THE IN VITRO ANTIPLASMODIAL PROPERTIES OF NOVEL AMINOQUINOLINES AND METAL COMPLEXES OF THIOMETHYL AND METHYLTHIOMETHYL AINLINE DERIVATIVES



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A Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfillment of the requirements for the degree of Master of Science in Medicine

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DECLARATION

I, Shamillah Holland, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at this or any other University.

.....

Shamillah Holland

.....day of.....2016

DEDICATION:



"March on. Do not tarry. To go forward is to move toward perfection. March on, and fear not the thorns, or the sharp stones on life's path."

-Kahlil Gibran

To my Father,
To my Mother,

Thank you for your unrelenting support and constant source of love and encouragement through this journey, and through the journey of my life.

To my uncle, Clifford Jousten,

Thank you for giving me the gift of science and the passion for learning that has remained with me into adulthood.

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ABSTRACT

The spread of drug resistance in Africa to standard antimalarials has equally contributed to the burden of malaria. As a result new drug delivery targets and compounds need to be explored for malaria treatment. This could include the use of metallo-based antimalarials and compounds capable of inducing oxidative stress in the parasite.

A series of aminoquinoline and quinolin-8-yl derivatives, as well as metal complexes of thiomethyl (**MA**) and methylthiomethyl (**MT**) aniline derivatives were evaluated for antimalarial activity using the tritiated hypoxanthine incorporation and parasite lactate dehydrogenase assay against the chloroquine-sensitive 3D7 *P. falciparum* strain. The compounds were investigated to determine a possible mechanism of action through monitoring the inhibition of haemozoin formation, oxidative stress pathways, iron and copper chelating properties. The toxicity was evaluated by examining the effect on haemolysis and human kidney epithelium and erythroleukemia cells.

The aminoquinoline derivatives displayed moderate antimalarial activity in comparison to quinine (IC_{50} value: 0.0964 ± 0.002 μM). This was theorized to have been produced through a pro-oxidant effect that had no effect on the integrity of the red blood cell membrane. Compounds **III** and **V,** both aminoquinoline derivatives displayed antioxidant activity by scavenging of free radicals and inhibiting lipid peroxidation, possibly by their gallic acid and 8-hydroxyquinoline components.

The copper complexes of MA and MT aniline derivatives displayed promising antimalarial activity when compared to quinine with its activity directed towards the parasite and not the host red blood cells. **CupMeO2MT** (IC_{50} value: 2.66 ± 0.12 μM) and **Cu4MeO2MA** (IC_{50} value: 2.82 ± 0.38 μM) were the most active, and were theorized to have inhibited parasite growth through a pro-oxidant effect. The derivatives bound to nickel and cobalt were able to scavenge free radicals, while the complexes bound to copper were able to inhibit lipid peroxidation. **CupMeO2MT** (IC_{50} value: 12.25 ± 1.97 μM) was found to be the most active in the MA and MT groups, although three fold less potent than camptothecin.

This study revealed the use of multi-component systems, as well as the use of complexing metals to compounds in the hope of finding novel compounds which could reveal new drug targets in the field of malaria research. Further studies would need to be conducted on the possible pro-oxidant effect of these compounds with a focus on the means of maintaining the hosts oxidative equilibrium.

LIST OF PUBLICATIONS AND CONGRESSES ATTENDED

Publications:

S Holland, RL van Zyl, P Khakhlary, JB Baruah. Antimalarial properties of salts and cocrystals of aminoquinolines with hydroxyaromatic acid. [Accepted for poster presentation at the World Congress of Pharmacology, Cape Town, July 2014] Poster 323. [Published in Basic & Clinical Pharmacology & Toxicology 2014, 115(S1):100]. [http://onlinelibrary.wiley.com/doi/10.1111/bcpt.12259_3/epdf]

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S Holland, RL van Zyl, P Khakhlary, JB Baruah. An investigation into the antiplasmodial and possible mechanism of action of salts and cocrystals of amnioquinolines with hydroxyaromatic acids. Abstract published in Southern African Journal of Infectious Diseases 2015, 30(1): 98.

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LIST OF ABBREVIATIONS

°C: Degrees celcius

Abs/ABS : Absorbance

APAD: Acetylpyridine adenine dinucleotide

CPM: Counts per minute

DDT: Dichlorodiphenyltrichloroethane

DMSO Dimethyl sulfoxide

DMEM: Dulbecco's Modified Eagle's Medium

DNA: Deoxyribonucleic acid

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EDTA: Ethylene diamine tetra acetic acid

FIC_{50} = Fractional IC_{50}

g: Gravitational constant

g: Gram

GIT: Gastrointestinal tract

HEPES: N-2-hydroxyethyl-piperazine-N-2-ethane-sulfonic acid

HPLC: High performace chromatography liquid

IC_{50}: Concentration that inhibited 50% parasite growth or biological activity

LDH: Lactate dehydrogenase

L: Litre

LMWA: Low molecular weight anti-oxidants

M: Molar

MA: Thiomethylaniline

mg: Milligram

ml: Milliliter

mM : Millimolar

MT: Methylthiomethylaniline

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

nM: Nano molar

$NaHCO_3$: Sodium bicarbonate

NaOH: Sodium hydroxide

NBT: Nitrotetrazolium blue chloride

O_2 : Oxygen

PBS: Phosphate buffer solution

PES: Phenazine ethosulphate

pLDH: Parasite lactate dehydrogenase

RBC: Red blood cell

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RPMI: Roswell Park Memorial Institute

SI_{pf}: Safety index

$SI_{nc:}$: Selectivity index

TETA: Triethylenetetramine

TTM: Tetrathiomolybdate

TRIS: Tris(hydroxymethyl)aminomethan

UV-VIS: Ultra-visible

µl: Micro litre

µM: Micro molar

(w/v): weight/volume

(v/v): volume/volume

WHO: World Health Organization

CHAPTER 1

1 Introduction

1.1 Malaria: Prevalence and spread

Malaria remains rampant on many continents on the global map, none more so than Africa, which accounted for 88% of the 214 million new cases of malaria worldwide (WHO, 2015). According to World Health Organization statistics, there were approximately 438 000 malaria deaths worldwide, 90% of which occurred in the African region. In 2015 an estimated 306 000 children died before their fifth birthday due to malaria globally, the majority of whom reside in the African region (WHO, 2015). Countries of Asia have also been affected by this devastation, to some extent as a result of its poor economic status in parts of the continent. Malaria is a disease that targets the vulnerable population groups who cannot afford treatment, or who have limited access to healthcare, thus keeping families in poverty. It is also a disease that targets the immunocompromised individuals of a population, in Africa the HIV pandemic has been superimposed on the long standing malaria pandemic which has brought about challenges to be faced with regards to the disease management and treatment.

Prevention and treatment programmes are steered by information obtained by specified surveillance systems that are implemented in all areas at risk for malaria. The information obtained from these surveillance systems may be used to assist in distributing resources effectively to populations most in need of assistance, to determine the most appropriate method for control and elimination as well as to identify disease patterns (WHO: Disease surveillance for malaria elimination, 2012).

1.2 Malaria: The disease

1.2.1 Types of malaria

Malaria is caused by an infection with the parasite *Plasmodium*. There are five species of *Plasmodium* that are known to infect humans, *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*. Although all five species affect humans, both *P.*

falciparum and *P. vivax* predominate in areas at risk for malaria with *P. vivax* estimated to be responsible for 13.8 million malaria cases globally in 2015 (WHO, 2015)

When comparing the two most prevalent types of malaria, *P. falciparum* predominates in Africa and causes the most severe infection and has been reported to cause the majority of deaths related to malaria globally. *P. vivax* infections are more common in areas outside of Africa, and have a wider distribution than *P. falciparum*. *P. vivax* has the ability to progress in the mosquito at much lower temperatures and survive in areas with cooler climates which differs when compared to *P. falciparum*, which thrives at higher temperatures, which would explain the difference in the area it predominates. With each species there are variances in the severity of the illness they cause as well as the ability to control the spread of the parasite (WHO, 2012).

1.2.2 Malaria life cycle: Mosquito and human stages

Malaria is transfered to human hosts by the bite of an infected *Anopheles* female mosquito and undergoes an intricate sexual developmental cycle in the insect host (Molina-Cruz *et al.*, 2012). There are 400 different species of the *Anopheles* mosquito and 30 have been identified to be of major importance with regards to malaria infection. Although there are different species of mosquitoes in one particular area, there is usually a dominant type responsible for infection and this is called the primary vector, control efforts are usually aimed at this primary vector. The various mosquito species differ in their ability to effectively transmit malaria, with some species being highly efficient in small numbers and others being a greater threat in larger numbers. The *Anopheles* species responsible for infection in Africa differ from those responsible for infection outside of Africa (WHO, 2012). The behavior and number of mosquito vectors, as well as the specific reservoir of the parasite determines transmission rates of malaria for a specific area (Barnes, 2012). The diversity in the parasite, the type of vector predominant as well as taking into account each contributing factor to the prevalence and spread in that particular area demonstrates the challenge faced in creating control strategies that are unique to each area.

The malaria life cycle consists of two different developmental stages, namely the asexual intra-erythrocytic (4) and sexual gametocyte stages (5) (Figure 1.1). The asexual developmental stage of the parasite occurs within red blood cells of the human host and results in the pathology and symptoms of malaria (Baker, 2010). The sporozite form of the parasite is found in an infected mosquitoes' salivary gland and is inoculated into the human host when the mosquito probes the skin in preparation for a blood meal. These sporozites enter liver cells where they multiply to form merozites. Merozites then invade red blood cells and multiply causing them to burst and infect new red blood cells. Some merozites that invade the red blood cells then develop into gametocytes, which is part of the sexual development stage (Barnes, 2012) The sexual developmental stage of the parasite consists of male and female gametocytes that initially develop within the human host, are then transferred to the mosquito vector, and are a requirement for transmission of the disease (Baker, 2010).

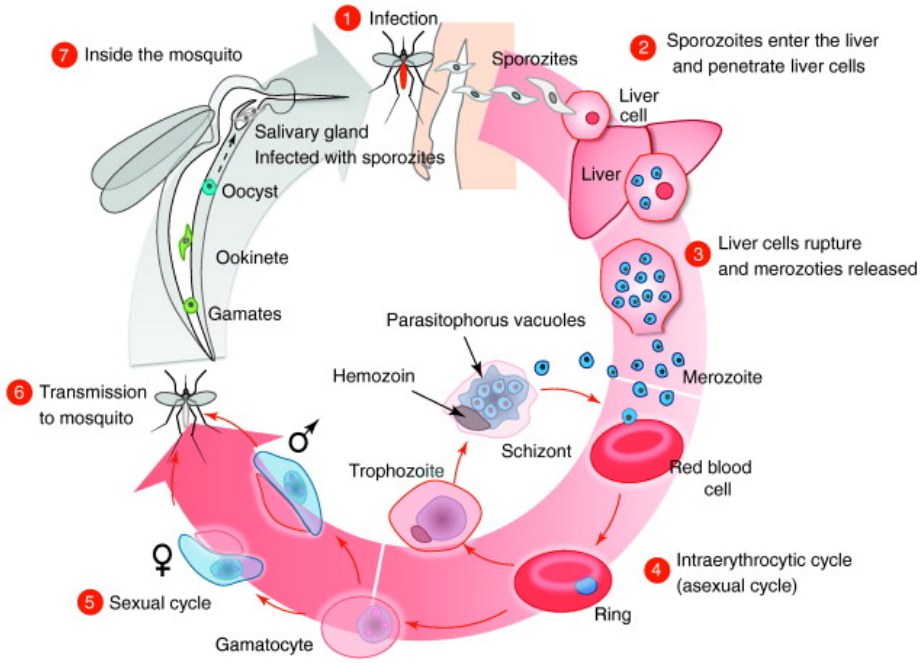


Figure 1.1: The life cycle of *P. falciparum (Cho et al., 2012).*

1.2.3 Symptoms and classification

Individuals at risk for acquiring malaria are those who are non-immune travellers to malaria areas. Pregnant women, children under the age of five, as well as the elderly and populations who are immunocompromised are considered especially vulnerable to infection (SA DOH, 2010). The initial symptoms of malaria are non-specific in

uncomplicated malaria and as such are often mistaken for a systemic viral illness. As a result malaria may be over-diagnosed based on the symptoms alone in endemic areas, which leads to mistreatment, or it is undiagnosed which leads to a more severe malaria developing and contributes to the spread of the disease (WHO Treatment Guidelines, 2015). Due to the non-specific nature of the initial presentation of the disease the confirmation or exclusion of the disease cannot be made clinically alone, and laboratory tests would need to confirm the presence of infection (SA DOH, 2010).

Signs and symptoms of uncomplicated malaria may present a week after exposure, which include headache, fatigue as well as abdominal discomfort and later develop into fever, chills, perspiration and malaise. At this early stage where there is no evidence of vital organ dysfunction, patients that are readily treated progress to full recovery, provided the treatment is prompt and the correct treatment is initiated. Although in certain instances where these treatment protocols are not followed patients develop severe malaria within a short period of time. Severe malaria presents as, coma or cerebral malaria, metabolic acidosis, severe anaemia, acute renal failure or acute pulmonary oedema. Treatment failure at this stage of the disease results in death in the majority of cases (WHO Treatment Guidelines, 2015).

1.3 Oxidative stress

1.3.1 Oxidation and reduction

Chemically a compound that is able to accept an electron is classified as an oxidant or oxidizing agent, and inversely, any compound able to donate an electron is classified as a reductant or reducing agent. Reduction of a chemical is defined as the gain of electrons, and oxidation is in turn defined as the loss of electrons. In order to maintain equilibrium, oxidation is not possible without reduction elsewhere in a system (Kohen and Nyska, 2002). Reductant and oxidant are used in chemical terms, where as oxidant and pro-oxidant are used in the context of biological systems.

Biologically an anti-oxidant may be described as a substance that prevents or delays pro-oxidant induced oxidation on an oxidizable substrate. A pro-oxidant in terms of biology may be defined as a toxic substance or as mentioned earlier ROS, that has

the ability to cause oxidative damage to biological targets such as lipids, proteins and nucleic acids, and may result in the development of specific disease states. Pro-oxidants may be classified as radicals or non-radicals. An anti-oxidant can effectively reduce a pro-oxidant with its formed products to a level of having little to no toxicity to prevent biological oxidative damage (Prior and Cao, 1999). The tightly regulated equilibrium between anti-oxidants and pro-oxidants is known as redox potential and is specific to each organelle and biological site within the cell as well as the organism. The balance of this equilibrium within the cell of a living organism can alter changes in signal transduction, DNA and RNA synthesis, protein synthesis, enzyme activation as well as regulation of the cell cycle. Life sustaining processes such as ligand binding, DNA binding and nuclear translocation are also all controlled through redox potential (Schafer and Buettner, 2001). Most transcription factors needed for protein synthesis are found only in the reduced state, as opposed to cell death that is initiated by oxidizing environments. Therefore the redox state of a cell would essentially determine if a cell will proliferate, differentiate or die (Schafer and Buettner, 2001).

1.3.2 Oxidative damage: Targets and types

There is a constant build up of oxidative damage to cellular components that results in changes in cellular function due to the movement of ROS between endogenous and exogenous sources. Lipid membranes, DNA as well as enzymes involved in protein synthesis are among the most vulnerable biological targets (Kohen and Nyska, 2002) (Figure: 1.2).

Cellular membranes are particularly prone to oxidative damage due to their high levels of unsaturated fatty acids, the damage caused to lipids, described as lipid peroxidation occurs in three stages and results in a loss of fatty acids. This process involves the formation of a fatty acid radical as a result of the loss of a hydrogen atom and this initial event follows a sequence of events in a chain like manner that results in the peroxidation of all the unsaturated lipid in the membrane (Halliwell and Gutteridge, 1989).

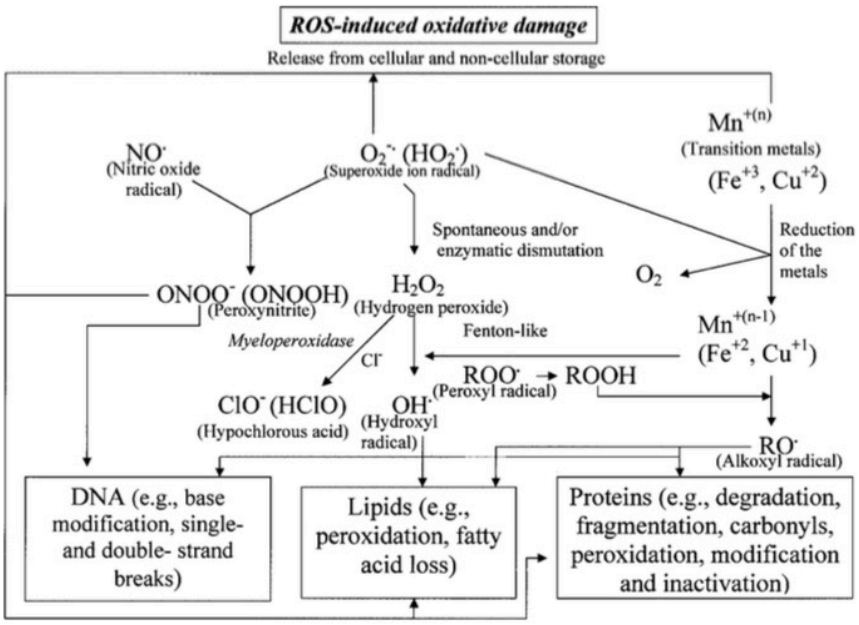


Figure 1.2: Reactive oxygen species (ROS) induced oxidative damage (Kohen and Nyska, 2002).

Proteins serve as a major target for ROS with nitrogen-reactive radicals causing severe damage to proteins. Proteins interact directly with ROS or indirectly with molecules such as sugars and lipids, generating products that may then react with the protein. Interactions with ROS include, peroxidation, damage to specific amino acid residues, changes in their tertiary structure, degradation and fragmentation. The result of this protein damage includes the loss of enzymatic activity, altered cellular functions such as energy production, and changes in the type and level of cellular proteins. Consequences of protein oxidation cause changes in their function such as chemical fragmentation, inactivation and increased proteolytic degradation (Levine and Stadtman, 2001). DNA could be considered the most biologically significant target with regards to oxidative damage, and continuous oxidative attack results in the development of age related cancers of the breast, colon, rectum and prostate. ROS interacting with DNA may cause the modification of DNA bases, single and double stranded DNA breaks, loss of purines, damage to the deoxyribose sugar as well as damage to the DNA repair system (Halliwell, 2000).

1.3.3 The role of the anti-oxidant

The cell is under steady fluctuations of endogenous and exogenous sources of oxidative stress as a result various defense mechanisms have been put into place for

protection against these reactive metabolites. These mechanisms may be divided into direct as well as indirect methods (Becker *et al.*, 2004). Indirect methods may involve the control of endogenous production of ROS for example by altering the activation of enzymes that produce oxygen metabolites. Organisms employ an efficient repair system consisting of small molecules as well as enzymes that are able to efficiently repair oxidative damage on macromolecules. Physical defense mechanisms such as membranes also play a significant role in the cells methods employed to cope with oxidative stress (Kohen and Gati, 2000).

Although there are a variety of methods employed by the cell to deal with oxidative stress, the use of an anti-oxidant is extremely vital due to the fact that its mechanism of action involves the direct removal of pro-oxidants. The anti-oxidant system found in the body may be divided into two major groups, namely, anti-oxidant enzymes and low-molecular weight anti-oxidants (LMWA). The anti-oxidant enzymes are composed of direct acting proteins such as super oxide dismutase, amongst others that differ in their structure and co-factors as well as other enzymes that act by supporting the activity of the antioxidants. LMWA's employ direct and indirect methods in counteracting oxidative stress. One of the most significant direct methods employed by LMWA's is through the scavenging of free radicals, which prevent attacks on biological targets (Kohen, 1999).

1.3.4 Oxidative stress in the malaria parasite

Redox reactions and processes governed by redox potential are essential to the survival of all living organisms. This tightly regulated balance between antioxidant and pro-oxidant molecules or redox state, controls life sustaining processes such as metabolism, and cellular respiration. A considerable decrease in the redox state may lead to pathological events and is defined as redosis, and an increase in the redox state would in turn be defined as oxidative stress and may lead to oxidative damage to biological targets (Shapiro, 1972). The malaria infected RBC is under constant interaction with its human host, as such there is a delicate balance between pro-oxidant and anti-oxidant molecules which needs to be maintained as the host as well as the parasite have the ability of producing both types of molecules (Becker *et al.*, 2004).

Reactive oxygen species (ROS) are pro-oxidants capable of causing damage to biological targets such as lipids, DNA and protein. These ROS are generated by the malaria parasite as a result of its high metabolic rate due to its rapid multiplication. The detoxification of these ROS pose a challenge to red blood cells infected with the parasite, and results in oxidative stress within the infected cell. Host haemoglobin is the most abundant source of amino acids for the malaria parasite, and is therefore needed for protein synthesis which is life sustaining to the parasite, and the degradation of host haemoglobin is one of the most significant causes of oxidative stress within the parasite, as such it is considered an endogenous source of oxidative stress. Within the red blood cells of the host, there are high concentrations of iron, changes in the levels of oxygen, as well as ROS derived from the host's immune response and is considered an exogenous source of oxidative stress for the parasite. There are various other factors that contribute to the oxidative stress within the parasite and the parasite is equipped with mechanisms in place to deal with this oxidative stress and maintain a state of redox equilibrium (Becker *et al.*, 2004).

1.4 Preventative measures

Although malaria is a potentially fatal disease, it is both treatable as well as preventable given the correct and timeous implementation of treatment and diagnosis coupled with the correct preventative measures employed. Individuals travelling to malaria areas would need to determine the need for chemoprophylaxis, or whether other non-drug preventative measures may be used to avoid malaria infection. The decision whether to use chemoprophylaxis or non-drug measures is a complex one, as identifying malaria transmission areas are difficult due to the fact that these transmission patterns are constantly changing as a result of for example climate change, amongst others (Blumberg, 2015) .

1.4.1 Non-drug preventative measures

Although there are drug measures that may be put into place, these are not fool proof and non-drug preventative measures need to always be applied. Non-drug preventative measures include the use of indoor residual spraying as well as the use of insecticide treated nets and long lasting insecticidal bed nets. The increased unselective use of insecticide sprayings in previous years such as DDT

or dichlorodiphenyltrichloroethane has led to the emergence of insect resistance and as such campaigns have been put into place to regulate the use of such products in order to decrease the increasing insect resistance. The use of insecticide treated nets as well as long lasting insecticide treated nets have effectively decreased transmission levels, however recent studies have shown the development of resistance to these long lasting compounds as well (Biot *et al*., 2011). By 2014, more than half of the population was protected by insecticide treated nets, compared to less than 2% in 2000. In 2014, 116 million people were protected by indoor residual spraying, including 55 million people in Africa (WHO, 2015).

1.4.2 Drug preventative measures

The choice of antimalarial agent to use for treatment and prophylaxis of malaria is dependent on the severity of the illness, pattern of drug resistance, as well as the geographical area in which the malaria was acquired (SA DOH, 2010). The need for prophylaxis is based on the level of risk for malaria as well as being part of special groups identified as being at risk for malaria, as previously discussed which increases the need for prophylaxis. Chemoprophylaxis may denote complete prevention of infection or a suppression of the parasitaemia and its symptoms or clinical prophylaxis. Antimalarials that work on the erythrocytic stages of the parasite and terminate the asexual stages of the parasite are classified as blood schizontocides. These blood schizontocides reduce the clinical symptoms of the disease. Antimalarials that work on the exo-erythrocytic forms of the parasite are classified as tissue schizontocides (Blumberg, 2015).

1.4.2.1 Mefloquine

Mefloquine is used for long-term prophylaxis and for individuals requiring prophylaxis for longer than 6 months. Mefloquine is a quinoline methanol derivative, structurally related to quinine. Mefloquine is classified as a blood schizontocide that affects the asexual stages of the parasite in *P.falciparum* and has also shown activity against the *P.vivax* gametocytes with no effect on the hepatic stages of the parasite (Rossiter and Association, 2014). Adverse effects associated with mefloquine include various central nervous system effects such as insomnia, strange dreams and mood

changes which usually impact on the decision to take mefloquine as well as other adverse effects such as nausea, diarrhoea and headaches. As a result of its side-effect profile, compliance with mefloquine is very poor. An oral dose of mefloquine is initiated one week before travelling to a malaria area and is continued for four weeks after the last possible exposure (Rossiter and Association, 2014).

1.4.2.2 Doxycycline

Doxycycline is effective against all four species of the human malaria parasite, and is the preferred short-term prophylaxis for travellers to drug resistant malaria areas. Doxycycline acts by inhibiting protein synthesis by binding to the 30S ribosomal subunit (Gaillard *et al.*, 2016). They are relatively slow acting as antimalarials and may be used not only for prophylaxis but also in conjunction with conventional antimalarials agents to increase recovery rates (Staines and Krishna, 2012). Doxycycline prophylaxis is started a day before entering the malaria area, and continued daily while in the malaria area as well as daily for four weeks after leaving the area. Doxycycline has an adverse effect on bone formation and therefore should not be used for individuals under the age of 8. The drug should also be avoided in pregnancy as well as in breast-feeding females. Adverse effects of the drug include gastrointestinal symptoms as well as candida infections of the vagina and gut, and infections may be severe enough to discontinue use (Rossiter, 2010).

