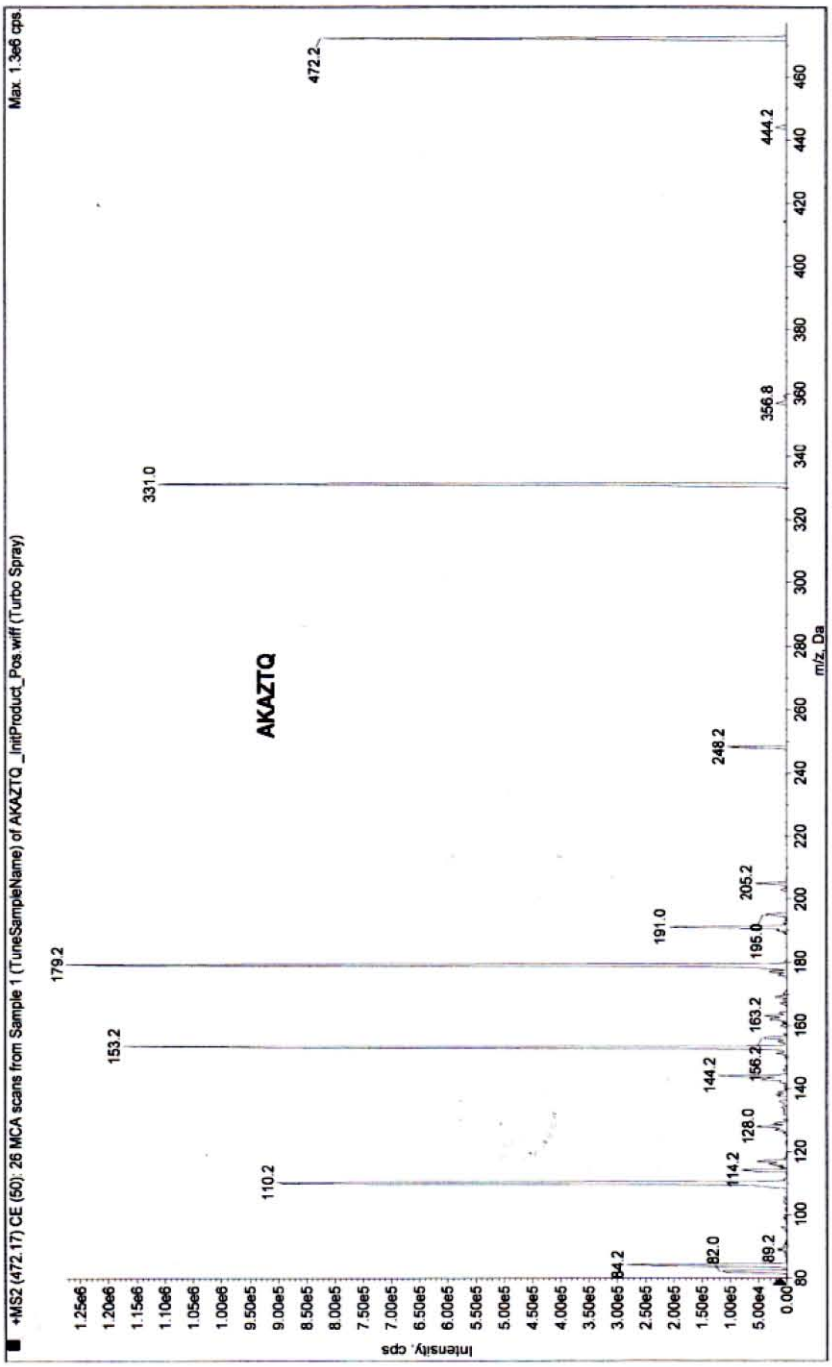


Figure A.66: Typical mass spectrum of AKAZTQ detection by LC-MS/MS.

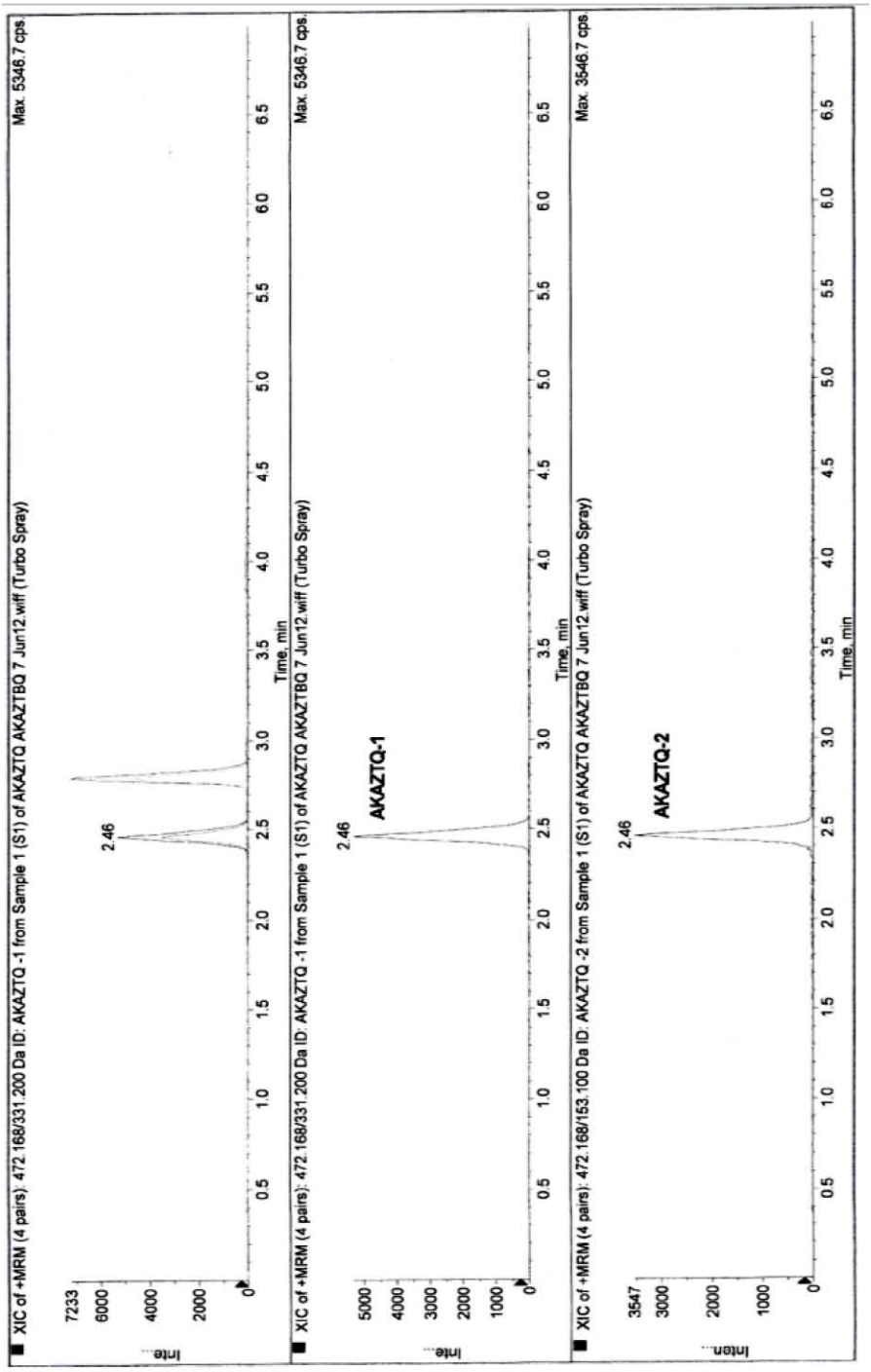


Figure A.67: Typical LC/MS/MS chromatograms in MRM mode for the analysis of AKAZT Q.