1.4.2.3 Atovaquone-proguanil

Atovaquone-proguanil is a fixed dose combination of atovaquone, an inhibitor of cytochrome b, and proguanil, a dihydrofolate reductase inhibitor. Both of these components interfere with the plasmodial pyramidine synthesis (Rossiter, 2010). Atovaquone-proguanil has the best safety profile when compared to doxycycline and mefloquine and is a shortened regimen, as such the compliance is improved and it is therefore the preferred choice for individuals travelling for short periods of time. It has a mild adverse effect profile with nausea being the most common symptom. The combination is usually taken one day before travelling to the malaria area and continued daily for up to a week from returning from the malaria area (Blumberg, 2015).

1.5 Treatment

Antimalarial combination therapy includes the concurrent use of two or more blood schizontocidal medicines with independent mechanisms of action, as well as different biochemical targets in the parasite. The justification for combination therapy is that it is often more effective to use a combination therapy rather than a monotherapy as it is theorized to delay or prevent the emergence of resistance, and in rare incidences, if a mutant parasite is resistant to one of the drugs in the combination, the second drug would prove effective (Biot *et al*., 2011). The choice of chemotherapy is dependent on the severity of the disease, the known or suspected resistance pattern of the area, the species of the parasite as well as patient characteristics. In order for treatment to be effective it is essential to differentiate between uncomplicated malaria and severe malaria to ensure the correct treatment protocols are followed (Blumberg, 2015). The most ideal weapon to combat malaria would be the development of a vaccine that would prevent infection and/or transmission and thus eradicate the disease. The use of a vaccine would allow for long-term prophylaxis, but as a result of the complexity of the parasites' defense against the host's immune system, the development of a vaccine has been less than ideal (Wells *et al.*, 2009).

1.5.1 Artemether-lumefantrine

Artemisinin-based combination therapies for the treatment of uncomplicated *P. falciparum* infection are comprised of artemisinin and its derivatives, artesunate, artemether or dihydroartemisinin. There are minor differences in oral absorption and bioavailability between the different artemisinin derivatives, which do not show any clinical significance to the combinations used, but the partner compound the artemisinin is combined with would determine the overall efficacy of the combination. Artemisinin produces prompt clearance of parasitaemia and fast improvement of symptoms by reducing parasite numbers during the asexual stage of the development cycle. Artemisinin has the advantage of rapid clinical and parasitological response, improved cure rates, decreased malaria transmission and the potential to delay drug resistance (WHO Guidelines for malaria treatment, 2015).

Artemether-lumefantrine is a fixed dose combination of artemether, a semi-synthetic artemisinin derivative and lumefantrine, a dichlorobenzylidine derivative, both of

which exhibit rapid schizonticidal activity during the asexual stage of the parasite life cycle. This combination has the advantage of a shorter treatment regimen as well as being more tolerable when compared to the alternative treatment option, quinine (Blumberg, 2015). Artemether-lumefantrine is administered over a period of three days, and the number of tablets to be administered is calculated according to a grade system based on patient weight. Adverse effects are generally well tolerated and include sleep disturbances, headache, dizziness, as well as gastrointestinal disturbances. Although this combination is well tolerated its efficacy in severe malaria has not been established and should only be used in instances of clear uncomplicated infection, it should also not be used for prophylaxis (Rossiter and Association, 2014).

1.5.2 Quinine

Quinoline-based drugs have been widely used as antimalarials, drugs belonging to this group include quinine, chloroquine, amodiaquine, piperaquine, primaquine and mefloquine. These drugs target the catabolism of the hosts' haemoglobin by the parasite by specifically inhibiting haemozoin formation by the parasite (Egan, 2006). Oral quinine is the recommended treatment for uncomplicated malaria in pregnant women and infants and when artemether-lumefantrine is not available (Blumberg, 2015). Quinine is used in combination with either clindamycin in the case of an infant or pregnant female, or in combination with doxycycline in other instances for both uncomplicated, as well as severe cases of malaria (Blumberg, 2015). Quinine is a fast acting blood schizontocide with selected gametocytocidal activity, and shows no exoerythrocytic activity. Although quinine remains a significant antimalarial drug, its use is contested by its reduced tolerability and poor compliance with complex dosing regimens (Achan *et al*., 2011). As such it is reserved for instances where an artemisinin based combination may not be used. Minor adverse effects associated with quinine use are, cinchonism, which includes a mild hearing impairment, tinnitus, headache, nausea and slight visual disturbances. These adverse effects are dose related. Major adverse effects of quinine use include arrhythmias, hypersensitivity and hypoglycaemia (Rossiter, 2010).

1.6 Resistance and treatment failure

The WHO defines “drug resistance” as the ability of a parasite strain to survive or multiply despite the administration and absorption of a drug given in doses equal or higher than those usually recommended, but within the tolerance of the subject (WHO, 1967). The pattern of malaria disease is one that is always changing, as with all components of medicine, as a result of various factors related to malaria evolving, the development of resistance has been documented in all classes of antimalarial medication. This resistance includes the class of artemisinin derivatives that are used globally for malaria treatment as a recommended first line treatment by the WHO, which poses a significant threat to the possibility of malaria elimination (WHO Guidelines for malaria treatment, 2015). Drug resistance results in an increase in the clearance time of asexual parasites in blood, which then permits the production of gametocytes that are responsible for the transmission of the resistant genotype. Drug resistance has been documented not only on the basis of antimalarials, but has also been documented specifically in three of the five malaria species known to effect humans, namely, *P. falciparum.*

*P .viva*x and *P. malariae*. Cross-resistance and multi-drug resistance have both further complicated the impact of resistance that has emerged with antimalarial compounds. Cross-resistance may occur between drugs belonging to the same class, or chemical family and thus have similar mechanisms of action. An example of cross-resistance documented is between halofantrine and mefloquine. Multi drug resistance is observed when the parasite is resistant to more than two antimalarial compounds of different classes with different mechanisms of action (WHO, 2010).

A clear distinction needs to be made between treatment failure and antimalarial drug resistance. Treatment failure is the failure to clear malarial parasitaemia or resolve clinical disease following treatment with an antimalarial drug. Thus drug resistance may result in treatment failure, but not all treatment failure is as a result of drug resistance. Various factors contribute to treatment failure such as, incorrect dosing, non-compliance on the part of the patient, poor drug quality, drug interactions, poor drug absorption as well as incorrect diagnosis (Bloland and Organization, 2001). Resistance occurs through spontaneous mutations that result in reduced sensitivity to a given drug, or a particular class of drugs (Bloland and Organization, 2001).

Factors that contribute to the spread of resistance are, poor treatment practices, inadequate patient compliance, the widespread availability of artemisinin monotherapies available as well as substandard forms of the drug (WHO: World Malaria Report, 2015).

1.7 Metal homeostasis: a susceptible target

The high incidence of malaria documented globally as well as the development of drug resistant strains of *Plasmodium* has highlighted malaria as a major health concern, as such new avenues need to be explored for possible treatment alternatives. One such avenue is the idea of metabolic immunity where is that if it is possible to deprive a host of a nutrient essential to the survival of an invading organism it might serve as a host defense mechanism and essentially provide a therapeutic strategy, provided it is not detrimental to the host (Cabantchik *et al.*, 1999). In addition, the effects of trace elements are highly dependent on one another and can have a cascade effect in inhibiting the parasite (Tapeiro *et al*., 2003).

1.7.1 Iron

Iron is needed by nearly all protozoa, fungi and bacteria, in order to grow and divide and it is obtained from the host. Parasites require iron from their host for processes such as DNA synthesis, carbohydrate metabolism, mitochondrial function and electron transport, amongst others (Mabeza *et al*., 1999). The intraerythrocytic parasite multiplies by forming 16-32 daughter cells with each cycle of growth and this process demands a high concentration of the hosts iron; which are accumulated during the trophozoite stage in order to synthesise the required DNA during the schizont stage of the parasite. One of the enzymes critical for DNA synthesis is ribonucleotide reductase, which is an iron containing enzyme that catalyzes the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, which are the precursors for DNA synthesis (Munro and Silva, 2012). The preservation of iron balance is not only a general requirement for the growth and proliferation of all cells, but it is also critical for the regulation of immune function. The condition of iron overload as well as iron deficiency appear to exert indirect effects on the immune system by altering the proliferation of T-cells and B-lymphocytes, as well as having a cytotoxic effect by catalyzing the production of reactive oxygen species.

As a result, altering the iron balance or homeostasis could have an effect on immune function (Mabeza *et al*., 1999).

1.7.2 Copper

Copper is an essential micronutrient needed as a cofactor for many enzymes involved in metabolic metabolism required for survival of the parasite as well as the human host. The reduction-oxidation property of copper permits it to receive and donate electrons, which renders it potentially dangerous. Organisms thus have specific cellular mechanisms in place to for isolation and detoxification to ensure that free ionic copper is kept at a low concentration (Rasoloson *et al.*, 2004). Studies conducted on *Plasmodium* infected red blood cells have shown a decrease in copper content, which would suggest that the parasite implements an efflux mechanism for its metabolism of copper. The copper required by the parasite for growth is not imported from serum, but is sourced directly from the host red blood cell (Goldring, 2004). Cuprozinc superoxide dismutase is the most prevalent copper-containing enzyme in the red blood cell, and is believed to provide a possible copper source for the malaria parasite. During the ingestion of the host cell cytoplasm cuprozinc superoxide dismutase along with haemoglobin is taken up via the cytosome and is transported into the parasites food vacuole where digestion occurs, the fate of this digested copper is not known (Rasoloson *et al.,* 2004). Although this is so studies conducted on the intracellular copper chelator neocuproine, proved that when the parasite was starved of copper in the presence of the copper chelator, its growth rate declined (Choveaux *et al.*, 2012).

1.8 Chelation therapy

Although trace elements and metals are essential to the sustenance of life, if they are found in excess they exert toxic effects. In the case of both copper and iron, when in excess they have the ability to initiate the Fenton type reaction to produce free radicals, which are toxic for the body (Valko *et al.*, 2005). Therefore there is a clinical need for a means of depleting these trace elements that are in excess and chelating agents have been designed and used to do so (Ding *et al*., 2011).

A chelating agent may be described as a polydentate ligand that is capable of occupying two or more sites in a co-ordination sphere. A ligand is an ion or molecule that co-ordinates to a metal atom or metal ion to form a complex (Brown and Lemay Jr, 2000). Therefore a chelating agent is a metal atom or ion that has the ability to form “bonds” or occupy a “space” at two or more sites within a co-ordination sphere, which then forms a stable complex. The formation of complexes between chelating agents and metals should prevent or reverse metal binding in biological ligands. Knowledge of the structural and binding features of relevant metal ions, as well as the mechanism by which they might participate in biological ligand complexes being formed has contributed to the design of a new therapeutic scheme based on the chemistry of metal ion chelators (Tapiero *et al.*, 2003).

1.9 Chelating agents

The proposed antimalarial action of iron chelator’s involve their penetration into the infected parasite erythrocyte, binding iron within the parasite cytosol and from the labile iron pool within the host red blood cell which are utilized for plasmodial growth (Sekhon and Bimal, 2012). Studies previously conducted on the growth of *P. falciparum* in cultured red blood cells in the presence of the iron chelating agent desferrioxamine have demonstrated that iron is an essential nutrient during the asexual phase of the parasites development (Van Zyl *et al.*, 1992). When the parasite is deprived of iron its growth and replication is inhibited at a maximal effect during the late trophozite stage of development. During the asexual stage of growth, parasites have no transferrin receptor pathway to take up iron into the parasite, but a non-transferrin iron pathway has been proposed (Sanchez-Lopez and Haldar, 1992) . Transferrin is the most important iron source for the production of haeme proteins, which are used for cellular proliferation. In addition, the unique means by which parasites metabolise iron make them susceptible to a drug-induced iron deficiency scenario (Mabeza *et al*., 1999). The mechanism by which iron chelators exert their antimalarial effect varies and they may either isolate iron necessary for plasmodial replication, or it may be through the direct withholding of other essential trace elements such as copper and calcium, which are essential to parasite metabolism. The cytochromes in the electron transport chain of almost all organisms contain haeme, inhibition of parasitic haeme synthesis could possibly disrupt electron transport and ultimately result in the arrest of mitochondrial respiration and the

accumulation of ATP and eventually result in death of the parasite (Mabeza *et al*., 1999). Due to the parasite's obvious need for iron to complete life-sustaining processes, the use of an iron chelator would cause "iron starvation", thereby inhibiting those life sustaining processes and killing the parasite (Weinberg and Moon, 2009).

Neocuproine, a known copper chelating agent, has been proven to specifically inhibit parasite growth at the ring-to-trophozoite stage phase. One proposed mechanism of action for neocuproine is that it would enter an infected red blood cell via diffusion and then chelate copper from copper-containing enzymes, thereby decreasing the parasite copper supply and ultimately decreasing its metabolism. The antimalarial activity of diethyldithiocarbamate has been shown as a result of its potent inhibition of cuprozinc superoxide dismutase, which is a rich source of copper for the malaria parasite (Rasoloson *et al*., 2004).

1.10 Metal bound chelates

Metals may also be used to enhance the efficacy of organic drugs. With this in mind a series of studies were conducted by including a transition metal into the molecular structure of the antimalarial agent, chloroquine, which enhanced the *in vitro* activities of the metal complex by 4.5 fold when compared to the standard drug (Biot *et al*., 2012). The *in vitro* antimalarial activities of the copper (II) complexes of pyridine-2-carboxamidrazones have clearly shown the advantage of copper complexation and the requirement of a four coordinate geometry as being a key structural feature for designing a metal-based antimalarial (Sekhon and Bimal, 2012). Both commonly used quinolone and artemisinin classes of drugs have been proposed to interact with metals to kill malaria parasites through free-radical intermediates (Rasoloson *et a*l., 2004).

Both copper and iron are classified as reactive metals and have the ability to participate in the Fenton reaction. The Fenton reaction results in the generation of excessive free radicals that increase oxidative stress and increase lipid peroxidation. Under normal physiological conditions, copper does not participate in Fenton chemistry, however when bound to membrane-permeant copper chelators, it may increase its redox activity to induce oxidative damage in *Plasmodium*. The malaria

parasite lacks a good defense mechanism against oxidative stress, as such the combination of a metal and chelator complex may provide a beneficial strategy to enhance the activity of a metal chelator (Rasoloson *et al.*, 2004).

1.11 8-Hydroxyquinolines

8-Hydroxyquinolines such as clioquinol have shown great potential as anti-proliferative agents, as well as in the field of neurodegenerative diseases such as Alzheimer's disease (Zhou *et al.*, 2011). 8-Hydroxyquinolines possess anti-oxidant properties and are lipophilic chelates that have been reported to inhibit RNA polymerase, and have the ability to chelate both copper and iron. The proposed mechanism of antimalarial action of the 8-hydroxyquinolines is that they form complexes with iron extracellularly which enters the infected red blood cells and rapidly produces a lethal free radical mediated intracellular reaction (Mabeza *et al*., 1999). In a study conducted on the *in vitro* inhibition of *P. falciparum*, the 8-hydroxyquinoline derivatives showed a greater degree of inhibition when compared to chloroquine (Biswas, 2003).

1.12 Quinolin-8-yl derivatives

Quinolin-8-yl derivatives have been shown to possess antiparasitic properties in leishmaniasis and trypanosomiasis chemotherapy, and have the ability to form complexes with copper and zinc (Da Silva *et al.*, 2007). Although they possess antiparasitic properties in leishmaniasis and trypanosomiasis, formal studies to evaluate their antiplasmodial properties have not been conducted, and therefore there is a need to complete such a study.

1.13 Methylsulfanyl aniline chelates

Methylsulfanyl aniline chelators are a fairly new group of compounds and therefore there is not a vast amount of information regarding their mechanisms of action with regards to antimalarial activity. Although their antimicrobial activity has shown promise (Olalekan *et al.*, 2014). As such research would need to be conducted to

determine their potential as antimalarials and to elucidate their mechanisms of action for possible antiplasmodial properties.

1.14 Chelation therapy in malaria

The failure of vector eradication programs, lack of vaccine development, as well as the apparent spread of resistance has created a great need for research into the development of new treatment strategies that may be employed to target malaria. The knowledge of a compound's mechanism of action may assist in the understanding of the function and effect a complex may have within the body/microorganism, as well as provide a means by which to redesign the structure to increase biological activity and avoid parasite resistance (Biot *et al*., 2011).

One promising strategy is the possible use of novel metal chelating agents. They are able to target vital processes that are metal dependent, as well as metal ion pools within the parasite that have been shown to be more susceptible to metal starvation than the host (Weinberg and Moon, 2009). Although the use of metal chelating agents in malaria is not a new concept, there is a need to re-visit and re-design the concept. The success of employing chelating agents in the fields of cancer therapy and Alzheimers disease cannot be ignored, and thus a possible approach for malaria therapy would be to explore and identify agents that have had success in other diseases. These compounds might act against other systems found in the malaria parasite and discover new targets for therapy. If the possibility of discovering new compounds is not feasible, the possibility of combining new compounds with antimalarials already on the market to enhance activity or reduce resistance may be an alternative (Rosenthal, 2003).

With this in mind the aim of this study was to investigate the antimalarial activity of a series of novel aminoquinoline derivatives and metal chelates as well as to determine the mechanism of action by which they produce their effect.

1.15 Study Objectives

- To assess the *in vitro* properties of the novel aminoquinolines, quinolin-8-yl and metal complexes of thiomethyl and methylthiomethyl aniline derivatives against the asexual parasite and determine the effect of combining the most active compounds with quinine.

- To investigate the mechanism of action of the novel compounds by monitoring oxidative stress pathways within the parasite, inhibition of haemozoin formation, iron and copper chelation properties.

- To determine preliminary toxicological and antiproliferative properties of the compounds against human red blood cells, human kidney epithelium (HEK-293) and human erythroleukemia (K562) cell lines.

CHAPTER 2

Materials and Methods

2.1 Test compounds

The five amminoquinoline derivatives and six quinolin-8-yl derivatives were synthesised, characterised and the purity and validation of the structures completed by Prof J. Bikash Baruah and Prithiviraj Khakhlary, Indian Institute of Technology, Assam, India (Appendix A). These derivatives were synthesised by reacting an amminoquinoline and respective acid. Using X-ray single crystal diffraction the structures of the compounds were determined and characterised.

A total of 31 methyl(thiomethyl)aniline compounds and 24 thiomethyl aniline compounds were synthesised and characterised by Dr G. Watkins and Dr D. Beukes, Rhodes University, South Africa (Olalekan *et al.*, 2014). These compounds were bound to various metal chelates in order to determine the effect the bound metals at different positions on the hydroaromatic ring, would have on the biological activity of the compounds.

The 66 novel compounds were dissolved in dimethyl sulfoxide (DMSO) to prepare 10 mM stock solutions. All of the compounds dissolved easily under sterile conditions and as such no physical changes were required such as heating, in order for the compounds to go into solution. Once dissolved the sterile compounds were aliqouted into small volumes and stored at -20°C to increase the stability of the solution and increase the storage time. Stock solutions of all controls used for all experiments were made up to 10 mM in the appropriate solvent and stored at -20°C until needed.

For each experiment the compounds were screened at 50 μM with the exception of the copper chelation assay (Section 2.4.5.3) in which the compounds were screened at 200 μM. Absorbances for all plates were determined using a UV-VIS spectrophotometer (Labsystems iEMS Reader MF) and recorded in Ascent™ software version 2.6. Thereafter, the results were analysed and compounds showing potential activity of greater than 50% or inhibition more than 50% were studied

further. Lead compounds were serially diluted and log sigmoid dose response curves were generated in order to determine the concentration of the compound at which there was 50% inhibition of biological activity (IC_{50}) using the computer software Graphpad Prism® version 5.0. All assays were repeated at least three times on all compounds to ensure the reproducibility and reliability of the results.

All reagents and chemical controls used in this study were purchased from reputable companies and were of the highest quality and standard.

2.2 *In vitro* culturing of *P. falciparum*

2.2.1 Culture maintenance

A chloroquine-sensitive 3D7 strain of *P. falciparum* was cultured *in vitro* and maintained daily according to the method described by Jensen and Trager (1977). A thin blood smear of the culture was made daily and fixed using 100% methanol and then stained using a rapid staining diagnostic kit (Clinical Sciences Diagnostics) to visualize the parasite which stained the parasites purple, which allowed for easy detection. The slide was examined under oil immersion (1000x) with a Nikon Optiphot light microscope and the % parasitaemia and stage of the parasite's life cycle were determined. Percentage parasitaemia of the flask was maintained at below 5% with a 5% haematocrit. Spent media was aspirated and replaced with fresh media daily (Section 2.2.2). Freshly washed erythrocytes (Section 2.2.5) were added when parasites where mainly in the schizont stage and gassed daily with a mixture of 92% N_2, 5% CO_2, 3% O_2 and incubated at 37°C. When the majority of parasites in the flask were in the ring phase, the culture was synchronized using D-sorbitol every second day (Section 2.2.4) (Lambros and Vanderberg, 1979).

2.2.2 Preparation of incomplete culture media and experimental media

Incomplete media used for culturing purposes consisted of 10.4 g Roswell Park Memorial Institute RPMI-1640, 5.9 g N-2-hydroxyethyl-piperazine-N-2-ethane-sulfonic acid (HEPES), 4 g glucose, 44 mg hypoxanthine and 100 µl of 50 mg/ml gentamicin. This was dissolved 1 L in sterile Millipore™ water. Experimental media was made up in the same way except for exclusion of the hypoxanthine and gentamicin.

2.2.3 Preparation of complete experimental and complete culture media

Incomplete culture media was supplemented with 5% (v/v) Albumax™ and 5% $NaHCO_3$ (w/v). A 5% (w/v) stock solution of Albumax™ was prepared by dissolving 5 g of Albumax™ in 100 ml of RPMI-1640 media. The 5% (w/v) $NaHCO_3$ solution was prepared by dissolving 5 g $NaHCO_3$ in 100 ml of autoclaved Millipore™ water. Both solutions were filtered through low-protein binding 0.22 µM filters and stored at -20°C and 4°C, respectively until needed.

2.2.4 Synchronisation of the culture

Parasite cultures needed to be predominantly in the ring stage of the asexual life cycle in order to complete the planned experimental protocols (Jensen and Trager, 1977). Parasites grown in culture loose their natural synchronicity, as such they needed to be treated with sorbitol to achieve phase synchronicity (Lambros and Vanderberg, 1979). Sorbitol is a sugar alcohol that has the advantage of inducing haemolysis as it remains osmotically active in the infected red blood cell. While doing so, it is not metabolised by the parasite and affects mainly the trophozoite and schizont stages of asexual development, as a result of their increase in red blood cell permeability (Wagner *et al.*, 2003).

Cultures with a high percentage of parasites in the ring stage were centrifuged at 400*g* for 5 minutes and the supernatant discarded. The pellet was incubated at 37°C with a 5% (w/v) D-sorbitol (Sigma-Aldrich®) for 20 minutes. Thereafter the suspension was centrifuged at 400*g* for 5 minutes and the pellet was re-suspended in incomplete culture media, gassed and incubated at 37°C. The 5% (w/v) D-sorbitol solution was prepared by dissolving 5 g in 100 ml of autoclaved Millipore™ water. The solution was sterilised through a 0.22 μm filter and stored at 4°C until needed.

2.2.5 Preparation of red blood cells

Whole blood was obtained weekly from healthy drug free volunteers within the Department of Pharmacy and Pharmacology (Appendix B1, clearance certificate number: M140669) and stored in anticoagulant tubes containing acid citrate dextrose-B solution. The red blood cells (RBCs) were washed after collection by centrifuging at 400*g* for 5 minutes, the buffy coat was aspirated and the pellet re-

suspended in sterile phosphate buffered saline (PBS) (pH 7.2). This process was repeated three times and the pellet was then suspended in incomplete experimental media and stored at 4°C for up to a week. PBS (pH 7.2) was prepared by dissolving: 8 g NaCl, 0.3 g KCl, 0.73 g $Na_2HPO_4.2H_2O$, 0.2 g KH_2PO_4 in 1 L of water and then steam autoclaved (121°C at 1.2 kg/cm^2 for 20 minutes) to achieve sterility and was stored at 4°C until needed.

2.3 Antimalarial activity

2.3.1 The tritiated hypoxanthine incorporation assay

The tritiated hypoxanthine incorporation assay is based on the inhibition of uptake of a radiolabeled nucleic acid precursor, hypoxanthine. Within the RBCs, the malaria parasite is unable to synthesise purines that are essential for nucleic acid synthesis and life sustaining for parasite growth. Therefore hypoxanthine is obtained from the mammalian host by salvage pathways. The incorporation of the hypoxanthine isotope into parasitic DNA offers a direct measure of the number of parasitised RBCs in the culture, which then allows the parasite growth to be quantified, the antimalarial activity was determined (Desjardins *et al.*, 1979).

2.3.1.1 Preparation of the parasites

Parasites mainly in the ring stage of the life cycle were treated with D-sorbitol (Section 2.2.4), in order to achieve synchronicity. A Giemsa-stained thin blood smear was examined microscopically. A total of ten fields were examined determining the number of red blood cells and infected RBCs. % Parasitaemia of the culture was calculated using Equation 2.1.

$$\%\ Parasitaemia = \frac{\text{Total number of infected RBCs}}{\text{Total number of infected + uninfected RBCs}}\ x100$$

Equation 2.1: Percentage parasitaemia.

The parasitaemia of the culture was adjusted to 0.5 % using washed RBCs (Section 2.2.5) and the haematocrit of the experimental parasite suspension was adjusted to 1% using complete experimental media (Section 2.2.3).

2.3.1.2 Experimental protocol

A volume of 25 µl of the 10 mM test compound and the positive controls chloroquine diphosphate (Sigma-Aldrich®) and quinine hydrochloride dehydrate (Sigma-Aldrich®) were diluted in incomplete experimental media (10 times dilution factor). The compounds and controls were serially diluted and plated out in triplicate in a sterile 96 well plate (Figure 2.1). Row H was considered as the control row, with the first four wells used as a RBC control and the last eight wells used as the drug-free parasite control, where 25 µl incomplete experimental media was added to these wells instead of drug. A volume of 200 µl of a 1% haematocrit of un-infected RBCs was added to the first four wells of row H. Thereafter, 200 µl of the infected RBC suspension was added to the remainder of the plate. The plate was then placed in a sterile candle jar in a humidified environment where the optimal gas concentration (92% N_2, 5% CO_2, 3% O_2) was achieved by lit candles which consumed the oxygen, before being incubated at 37°C for 24 hours (Desjardins *et al.*, 1979).

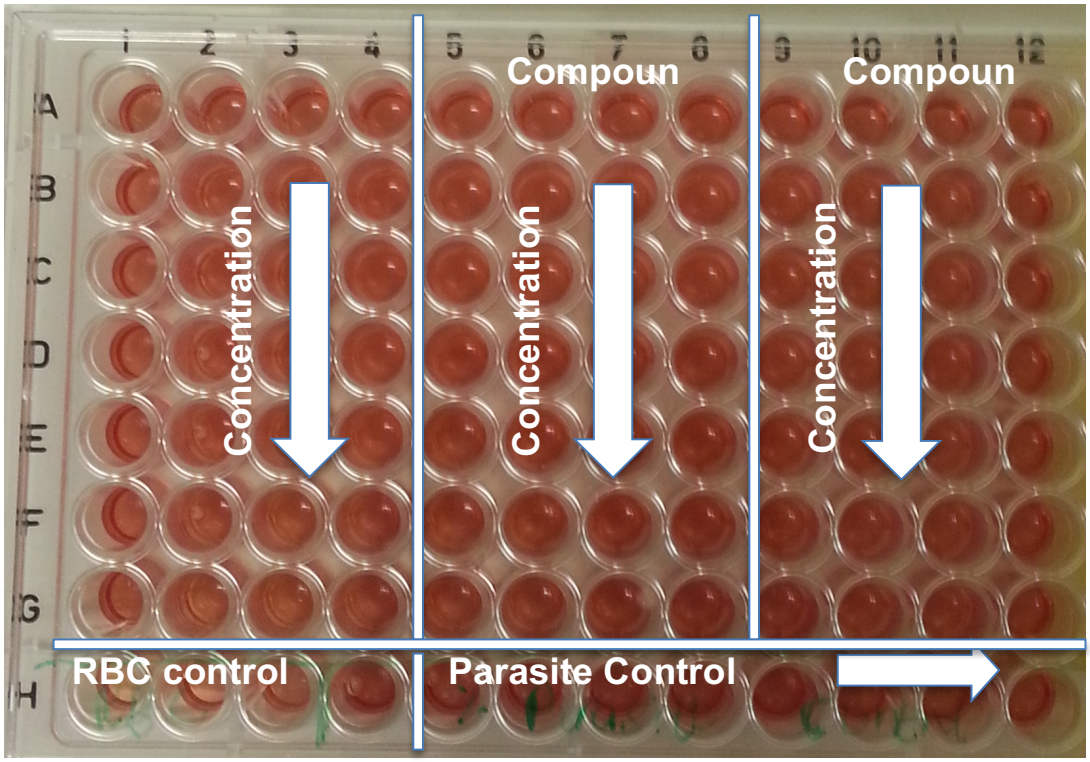


Figure 2.1: Plate set-up for the titriated hypoxanthine incorporated assay.

Thereafter, the [^{3}H] isotope (Amersham Pharmacia Biotech) solution was prepared in

incomplete experimental media at a ratio of 1:270. Of the latter solution, 25 µl was added to all the wells of the plates and re-incubated for a further 24 hours at 37°C. The [^{3}H]-DNA was harvested onto glass fibre filter mats (Wallac™) using a Titertek® semi-automated cell harvester. The filter mats were left to dry overnight and were then transferred to sample bags and filled with 10 ml of liquid beta scintillation fluid (Wallac™) and the harvested mats were read in a Wallac™ beta scintillation counter.

Radio-label ^{3}H uptake was expressed in counts per minute (CPM). This was used to measure the % parasite growth of the test compounds as per Equation 2.2.

$$\%\ Parasite\ growth = \frac{CPM_{drug} - CPM_{mean\ \mathrm{RBC\ control}}}{CPM_{mean\ parasite\ control} - CPM_{mean\ \mathrm{RBC}\ control}}\ x100$$

Equation 2.2: Percentage parasite growth determined using the hypoxanthine incorporation assay.

During the course of this study the Wallac™ beta scintillation counter used to quantify the antimalarial activity of the compounds broke down and was made redundant. Thus requiring an alternative means of quantifying the antimalarial activity of the chosen compounds to be established. Initially the sybr green 1 fluorescent assay (Johnson *et al.*, 2007) was established and validated in the Pharmacology division. All compounds were screened using this assay, but it was finally decided to use the parasite lactate dehydrogenase (pLDH) assay (Makler *et al.*, 1998). It was observed that the three assays generated similar results, but the pLDH assay generated more reproducible results and was less intensive for high-throughput evaluation of novel compounds than the sybr green.

2.3.2 Parasite lactate dehydrogenase assay for drug sensitivity

The pLDH assay for drug sensitivity works on the principle that host lactate dehydrogenase is distinguishable from that of the parasite DH when using the substrate 3-acetyl pyridine adenine dicunleotide (APAD) which can only be used by the parasitic LDH. This colourimetric assay is quantitated following the reduction of the nitro tetrazolium to form a blue formazan salt (Makler *et al.*, 1993).

2.3.2.1 Experimental protocol

Parasites were prepared in the same manner as the tritiated hypoxanthine incorporation assay (Section 2.3.1.1) with the same plate set-up with the exception of Row H, where the first three wells were treated as a blank and the following nine wells as a drug free parasite control (Figure 2.2). The parasite suspension plated out to the appropriate wells consisted predominantly of the ring stage with a 2% parasitaemia and 2% haematocrit. A volume of 100 µl parasitised RBCs in complete experimental media was plated out in a sterile 96 well plate. This plate was incubated in a candle jar for 24 hours at 37°C (Section 2.3.1.2) without the addition of test controls or compounds. Thereafter, 12.5 µl of the test compound and positive control, quinine prepared in incomplete experimental media (9 times dilution factor), was added to the wells during the trophozite stage. The candle jar was then incubated for a further 48 hours.

Thereafter, the plates were frozen at -70°C for an hour, after which they were thawed for 1-2 hours under sterile conditions. The plate was then shaken at 1200 rpm for 5 minutes and 25 µl of the lysate was transferred to a new 96 well plate. To which 100 µl of Malstat™ reagent (Section 2.3.2.2) and 20 µl of the nitro blue tetrazolium/phenazine ethosulfate (Section 2.3.2.2) solution were added. The plate was then shaken for 30 seconds at 1200 rpm and allowed to incubate for 40 minutes at 37°C. Thereafter, 50 µl of 5 % (v/v) acetic acid was added to all the wells to stop the colour reaction. The reaction solution was read at 620 nm. The % parasite growth was calculated according to Equation 2.3.

$$\%\ Parasite\ growth = \frac{ABS_{compound} - ABS_{blank}}{ABS_{100\%\,\mathrm{control}} - ABS_{blank}}\ x\,100$$

Equation 2.3: Percentage parasite growth determined using the pLDH assay.

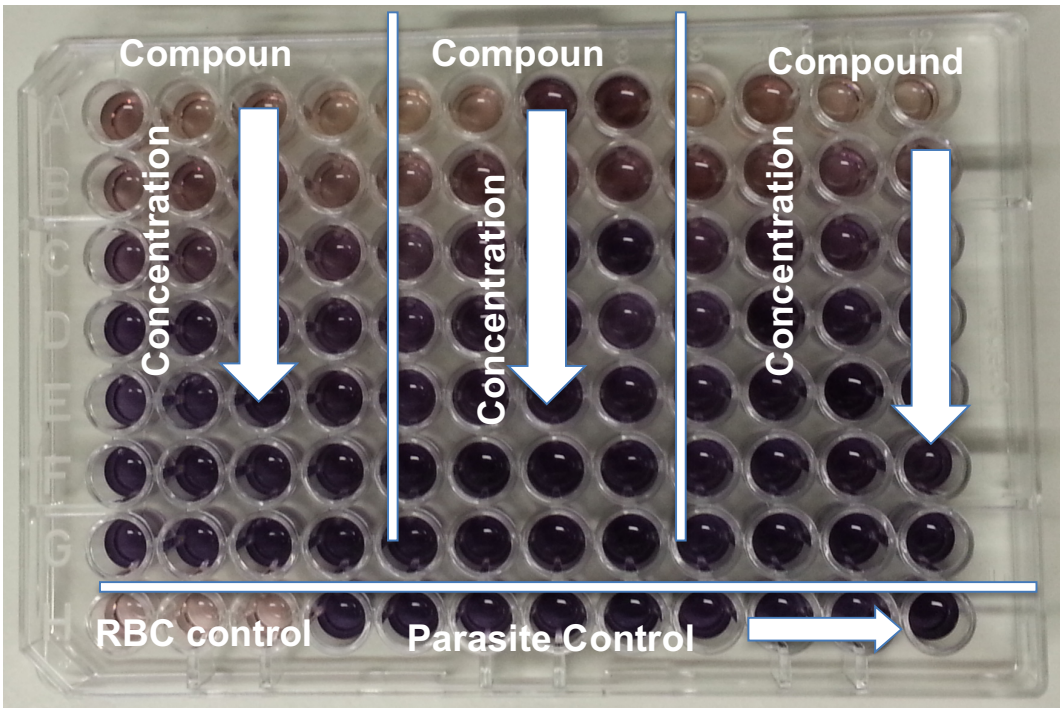


Figure 2.2: Plate set-up for the pLDH assay with resultant colour indicating parasite growth and light pink indicating no growth.

2.3.2.2 Preparation of pLDH reagents

The Malstat™ reagent (pH 9.1) was prepared with 0.055 M Tris base, 0.055 M L-lactate 0.037 mM APAD and 200 µl Tritonx-100 ™ made up to 100 ml in Millipore™ water and stored at 4°C up to a week. The NBT/PES stock was prepared weekly with a ratio of 1:1 of 0.0025 mM NBT and 289 nM PES (Sigma-Aldrich®) and stored in the dark at 4°C.

2.3.3 Combination studies

Based on the pLDH assay, the two most active compounds were studied further in combination with the standard antimalarial, quinine. Solutions of quinine with the test compound were prepared at concentrations 18 times higher than the final concentration required for the experiment (Section 2.3.2.1) in order to take into account for the nine times dilution factor of the experiment and the two times dilution factor of combining the two solutions in equal volume.

The two compounds were combined with quinine in various ratios (Table 2.1) and

serially diluted seven times in order to generate log sigmoid dose response curves to determine the IC_{50} value of the combination using Graphpad Prism® software version 5.0.

Table 2.1: Percentage concentration of standard antimalarial quinine (X) and compound (Y).

Ratio	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
X	100	100	50	25	2.5	0.25	0.025	0.0025	0
Y	0	0.031	0.31	1.25	5	17.5	25	100	100

Based on the IC_{50} values of the combination as well as the drug alone, relative ratios or fractional IC_{50} values (FIC_{50}) were determined (Equation 2.4). The sum of the FIC_{50}values (**Σ**FIC_{50}) were used to generate isobolograms (Figure 2.3) in order to determine the degree of interaction between the compound and quinine and this was categorized as being additive, synergistic or antagonistic (Berenbaum, 1989). ΣFIC_{50}> 1.25 indicates an antagonistic interaction, 0.75 >ΣFIC_{50}< 1.25 indicates an additive interaction, ΣFIC_{50}< 0.75 indicates a synergistic interaction (Figure 2.2) (Bell, 2005).

$$\text{FIC}_{50} = \frac{IC_{50}\ \text{value of compound in combination}}{IC_{50}\ \text{value of compound alone}}$$

Equation 2.4: FIC_{50} values for combination experiments.

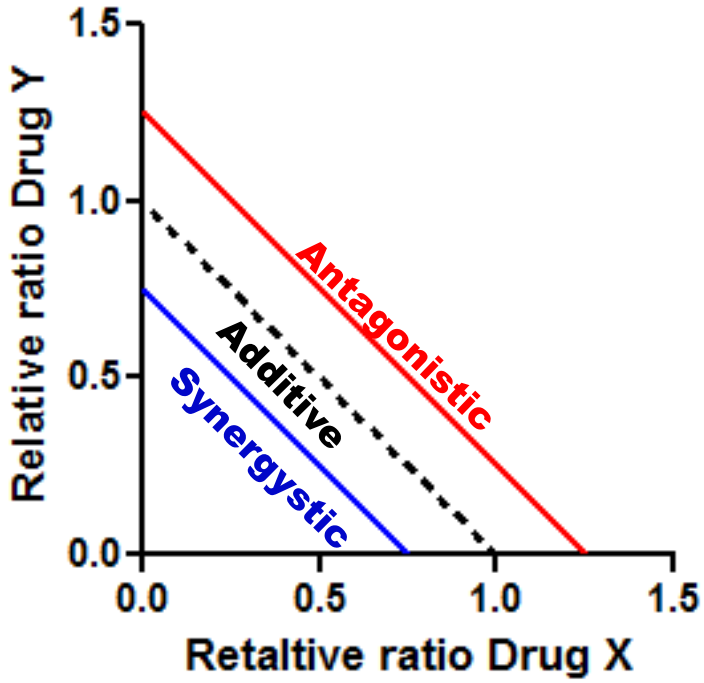


Figure 2.3: Isobologram depicting the possible pharmacological interaction between the combination of quinine and the chosen test compound.

2.4 Mechanism of action

2.4.1 Antimalarial activity mechanism of action

2.4.1.1 β-Haematin inhibition assay

β-Haematin is a synthetic form of the malaria pigment haemozoin, and the chemical synthetic process can be used to mimic the formation of haemozoin in the food vacuole of the parasite (Egan, 2006). The presence of haemozoin in the food vacuole of the malaria parasite is significant as it signifies the breakdown of haemoglobin a food source for the parasite. The β-haematin inhibition assay was used to determine the ability of the compounds to inhibit the formation of β-haemin under acidic conditions as a possible mechanism of action similar to that of chloroquine (Chemaly *et al.*, 2007).

2.4.1.2 Experimental protocol

A volume of 12.5 µl of the test compounds and the positive control, chloroquine prepared in DMSO (eight times dilution factor) were added to a non-sterile 96 well plate in quadruplicate wells, with one of the four wells serving as a compound colour control (Figure 2.4). Row H was used as a control row, and 12.5 µl DMSO with one as the blank and the remainder as the 100% β-haematin control. Thereafter 12.5 µl of a 1 mg/ml stock solution of haemin chloride dissolved in DMSO was added to all the wells except the blank, to which DMSO was added. A volume of 25 µl of Millipore™ water was then added to all the wells. β-haematin formation was initiated by the introduction of 50 µl 0.5 M acetate buffer (pH 4.4). The acetate buffer consisted of 0.5 M sodium acetate and 0.03% (v/v) glacial acetic acid made in a 1:1 ratio to a final volume of 1 L.

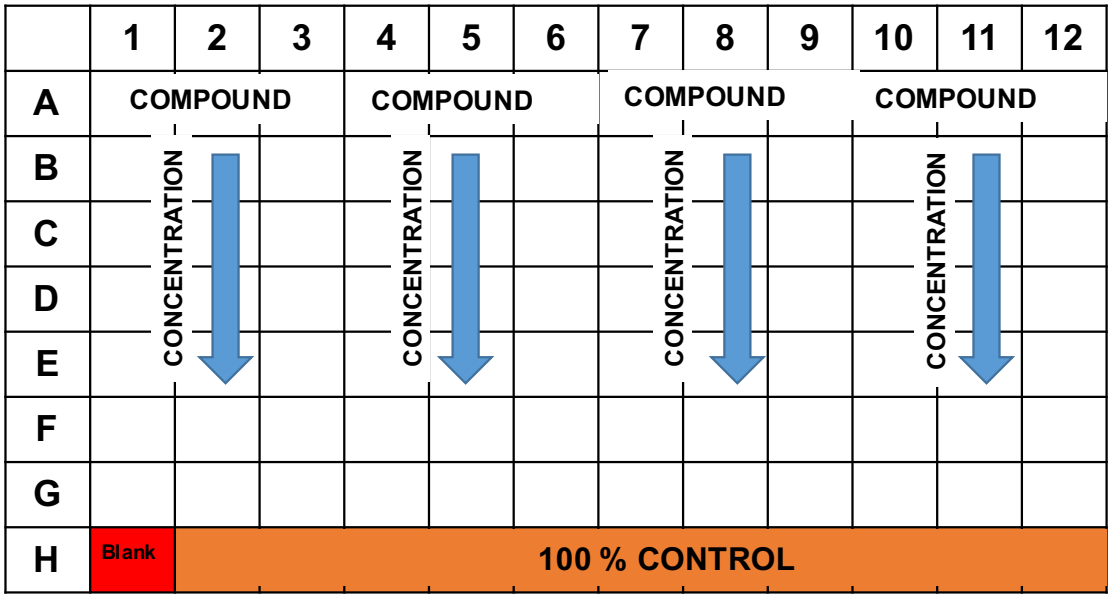


Figure 2.4: Plate set-up for the β-haematin inhibition assay.

After a 24 hour incubation period at 37°C, the DMSO-soluble solution in the wells was separated from the β-haematin crystals by centrifuging the plates at 1600*g* for 10 minutes. The supernatant consisting of unreacted haemin was discarded. In order to dissolve the washed β-haematin crystals, 150 µl of the supernatant was discarded and the crystals re-suspended in 150 µl 0.2 M NaOH solution. This solution was then diluted four-fold using 0.2 M NaOH to ensure all absorbance readings were <1. The reaction solution was read at 405 nm. The β-haematin inhibitory effects of the

compounds were calculated according to Equation 2.5.

$$\% \text{ Inhibition} = \frac{ABS_{compound} - ABS_{blank}}{ABS_{100\% \text{ control}} - ABS_{blank}} x\,100$$

Equation 2.5: Percentage β-haematin inhibition.

2.4.2 Anti-oxidant activities

2.4.2.1 The 2,2-diphenyl-1-picrylhydrazyl free-radical scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to evaluate the free radical scavenging activity of the test compounds. DPPH$^{\bullet}$ is a stable free radical with a characteristic absorption band. When it is in the presence of an anti-oxidant, protons are donated to DPPH$^{\bullet}$ resulting in the production of a non-radical stable form DPPH-H, with a decrease in absorption at 540 nm and visualise a colour change from purple to yellow (Figure 2.5)(Gülçın *et al.*, 2004).

Diphenylpicrylhydrazyl (free radical) Diphenylpicrylhydrazine (non-radical)

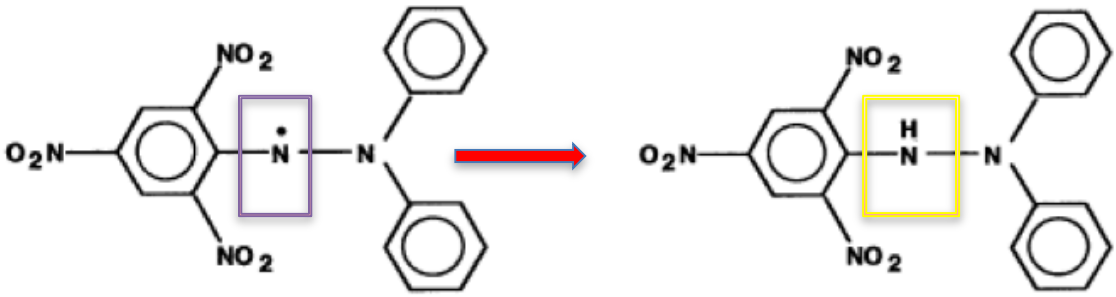


Figure 2.5: Reaction depicting the formation of the stable non-radical form of DPPH from the unstable radical form (Molyneux, 2004).

2.4.2.2 Experimental protocol

A volume of 50 μl of the test compounds and the positive control, Trolox™ prepared in DMSO (five times dilution factor) were added to the wells of a non-sterile 96 well plate in quadruplicate wells, with one well serving as a colour control for the compound. Row H (1-9) served as a 100% DPPH control and H (10-12) a blank to which 50 μl DMSO was added. Thereafter, 200 μl of a 96.16 μM $DPPH^{\bullet}$ solution prepared in HPLC grade methanol was added to all wells except the blank, to which 200 μl methanol was added. The $DPPH^{\bullet}$ stock prepared in HPLC methanol was stored at 4°C in the dark for a week. The plate was then incubated in the dark at room temperature for 30 minutes. Thereafter the reaction solution was read at 540 nm. The free radical scavenging effect of the compounds was determined using Equation 2.6.

$$\% \text{ Scavenging effect} = \frac{(ABS_{100\% \text{ control}} - ABS_{blank}) - (ABS_{compound} - ABS_{blank})}{ABS_{100\% \text{ control}} - ABS_{blank}} x100$$

Equation 2.6: Percentage free radical scavenging effect.

2.4.2.3 Lipid peroxidation inhibition assay

The lipid peroxidation inhibition assay measured the anti-oxidant activity of the compounds using thiocyante. This assay measures the absorbance of the stable complex formed between thiocyanate and the oxidative products of ferrous chloride and linoleic acid (Gülçın *et al.*, 2004).

2.4.2.4 Experimental protocol

The test compounds and the positive control, Trolox™ prepared in DMSO (10 times dilution factor) was plated out in quadruplicate wells in a non-sterile 96 well plate with one of these four wells being treated as a colour control. Row H was used as a 100% control row with 25 μl of DMSO added to all the wells (Figure 2.6). This was then reacted with 125 μl linoleic acid emulsion in all wells, except the blank and colour control wells, to which sodium phosphate buffer was added. A 2:1 suspension of linoleic acid (102.4 μl) and Tween-20 (51.2 μl) was freshly prepared in sodium

phosphate buffer (pH 7.0), which consisted of 0.23 mM NaH_2PO_4 and 0.23 mM $NaH_2PO_4.2H_2O$. A volume of 100 µl sodium phosphate buffer (pH 7.0) was added to all wells thereafter. The mixture was then shaken at 600 rpm using an orbital shaker (Thermo-Shaker MB100-4P) for a minute and incubated for 24 hours at 37°C. Thereafter, 25 µl of the latter reaction was reacted with 150 µl of 70% (v/v) ethanol, 40 µl 3.9 mM ammonium thiocynate, and 40 µl 5 mM ferrous chloride in 3.5% (v/v) hydrochloric acid. The reaction solution was read at 492 nm. The % lipid peroxidation inhibition of the compounds screened at 50 µM was calculated according to Equation 2.7.

$$\%\ \text{Lipid peroxidation inhibition} = \frac{(ABS_{100\%\,control} - ABS_{blank}) - (ABS_{compound} - ABS_{compound/blank})}{ABS_{100\%\,control} - ABS_{blank}} x\,100$$

Equation 2.7: Percentage lipid peroxidation inhibition.

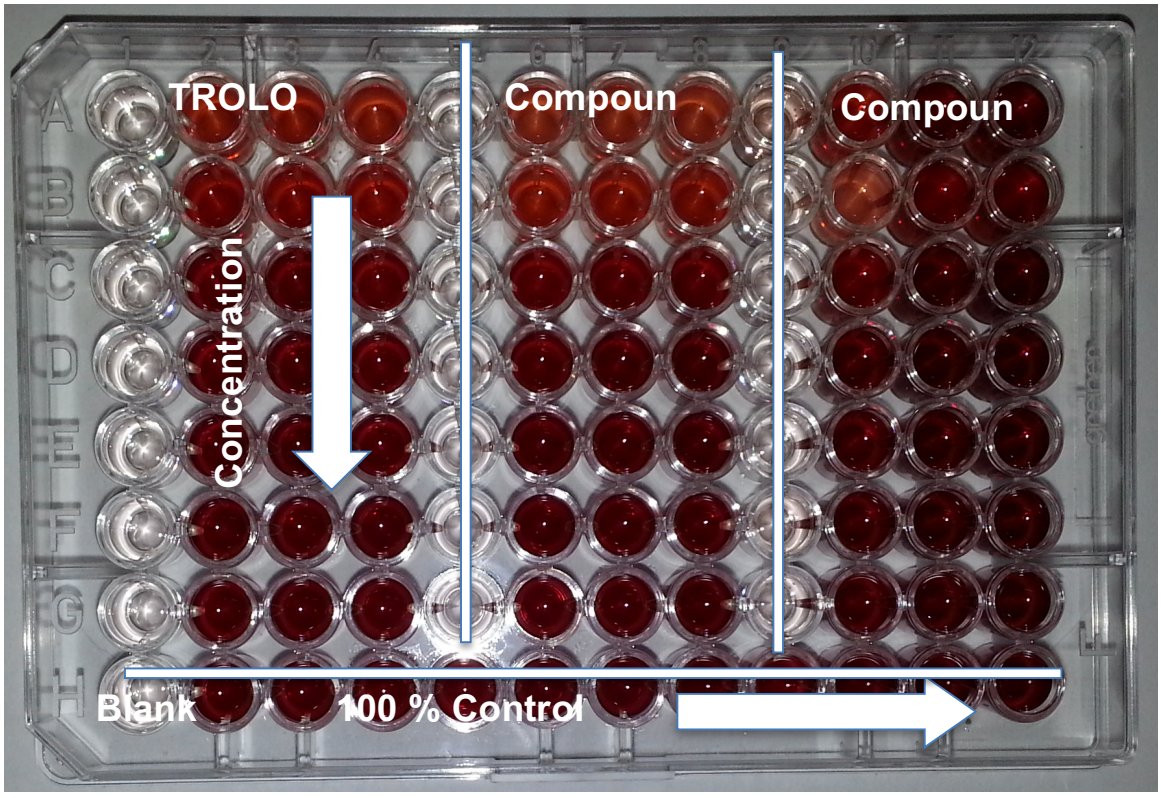


Figure 2.6: Plate set-up for the lipid peroxidation inhibition assay.