A.5. AKAZTBQ Detection by LC-MS/MS

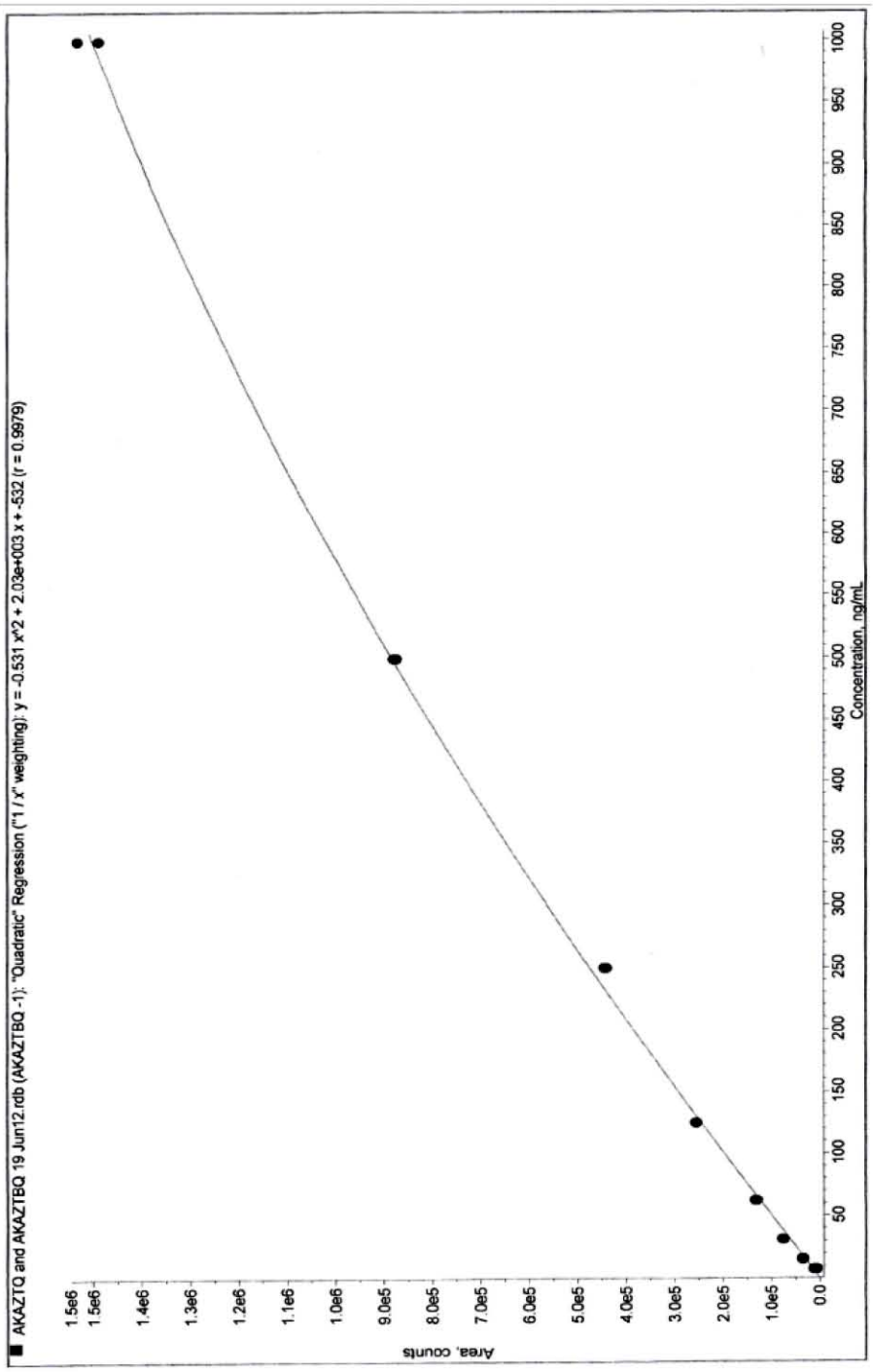


Figure A.68: Typical calibration curve for AKAZTBQ detection by LC-MS/MS.

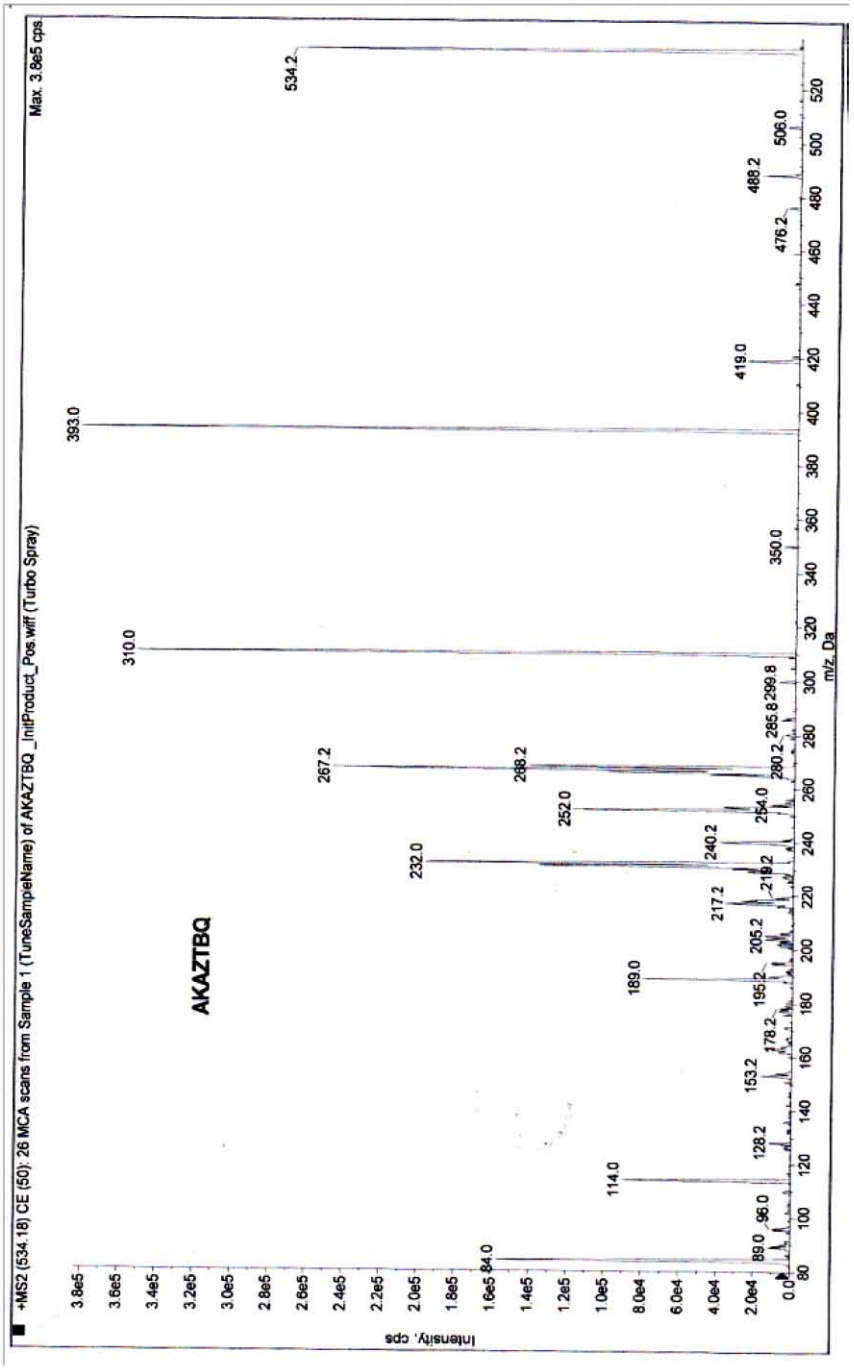


Figure A.69: Typical mass spectrum of AKAZTBQ detection by LC-MS/MS.