2.4.3 Toxicity evaluation assays

2.4.3.1 Red blood cell toxicity assay

The RB haemolysis assay was used to determine if the test compounds would have a direct effect on the parasite or the RBC membrane, the latter would indicate an effect on the host cell and a false positive for antimalarial activity (Hayat *et al.*, 2011).

2.4.3.2 Experimental protocol

Chloroquine and quinine, both negative controls were prepared (10 times dilution factor) in incomplete experimental media (Section 2.2.2) and plated out (20 μl) in quadruplicate into a sterile 96 well plate with one of the latter four wells treated as a colour control well. Row H was used as a control row with the first half of the row serving as a 0% lysis control using complete experimental media and the second half as a 100% lysis positive control using 0.2% (v/v) Triton-X100 prepared in PBS (pH 7.2) (Section 2.2.5). Thereafter, 180 μl of complete experimental media (Section 2.2.2) was added to the colour control wells. A 1% haematocrit of washed RBCs (Section 2.2.5) that were (not older than three days to ensure the optimal RBC membrane integrity) were incubated in complete experimental media. Of this suspension, 180 μl was added to all the wells except the colour control wells for the compounds and the plate was then incubated for 48 hours at 37°C.

Thereafter, the plate was shaken at 1200 rpm for 5 minutes and centrifuged at 400*g* for 5 minutes to sediment the intact RBCs. The supernatant (50 μl) was transferred to a new non-sterile 96 well plate, to which 150 μl Millipore™ water was added. The released haemoglobin was read at 540 nm. The % RBC haemolysis of the compounds screened at 50 μM was calculated according to Equation 2.8.

$$\%\ \text{RBC haemolysis} = \frac{(ABS_{compound} - ABS_{blank}) - (ABS_{0\%\,\text{lysis}})}{ABS_{100\%\,\text{lysis}} - ABS_{0\%\,\text{lysis}}} x\,100$$

Equation 2.8: Percentage red blood cell haemolysis.

2.4.3.3 The 13-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide based cellular viability assay

The 13-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) based cellular viability assay utilizes a change in colour as an expression of the cleavage of the yellow MTT by active mitochondria to form a dark blue formazan product which is quantitatively measured (Mosmann, 1983). This assay was used as a means of producing a preliminary toxicity profile for the novel compounds in comparison to the positive controls.

2.4.3.4 Culture maintenance

Human kidney epithelium cells (HEK-293) (Appendix B3, Ref W-CJ-130301-4) were continuously maintained *in vitro* at 37°C in 5% CO_2 in a tissue culture flask fitted with a porous membrane on the lid, according to the established departmental protocols. The cells were maintained with complete Dulbecco's Modified Eagle's Medium (DMEM) culture medium (Section 2.4.3.6) which was replenished twice a week. HEK-293 cells are an attachment cell line and was sub-cultured using trypsinization at least twice a week to allow for optimal growth of new cells.

The degree of confluency or cell density was determined using the light microscope (400x and 100x). Spent medium from the tissue culture flask was aspirated when the cells were more than 80% confluent. These cells were washed with PBS (Section 2.2.5) and thereafter 3 ml of 0.25% trypsin 0.1% Versense-EDTA (Sigma-Aldrich®) was added to cover the cells. After 5-10 minutes, 3 ml complete culture medium was added to detached cells to neutralize the trypsin. Based on the experiment schedule that week and the density/number of cells required, the cell suspension was re-seeded in 25 ml complete culture medium and incubated at 37°C in 5% CO_2.

2.4.3.5 Preparation of incomplete culture media

Incomplete culture medium was prepared by supplementing 13.5 g/L of high glucose DMEM (Sigma-Aldrich®) with 3.7 g/L $NaHCO_3$ (Merck) prepared in Millipore™ water and filtered through low-protein binding 0.22 µm filters and stored at 4°C until needed.

2.4.3.6 Preparation of complete culture and experimental medium

Complete culture medium was prepared by adding 10% foetal heat inactivated calf

serum (Sigma-Aldrich®), 1 ml of 200 mM L-glutamine (Sigma-Aldrich®), 1 ml 0.026 mM carbapenicillin and 1 ml 10 mM sodium pyruvate (Sigma-Aldrich®) to 100 ml incomplete culture medium (Section 2.4.3.2.4). Foetal calf serum (Sigma-Aldrich®) was thawed to room temperature, it was then heat inactivated at 56°C for 45 minutes, then aliquoted out and stored at -20°C until needed.

Complete experimental medium consisted of the same components with the exception of carbapenicillin.

2.4.3.7 Experimental protocol

In order to determine the cell number/ml in the suspension the trypsinized cell suspension (Section 2.4.3.4) was stained in a 1:1 ratio with sterile 0.2% (w/v) trypan blue (Sigma-Aldrich®) in PBS (Section 2.2.5). The stained cells were counted using a haematocytometer and light microscope at 100x magnification. To determine the number of viable cells for the assay, the numbers of dead cells as well as viable cells were noted. Dead cells stained blue due to their ruptured membranes and as such they have the ability to absorb the trypan blue stain. Whilst live cells had an intact membrane and were transparent under the microscope.

The suspension was adjusted to 0.55 million cells per ml using incomplete experimental medium (Section 2.4.3.6). A volume of 180 µl of this suspension was plated out in all the wells of a sterile 96 well plate, except the colour control wells for the compounds and the untreated wells, to which 180 µl of experimental medium was added (Figure 2.7). The plate was incubated at 37°C in 5% CO_2 for 2 hours in order for the cells to adhere to the surface of the well.

Thereafter, 20 µl of the test compound and positive control, camptothecin (prepared in incomplete experimental media, 10 times dilution factor) were plated out in quadruplicate, with one of the four wells serving as a colour control. The last row was treated as a 100% untreated control with a blank in the first three wells, to which 20 µl incomplete experimental medium was added. The plate was incubated in a humidified environment for 44 hours at 37°C in 5% CO_2.

After the incubation period, 40 µl MTT was added to all the wells and the plate was incubated under the same conditions (as mentioned previously) for 2 hours. The MTT (USB) (5mg/ml) was made up in PBS (Section 2.2.5) and sterilized through a 0.2 µm

syringe filter and stored at 4°C in the dark as it is light sensitive. Thereafter the plate was centrifuged at 400*g* for 5 minutes and 180 µl of the supernatant was discarded and replaced with 150 µl DMSO to dissolve the insoluble formazan crystals. The plate was then shaken at 600 rpm for 2 minutes to ensure the crystals were completely dissolved. The reaction solution was read at a test wavelength of 540 nm and reference wavelength of 690 nm. The % cell viability of the compounds at 50 µM was calculated according to Equation 2.9.

$$\%\ Cell\ viability = \frac{ABS_{drug\ (540nm)} - ABS_{drug\ (690\ nm)}}{mean\ ABS_{drug\ free\ control\ (540nm)} - mean\ ABS_{drug\ free\ control\ (690\ nm)}} x\ 100$$

Equation 2.9: Percentage cell viability as determined by the MTT assay.

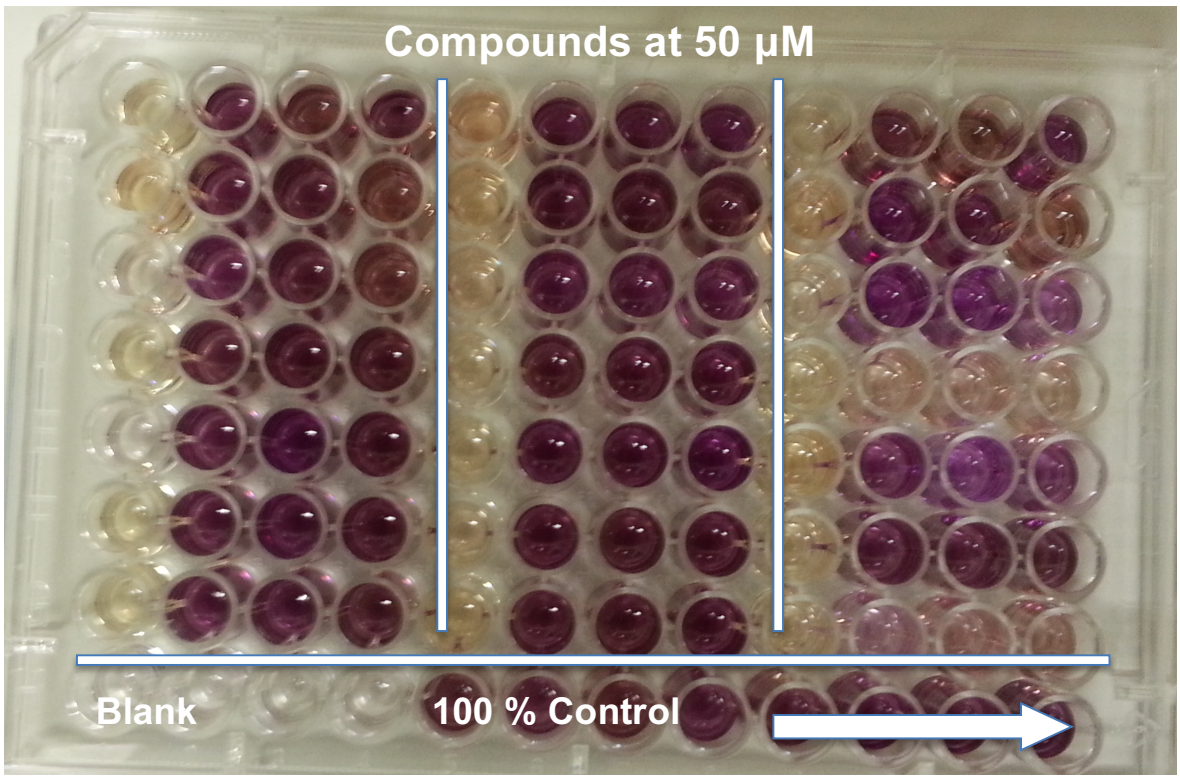


Figure 2.7: Plate set-up for the MTT assay.

2.4.4 Anti-proliferative activity

2.4.4.1 Culture maintenance

A human erythromyeloblastoid leukemia cell line, namely K562 was cultured and maintained *in vitro* at 37°C in 5% CO_2 in a tissue culture flask fitted with a porous membrane on the lid, according to the established departmental protocol. The cells were maintained with complete DMEM culture medium (Section 2.4.3.6) in a similar manner to the HEK-293 cells (Section 2.4.3.4) with the exception of the sub-culturing method. The K562 is a suspension cell line and as such there was no need to trypsinize the cells. In order to sub-culture the cell suspension was centrifuged at 400*g* for 5 minutes. Thereafter, 90% of the spent culture medium was aspirated and replaced with fresh pre-warmed complete culture medium.

2.4.4.2 Experimental protocol

The MTT assay was used to determine the anti-proliferative activity of the test compounds and was carried out and analysed in the same manner as that of the HEK-293 cell line (Section 2.4.3.7).

2.4.5 Chelating potential

2.4.5.1 Iron chelation assay

Iron is vital to both the host as well as the malaria parasite. Parasites require iron from their host for processes such as DNA synthesis, carbohydrate metabolism, mitochondrial function and electron transport, amongst others (Mabeza *et al.*, 1999). Due to the parasite's obvious need for iron to complete life-sustaining processes, the use of an iron chelator would cause "iron starvation", thereby inhibiting essential metabolic processes and killing the parasite (Weinberg and Moon, 2009). Ferrozine forms complexes with Fe^{2+}, and when exposed to a chelating agent, the complex formation is disrupted and the red/purplish colour of the complex decreases, which allows for quantification of the iron chelating ability of the novel compounds (Gülçın *et al.*, 2004).

2.4.5.2 Experimental protocol

The test compounds and positive control, EDTA (20 μl) prepared in DMSO (7.5 times dilution factor) was plated out in quadruplicate wells into a non-sterile 96 well plate with one of these four wells serving as a colour control for the compound. Row H was used as the control row with eight of the wells used as a 100% ferrozine-fe^{2+}complex or positive control, and four of the wells were the blanks. A volume of 20 μl DMSO was added to these wells instead of compound. Thereafter, freshly prepared 0.48 M iron chloride (30 μl) prepared in 649 μM ammonium acetate was added to all the wells, except the blank to which Millipore™ water was added. A volume of 30 μl ammonium acetate was added to the colour control wells, as well as the blank. The plate was then incubated for 30 minutes at room temperature in the dark.

Thereafter 100 μl of 1.2 mM ferrozine was added to all wells, except the blank and colour control wells. To these wells, 100 μl Millipore™ water was added. The plate was incubated in the dark at room temperature for 10 minutes. The reaction solution was read at 550 nm.
The % Iron chelation was calculated according to Equation 2.10.

$$\%\ \text{Iron chelation} = \frac{(ABS_{100\%\ control} - ABS_{blank}) - (ABS_{compound} - ABS_{blank})}{ABS_{100\%\ control} - ABS_{blank}} x100$$

Equation 2.10: Percentage Iron chelation.

2.4.5.3 Copper chelation assay

The copper I and copper II assay worked on the principle of exposing the potential copper chelator to a solution of either Cu^{1+} or Cu^{2+} under specified pH conditions to ensure the stability of the cationic state. The assay measured the potential chelators ability to compete with neocuproine to form a stable complex with the Cu^{1+} or Cu^{2+} ions, respectively and in doing so exhibiting its chelating potential (Říha *et al.*, 2013).

2.4.5.4 Experimental protocol copper (I)

A volume of 50 μl of the test compound as well as the positive control tetrathiomolybdate (TTM) were prepared in DMSO to an initial concentration of 200 μM (six times dilution factor) and plated out in quadruplicate into a non-sterile 96 well plate with one well serving as a colour control well for the compounds. The plate set-up was similar to that of the iron chelation assay (Section 2.4.5.2; Figure 2.8). To the blank wells, 50 μl Millipore™ water was added instead of compound. Thereafter, 50 μl $CuSO_4.5H_2O$ (1 nM) (Section 2.4.5.5) was added to all the wells, followed by 100 μl acetate buffer to all the wells. The acetate buffer (pH 4.5-5.5) was prepared by mixing 0.1 M sodium acetate solution with 0.1 M acetic acid. Thereafter, 50 μl 5 mM hydroxylamine was added to all the wells and the plates were incubated in the dark. After 15 minutes, 50 μl 5 mM neocuproine was added to all wells, except the colour control and blank wells, to which 50 μl Millipore™ water was added. The reaction solution was read at 450 nm. The % copper chelation was calculated according to Equation 2.11.

$$\%\ \text{Copper chelation I/II} = \frac{(ABS_{100\%\ control} - ABS_{blank}) - (ABS_{compound} - ABS_{blank})}{ABS_{100\%\ control} - ABS_{blank}} x100$$

Equation 2.11: Percentage copper chelation (I/II).

2.4.5.5 Preparation of copper sulphate pentahydrate

A 5 mM stock solution of $CuSO_4.5H_2O$ Millipore™ water and stored at 4° C covered in foil. This solution was then further diluted for each experiment using hydroxylamine to prepare a working 1 nM Cu^{1+} solution and Millipore™ water for a 1 nM Cu^{2+} solution.

2.4.5.6 Experimental protocol copper (II)

A volume of 50 μl of the test compound as well as the positive control, triethylenetetramine (TETA; 200 μM in DMSO, six times dilution factor) were plated out in quadruplicate into a non-sterile 96 well plate with the same plate set-up as the copper (I) assay (Section 2.4.5.4). A volume of 50 μl $CuSO_4.5H_2O$ (Section 2.4.5.5) was added to all the wells, followed by 100 μl HEPES buffer (pH 6.8-7.5) to all the

wells. The plate was then incubated in the dark at room temperature for 15 minutes. Thereafter 50 µl 5mM hydroxylamine was added to all the wells to reduce non-chelated cupric ions to cuprous ones and the plates were incubated in the dark for a further 15 minutes. Thereafter 50 µl 5 mM neocuproine was added to all wells, except the colour control and blank wells, to which 50 µl Millipore™ water was added. The plate was then incubated for 15 minutes in the dark at room temperature and the reaction solution was read at 450 nm.

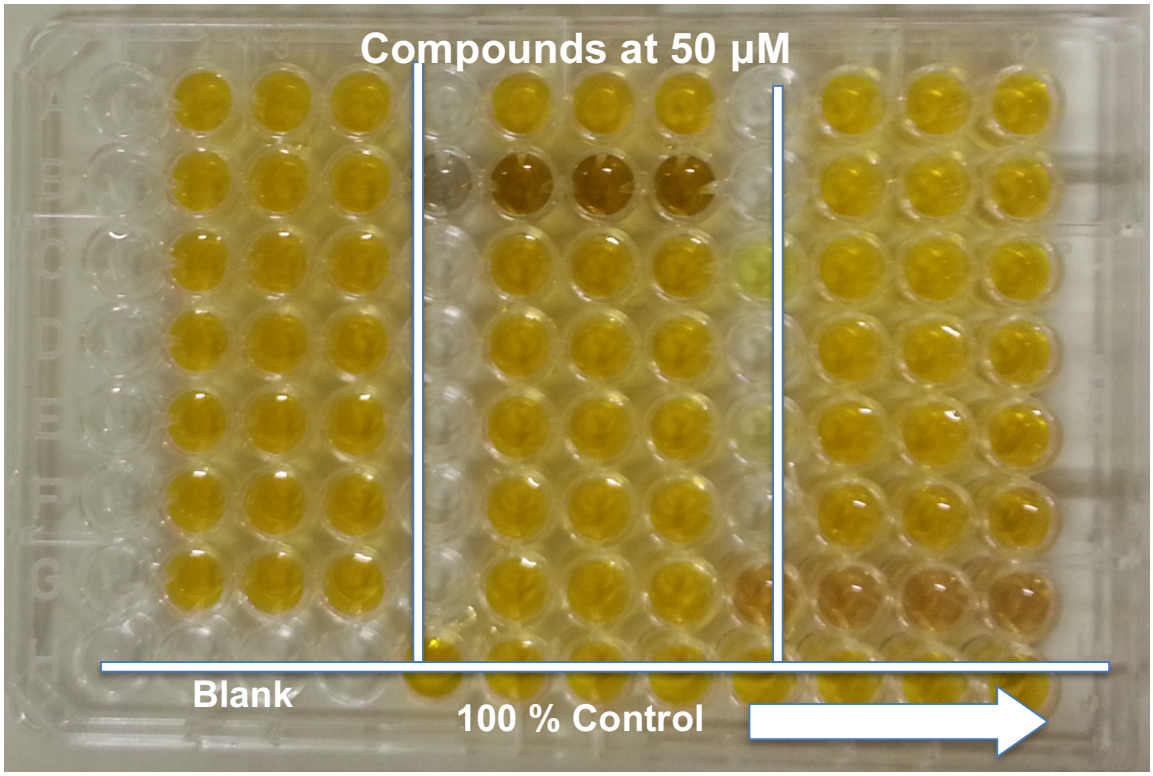


Figure 2.8: Plate set-up for the copper (I/II) chelation assay.

2.5 Data and statistical analysis

The mean and standard deviation (s.d) of the triplicate runs were determined. The Mann-Whitney U test was used to determine the statistical differences or similarities between the compounds and the positive controls with a p value < 0.05 considered significant.

Further analysis was conducted to determine the selective activity of the compounds against *P. falciparum* compared to their cytotoxicity using the HEK-293 cell line, by calculating a safety index (SI_{pf}) of the compounds. Compounds with a SI_{pf} value of

>10 were regarded as non-toxic (Zirihi *et al.*, 2005) (Equation 2.11). The selective activity of the compounds against the HEK-293 cell line or non-cancerous cells as compared to the K562 cell line or cancerous cells were determined by calculating the selectivity index of the compounds. A SI_{nc} (Equation 2.12) of >10 was considered more favourable towards HEK-293 cells and a $SI_{n.c}$ of <10 was considered more favourable towards K562 cells.

$$SI_{pf} = \frac{\text{Cytotoxicity } (IC_{50})}{\text{Antimalarial activity } (IC_{50})}$$

Equation 2.11: Selectivity index for *P. falciparum* compared to HEK-293 cells

$$SI_{nc} = \frac{Cytotoxicity\ of\ HEK\text{-}293\ cell\ line\ (IC_{50})}{Cytotoxicity\ of\ K562\ cell\ line\ (IC_{50})}$$

Equation 2.12: Selectivity index for normal cells as compared to cancer cells.

CHAPTER 3

Aminoquinoline and quinolin-8-yl derivatives

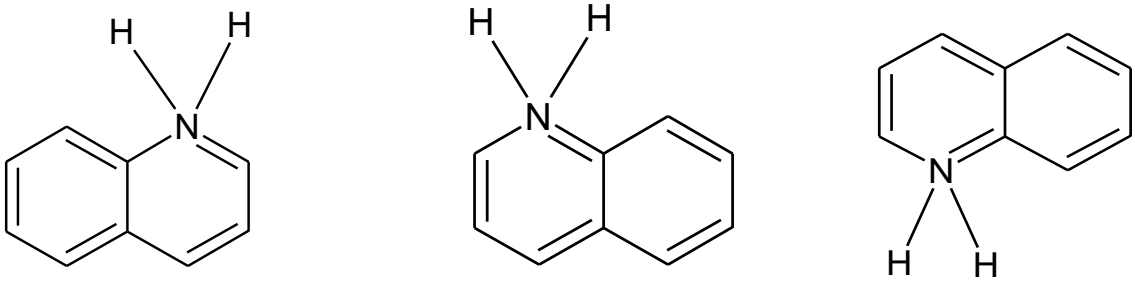
3.1 Introduction

Historically quinoline based antimalarials have been at the forefront of malaria treatment and drug discovery dating back to the 17th century. Prior to World War II compounds such as quinine, pamaquine and chloroquine were developed. These were followed by amodiaquine and primaquine. Quinine was first isolated from the *Cinchona* tree bark in the late 1600's and during the 20th century chloroquine, the most widely known drug from this group provided hope for eradication of the disease that plagued society since the age of Hippocrates 400 B.C (Meshnick and Dobson, 2001). Pamaquine was the first 8-aminoquinoline antimalarial used in combination with quinine and was able to target the parasitic gametocytes (Figure 3.1). This drug was then used for preventing relapses from occurring in *P. vivax* infections specifically during the liver stage of the disease. A relapse infection occurs when dormant liver stage forms known as hypnozoites become active and differentiate into merozoites, which burst and cause a blood-stage infection. In so doing the 8-aminoquinolines have the unique ability to exhibit activity on gametocytes as well as hypnozoites (Waters and Edstein, 2012). Although this is so, pamaquine showed little effectiveness against blood-induced infection, as such it was a poor blood schizontocide, and one of the more significant disadvantages was its high toxicity levels which ultimately led to its discontinuation of use therapeutically. This led to the development of primaquine, which displayed the highest efficacy and lowest toxicity levels when compared to other 8-aminoquinolines in development at the time such as pentaquine or isopentaquine (Vale *et al.*, 2009).

The particular manner in which antimalarials consisting of a quinoline scaffold produce their effect is not completely understood, but there are a few proposed mechanisms of action. These include the inhibition of protein synthesis, the inhibition of food vacuole phospholipases, the inhibition of aspartic proteinases, effects on

DNA and RNA synthesis and the prevention of haemozoin formation being the most favoured of all the proposed mechanisms of action. In so doing these drugs have the ability to act during the blood stages, as well as the hepatic stages of the asexual stages of development (Egan *et al.*, 2000). Chloroquine, a 4-aminoquinoline derivative is proposed to be specifically active against the process during which the parasite actively degrades haemoglobin (Figure 3.1). In order for the parasite to obtain essential amino acids needed for growth, the parasite digests the haemoglobin within red blood cells within the acidic digestive food vacuole. During the digestion process the parasite produces toxic haem which is polymerized to form inert haemozoin. Chloroquine and other 4-aminoquioline derivatives are proposed to inhibit the formation of haemozoin as a mechanism of action (O'neill *et al.*, 2012).

Although compounds consisting of a quinoline scaffold have proven successful against *Plasmodium* infections, the threat of the expansion of resistance is still very real with resistance developed against standard antimalarials such as chloroquine and mefloquine (WHO., 2015). However the quinoline backbone still provides a starting point for further derivatisation of novel antimalarial compounds. Thus the first set of compounds used in this study were structurally similar including the quinoline scaffold and comprised of a series of salts, adducts of 5-aminoquinoline and 8-aminoquinoline, with hydroxyaromatic carboxylic acids and functionalized quinoline derivatives (Figure 3.1).



4-aminoquinoline 8-aminoquinoline 5-aminoquinoline

Figure 3.1: Structures of 4-aminoquinoline, 8-aminoquinoline and 5- aminoquinoline.

3.2 Objectives

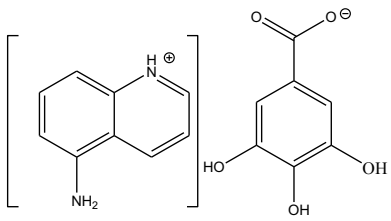
- To assess the *in vitro* properties of the novel aminoquinolines and quinolin-8-yl derivatives against the asexual stages of the parasite using the tritiated hypoxanthine incorporation assay.
- To investigate the mechanism of action of the novel compounds by monitoring oxidative stress pathways within the parasite, inhibition of haemozoin formation as well as iron and copper chelation properties.
- To determine preliminary toxicological and antiproliferative properties of the compounds against human red blood cells, human kidney epithelium (HEK-293) and human erythroleukemia (K562) cell lines.

3.3 Methodology

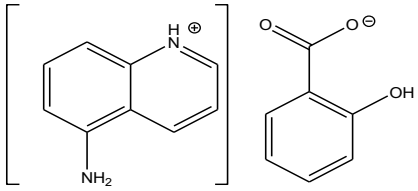
3.3.1 Chemical synthesis of derivatives

3.3.1.1 Aminoquinoline derivatives

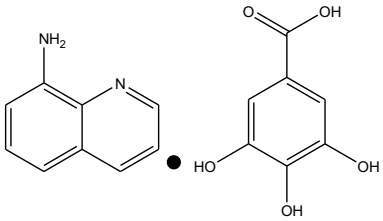
The series of aminoquinoline derivatives were synthesized and the purity as well as validation of the structures were completed by Prof J. Bikash Baruah and Prithiviraj Khakhlary from the Indian Institute of Technology, Assam, India. Salts were prepared for compounds **I**, **II**, **IV** from a 1:1 mole ratio mixture of 5-aminoquinoline (Figure 3.1) and its corresponding hydroxyaromatic acid. Compound **I** was formed from 5-aminoquinoline and gallic acid, compound **II** from 5-aminoquinoline and 2-hydroxybenzoic acid, compound **IV** was formed from 8-aminoquinoline with 1-hydroxy-2-napthoic acid (Figure 3.2). An adduct was formed for compound **III** with 8-aminoquinoline and gallic acid. In general, quinoline interacts with aromatic carboxylic acid and has the ability to form hydrogen bonded adducts, and in aminoquinolines there are two potential sites to act as hydrogen bond acceptors depending on the acidity of the carboxylic acid it is interacting with. Thus they have the ability to either form a hydrogen bonded adduct or hydrogen bonded salt. A salt forms when there is a proton transfer between the carboxylic acid and the aminoquinoline. The result of a proton being transferred is based on the acidity of the acid, if the difference between the pH of the acid and base is < 2/3 a salt is formed, and if it is >0 an adduct will be formed (Figure 3.3)(Childs *et al.*, 2007).



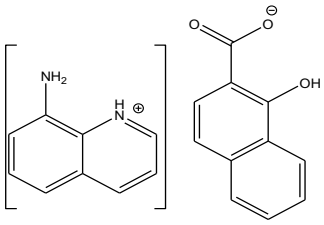
5-aminoquinolinium gallate **(5AqGA) (I)**



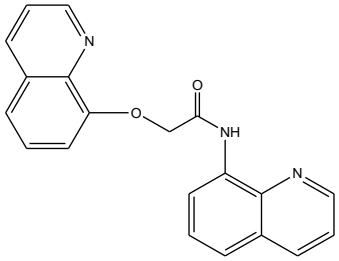
5-aminoquinolinium-2-hydroxybenzoate **(5AqOBA) (II)**



Co-crystal of 8-aminoquinoline and gallic acid **(8AqGA) (III)**



5-aminoquinolinium1hydroxynapthalene-2-carboxylate **(8Aq1H2NA) (IV)**



N-(quinolin-8-yl)-2-(quinolin-8-yloxy)acetamide **(8Hq8Aqmide) (V)**

Figure 3.2: The five aminoquinoline derivatives used in this study (Barauh, personal communication).

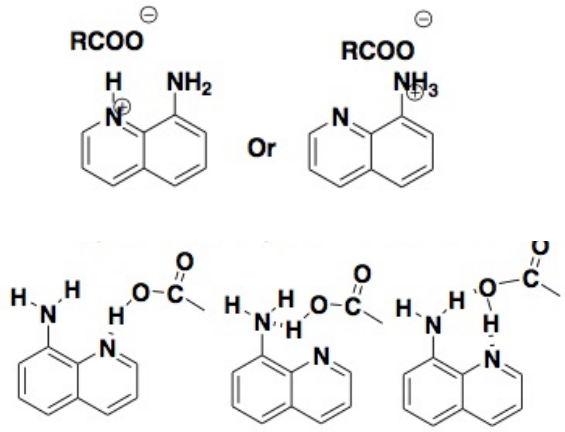


Figure 3.3: Examples of possible ways for salt or adduct formation by 8-aminoquinoline with a carboxylic acid.

Biologically, the manner in which amides are constructed determine their biological effect. In the case of proteins, their activity is determined by amide bonds and their weak interactions. To achieve a particular effect the geometrical features of the amide backbone is important. The geometrical features of 8-aminoquinoline and 8-hydroxyquinoline were used to form a salt namely compound **V** as a result of the nitrogen atoms present on the oxyquinoline being protonated. (Kalita and Baruah, 2010).

3.3.1.2 Quinolin-8-yl derivatives

4-Nitro-N-(quinolin-8-yl)benazamide has proven to be a biologically active amide by exhibiting potential as inhibitors of vascular endothelial growth in cancer therapy. This amide has various unique structural features such as two distinguishable ends for supramolecular reactions. As well as a rotatable amide bond which allows for various packing patterns to form and create different arrangements within its lattice and in so doing allows for the conversion into different polymorphs (Yang *et al.*, 2010). The nitrogen atom present on the quinoline has the ability to act as a proton acceptor and in so doing has the ability to form a salt. Compounds **S3** and **S5** were polymorphs synthesized from 4-nitro-N-(quinolin-8-yl)benazamide by reacting 8-aminoquinoline and its corresponding acid chloride (Khakhlary and Baruah, 2014). Compounds **S1** and **S2** were prepared by reacting 8-aminoquinoline with bromoacetyl bromide followed by reacting the product of this reaction with phenolic compounds (Figure 3.4). Compound **S4** was prepared by reacting 1-napthyl isocyanate with a mixture of 8-aminoquinoline and compound **S6** was prepared by reacting 1-napthyl isocyanate with a mixture of 5-aminoquinoline. X-ray single crystal diffraction data was collected and used to characterize the compounds (Baruah, personal communication).

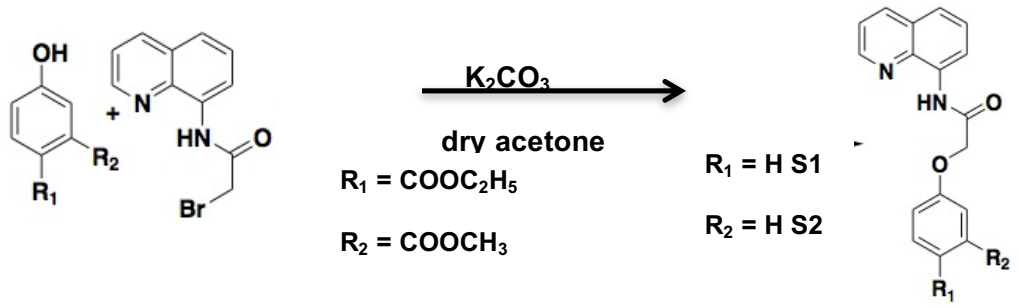
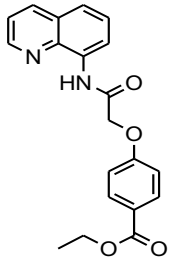
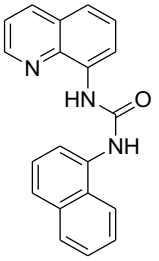
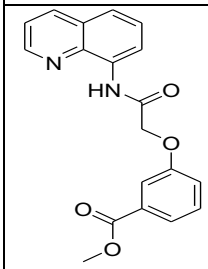
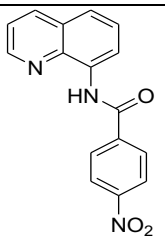
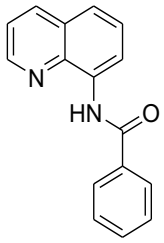
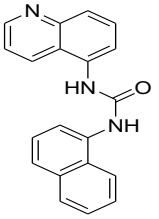


Figure 3.4: Synthesis of **S1** and **S2.**

Table 3.1: The six quinolin-8-yl derivatives used in this study (Baruah, personal communication).

Structure	Compound name	Structure	Compound name
	ethyl 4-[2-oxo-2-(quinolin-8-ylamino)ethoxy]benzoate (**S1**)		1-naphthalen-1-yl-3-quinolin-8-ylurea (**S4**)
	methyl 4-[2-oxo-2-(quinolin-8-ylamino)ethoxy]benzoate (**S2**)		4-nitro-*N*-(quinolin-8-yl)benzamide (**S5**)
	*N*-(quinolin-8-yl)benzamide (**S3**)		1-naphthalen-1-yl-3-quinolin-5-ylurea (**S6**)

3.3.2 Pharmacological evaluations

To achieve the objectives of this chapter the following assays were undertaken:

1) Antimalarial activity of the compounds was determined using the titriated hypoxanthine incorporation assay where compounds were screened at 50 μM. Compounds showing inhibition of >50% were serially diluted and IC_{50} values obtained (Section 2.3).
2) The inhibition of haemozoin formation of the compounds was determined (Section 2.4.1)

3) The anti-oxidant activity of the compounds were determined using the DPPH free radical scavenging assay, as well as the lipid peroxidation inhibition assay (Section 2.4.2).
4) A toxicity evaluation was determined using the RBC toxicity assay, as well as the MTT-based cellular viability assay (Section 2.4.3)
5) The anti-proliferative activity of the compounds was determined using the MTT based cellular viability assay (Section 2.4.4)
6) The metal chelation potential of the compounds were determined using the iron and copper chelation assays (Section 2.4.5)

3.4 Results

3.4.1 *In vitro* antimalarial activity and β-haematin inhibitory activity

The screening of the compounds at 50 μM resulted in parasite growth in the range of 0.1% - 95.4% when compared to chloroquine (0.0005 %) and quinine (0.064%)(Table 3.2). Of the 11 compounds screened, IC_{50} values were obtained for 5 compounds with compounds **I** (IC_{50} value: 25.1 ± 1.3 μM) and **V** (IC_{50} value: 25.2 ± 3.6 μM) displaying the highest potential activity within this group. Both compounds inhibited the parasite growth to a similar degree, but when compared to quinine (IC_{50} value: 0.085 ± 0.005 μM) and chloroquine (IC_{50} value: 0.0065 ± 0.0006 μM) the activity was moderate. The order of decreasing activity for the five compounds against the 3D7 strain is as follows: **I** > **V** > **II** > **III** > **IV**.

In order to elucidate a possible mechanism of action as described for the 4-aminoquinoline, such as chloroquine, the inhibitory effects of the derivatives were evaluated. Compound **I** was shown to possess negligible activity to inhibit haemozoin formation when compared to chloroquine (92.0 ± 1.87%), with the remainder of the compounds not having any inhibitory effect (Table 3.2).

Table 3.2: Antimalarial activity and β-haematin inhibitory activity of the aminoquinoline (**I-IV**) and quinolin-8-yl derivatives (**S1-S6**).

Compound	**Antimalarial Activity** (% Parasite growth at 50 μM ± s.d)	**Antimalarial Activity** (IC_{50} ± s.d μM)	**β-Haematin Activity** (% Inhibition at 50 μM ± s.d)
I	6.38 ± 0.97	**25.08 ± 1.3**	10.76 ± 1.86
II	27.13 ± 2.22	34.6 ± 3.19	0.10 ± 0.01
III	69.16 ± 3.19	75.38 ± 5.20	0.10 ± 0.01
IV	67.76 ± 7.11	104.09 ± 16.52	0.10 ± 0.01
V	0.10 ± 0.01	**25.19 ± 3.62**	0.10 ± 0.01
S1	76.54 ± 6.14	n.d	0.10 ± 0.01
S2	76.76 ± 4.48	n.d	0.10 ± 0.01
S3	80.87 ± 3.44	n.d	0.10 ± 0.01
S4	79.45 ± 12.47	n.d	0.10 ± 0.01
S5	95.44 ± 2.36	n.d	0.10 ± 0.01
S6	85.24 ± 5.70	n.d	0.10 ± 0.01
Quinine	0.068 ± 0.003	0.085 ± 0.005	0.2 ± 1 *
Chloroquine	0.0009 ± 0.0005	0.0065 ± 0.0006	92.0 ± 1.87

n.d: not determined; * IC_{50} (Taken from Chen, 2010)

3.4.2 Toxicity evaluation

3.4.2.1 Red blood cell toxicity

Varying degrees of haemolysis were displayed by the compounds ranging between 0.06% and 3.67% when screened at a concentration of 50 μM (Table 3.3). Compound **V** (3.67%) displayed the most haemolytic activity from the group, and this was slightly more haemolytic in comparison to chloroquine (2.09%) at 50 μM.

3.4.2.2 Cytotoxic effects against the HEK-293 cell line

Two of the eleven compounds screened for potential toxicity against the human kidney epithelium, HEK-293 cell line, were found to be toxic when screened at 50 μM (Table 3.3). Compound **I** (IC_{50} value: 23.61± 4.31μM) displayed the most toxic effect when compared to the positive control camptothecin (IC_{50} value: 0.11 ± 0.01 μM).

3.4.2.3 Safety index of compounds against *P. falciparum*

The safety index of the compounds (SI_{pf}) that displayed antimalarial activity was determined by comparing their cytotoxic activity on HEK-293 kidney cells to their antimalarial activity. This was achieved using the IC_{50} values of the two assays as a means of comparing the degree of effect on the human kidney epithelium and malaria parasite. Of the five compounds active against *P. falciparum*, compound **I** and **V** both had a safety index of <10, which indicated that the compounds were considered toxic to the human host (Section 2.5). The remainder of the compounds active against *P. falciparum* had no significant effect on cell viability using the HEK-293 cell line and the safety index could only be estimated. These compounds all had safety indexes estimated at <10 and were therefore considered toxic (Table 3.3).

3.4.3 Anti-proliferative activity

The anti-proliferative activity of the compounds was determined using the MTT-based cellular viability assay on a K562 leukemic cell line (Section 2.4.4.2). Two of the aminoquinoline derivatives displayed slight anti-proliferative activity when compared to the positive control camptothecin (IC_{50} value: 4.77 ± 0.28 μM). Compound **I** (IC_{50} value: 59.3 ± 13.3 μM) displayed the most activity within the group, but was fifteen fold less potent than camptothecin (Table: 3.3). The compounds did not display any significant anti-proliferative activity compared to camptothecin (p= 0.1).

3.4.4 Selectivity index of compounds against HEK-293 cells and K562 cells

The selectivity index of the compounds was determined as a means of measuring the effect the compounds had on HEK-293 cells non-cancerous cells as compared to K562 cancer cells (Section 2.5). Both compounds **I** and **V** displayed activity against

both cell lines (Table 3.3). In contrast when looking at the selectivity index (SI_{nc}) for the compounds for K562 cancer cells over non-cancerous HEK-293 cells, no selectivity for compounds **I** and **V** were noted with very small SI_{nc} values of 0.40 and 0.36, respectively (Table 3.3).

Table 3.3: Toxicity profile of the aminoquinoline (**I-IV**) and quinolin-8-yl derivatives (**S1-S6**).

Compound	% Haemolytic activity at 50 µM (% ± s.d)	% Cell viability (HEK-293) at 50 µM [IC_{50} ± s.d µM]	% Cell viability (K562) at 50 µM [IC_{50} ± s.d µM]	Safety Index (SI_{pf})	Selectivity index (SI_{nc})
I	1.29 ± 0.67	**8.43 ± 2.11 [23.61 ± 4.31]**	**36.34 ± 19.27 [59.3 ± 13.34]**	**0.94**	0.40
II	3.64 ±1.20	55.39 ± 6.55	62.22 ±15.14	> 1.44**	>1.00*
III	1.01 ± 0.33	52.96 ± 3.57	63.96 ± 7.33	> 0.66**	>1.00*
IV	0.15 ±1.58	51.51 ± 6.36	61.53 ± 12.56	> 0.48**	>1.00*
V	3.67 ± 0.30	**0.10 ± 0.01 [38.18 ± 6.67]**	**44.25 ± 20.62 [105.23 ± 10.01]**	**1.51**	0.36
S1	0.85 ± 0.26	51.44 ± 2.15	50.69 ±12.33	>1.00**	>1.00*
S2	0.85 ± 1.1	51.63 ±0.33	47.82 ±10.35	>1.00**	>1.00*
S3	0.38 ± 0.25	75.43 ± 5.57	52.02 ± 6.68	>1.00**	>1.00*
S4	0.54 ± 0.17	53.88 ±11.74	85.28 ±11.1	>1.00**	>1.00*
S5	0.06 ± 0.24	54.64 ±14.68	52.17 ±14.6	>1.00**	>1.00*
S6	0.67 ± 0.53	52.17 ± 6.87	65.79 ± 15.634	>1.00**	>1.00*
Chloroquine	2.09 ± 0.59	n.d	n.d	n.d	n.d
Quinine	8.22 ± 2.17	n.d	n.d	n.d	n.d
Camptothecin	n.d	21.89 ± 6.62 [0.11 ± 0.01]	22.5 ± 3.28 [4.77 ± 0.28]	n.d	0.02

*Selectivity index calculated using 50 µM for IC_{50} ; ** Safety index calculated using 50 µM for IC_{50}

3.4.5 Anti-oxidant activity

3.4.5.1 DPPH free radical scavenging activity

Of the eleven compounds screened at 50 µM for free radical scavenging activity, two compounds showed potent activity, with the overall free radical scavenging activity of the group varying between 0.1% and 96.45% (Table 3.4). The 8-hydroxyquinoline derivative compound **V**, displayed the most free radical scavenging activity (IC_{50} value: 3.09 ± 0.16 µM) being six fold more potent than the positive control, Trolox™ (IC_{50} value: 19.15 ± 1.14 µM) (Figure 3.6). Compound **III** (IC_{50} value: 6.24 ± 0.26 µM) was three fold more potent than the positive control Trolox™. Compound **V** was two-fold more potent than compound **III** in scavenging free radicals. Compounds **III** and **V** were not significant when compared to that of Trolox™ with p = 0.1.

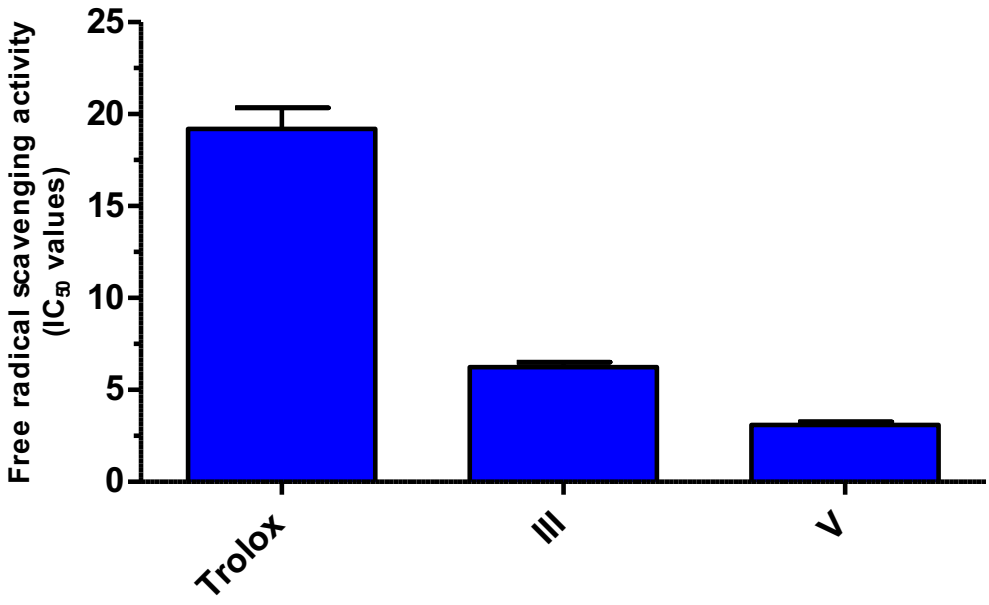


Figure 3.5: The free radical scavenging effect of compound **III** and **V** compared to Trolox™.

Table 3.4: Free radical scavenging activity of the aminoquinoline (**I-IV**) and quinolin-8-yl derivatives (**S1-S6**).

Compound	**% Free radical scavenging activity** (at 50 µM % ± s.d)	**Free radical scavenging activity** (IC_{50}± s.d µM)
I	18.48 ± 3.71	n.d
II	21.37 ± 0.60	n.d
III	96.45 ± 0.29	**6.24 ± 0.26**
IV	6.12 ± 0.90	n.d
V	96.33 ± 0.43	**3.09 ± 0.16**
S1	0.10 ± 0.01	n.d
S2	0.10 ± 0.01	n.d
S3	0.10 ± 0.01	n.d
S4	0.10 ± 0.01	n.d
S5	0.10 ± 0.01	n.d
S6	0.10 ± 0.01	n.d
Trolox™	88.09 ± 10.82	19.15 ± 1.14

3.4.5.2 Lipid peroxidation inhibition activity

Two of the aminoquinoline derivatives displayed lipid peroxidation inhibition activity when screened at 50 µM with the group displaying an average inhibition activity between 0.1% and 86% (Table 3.5). When serially diluted, compound **V** (IC_{50} value: 8.85 ± 1.13 µM) and compound **III** (IC_{50} value: 13.64 ± 1.83 µM) showed promising lipid peroxidation inhibition activity comparable to that of the positive control Trolox™ (IC_{50} value: 5.53 ± 0.54 µM), (Figure 3.6). The compounds did not display any significant activity when compared to Trolox™ with p = 0.1.

Table 3.5: Inhibition of lipid peroxidation activity of the aminoquinoline (**I-IV**) and quinolin-8-yl derivatives (**S1-S6**).

Compound	Inhibition of lipid peroxidation inhibition activity (at 50 µM % ± s.d)	Inhibition of lipid peroxidation inhibition activity (IC_{50}± s.d µM)
I	29.02 ± 11.27	n.d
II	9.05 ± 7.99	n.d
III	71.27 ± 1.8	**13.64 ± 1.83**
IV	33.01 ± 8.23	n.d
V	86.0 ± 2.97	**8.85 ± 1.13**
S1	0.10 ± 0.01	n.d
S2	22.67 ± 1.70	n.d
S3	18.56 ± 5.65	n.d
S4	16.55 ± 8.38	n.d
S5	28.86 ± 5.79	n.d
S6	23.99 ± 9.11	n.d
Trolox™	70.35 ± 2.84	5.53 ± 0.54

3.4.6 Chelating potential

3.4.6.1 Iron chelation activity

None of the compounds screened for iron chelation activity displayed effective iron chelation activity. Compound **V** showed the most activity within the group at an average of 22.38%, when compared to the positive control EDTA averaging at 64.55% (Table: 3.6).

3.4.6.2 Copper chelation activity

Of the 11 compounds screened for copper (I) and (II) chelating potential, compound **V** showed the most activity at 34.02% and 37.07% respectively, although this was

insignificant in comparison to the positive controls Tetrathiomolybdate (TTM) (93.95%) and Triethylenetetramine (TETA) (92.54%)(Table 3.6).

Table 3.6: The Iron and copper chelating potential of the aminoquinoline (**I-IV**) and quinolin-8-yl derivatives (**S1-S6**).

Compound	**% Iron chelation** (200 µM ± s.d)	**% Copper (I) chelation** (200 µM ± s.d)	**% Copper (II) chelation** (200 µM ± s.d)
I	0.10 ± 0.01	0.97 ± 0.12	12.58 ± 2.15
II	0.10 ± 0.01	1.49 ± 0.21	0.1 ± 0.01
III	0.10 ± 0.01	4.55 ± 0.59	7.41 ± 1.09
IV	0.10 ± 0.01	4.50 ± 0.71	9.36 ± 1.22
V	**22.38 ± 4.96**	**34.02 ± 1.41**	**37.07 ± 0.45**
S1	13.59 ±1.77	5.08 ± 0.56	8.02 ± 1.18
S2	21.37 ± 4.29	2.02 ± 0.66	19.45 ± 1.38
S3	0.10 ± 0.01	1.22 ± 0.39	4.6 ± 1.28
S4	20.3 ± 5.73	1.00 ± 0.10	7.52 ± 1.2
S5	18.57 ± 4.64	1.51 ± 0.33	27.9 ± 8.58
S6	17.0 ± 4.81	4.51 ± 0.15	0.10 ± 0.01
EDTA	64.55 ± 8.39	n.d	n.d
TTM	n.d	93.95 ± 0.93	n.d
TETA	n.d	n.d	92.54 ± 0.27

3.5 Discussion

3.5.1 *In vitro* antimalarial activity of the aminoquinoline derivatives

The compounds that displayed antimalarial activity against the 3D7 strain were screened for activity against a chloroquine-resistant FCR-3 strain using the tritiated hypoxanthine incorporation assay (Section 2.3.1) and compared to quinine (IC_{50} value: 0.17 ± 0.03 µM), as well as to the 3D7 strain results (Figure 3.7). Overall the compounds showed more potent activity against the FRC-3 strain than the 3D7 strain, although their activity was not comparable to quinine. Compound **I** showed the

most potent activity within the group against both strains, although four fold more potent against the FCR-3 strain (IC_{50} value: 5.91 ± 1.12 μM) as opposed to the 3D7 strain (IC_{50} value: 25.1 ± 1.3 μM). Compound **I** is a salt derived from 5-aminoquinoline and gallic acid with the latter a benzoic acid derivative which belongs to a large family of secondary metabolites known as polyphenols. Polyphenolic compounds are divided into two major sub groups, flavonoids and phenolic acids that include hydroxycinnamic acids as well as hydroxybenzoic acids. Polyphenols are an important class of natural compounds and have been studied for their potential activity in a variety of chronic diseases, as well as specifically, their potential use in neurodegenerative diseases (Daglia, 2012).