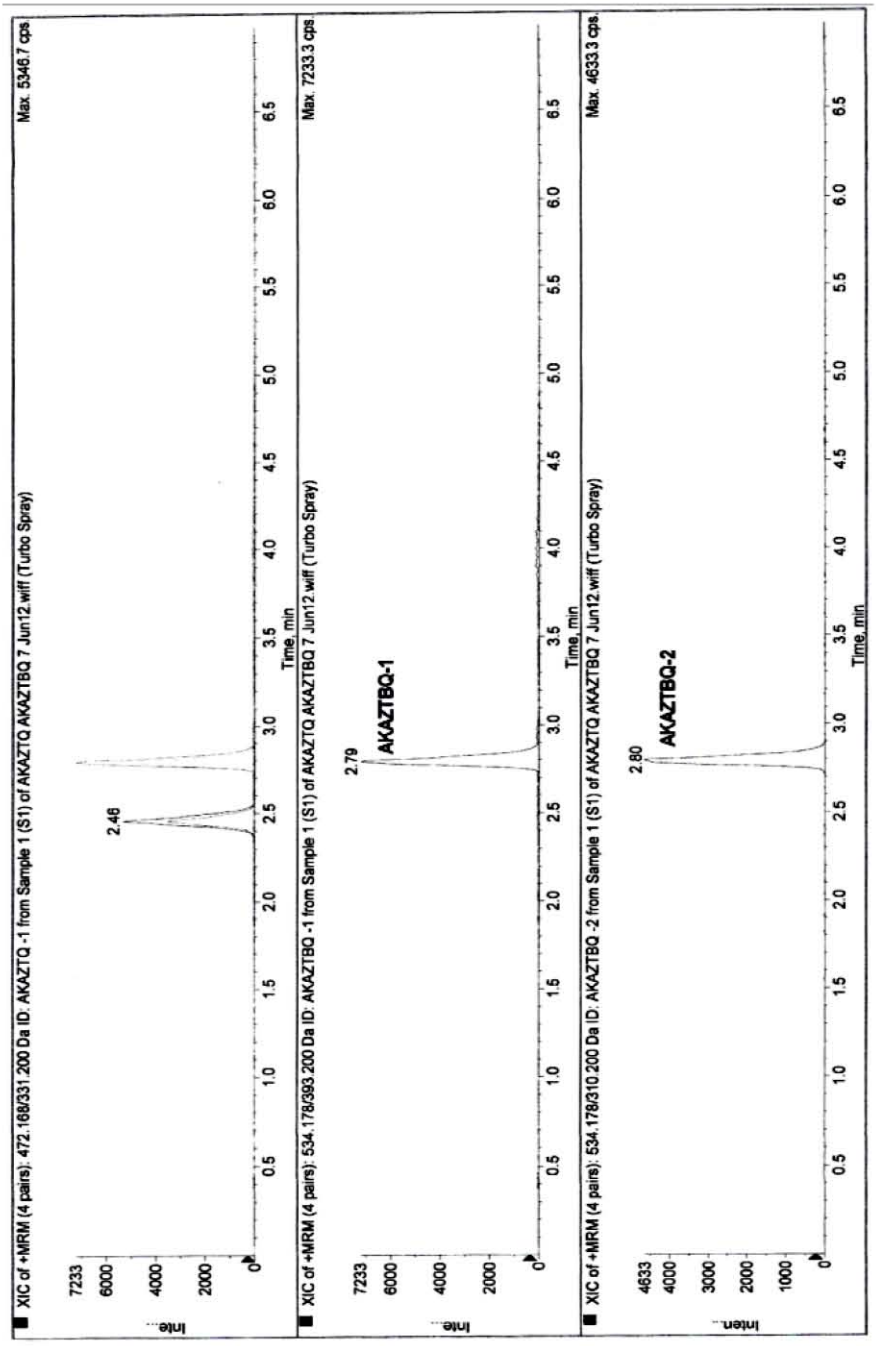


Figure A.70: Typical LC/MS/MS chromatograms in MRM mode for the analysis of AKAZTBQ.

A.6. D13 Detection by LC-MS/MS

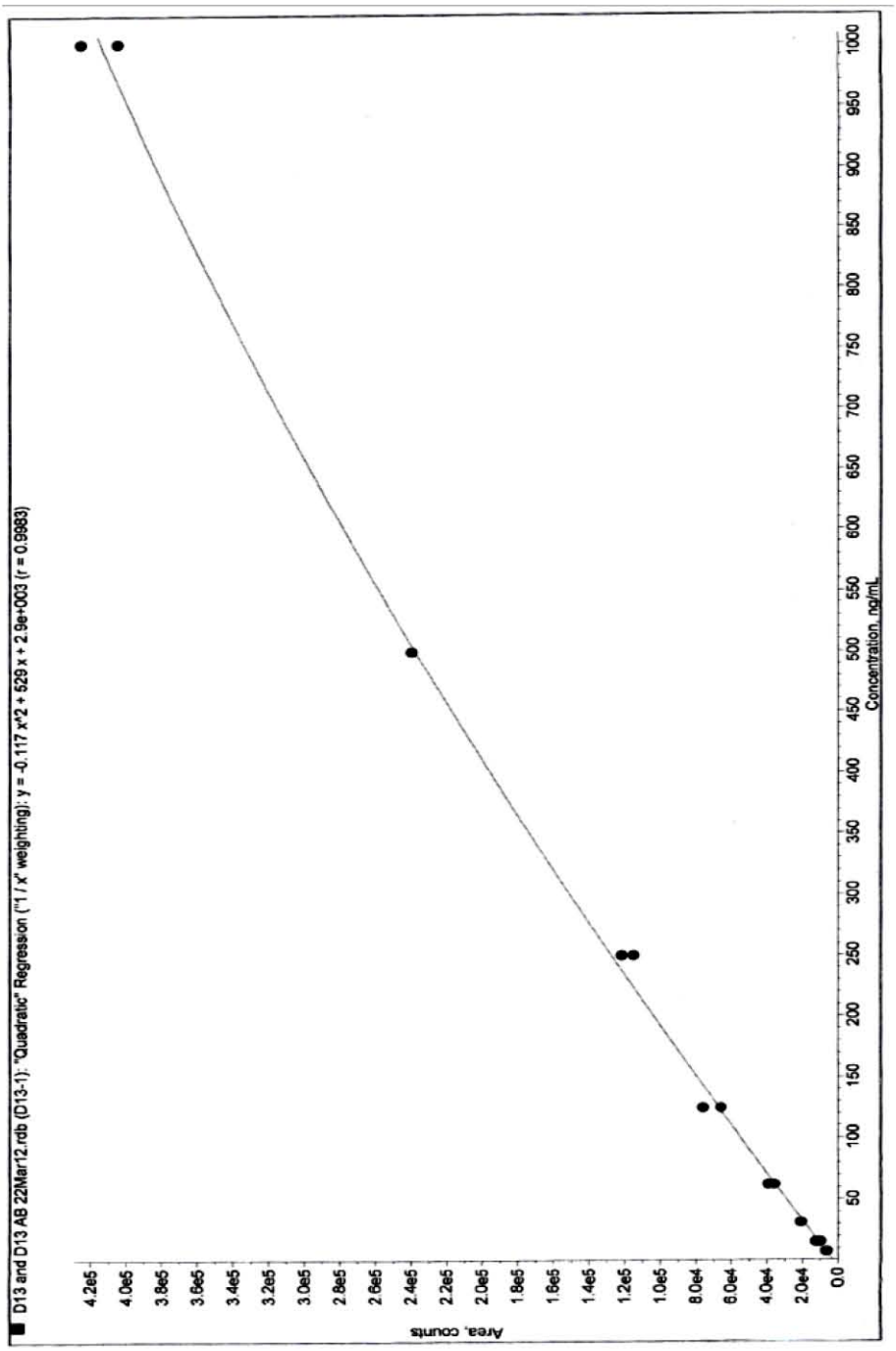


Figure A.71: Typical calibration curve for D13 detection by LC-MS/MS.