Gallic acid has proven successful in other protozoan diseases such as *Trypanosomacruzi* through the inhibition of cellular respiration (Cortes *et al.*, 2015). Another proposed mechanism in which gallic acid inhibits the formation of *Trypanosomacruzi* is through ROS which may be involved in gallic acid-induced apoptopic cell death (Kayser *et al.*, 2002). Studies have shown that gallic acid and its derivatives display antimalarial activity against a 3D7 strain of *P. falciparum* (Ndjonka et al., 2012). Although the precise mechanism in which gallic acid produces its antimalarial effect has not been not defined, the proposed mechanism of action by which gallic acid inhibits malaria parasite growth is through the increase of RBC oxidation (Jongsakul, 2010).

Compound **II** is a salt derived from 5-aminoquinoline and 2-hydroxybenzoic acid, and although similar to compound **I** in the components used to form the salt, both composed of polyphenolic acids, the use of gallic acid in place of hydroxbenzoic acid improved the antimalarial activity by almost two-fold against both strains (Figure 3.6). Compound **III** is an adduct or co-crystal formed from 8-aminoquinilone and gallic acid and is structurally similar to compound **I** as it is composed of gallic acid. Although structurally similar, their activity varied in that compound **I** (IC_{50} value: 25.1 ± 1.3 μM) was three-fold more potent at inhibiting 3D7 parasite growth than compound **III** (IC_{50} value: 75.4 ± 5.2 μM) (Figure 3.6).

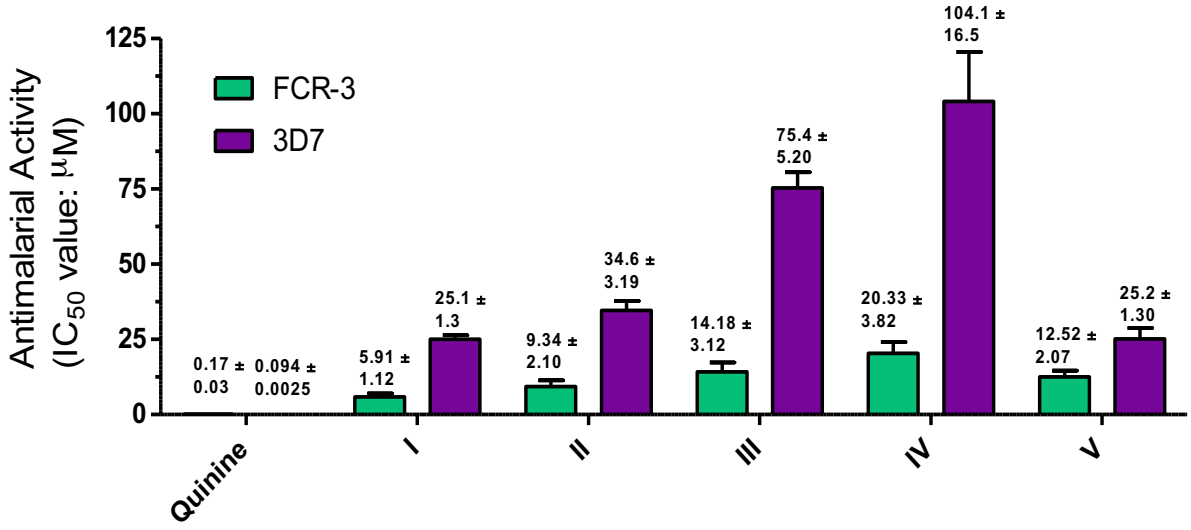


Figure 3.6: *In vitro* antimalarial activity of the aminoquinoline derivatives in comparison to quinine.

Compound **V** is a salt derived from 8-aminoquinoline and an 8-hydroxyquinoline and its antimalarial activity was similar to compound **I** against the 3D7 strain, but not as potent against the FCR-3 strain (Figure 3.7). 8-Hydroxyquinolines are known to produce an antimalarial effect through their ability to chelate iron and essentially starve the malaria of iron and copper, which it needs for life sustaining processes such as DNA replication amongst other things (Van Vuuren and Van Zyl, 2014). Primaquine, an 8-aminoquinoline derivative is used as an antimalarial and the proposed mechanism of action is through the production of highly reactive metabolites, thereby increasing oxidative stress (Vale *et al.*, 2009). Ring-substituted 8-aminoquinoline derivatives have been shown to display potent antimalarial activity (IC_{50}: 39ng/ml) superseding that of chloroquine (Jain *et al.*, 2004). Antimalarial activity studies completed on chloroquine-resistant as well as chloroquine sensitive strains revealed 8-hydroxyquinoline was more effective than chloroquine, by totally inhibiting the parasite growth at 1 µM (Biswas, 2003). Compound **V** is composed of two components, an 8-hydroxyquinoline which has proven to display potent antimalarial activity, and an 8-aminoquinoline used as an antimalarial. What is significant in this regard is the fact that the salt was derived from two compounds that individually have the ability to act as antimalarials, yet the combination of the two in salt form failed to produce a potent antimalarial effect.

When comparing the sensitivity of quinine towards both strains, it displayed a slightly more favourable activity against the 3D7 chloroquine-sensitive strain as opposed to the chloroquine-resistant FCR-3 strain. What is important to note is that although the compounds all contained an aminoquinolne scaffold, similar to that of chloroquine, they were vastly more active against the chloroquine resistant strain as opposed to the chloroquine sensitive strain. One of the main proposed features of chloroquine is its ability to interfere with the formation of haemozoin, this was not a mechanism of action displayed by any of the compounds (Egan *et al.*, 2000) (Table 3.2). The acidic nature of the digestive food vacuole of the parasite provides a pharmacological parameter for the sensitivity of this drug target. Studies have shown that there is a difference in the pH between a chloroquine-sensitive HB3 strain as well as a chloroquine-resistant Dd2 strain, as such the pKa of potential antimalarials with an aminoquinoline scaffold for example would play a role in the degree of efficacy of the compound (Dzekunov *et al.*, 2000). Further studies would need to be conducted on the pharmacological properties of the compounds relating to pH as a means of determining the reason for variation in activity of the compounds against the two different strains.

3.5.1.2 Pro-oxidant as an antimalarial

The meticulously regulated redox equilibrium is needed for stable functioning of the parasite within its host, and the manipulation of this redox state provides a possible target for antimalarial activity (Müller, 2004). Ascorbic acid, a known anti-oxidant in humans is known to exert a pro-oxidant effect on *Plasmodium*-infected RBC's. Pro-oxidant drugs cause the increased production of oxygen radicals inside the infected RBC, as well as to leave the parasite more prone to attack by oxygen radicals (Winter *et al.*, 1997). The manner in which pro-oxidant drugs would produce an antimalarial effect is directed at the parasites' digestive food vacuole. Redox-active iron is generated within the digestive food vacuole as haemoglobin is digested and amino acids are produced for protein synthesis. The pro-oxidant drug would serve to reduce iron to its ferrous state, oxygen to hydrogen peroxide and the resultant ferrous iron would induce the conversion of hydrogen peroxide to highly reactive hydroxyl radicals (Figure 1.2). Thereby causing an increase in oxidative stress on the infected RBC (Jortzik and Becker, 2012).

Primaquine, an 8-aminoquionline derivative is used in the treatment of *P. vivax* infections. The mechanism of action by which it produces its antimalarial effect is through its metabolism producing reactive metabolites that result in the build up of free radicals, hydrogen peroxides and superoxides, which disrupt the redox equilibrium and cause an increase in oxidative stress on the parasite (Waters and Edstein, 2012). Previous studies on 5-aminoquinolines using mice infected with *P. berghei* revealed that the 5-aminoquinoline scaffold is unable to produce an inhibitory effect on malaria parasite growth at the highest dosage level tested, 640 mg/kg (Khan and Lamontagne, 1979). As such one could deduce the role gallic acid would play in the salt, **Compound I**, composed of gallic acid and 5-aminoquinoline in contributing towards the inhibition of parasite growth against both the FCR-3 (IC_{50} value: 5.91 ± 1.12 µM), as well as the 3D7 strain (IC_{50} value: 25.1 ± 1.3 µM) through a proposed pro-oxidant mechanism of action. Plant species composed of gallic acid have displayed antimalarial activity against a K1 strain, with IC_{50} <50µg/ml with a proposed pro-oxidant effect (Jongsakul, 2010). Compound **V** did not display any iron or copper chelating ability (Table 3.6). As such one could deduce that the pro-oxidant effect of the 8-aminoquinoline component within compound **V** could have contributed to its slight antimalarial effect.

The challenge with regards to the use of pro-oxidants in the treatment of malaria is the effect of the pro-oxidant on the human host, and the ability of the pro-oxidant to display specific activity targeted at the infected RBC's as opposed to uninfected RBC's of the host. Haeme is an iron-containing component part of haemoglobin, its hydrophobic nature allows for easy access into cellular membranes and as such it has the ability to compromise vascular cell integrity. The consequences of this have been linked to various pathological conditions (Jeney *et al.*, 2002). Therefore careful study would need to be conducted to ensure the use of the pro-oxidant as an antimalarial would not result in the development of other pathological conditions.

3.5.2 Mechanism of action: Anti-oxidant activity

Compound **V** is a salt derived from 8-hydroxyquinoline and 8-aminoquinoline and displayed stronger free radical scavenging activity than the positive control Trolox™ (Table 3.4). Compound **III** also displayed potent free radical scavenging activity more

potent than Trolox™. The same compounds displayed anti-oxidant activity through the inhibition of lipid peroxidation (Table 3.5). Although both compounds displayed anti-oxidant activity they both expressed this activity through their ability to scavenge free radicals, rather than through the inhibition of lipid peroxidation. When considering the components that form the salt for compound **V**, the 8-hydroxyquinoline nature of the compound would have contributed to its ability to act as an anti-oxidant through the scavenging of free radicals as well as the inhibition of lipid peroxidation.

3.5.2.1 8-Hydroxyquinoline: Anti-oxidant activity

8-Hydroxyquinolines are one of the most significant derivatives of quinoline, and have proven successful in a variety of diseases such as *Mycobacterium tuberculosis*, *Escherichia coli*, as well as displaying antiviral activity and anti-inflammatory activity. Novel 8-hydroxyquinoline derivatives have proven successful as anti-oxidants employed in the treatment of alzheimers disease (Prachayasittikul *et al.*, 2013). 8-Hydroxyquinolines participate in radical scavenging reactions as electron donors of hydroxyl groups to form stable radicals. The anti-oxidant mechanism of action 8-hydroxyquinolines displays is dependent on the stability of the phenoxyl radical formed within in the reaction as well as the positioning of substituents, which may effect the stability and action of the phenoxyl radical. According to studies conducted the manner in which 8-hydroxyquinolines would produce its anti-oxidant effect is through its ability to form stable metal complexes with transition metals such as iron and copper involved in the production of ROS within the body (FernáNdez-Bachiller *et al.*, 2010).

3.5.2.2 Gallic acid: Anti-oxidant and pro-oxidant activity

Gallic acid is a known anti-oxidant in emulsion and lipid systems as well as a free radical scavenger, and has been known to show stronger anti-oxidant activity than several water-soluble anti-oxidants such as ascorbic acid (Yilmaz and Toledo, 2004). The neighbouring positioning of the three hydroxyl groups in the aromatic ring allows this molecule to interfere with the redox equilibrium that involves the formation of quinoid products as a result of their oxidation (Serrano *et al.*, 1998). Although structurally similar in that both compound **I** and compound **III** are composed of a

known anti-oxidant, gallic acid, the adduct formed from gallic acid and 8-aminoquinoline proved a stronger anti-oxidant than the salt it formed with 5-aminoquinoline. Various potent antioxidants such as ascorbic acid, as well as other phenolic compounds have proven to display anti-oxidant as well as pro-oxidant activity under specified conditions (Yen *et al.*, 2002). What is significant in this regard is that gallic acid displayed the ability to act as a pro-oxidant as mentioned previously with regards to its mechanism of action in inhibiting malaria parasite growth, as well as to act as an anti-oxidant.

In this circumstance with regards to the chemical structure of compound **I** and compound **III** one cannot ignore the difference in the manner in which a salt would react during an oxidation reaction as oppose to a co-crystal. During the reaction between an organic acid and organic base the outcome as to whether a salt or co-crystal is formed is determined by the occurrence of a proton transfer form the organic acid to the organic base. If a proton transfer occurs, a salt will be formed, if no proton transfer occurs a co-crystal will be formed. Another means of expressing this is if the proton is found on the base the compound formed is a salt, if the proton is left on the acid the compound formed will be a co-crystal (Childs *et al.*, 2007). In this instance with regards to compound **I** that was categorized as a salt through X-ray single crystal diffraction, a proton was donated from gallic acid to 5-aminoquinoline, and in compound **III** there was no proton transfer from gallic acid to 8-aminoquinoline. When considering the pro-oxidant and anti-oxidant nature of the manner in which gallic acid, it is important to note the formal charge on gallic acid is neutral, or 0. The outcome of a proton transfer occurring in the case of compound **I** would result in the gallic acid having a negative charge and in the case of compound **III** where there was no proton transfer and it would result in the gallic acid having a neutral charge or zero. In the case of free radical scavenging the antioxidant would need to act as a hydrogen donor in order to reduce the radical to a less toxic form (Greeff *et al.*, 2012). As such one could deduce or consider this difference in proton transfer as a contribution towards the difference in mechanism of action with regards to gallic acid in compound **I** acting as a pro-oxidant, and gallic acid in compound **III** behaving as an anti-oxidant.

This proposed mechanism of action assumes the aminoquinoline bound to the gallic acid within the crystalline structure had little effect on the pharmacological

mechanism of action with regards to its pro-oxidant and anti-oxidant nature. The use of a co-crystal structure allows two separate components to combine chemically through non-covalent interactions such as hydrogen bonding, van der Waals interactions as well as π-π stacking. The use of multicomponent systems within pharmacology has gained interest as it provides a means of improving on physiochemical properties of parent compounds such as increased solubility and stability. Although this is true, the physical and chemical properties of the resultant crystal, as well as whether an actual crystal could be formed between two separate entities cannot be predicted and further studies on each co-crystal formed would need to be conducted in order to retrieve the required information (Clements *et al.*, 2015).

3.5.3 Toxicity evaluation

The antimalarial activity of all five of the compounds were directed against the intra-erythrocytic parasite rather than affecting the integrity of the red blood cell of the host as the compounds displayed negligible haemolytic activity. According to the safety index calculated, the compounds were considered toxic.

3.5.3.1 Cytotoxic and anti-proliferative activity

Compound **I** and compound **V** displayed a cytotoxic effect on normal human kidney epithelial cells as well as slight anti-prolifertative activity against a leukemic cell line. Although both compounds had an effect on both cell lines, when comparing the effect on the two cell lines, their effect was slightly more favoured towards the cancer cells as opposed to the normal cells.

Oxidative stress and the production of ROS has been tightly linked to the development of cancer. This is as a result of two possible mechanisms, firstly, the induction of gene mutations as a consequence of cell injury, and secondly the effect of active oxygen on signal transduction and transcription factors. The effect of oxidative damage on previously discussed biological targets could have an effect on tumour suppressor genes such as p53. The manner in which anti-proliferative drugs produce their effect is through the production of a state of oxidative stress within the body, which in turn triggers apoptosis or cell death (Noda and Wakasugi, 2001). As

such these compounds are unable to distinguish between normal cells and cancerous cells, and it is the main reason the use of these compounds result in such damaging side effects.

The pro-oxidant mechanism of action theorized with regards to compound **I** and compound **V** could be applied in this instance as well, as it would explain the effect on both the HEK-293 cell line as well as the K562 cell line. Studies on gallic acids ability to prevent cell proliferation has resulted in various possible mechanisms of action, on different types of cancer cell lines. These include cell death occurring according to DNA fragmentation in a mouse cell lymphoma Wehi 231 cell line, as well as morphological characteristics such as cell shrinkage and chromatin condensation in a mouse fibroblast L929 cell line (Serrano *et al.*, 1998). Although 8-hydroxyquiniolines have proven to display anticancer activity, most of their anticancer activity is attributed to their ability to chelate metal ions such as copper and zinc, the ability to chelate metals was not a feature of Compound V (Prachayasittikul *et al.*, 2013).

3.5.4 Chelating potential

The chelation of copper and iron was not a feature of the quinolin-8-yl derivatives and aminoquinoline derivatives. Compound **V** which is composed of 8-hydroxyquinoline, a known chelator of copper and iron, did not retain its ability to do so when combined with 8-aminoquinoline in a salt form. Although phenolic compounds have the ability to chelate iron, which is closely linked to their anti-oxidant capabilities, compounds **I, II**, and **III** did not retain this ability to chelate iron, or copper (Ebrahimzadeh *et al.*, 2008)

3.6 Summary

- The quinolin-8-yl derivatives did not posses any of the pharmacological properties of their parent compounds and displayed little to no activity in any of the assays conducted.
- The salt and co-crystal formation of the aminoquinoline derivatives resulted in the expression of pharmacological mechanisms of action that could be explained by one of the components making up the salt.

- Compound **I** and compound **III** both composed of gallic acid displayed a pro-oxidant effect in its salt form, and a potent anti-oxidant effect in its co-crystal form, formed with 5-aminoquinoline and 8-aminoquinoline respectively.
- Compound **I** and compound **II** proved that using gallic acid in place of 2-hydroxybenzoic acid when forming the salt with 5-aminoquiniline allowed for slightly improved antimalarial activity.
- Compound **V** displayed antimalarial activity attributed to its 8-aminoquinoline component, and expressed potent anti-oxidant activity as a result of its 8-hydroxyquinoline component.
- With regards to the aminoquinoline derivatives used in the study, the antimalarial activity of the salts and co-crystals formed was not as potent when compared to the parent compounds making up the salt or co-crystal, as such they displayed slight activity, the same could be said for their anticancer activity. This was not the case with regards to the anti-oxidant activity with the compounds displaying more potent activity than the positive control, Trolox™.

CHAPTER 4

Methyl(thiomethyl)aniline and thiomethyl aniline metal bound chelates

4.1 Introduction

Metals play an integral role in a range of biological systems of living organisms. Metal homeostasis is sustained by a meticulously regulated system of uptake, storage and secretion and is maintained with strict limitations. Metal ion transporters assist in maintaining the required levels of the various metal ions in the different cellular compartments. Metals such as copper, cobalt, zinc, iron and manganese take part in various metabolic and signaling pathways in the human body (Tapiero *et al.*, 2003). Copper is present in most living organisms in the forms Cu(I) and Cu(II), with the bivalent form being the most stable oxidative state. Copper is essential to all living organisms as it acts as a co-factor for several proteins and enzymes involved in a variety of biological processes such as respiration, and mechanisms involved in protection from oxidative stress (Bertini and Cavallaro, 2008). Cobalt is a natural earth element that has trace amounts present throughout our environment, with the most common oxidation numbers of +2 or +3. Cobalt is considered an essential element since the body is unable to synthesize it. Cobalt makes up 4% of vitamin B12 that is needed for the development and initial myelination of the central nervous system. Nickel is a metal found in several oxidative states, although the most common in biological systems is the +2 state. Soluble nickel such as nickel carbonyl is fat-soluble and can freely cross membranes via diffusion or through calcium channels, and the estimated daily nickel intake is 35-300 μg (Valko *et al.*, 2005).

Metal complexes have provided a new avenue of approach to treatment regimens in the various fields of medical research such as in the field of cancer where previously there have been obstacles that have hindered progress. The most well known example of the use of metals in cancer research has been the development and use of cisplatin, a platinum coordinated compound, which has proven successful as an antitumour drug (Kostova, 2006). Success in this field has prompted researchers in other fields of disease to consider the use of coordinating metals to standard agents in order to improve the effect of these drugs. In the case of malaria, it has provided

an innovative approach to the obstacle currently faced, which is the development of resistance to standard antimalarials such as chloroquine. Studies have proven the potential of coordination of transition metals to chloroquine to improve the effect of the standard antimalarial and in so doing overcome resistance (Sekhon and Bimal, 2012). The introduction of copper into the formation of alkyl ligands such as hydroxynathoquinone ligands was found to be beneficial to the antimalarial activity against chloroquine-resistant strains of *P. falciparum* (Gokhale *et al.*, 2006). The use of coordinating nickel to an aromatic sulphur containing ligand has proven to be effective against *P. berghei* (Tella and Obaleye, 2011). Cobalt (III) complexes with chloroquine revealed the metal complex was more effective against the K1 strain of *P. falciparum*, as well as less toxic (Ajibade and Kolawole, 2010). When comparing the coordination of nickel or cobalt to the standard antimalarials mefloquine and chloroquine, cobalt proved safer and less toxic than nickel (Adediji *et al.*, 2009) .

The use of these metal complexes in medicine has provided advantageous and more effective methods of synthesizing compounds such as, reliable preparations of stable transition metal complexes with variable structures, the ability to select and modify the ligands affinities according to electron transfer, substitution rates and reduction potential (Biot *et al.*, 2012). Metal complexes have also allowed researchers to explore the biological effects of metals in the organisms being studied in order to develop efficient biological targeting methods (Biot *et al.*, 2012). When focusing specifically on metalloantimalarials, the metal coordination has proven to enhance pharmacological, as well as chemical properties such as potency, selectivity, chemical stability and lipophilicity (Bahl *et al.*, 2010). When developing metal complexes as potential agents it is important to understand that accumulation of metal ions in the body could lead to harmful effects, as such the biodistribution and clearance of these metals from the body would need to be ensured (Kostova, 2006).

Anilines that contain alkylthioalkylated components in their molecular structure have the ability to act as coordinating ligands due to the presence of the nitrogen, thioethersulphur and aniline. The presence of nitrogen and sulphur create a hard borderline and soft nature that permits the formation of stable complexes between them and metal ions and have found application as intermediates in the production of many organic compounds. In many biologically active organic compounds donor groups such as nitrogen, oxygen, sulphur and chlorine are commonly found and used

in pharmaceutical production of these compounds (Olalekan *et al.*, 2014). When developing novel compounds consisting of an aromatic ring and various substituents it is important to note the ability to modify the effect of the compound by varying the positioning of the substituents on the aromatic ring while maintaining the biologically desirable characteristics of the novel compound (Biot *et al.*, 2012).The use of metals coordinated to alkylthioalkylated anilines could prove useful with regards to the obstacles which has hindered malaria eradication, as such novel methyl(thiomethyl)aniline and thiomethyl aniline metal bound chelates were synthesized and chosen to be investigated on.

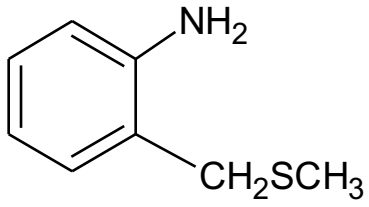
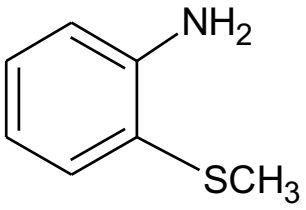
4.2 Objectives

- To assess the *in vitro* properties of the novel methyl(thiomethyl)aniline and thiomethyl aniline metal bound chelates against the asexual stages of the parasite and to determine the resultant effect of combining the most active compounds with quinine using the pLDH assay.
- To determine preliminary toxicological properties of the compounds against human red blood cells and human kidney epithelium.
- To investigate the mechanism of action of the novel compounds by monitoring oxidative stress pathways within the parasite, inhibition of haemozoin formation, iron and copper chelation properties and anti-proliferative activity as a means of determining potential targets to be modulated in the parasite.
- To determine the safety index of the compounds against *P. falciparum*.
- To determine the selectivity Index of compounds against HEK-293 cells and K562 cells.

4.3 Methodology

4.3.1 Chemical synthesis of derivatives

Ligands, 2-(methylthiomethyl)aniline (MT) and 2-methylthioaniline (MA) were synthesized according to the reported procedure (Olalekan *et al.*, 2014), with metal complexes with Cu(II), Co(II) and Ni(II) (Figure 4.1) (Table: 4.1).

Base Compound	
	
2MT: Methylthiomethoxyaniline	**2MA: Thiomethoxyaniline**

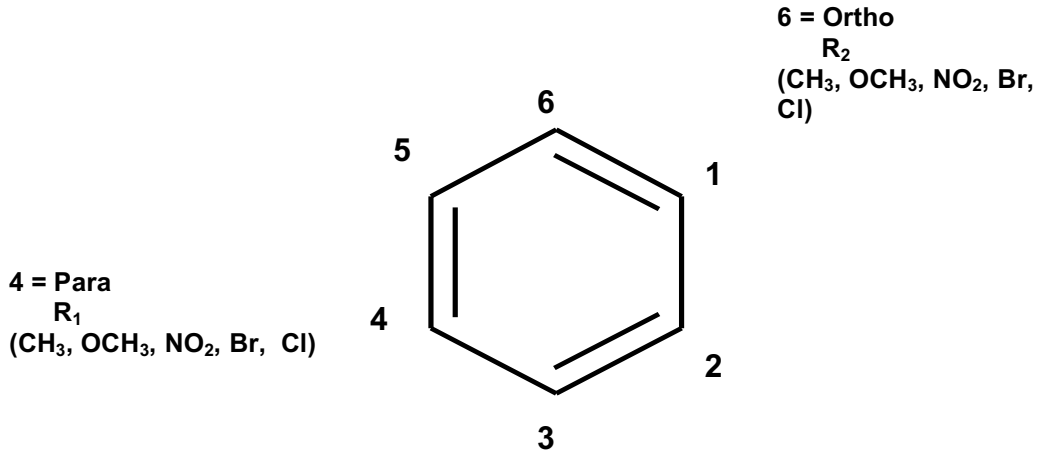


Figure 4.1: The base compound structures for the MT and MA derivatives and numbering system for *ortho* and *para* substituents.