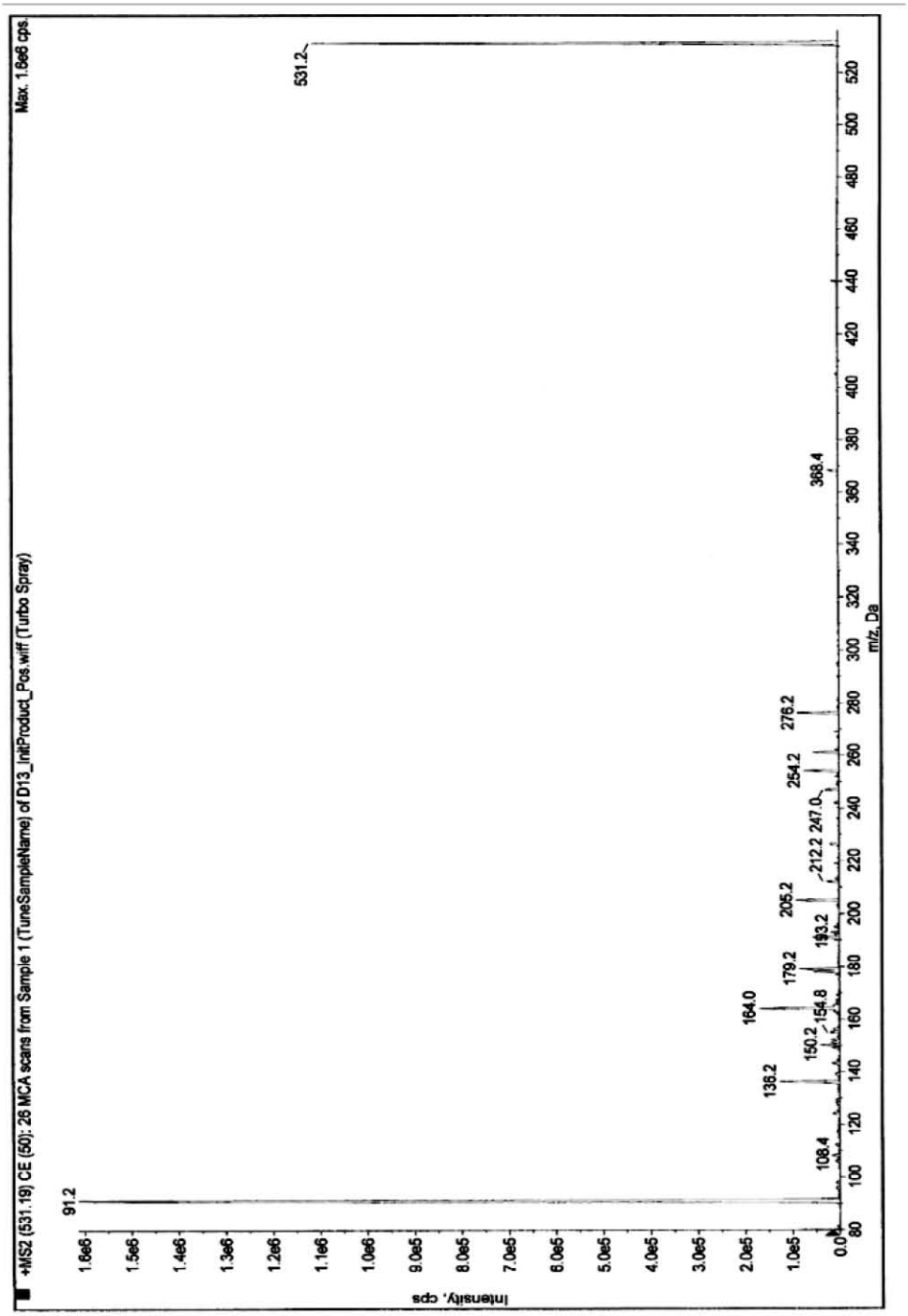


Figure A.72: Typical mass spectrum of D13 detection by LC-MS/MS.

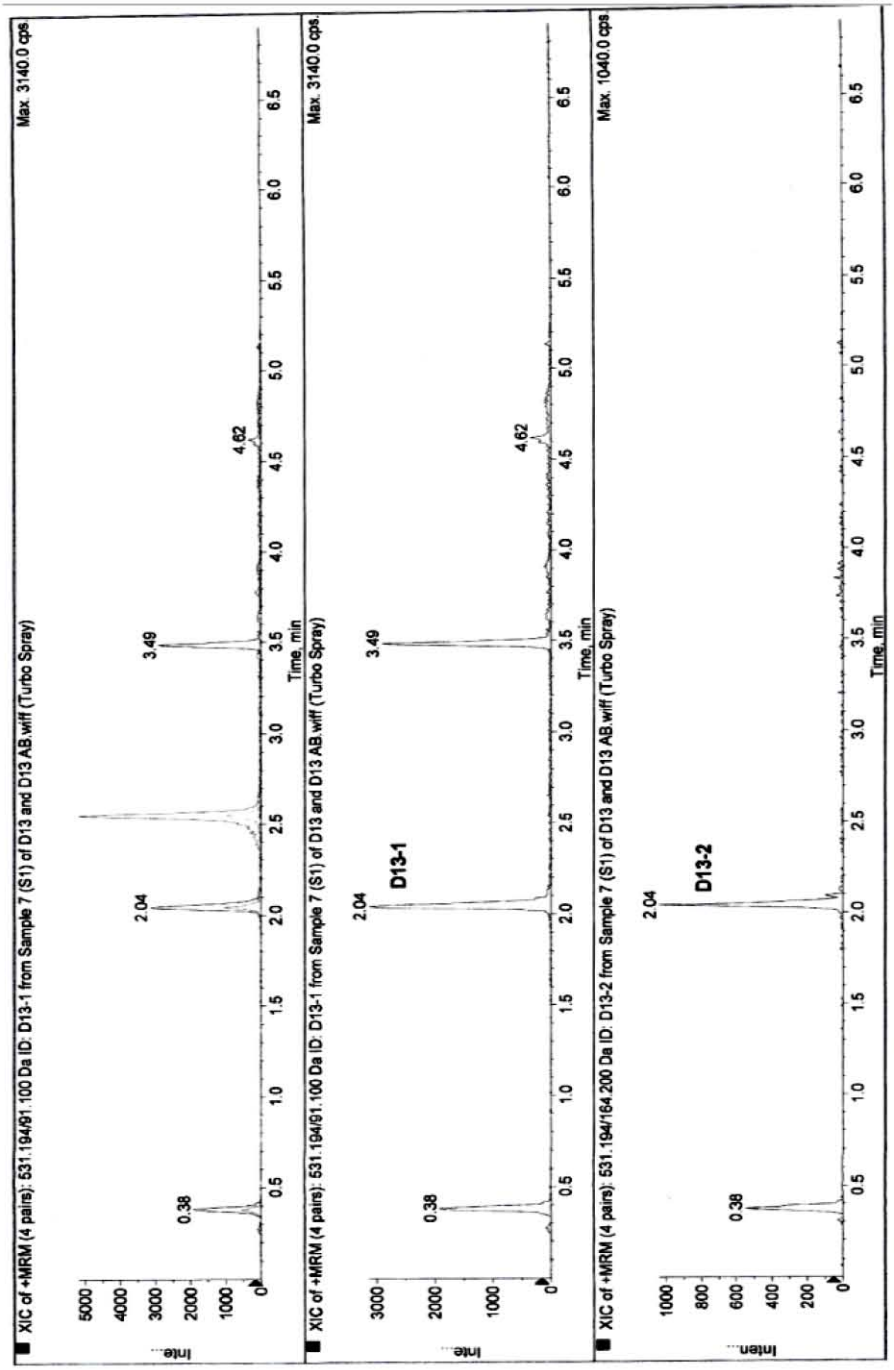


Figure A.73: Typical LC/MS/MS chromatograms in MRM mode for the analysis of D13.

A.7. 13AB.2HCl Detection by LC-MS/MS

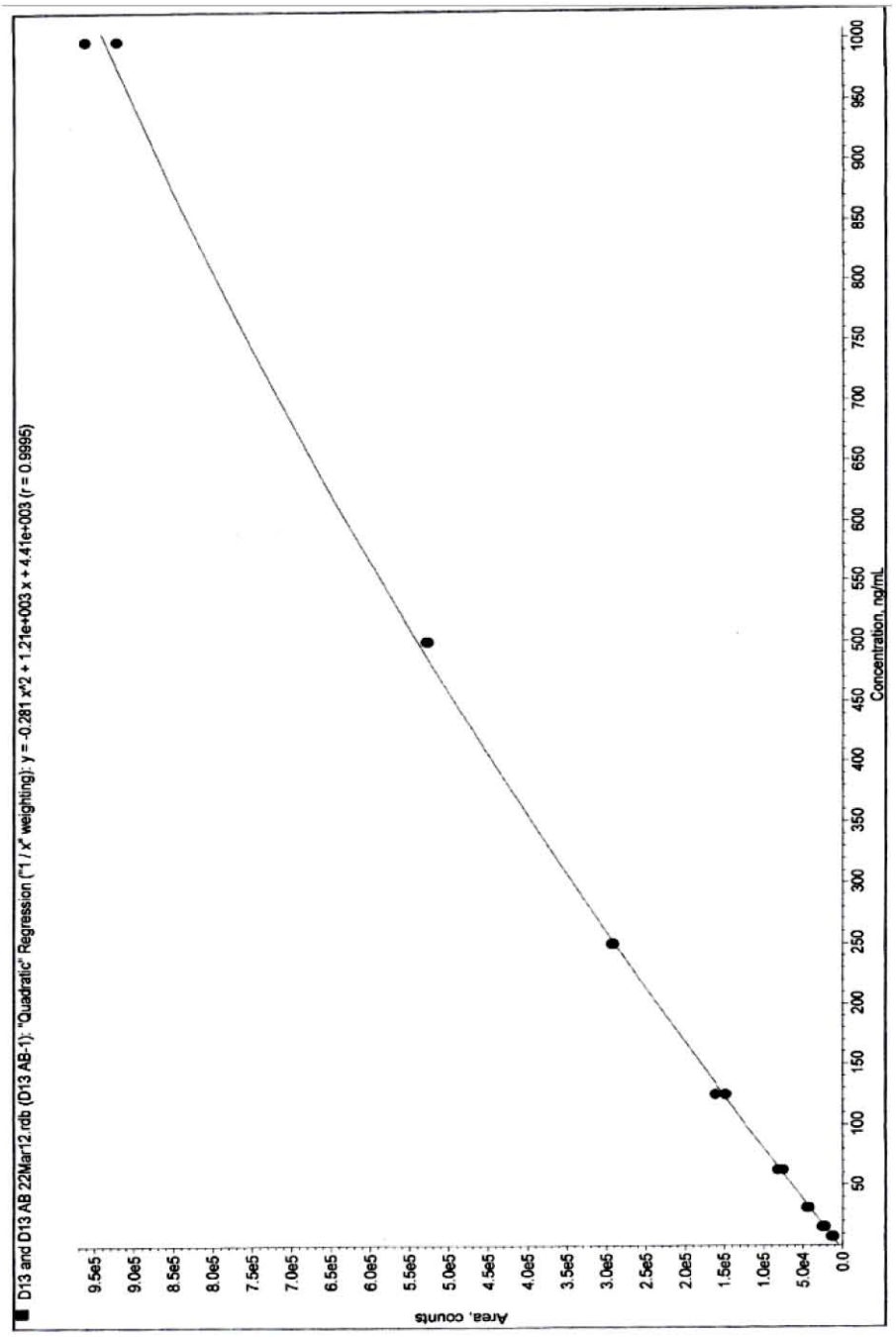


Figure A.74: Typical calibration curve for 13AB.2HCl detection by LC-MS/MS.

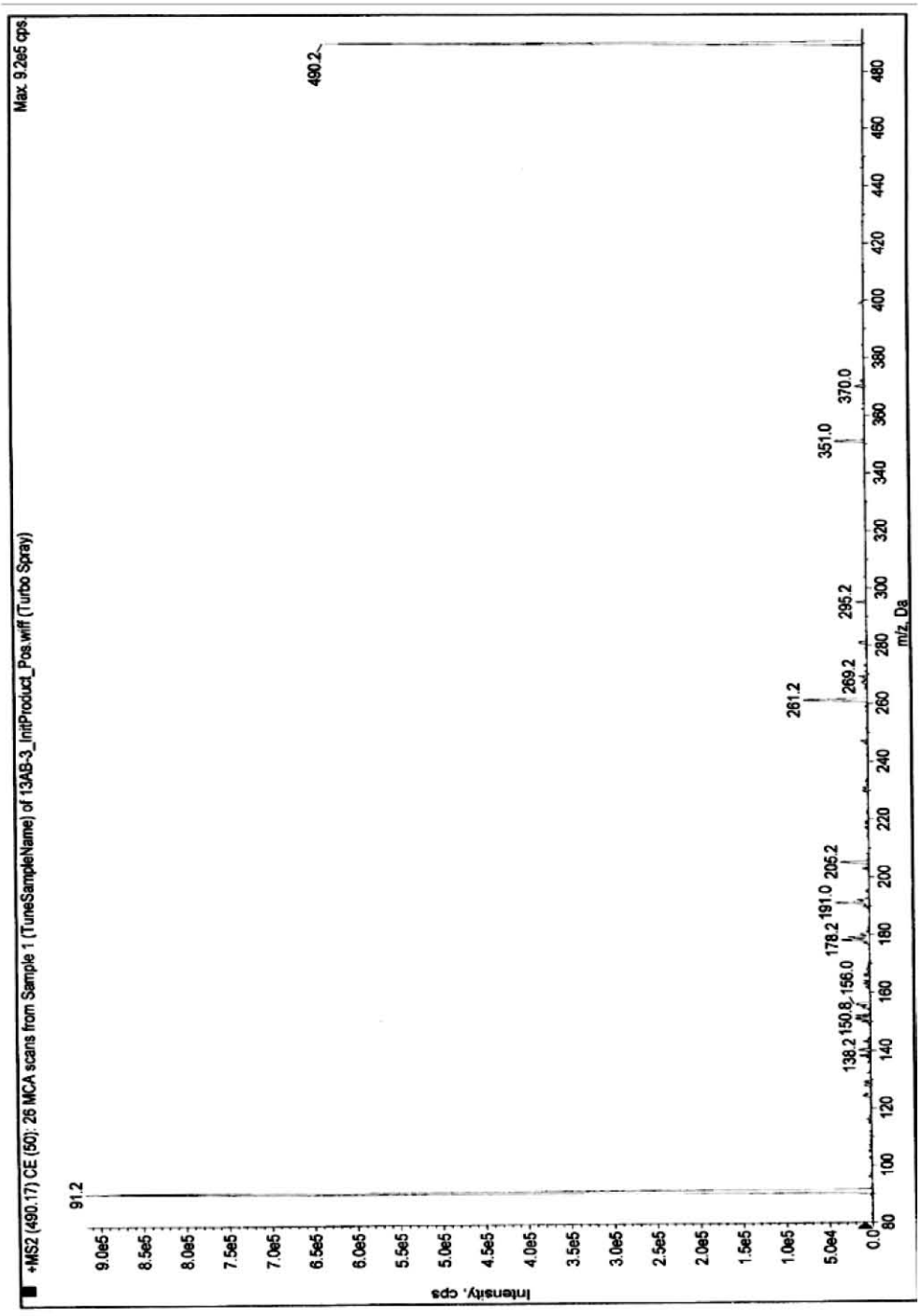


Figure A.75: Typical mass spectrum of 13AB.2HCl detection by LC-MS/MS.