Table 4.1: Methylthiomethoxyaniline and thiomethoxyaniline compounds with bound ligands.

Bound Structures			
Chemical formula	**Bound ligands**	**Chemical formula**	**Bound ligands**
2MT		**2MA**	
Cu2MT	R = Cu	Cu2MA	R = Cu
Co2MT	R = Co	Co2MA	R = Co
pCl2MT	R = Cu R_1= Cl	4Cl2MA	R = Cu R_1= Cl
oCl2MT	R = Cu R_2 = Cl	6Cl2MA	R = Cu R_2 = Cl
CupCl2MT	R = Cu R_1 = Cl	Cu4Cl2MA	R = Cu R_1 = Cl
CuoCl2MT	R = Cu R_2 = Cl	Cu6Cl2MA	R = Cu R_2 = Cl
CopCl2MT	R = Co R_1 = Cl	Co4Cl2MA	R = Co R_1 = Cl
pBr2MT	R_1 = Br	4Br2MA	R_1 = Br
oBr2MT	R_2= Br		
CupBr2MT	R = Cu R_1 = Br	Cu4Br2MA	R = Cu R_1 = Br
CuoBr2MT	R = Cu R_2= Br	Cu6Br2MA	R = Cu R_2 = Br
CopBr2MT	R = Co R_1 = Br		
CooBr2MT	R = Co R_2 = Br		
oNO_2MT	R_2 = NO_2		
Cup$NO_2$2MT	R = Cu R_1 = NO_2		
Cop$NO_2$2MT	R = Co R_1 = NO_2		
pMe2MT	R_1 = CH_3	4Me2MA	R_1 = CH_3
oMe2MT	R_2 = CH_3	6Me2MA	R_2 = CH_3
CopMe2MT	R = Co R_1 = CH_3	Co4Me2MA	R = Co R_1 = CH_3
CooMe2MT	R = Co R_2 = CH_3		
CupMe2MT	R = Cu R_1 = CH_3	Cu4Me2MA	R = Cu R_1 = CH_3
CuoMe2MT	R = Cu R_2 = CH_3	Cu6Me2MA	R = Cu R_2 = CH_3
pMeO2MT	R_1 = OCH_3	4MeO2MA	R_1 = OCH_3
oMeO2MT	R_2 =OCH_3	6MeO2MA	R_2= OCH_3
CopMeO2MT	R = Co R_1 = OCH_3	Co4MeO2MA	R = Co R_1 = OCH_3
CupMeO2MT	R = Cu R_1 = OCH_3	Cu4MeO2MA	R = Cu R_1 = OCH_3
CuoMeO2MT	R= Cu R_2= OCH_3	Cu6MeO2MA	R= Cu R_2= OCH_3
NioMeO2MT	R = Ni R_2= OCH_3	Ni6MeO2MA	R = Ni R_2= OCH_3
NipMeO2MT	R = Ni R_1 = OCH_3	Ni4MeO2MA	R = Ni R_1 = OCH_3
CooMeO2MT	R = Co R_2= OCH_3	Co6MeO2MA	R = Co R_2= OCH_3

4.3.2 Pharmacological evaluations

To achieve the objectives of this chapter, the following assays were undertaken:

1) Antimalarial activity of the compounds were determined using the pLDH assay where compounds were screened at 50 µM. Compounds showing inhibition of >50% were serially diluted and IC_{50} values obtained (Section 2.3).
2) The Inhibition of haemozoin formation of the compounds was determined (Section 2.4.1).
3) The anti-oxidant activity of the compounds was determined using the DPPH free radical scavenging assay, as well as the lipid peroxidation inhibition assay (Section 2.4.2).
4) A toxicity evaluation was determined using the RBC toxicity assay, as well as the MTT-based cellular viability assay (Section 2.4.3).
5) The anti-proliferative activity of the compounds was determined using the MTT based cellular viability assay (Section 2.4.4).
6) The metal chelation potential of the compounds were determined using the iron and copper chelation assays (2.4.5).

4.4 Results

4.4.1 *In vitro* antimalarial activity and β-haematin inhibitory activity

The *Plasmodium* lactate dehydrogenase assay (Section 2.3.2.1) as well as the tritiated hypoxanthine incorporation assay (Section 2.3.1) was used to screen the MT and MA derivatives for antimalarial activity. Of the 55 compounds screened 34 of the compounds displayed antimalarial activity with an IC_{50} value range between 2.66 µM and 57.5 µM when compared to quinine (IC_{50} value: 0.0964 ± 0.002 µM) (Figure 4.2). All of the compounds displayed significant antimalarial activity when compared to quinine ($p < 0.05$) (Figure 4.2). At 50 µM there was a range of parasite growth between 7.08% and 101.44% (Table 4.2). From the MT and MA groups, both methoxy substituted compounds, **CupMeO2MT** (IC_{50} value: 2.66 ±0.12 µM) and **Cu4MeO2MA** (IC_{50} value: 2.82 ± 0.38 µM) displayed the most promising activity by inhibiting parasite growth to a similar degree when compared to quinine (Figure 4.3). The inhibition of haemozoin formation was not a feature of the compounds when screened at 50 µM, with compound **4MeO2MA** displaying slight activity at 20.9% when compared to chloroquine at 92 % (Table 4.2).

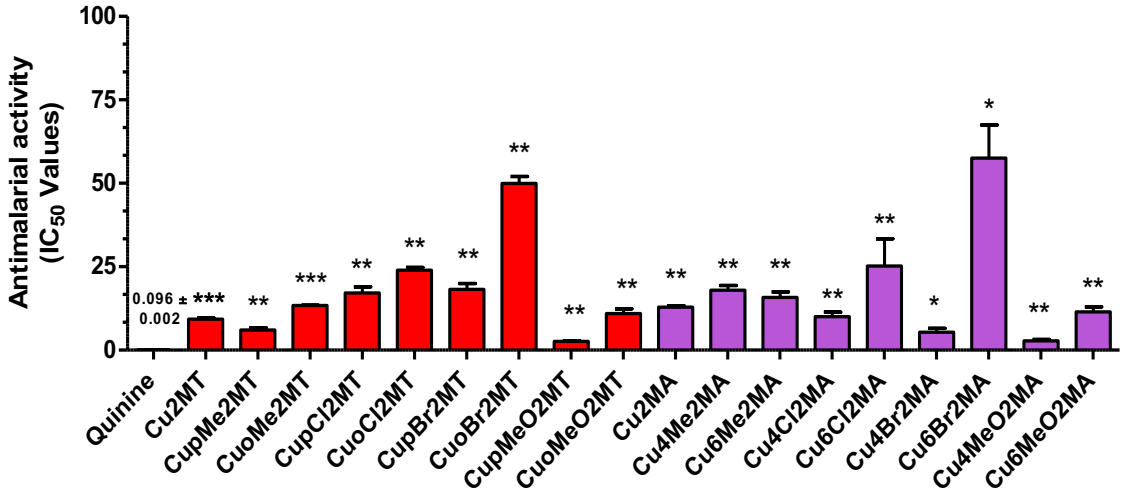


Figure 4.2: The *In vitro* antimalarial activity of the **MA** and **MT** derivatives

* p< 0.05 , ** p< 0.01, *** p< 0.001

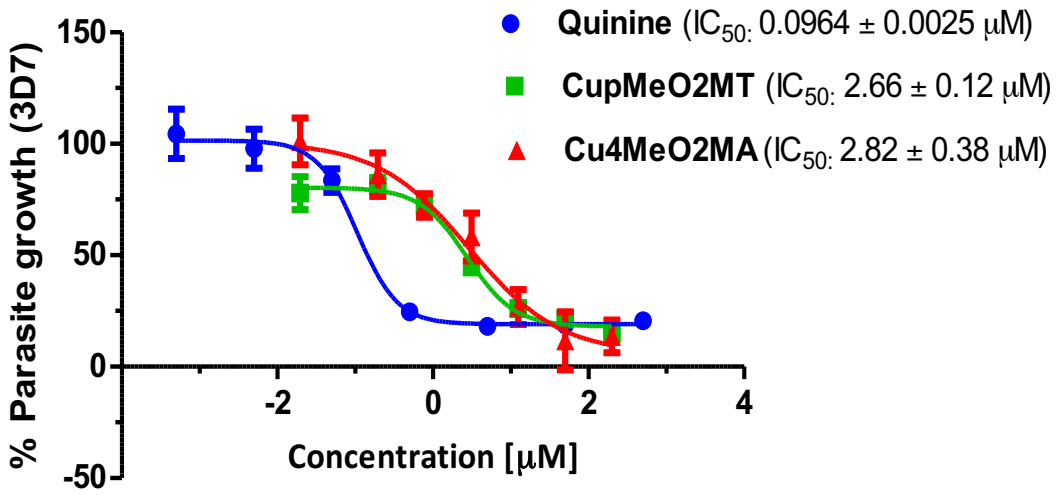


Figure 4.3: Log sigmoid dose response curve of quinine and the two most active compounds from the **MA** and **MT** group.

Table 4.2: Antimalarial activity and β-haematin formation inhibitory activity of the **MT** and **MA** derivatives.

Compound	**Antimalarial Activity 3D7** (% Parasite growth at 50 μM ± s.d)	**Antimalarial Activity 3D7 strain** (IC_{50}: μM ± s.d)	**β-Haematin Inhibition** (% Inhibition at 50μM ± s.d)
2MT	97.62 ± 6.16	n.d	0.10 ± 0.01
Cu2MT	24.0 ± 5.14	9.26 ± 0.33	0.10 ± 0.01
Co2MT	n.d	n.d	0.10 ± 0.01
pCl2MT	101.44 ± 7.87	n.d	0.10 ± 0.01
CupCl2MT	25.51 ± 4.99	17.17 ± 1.83	0.10 ± 0.01
CuoCl2MT	24.52 ± 4.23	23.92 ± 0.89	0.10 ± 0.01
CopCl2MT	n.d	n.d	0.10 ± 0.01
pBr2MT	101.38 ± 4.26	n.d	13.74 ± 3.63
CupBr2MT	33.93 ± 1.89	18.22 ± 1.75	n.d
CuoBr2MT	24.99 ± 7.48	49.99 ± 2.04	0.10 ± 0.01
CopBr2MT	n.d	n.d	0.10 ± 0.01
CooBr2MT	n.d	n.d	0.10 ± 0.01
oNO_2MT	n.d	n.d	0.10 ± 0.01
$CupNO_22MT$	n.d	n.d	0.10 ± 0.01
$CopNO_22MT$	n.d	n.d	0.10 ± 0.01
pMe2MT	87.25 ±12.45	n.d	8.35 ± 3.33
oMe2MT	89.31 ± 4.95	n.d	0.10 ± 0.01
CopMe2MT	n.d	n.d	0.10 ± 0.01
CooMe2MT	n.d	n.d	0.10 ± 0.01
CupMe2MT	21.5 ± 4.92	6.02 ± 0.66	0.10 ± 0.01
CuoMe2MT	20.5 ± 9.16	13.4 ± 0.05	0.10 ± 0.01
pMeO2MT	88.08 ± 8.90	n.d	0.10 ± 0.01
oMeO2MT	89.31 ± 4.95	n.d	0.10 ± 0.01
CopMeO2MT	n.d	n.d	n.d
CupMeO2MT	19.48 ± 6.41	**2.66 ± 0.12**	6.75 ± 0.58
CuoMeO2MT	7.08 ± 0.91	10.92 ± 1.46	0.10 ± 0.01
NioMeO2MT	n.d	n.d	0.10 ± 0.01
NipMeO2MT	n.d	n.d	0.10 ± 0.01
CooMeO2MT	n.d	n.d	11.56 ± 3.32
2MA	97.69 ± 10.42	n.d	4.08 ± 0.88
Cu2MA	12.39 ± 4.23	12.9 ± 0.39	13.57 ± 4.05
Co2MA	n.d	n.d	0.10 ± 0.01
4Cl2MA	94.52 ± 11.95	n.d	7.03 ± 2.46
6Cl2MA	96.14 ± 9.32	n.d	13.18 ± 3.46
Cu4Cl2MA	22.51 ± 7.19	10.07± 1.29	9.12 ± 2.55
Cu6Cl2MA	34.97± 9.17	25.19 ± 8.15	0.10 ± 0.01
Co4Cl2MA	n.d	n.d	0.10 ± 0.01
4Br2MA	96.81 ± 10.54	n.d	7.66 ± 1.08
Cu4Br2MA	37.47 ± 3.23	5.35 ± 1.20	0.10 ± 0.01
Cu6Br2MA	41.71 ± 7.79	57.5 ± 9.93	0.10 ± 0.01
4Me2MA	97.15 ± 9.93	n.d	7.02 ± 2.08
6Me2MA	98.09 ± 9.14	n.d	16.31± 5.4
Co4Me2MA	n.d	n.d	0.10 ± 0.01
Cu4Me2MA	21.29 ± 4.01	18.0 ± 1.40	14.67±1.51
Cu6Me2MA	18.53 ± 2.31	15.79 ± 1.65	0.10 ± 0.01
4MeO2MA	84.37 ± 15.33	n.d	**20.99 ± 3.22**
6MeO2MA	81.82 ± 11.01	n.d	14.02 ± 4.30
Co4MeO2MA	n.d	n.d	7.48 ± 4.24
Cu4MeO2MA	19.27 ± 4.55	**2.82 ± 0.38**	0.10 ± 0.01
Cu6MeO2MA	24.44± 3.57	11.44 ± 1.53	0.10 ± 0.01
Ni6MeO2MA	n.d	n.d	9.2 ± 6.77
Ni4MeO2MA	n.d	n.d	0.10 ± 0.01
Co6MeO2MA	n.d	n.d	9.13 ± 4.39
Quinine	19.5 ± 0.16	0.0964 ± 0.002	n.d
Chloroquine	14.4 ± 0.06	0.0321 ± 0.003	92.0 ± 1.87

n.d: not determined

4.4.2 Combination studies

Antimalarial combination studies were conducted on the two compounds in the group that displayed the most promising activity, those being compounds **CupMeO2MT** and **Cu4MeO2MA**, with the standard antimalarial quinine. The combination of quinine and **CupMeO2MT** yielded a ΣFIC_{50} = 1.56 which is indicative of an antagonistic interaction (Figure 4.4). The combination of quinine and **Cu4MeO2MA** also yielded an antagonistic interaction with a ΣFIC_{50} = 1.38 (Figure 4.5).

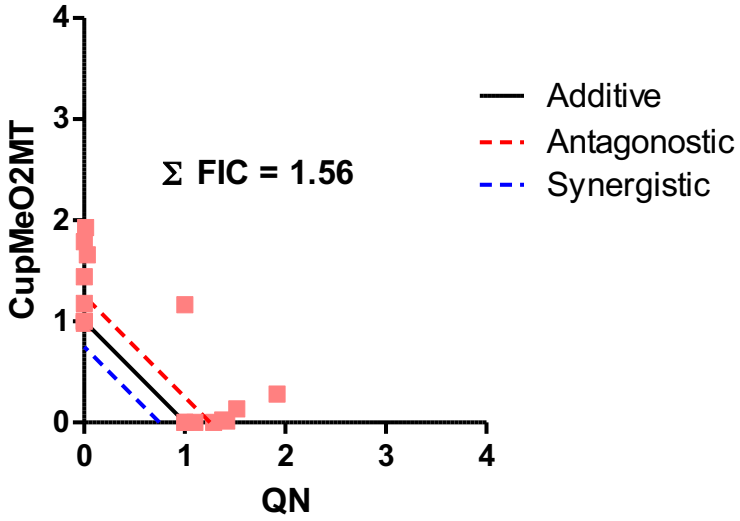


Figure 4.4: The isobologram of **CupMeO2MT** combined with quinine.

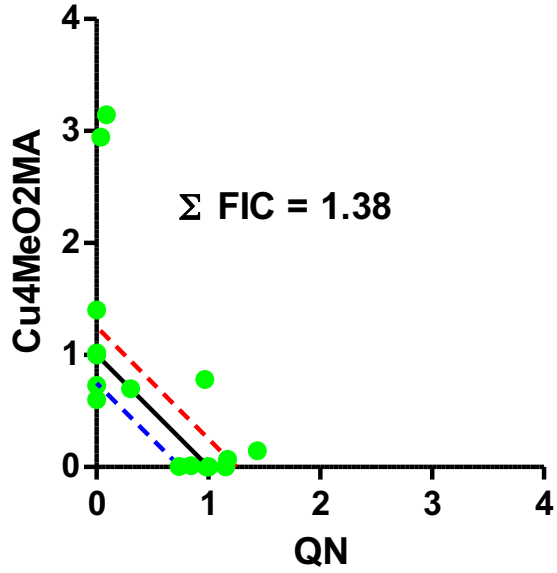


Figure 4.5: The isobologram of Cu4MeO2MA combined with quinine.

4.4.3 Toxicity evaluation

4.4.3.1 Red blood cell toxicity

The compounds were screened for haemolytic activity at a concentration of 50 µM and compared to the standard antimalarial chloroquine displaying activity of 2.09%. The compounds displayed negligible activity with a haemolytic activity range between 0.10% and 22.75% (Appendix C: Table C1;)

4.4.3.2 Cytotoxic effects against HEK-293

Of the total compounds screened for toxicity on non-cancerous cells from both the MT and MA groups, five MT derivatives and four MA derivatives displayed an effect on cell viability and resulted in a % cell viability of <50%. **CupMe2MT** (IC_{50} value: 11.23 ± 2.86 µM) was identified as the most toxic compound from the MT group, and **Cu4MeO2MA** (IC_{50} value: 46.97 ± 10.32µM) was the most toxic compound to non-cancerous cells in the MA group when compared to camptothecin (IC_{50} value: 0.11 ± 0.01µM) (Appendix C; Table C1,C2) Overall when comparing the two groups the MT derivatives were found to be more toxic than the MA derivatives (Figure 4.6). **CupMe2MT** was found to be four times more toxic than **Cu4MeO2MA** and 100 times less toxic to normal cells than camptothecin. The compounds did not display any statistically significant activity when compared to camptothecin ($p = 0.1$).

4.4.3.3 Safety index of compounds against *P. falciparum*

The safety index (SI_{pf}) of the compounds was determined by comparing the antimalarial activity of the compounds against their cytotoxic activity using the HEK-293 cell line (Section 2.4.3.7). A safety index of >10 was considered non-toxic to the human host. Of the 18 compounds which showed antimalarial activity, **CupMeO2MT** and **Cu4MeO2MA** revealed a safety index of >10 using the IC_{50} values obtained and were therefore considered non-toxic to human cells, with **Cu4MeO2MA** being less toxic than **CupMeO2MT** (Table 4.3). For the compounds that did not show a significant effect on the HEK-293 cell line at 50 µM their safety index could only be estimated. These compounds were therefore assumed not to have a toxic effect on human cells.

Table 4.3: Safety index of the MT and MA derivatives.

Compound	Safety Index (SI_{pf})
Cu2MT	1.62
CupMe2MT	1.72
CuoMe2MT	1.53
CupCl2MT	>2.91
CuoCl2MT	>2.19
CupBr2MT	>2.75
CuoBr2MT	>1.00
CupMeO2MT	**10.6**
CuoMeO2MT	4.24
Cu2MA	5.45
Cu4Me2MA	>2.79
Cu6Me2MA	4.58
Cu4Cl2MA	>4,95
Cu6Cl2MA	5.14
Cu4Br2MA	>9.35
Cu6Br2MA	1.55
Cu4MeO2MA	**18.3**
Cu6MeO2MA	7.31

4.4.4 Anti-proliferative activity

When screened for anti-proliferative activity on a leukemic K562 cell line, five MT derivatives and two MA derivatives displayed activity when compared to camptothecin (IC_{50} value: 4.8 ± 0.3μM) (Appendix C: Table C2) (Figure: 4.6). **CupMeO2MT** (IC_{50} value: 12.25 ± 1.97 μM) was found to be the most active in the MT group, although three fold less potent than camptothecin. **Cu2MA** (IC_{50} value: 24.07 ± 5.56 μM) displayed the most activity in the MA group, although it was negligible in comparison to camptothecin, as well as **CupMeO2MT**. The compounds did not display any statistically significant anti-proliferative activity when compared to camptothecin ($p \geq 0.1$).

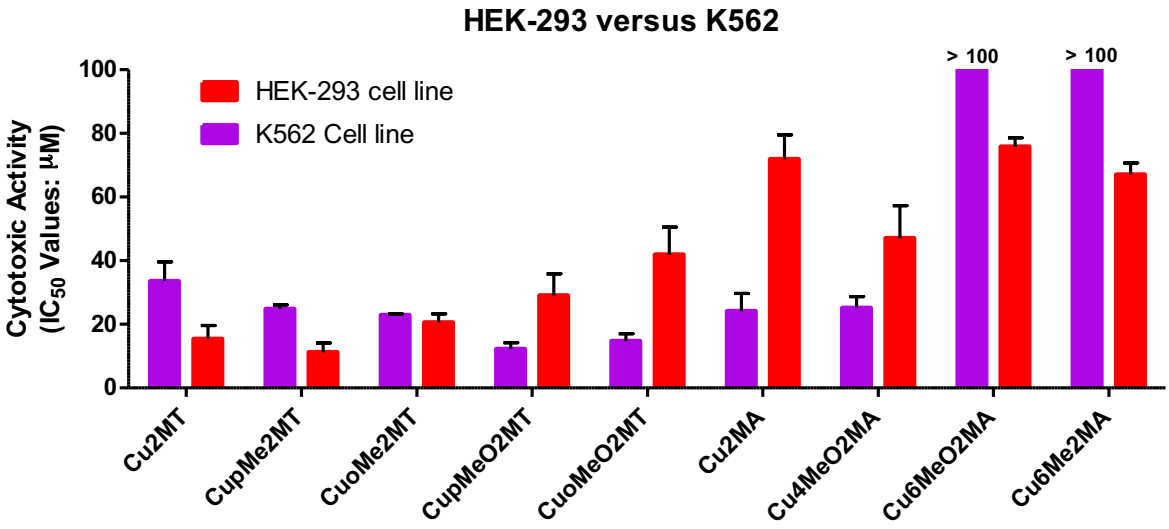


Figure 4.6: Effect on cellular viability of the MT and MA derivatives on the HEK-293 versus K562 cell line.

4.4.5 Selectivity index of compounds comparing HEK-293 cells and K562 cells

The selectivity index of the compounds was determined as a means of measuring the compounds effect on normalized kidney cells as opposed to leukemic cells (Table 4.4) (Section 2.5). Compounds from both the MA and MT groups displayed activity against both cell lines, with the exception of **Cu6Me2MA** and **Cu6MeO2MA** only displaying activity on the HEK-293 cell line. There was no correlation between the activity of the compounds on the two different cell lines with an r^2= 0.038 (Figure 4.7) (Table 4.5).

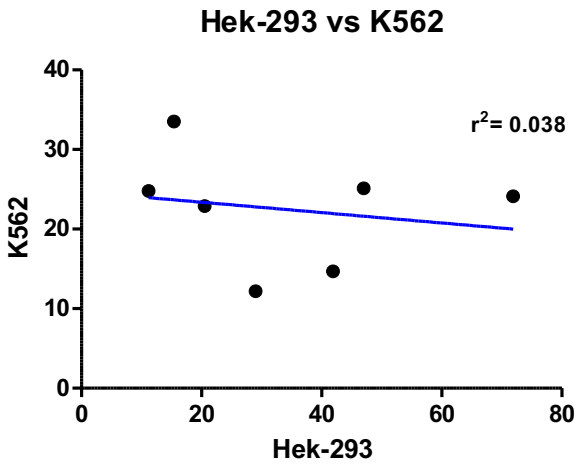


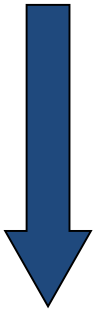
Figure 4.7: The linear regression line displaying the correlation between the HEK-293 cell line vs the K562 cell line.

Table 4.4: Selectivity Index: **HEK-293 versus K562 (Non-cancerous vs Cancer)**

Compound	Selectivity Index (IC_{50})
Cu2MT	0.46
CupMe2MT	0.45
CuoMe2MT	0.90
CupMeO2MT	2.38
CuoMeO2MT	2.85
Cu2MA	2.98
Cu6Me2MA	**>1.34**
Cu4MeO2MA	1.87
Cu6MeO2MA	**>1.52**

Table 4.5: Decreasing activity of the MT and MA derivatives on HEK-293 cells and K562 cells.