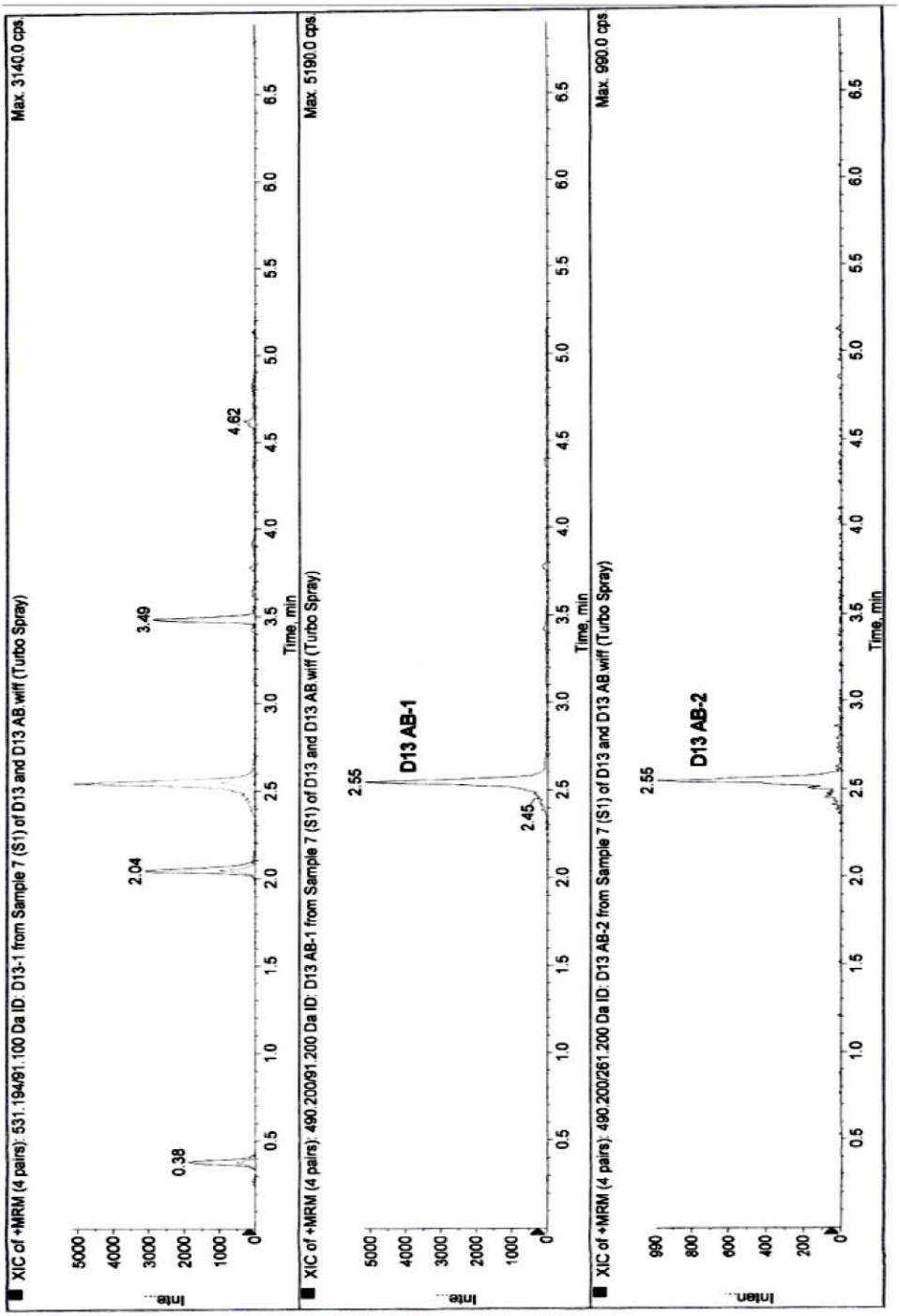


Figure A.76: Typical LC/MS/MS chromatograms in MRM mode for the analysis of 13AB.2HCl.

A.8. MMV394903 Detection by LC-MS/MS

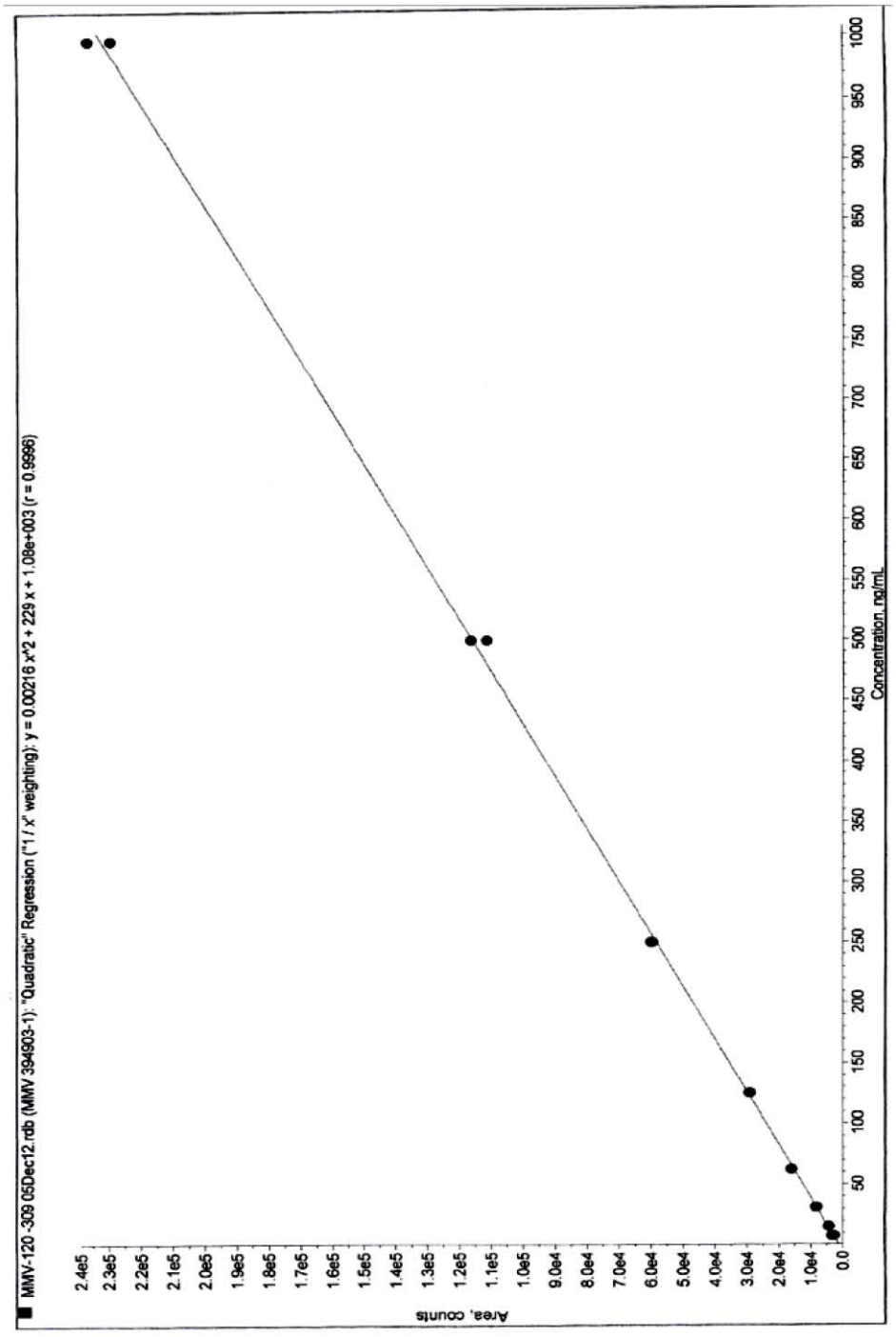


Figure A.77: Typical calibration curve for MMV394903 detection by LC-MS/MS.

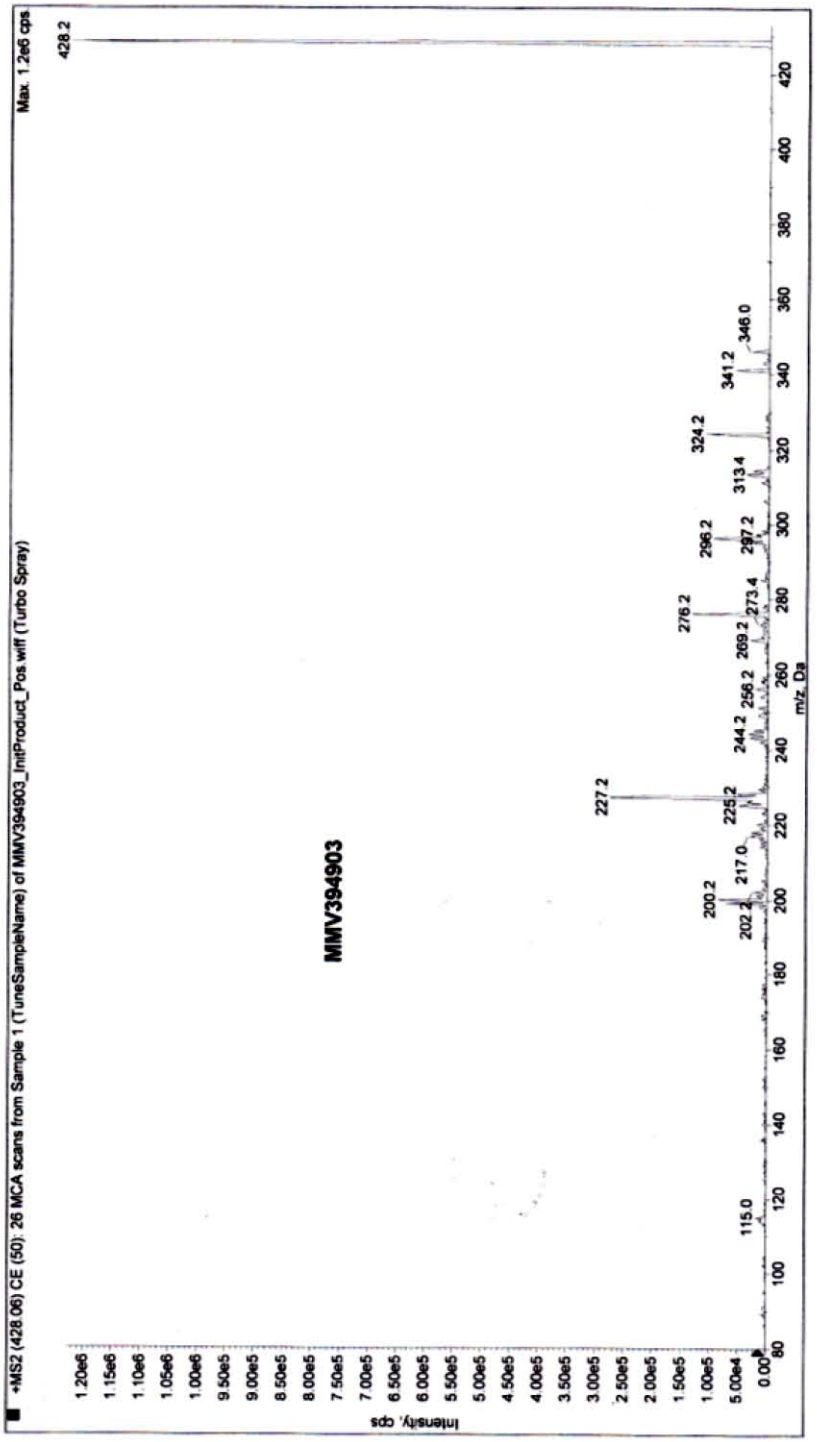


Figure A.78: Typical mass spectrum of MMV394903 detection by LC-MS/MS.

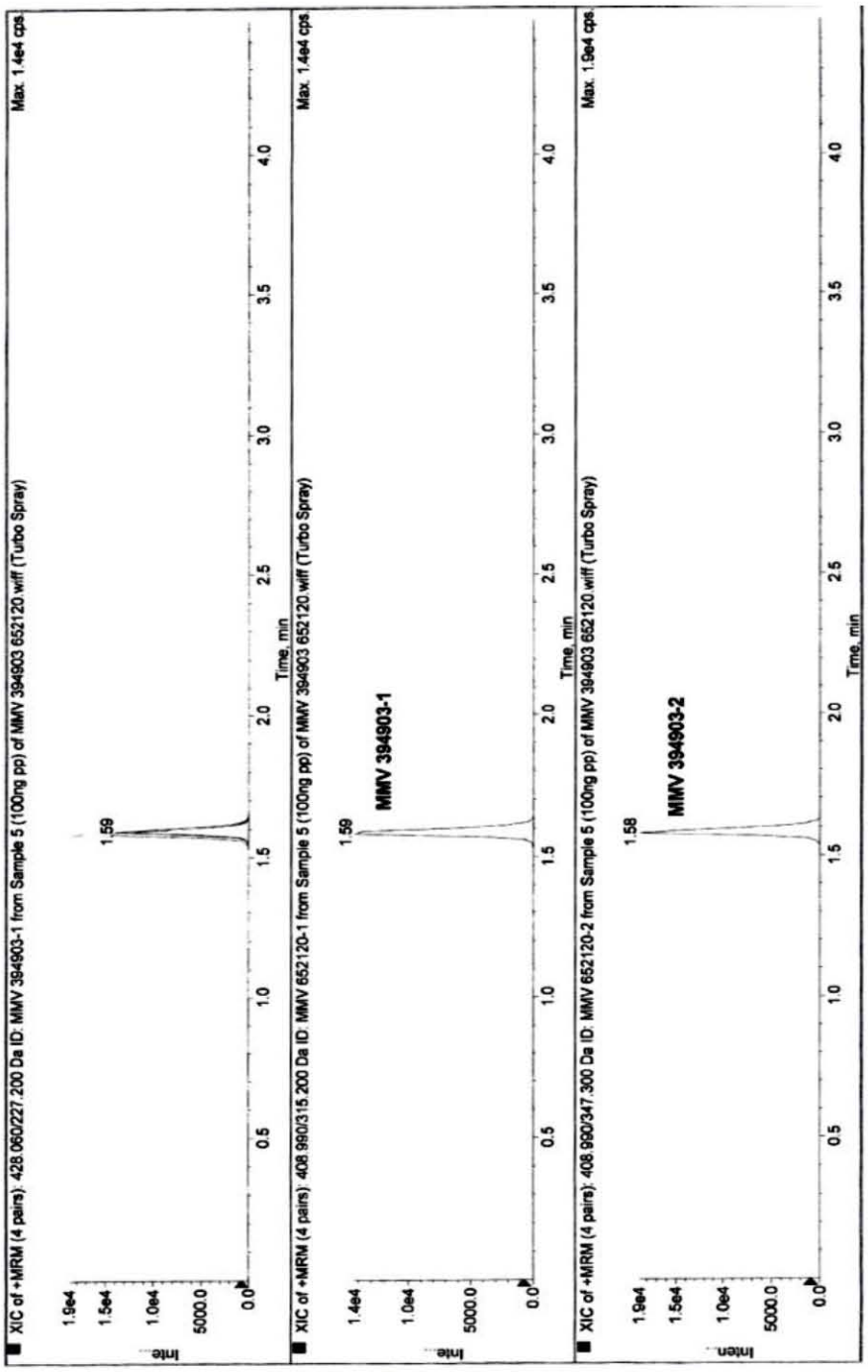


Figure A.79: Typical LC/MS/MS chromatograms in MRM mode for the analysis of MMV394903.

A.9. MMV652120 Detection by LC-MS/MS

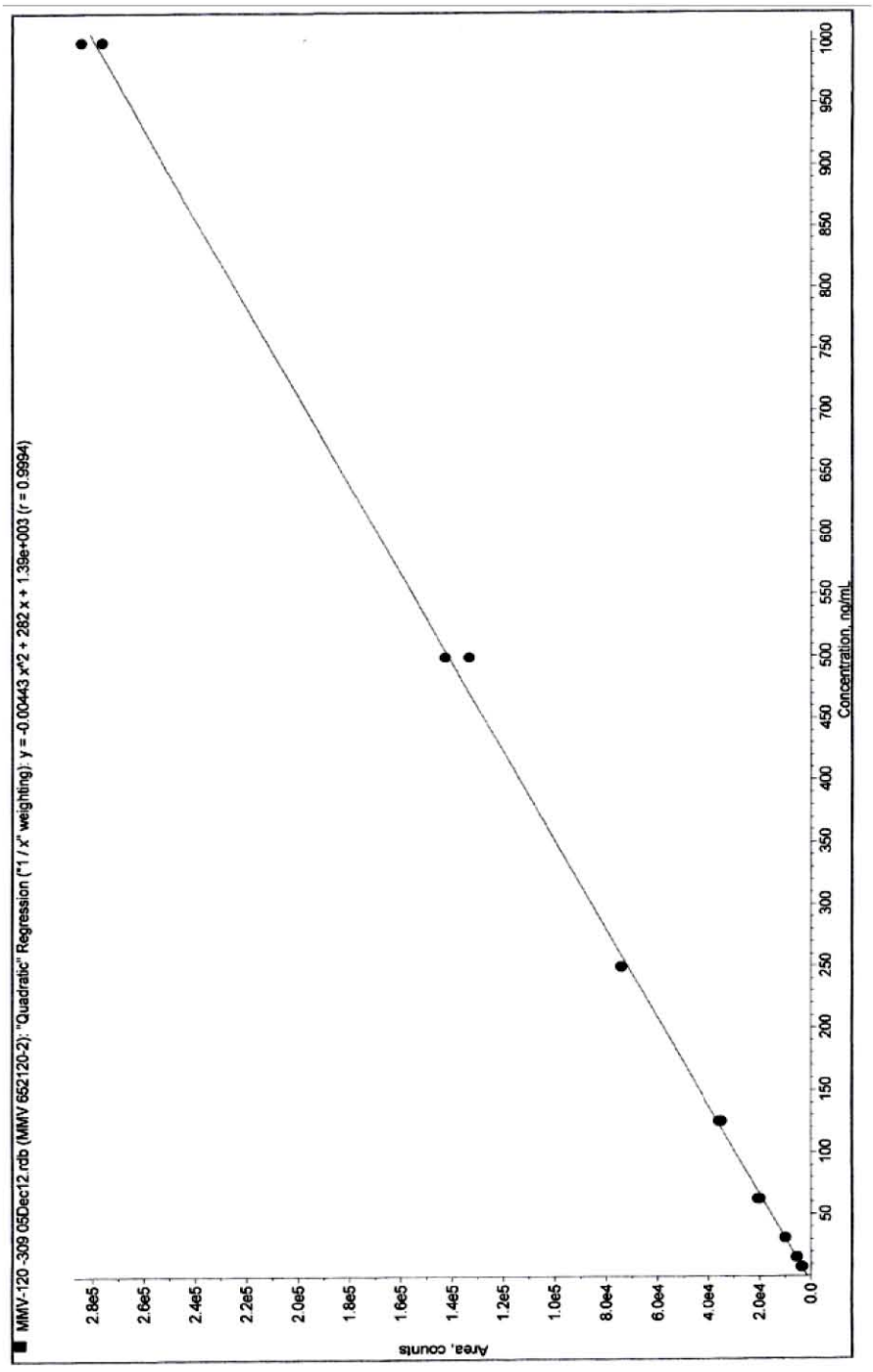


Figure A.80: Typical calibration curve for MMV652120 detection by LC-MS/MS.

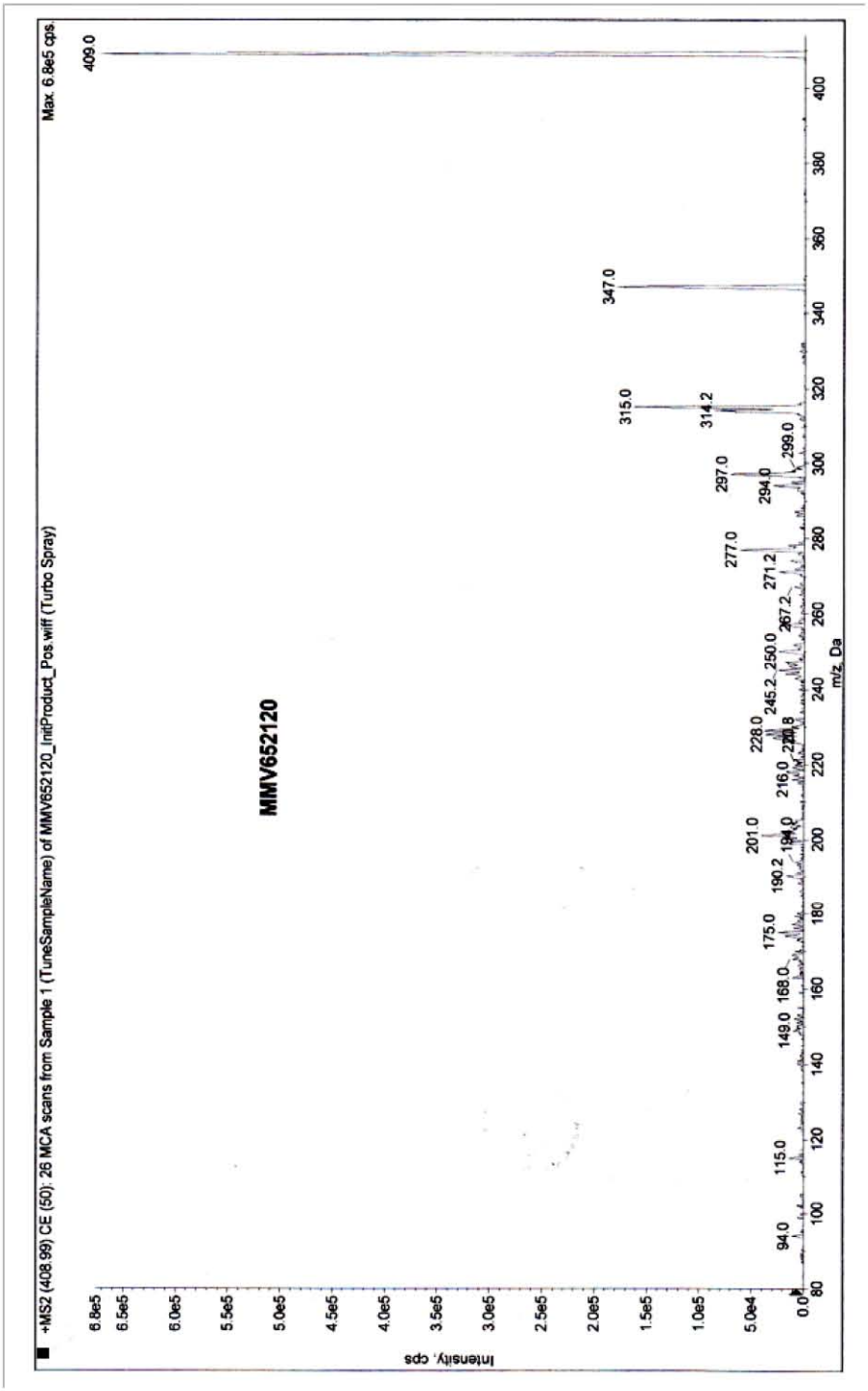


Figure A.81: Typical mass spectrum of MMV652120 detection by LC-MS/MS.

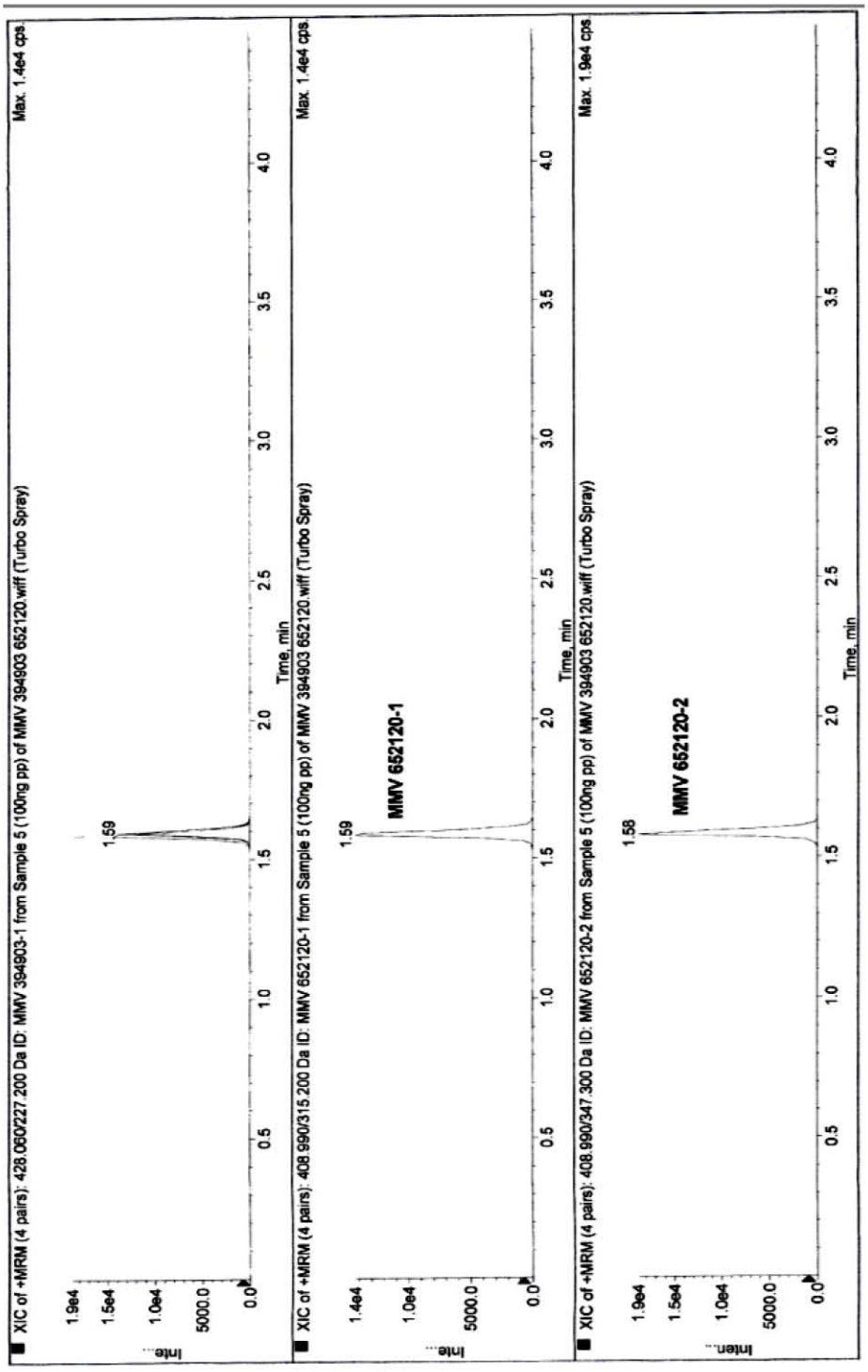


Figure A.82: Typical LC/MS/MS chromatograms in MRM mode for the analysis of MMV652120.