HEK-293 cell line		K562 cell line	
CupMe2MT	(IC_{50} value: 11.2 ± 2.9μM)	CupMeO2MT	(IC_{50} value: 12.2 ± 2.0μM)
Cu2MT	(IC_{50} value: 15.4 ± 4.2μM)	CuoMeO2MT	(IC_{50} value: 14.7 ± 2.3μM)
CuoMe2MT	IC_{50} value: 20.5 ± 2.8 μM)	CuoMe2MT	(IC_{50} value: 22.9 ± 0.4μM)
CupMeO2MT	(IC_{50} value: 29.0 ± 6.9μM)	Cu2MA	(IC_{50} value: 24.1 ± 5.6μM)
CuoMeO2MT	(IC_{50} value: 41.9 ± 8.6 μM)	CupMe2MT	(IC_{50} value: 24.8 ± 1.3μM)
Cu4MeO2MA	(IC_{50} value:47.0± 10.3μM)	Cu4MeO2MA	(IC_{50} value: 25.1 ± 3.6 μM)
Cu6Me2MA	(IC_{50} value: 67.0 ± 3.7 μM)	Cu2MT	(IC_{50} value: 33.5 ± 6.1μM)
Cu2MA	(IC_{50} value: 71.9 ± 7.6 μM)	Cu6Me2MA	(IC_{50} value: >100 μM)
Cu6MeO2MA	(IC_{50} value: 75.8 ± 2.8 μM)	Cu6MeO2MA	(IC_{50} value: >100 μM)



4.4.6 Anti-oxidant activity

4.4.6.1 DPPH free radical scavenging activity

The compounds were screened for free radical scavenging activity at 50 μM and a percentage free radical scavenging activity >60% warranted further studies and IC_{50} values were determined. There was a range of activity between 0.1% and 71.06% at

50 µM. Of the 53 compounds screened, four compounds all from the MA group of derivatives showed favourable activity when compared to the positive control Trolox™ (IC_{50} value: 19.15 ± 1.14 µM) (Appendix C: Table C4). **Ni4MeO2MA** (IC_{50} value: 9.63 ± 0.69 µM) was two-fold more potent than Trolox™ (Figure 4.8) at scavenging free radicals. There was no statistically significant activity comparable between the compounds and the positive control ($p \geq 0.1$).

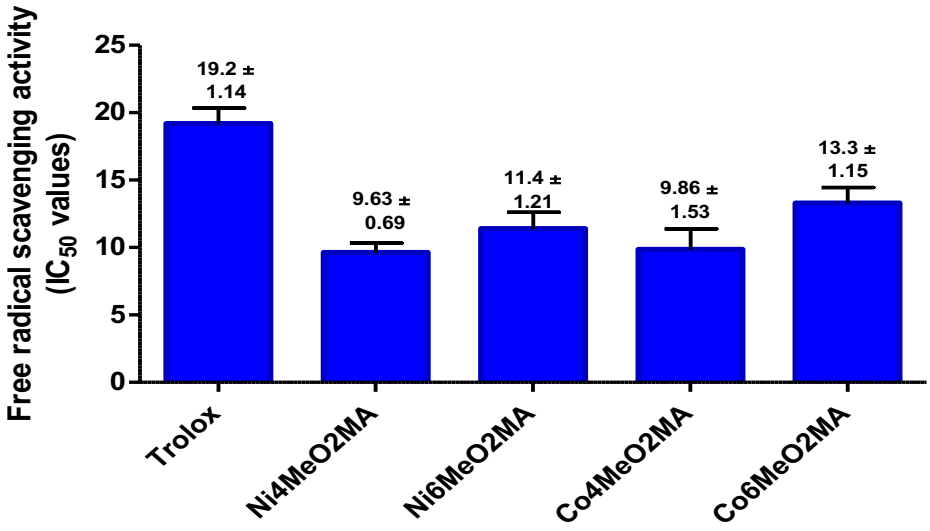


Figure 4.8: The free radical scavenging effect of the most active compounds in the group.

4.4.6.2 Lipid peroxidation inhibition activity

The lipid peroxidation inhibition activity of the compounds was determined at a concentration of 50 µM with an activity range between 0.10 % and 87.7% when compared to the positive control Trolox™ (IC_{50} value: 5.53 ± 0.54 µM) (Appendix C: Table C5). Of the 49 compounds screened, 37% of them displayed promising activity with **CupMeO2MT** (IC_{50} value: 5.57 ± 0.41µM) displaying the most favourable activity of the MT group and **Cu4Cl2MA** (IC_{50} value: 6.57 ± 1.71µM) displaying the most favourable activity of the MA group (Figure 4.9). When compared to the positive control, the compounds did not display any significant activity ($p \geq 0.1$). When the compounds were compared to each other, **CuoCl2MT** was found to display significant activity compared to **CuoBr2MT** with a $p = 0.0007$.

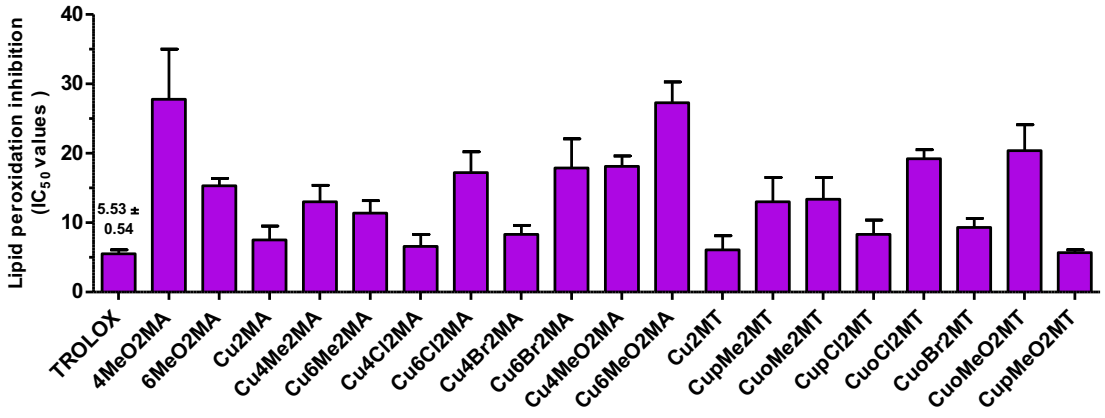


Figure 4.9: The lipid peroxidation inhibition activity of the MT and MA derivatives.

4.4.7 Chelating potential

4.4.7.1 Iron chelation activity

The base MT and MA compounds as well as ligands that were not bound to copper were selected to complete iron chelation activity studies. Of the 18 compounds examined none of them displayed favourable iron chelation activity with a range of activity between 4.5% and 18.38% when compared to the positive control EDTA which averaged at 79.29 % at 50 μM (Table: 4.6).

4.4.7.2 Copper chelation activity

The same MT and MA derivatives used for the iron chelation studies were selected to complete copper chelation studies on by examining their ability to chelate copper I and copper II. Of the compounds chosen to examine, none displayed promising copper chelation activity (Table 4.6). There was a range of copper I chelation activity between 0.1% and 4.04% when compared to TTM which averaged at 93.95% at 200 μM. For copper II there was a range of activity between 0.1% and 6.87% at 200 μM when compared to TETA, which averaged at 92.54%.

Table 4.6: The Iron and copper chelating potential of the MT and MA derivatives

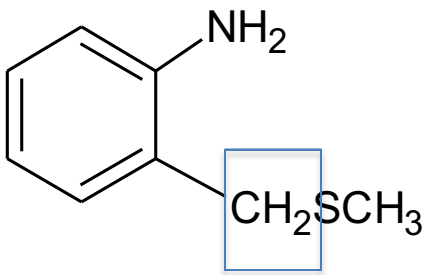
Compound	% Iron chelation (50 µM ± s.d)	% Copper (I) chelation (200 µM ± s.d)	% Copper (II) chelation (200 µM ± s.d)
2MT	14.77 ± 2.05	2.74 ± 0.47	1.94 ± 0.5
pMe2MT	16.57 ± 6.29	2.70 ± 0.12	**6.87 ± 0.41**
oMe2MT	**18.38 ± 4.64**	1.43 ± 0.29	1.59 ± 0.14
pMeO2MT	13.2 ± 2.85	2.29 ± 0.29	3.13 ± 0.37
OMeO2MT	18.09 ± 4.29	1.44 ± 0.17	3.48 ± 0.29
pCl2MT	14.1 ± 2.85	2.61 ± 0.14	1.63 ± 0.39
oCl2MT	13.78 ± 2.53	3.20 ± 0.48	2.90 ± 0.68
pBr2MT	14.88 ± 2.36	**4.04 ± 0.70**	3.28 ± 0.36
oBr2MT	12.09 ± 2.29	n.d	n.d
o$NO_2$2MT	13.42 ± 0.76	1.45 ± 0.34	2.52 ± 0.69
2MA	12.44 ± 1.82	1.35 ± 0.25	1.00 ± 0.13
4Me2MA	7.9 ± 3.72	0.48 ± 0.13	0.10 ± 0.01
6Me2MA	4.5 ± 0.57	0.10 ± 0.01	0.10 ± 0.01
4MeO2MA	15.17± 1.48	0.10 ± 0.01	1.51 ± 0.44
6MeO2MA	9.78 ± 3.5	0.10 ± 0.01	0.10 ± 0.01
4Cl2MA	16.75 ± 4.32	0.10 ± 0.01	0.10 ± 0.01
6Cl2MA	9.94 ± 3.06	2.03 ± 0.23	4.08 ± 0.86
4Br2MA	15.42 ± 2.4	0.54 ± 0.18	
EDTA	79.29 ± 17.85	n.d	n.d
TTM	n.d	93.95 ± 0.93	n.d
TETA	n.d	n.d	92.54 ± 0.27

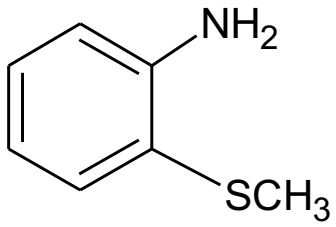
4.5 Discussion

4.5.1 *In vitro* antimalarial activity

When examining the structural differences between the **MT** and **MA** groups, it is important to note the only variation between the base compounds of two groups is the methylene group (CH_2), attached to the aromatic ring, resulting in differences

between the molecular weights and sizes of the compounds as well as differences in lipophilicity of the compounds (Figure 4.10).



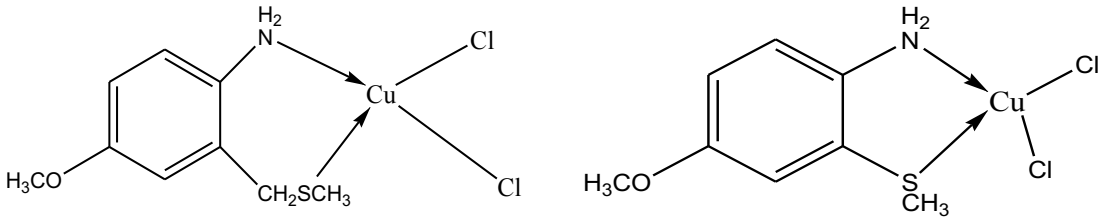


MT: 2-(methylthiomethyl)aniline **2MA: 2-methylthioaniline**

Figure 4.10: Base compounds for the **MT** and **MA** groups.

When examining the antimalarial activity of the compounds what is important to note is the chelation of copper into the base ligands for both the **MT** and **MA** groups resulted in the enhanced antimalarial activity in all instances. For example at 50 μM the base compound **2MT** displayed 97.62% parasite growth, and the chelation of copper into the base compound resulted in a substantial decrease in parasite growth to 24% (Table 4.2). When comparing the activity of the compounds between the two groups, differences in activity where seen in compounds chelated to copper and substituted with a methylene group (CH_3) at the *para* position, for example, **CupMe2MT** (IC_{50} value: 6.02 ± 0.66 μM) and **Cu4Me2MA** (IC_{50} value: 18.0 ± 1.40 μM) (Table 4.2; Figure 4.2).

Compounds **CupMeO2MT** (IC_{50} value: 2.66 ± 0.12 μM) and **Cu4MeO2MA** (IC_{50} value 2.82 ± 0.38 μM) displayed the most potent antimalarial activity (Table 4.2). These compounds are structurally very similar in that they are both substituted with a methoxy group (OCH_3) at the *ortho* position. The only structural difference between the two compounds is the methylene group attached to the aromatic ring in the **MT** group (Figure 4.11).



CupMeO2MT **Cu4MeO2MA**

Figure 4.11: Structure of CupMeO2MT and Cu4MeO2MA (Beukes, personal communication).

The **MT** and **MA** derivatives that displayed antimalarial activity against the chloroquine-sensitive 3D7 strain were assessed for activity against a chloroquine-resistant FCR-3 strain using the titriated hypoxanthine incorporation assay (Prof R L van Zyl personal communication) (Figures 4.10, 4.11). When comparing the overall activity of the two groups of derivatives to the two different strains, the majority of compounds displayed slightly more favourable antimalarial activity against the 3D7 strain, this slight difference in activity was also displayed by quinine (Figures 4.12, 4.13).

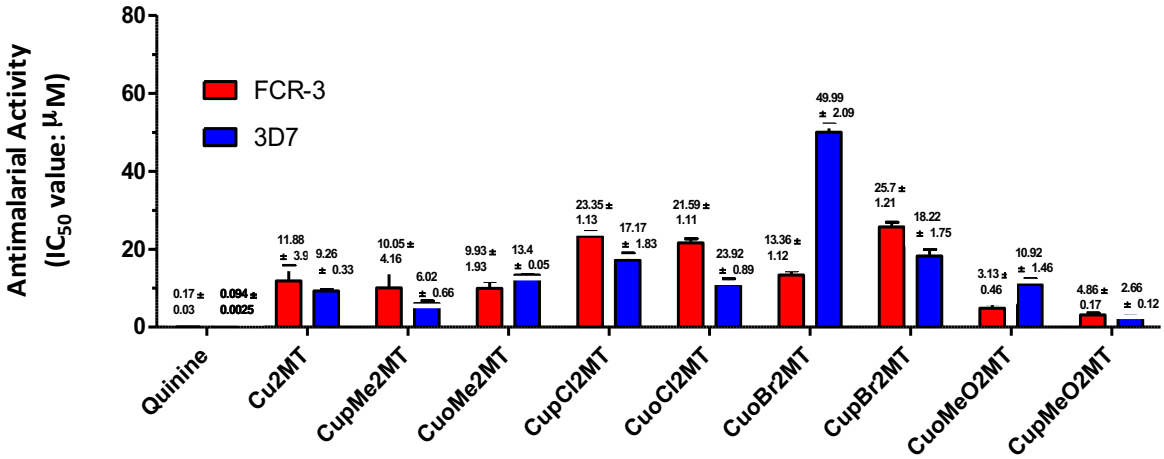


Figure 4.12: The comparison of the antimalarial activity of the (methylthiomethyl)aniline (**MT**) against a chloroquine-sensitive (3D7) and chloroquine-resistant (FCR3) strains.

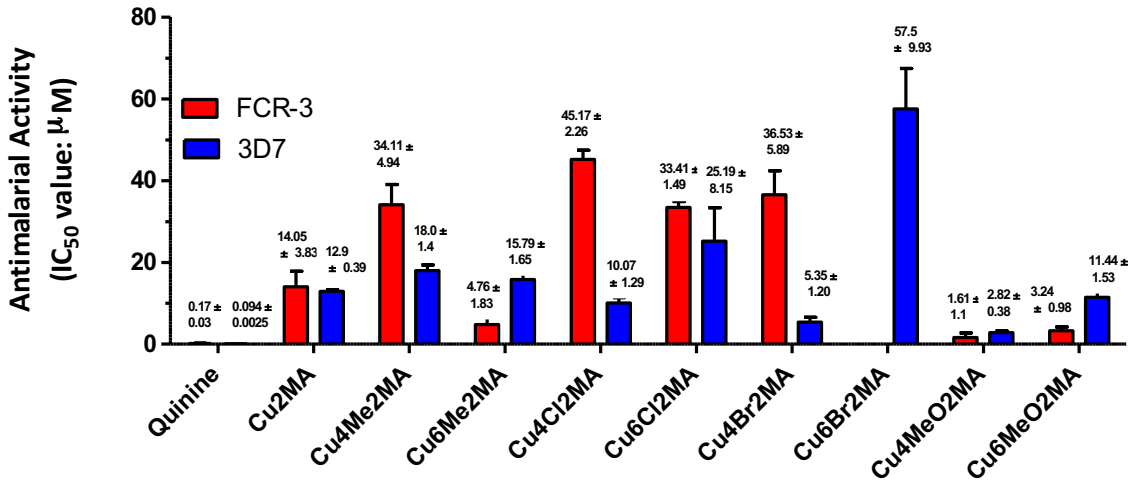


Figure 4.13: The comparison of the antimalarial activity of the methylthioaniline **(MA)** derivatives derivatives against a chloroquine-sensitive (3D7) and chloroquine-resistant (FCR3) strains.

Although the majority of compounds favoured the 3D7 strain, the most active compound, **Cu4MeO2MA**, displayed more potent activity against the FCR-3 strain. For the majority of compounds in the **MT** group, the activity was similar against both strains; but for the MA group there were apparent differences in the activity between the FCR-3 and 3D7 strain. (Figure 4.12; 4.13). When comparing the activity between the two groups against the two different strains, **CupMeO2MT** (IC_{50} value: 2.66 ± 0.12 μM) was the most active against the 3D7, whereas **Cu4MeO2MA** (IC_{50} value: 1.61 ± 1.1 μM) was the most active against the FCR-3 strain.

The compounds displayed similar activity against the FCR-3 strain regardless of whether substituents were bound at the *ortho* or *para* position, with the exception of the bromine substituent. For the FCR-3 strain the bromine substituent at the *ortho* position improved antimalarial activity by two-fold and for the 3D7 strain although the activity was moderate the *para* position improved the anti malarial activity three-fold.

4.5.1.1 Copper for therapeutic use

Trace metals such as copper have a close link to the survival of the malaria parasite, as studies have identified six copper-requiring protein orthologs needed for transport

mechanisms in the malaria parasite (Choveaux and Goldring, 2010). Due to the necessity of copper in the body of the host, the role it plays in disease states has been studied extensively (Tapiero *et al.*, 2003). The build-up of copper and disruption in copper homeostasis has been linked to the development and aggregation of Aβ peptide, which leads to amyloid formation and the progression of Alzheimers disease. The use of copper chelators has proven successful in this regard (Sabayan *et al.*, 2010). In addition, the use of a copper complex, Cu(II)-gamma-polyglutamic acid has been shown to mimic the action of insulin for potential use in diabetic treatment (Karmaker *et al.*, 2007).

Although these trace metals are essential for essential life sustaining processes to take place, their presence in excess in the body also allows them to participate in reactions that do not always serve a beneficial purpose to the human host. Copper is present biologically in two distinct oxidation states, either the oxidized state Cu(I) or the reduced stated Cu(II) metals (Jiang *et al.*, 2005). The intricate balance between copper necessity and toxicity is governed by homeostasis transport mechanisms, compartmentalization and binding capabilities at tissue and cell sites where they are needed. The ability of this metal to undergo redox cycling between the two oxidative states allows it to participate in redox reactions, and they are classified as redox active metals. As a result of its redox potential, copper in excess of the needs of cellular functioning participates in the production of ROS and in so doing assists in the direct oxidation of lipids, proteins and DNA (Valko *et al.*, 2005).

One of the proposed mechanisms of actions by which copper integrated into a napthoquinone derivative is through the production ROS resulting in an increase of oxidative stress placed on the parasite, resulting in an inhibition of growth (Gokhale *et al.*, 2006). The ability of transition metals to exist in different spin states permits the increase in the rate of biomolecule oxidation, and their ability to bind to dioxygen in proteins allows a link between the biomolecule being introduced and the dioxygen contained in the protein. In so doing, it has the ability to produce oxidative damage at a particular site (Buettner and Jurkiewicz, 1996). Copper complexes of pyridine-2-carboxamidraxones enhanced malarial activity of the parent compounds. One such proposed mechanism was the positive reduction potentials of Cu(I) which has the ability to activate intracellular oxygen, thereby resulting in ROS and inhibiting parasite growth (Gokhale *et al.*, 2003).

The compounds from both the **MA** and **MT** groups did not display an inhibitory effect on β-haematin formation (Table 4.2; Section 4.4.1), nor were they able to chelate iron and copper (Table 4.6; Section 4.4.7). It can therefore be proposed that these were not the mechanism of action by which the compounds produced their antimalarial effect. However, it could be proposed that the mechanism of action by which the copper bound **MT** and **MA** derivatives induced their antimalarial activity, was through their behaviour as a pro-oxidant.

The benzene ring is composed of localized and de-localized electrons, and has the ability to act as an electron withdrawing group, thereby contributing to the ability of **MA** and **MT** derivatives to act as a pro-oxidant (Hawley *et al.*, 1996). The incorporation of copper, could possibly contribute to the redox potential of the compound as it is a redox active metal, which was evident from the improved antimalarial activity improved when copper was bound to the base **MA** and **MT** compounds. Both compounds, **CupMeO2MT** (IC_{50} value: 2.66 ± 0.12 μM) and **Cu4MeO2MA** (IC_{50} value: 1.61 ± 1.1 μM) that displayed the most potent antimalarial activity were substituted with a methoxy OCH_3 groups at the *para* position (Table 4.1, Figure 4.3). Oxidation may be explained in terms of the loss of electrons, or through the gaining of oxygen (Brown and Lemay Jr, 2000). The methoxy functional group (OCH_3) is comprised of an oxygen atom which biologically may be unable to participate in electron donation, but through the incorporation of a transition metal it has the ability to participate in redox reactions (Aust *et al.*, 1985).

4.5.1.2 Substituent effect on pKa

With regards to the **MT** and **MA** derivatives, not only did the incorporation of metal into the scaffold improve activity but substituents placed on the benzene ring could have contributed to the physicochemical properties of the compound, and therefore have an effect on the activity on the compound.

The acidic digestive vacuole of the malaria parasite is a complex organelle optimized for the purpose of haemoglobin proteolysis, peptide transport, haematin polymerization and the detoxification of oxygen radicals (Banerjee and Goldberg, 2001). There is a pH gradient that exists between the external environment (pH 7.4)

and the acidic food vacuole (pH 5.0) and weakly basic drugs are proposed to accumulate easily in the acidic food vacuole (Figure 4.14) (Geary *et al.*, 1986). The level of drug accumulation that is possible to achieve is governed by the redox state of the drug in question. Protonated drugs are less permeable to biological membranes than un-protonated drugs (Hawley *et al.*, 1996) .

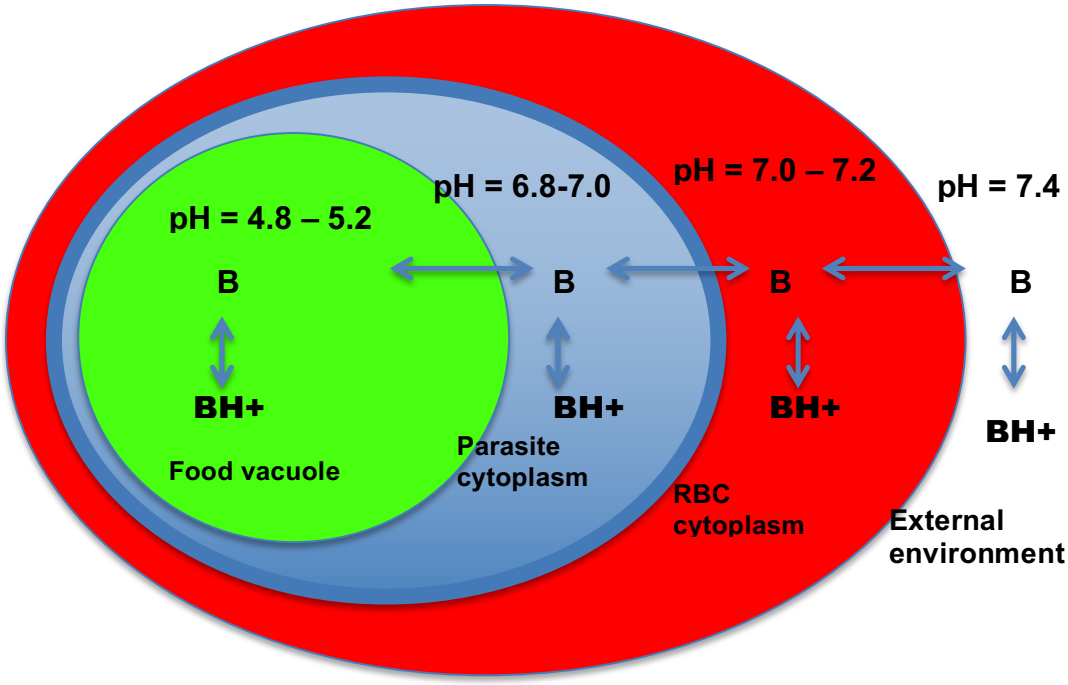


Figure 4.14: Schematic diagram illustrating the accumulation of a weak base against the pH gradient in *P. falciparum* (Krogstad *et al*., 1985)

There are various factors that play a role in the amount of drug that is reaching the acidic food vacuole, not only is redox state important but also the pKa of the compounds. Chemically, these two properties of drugs are related as the pH of a compound is determined through the ability to behave as a proton donor. When examining the **MA** and **MT** derivatives the substituents on the benzene ring would have an effect on the pKa of the compound. According to the Bronsted-Lowry acid/base theory, a compound that has the ability to act as a proton donor is considered an acid, and a compound that has the ability to act as a proton acceptor is considered a base (Brown and Lemay Jr, 2000). The most active compounds from the **MT** and **MA** groups, **CupMeO2MT** and **Cu4MeO2MA** (Table 4.2) were compounds that were both substituted with methoxy at the *para* position. The oxygen

found on the methoxy group composed of a lone pair of electrons has the ability to act as a proton acceptor. In doing so it could be theorized to increase the pKa making it less acidic, and therefore increasing the accumulation of the compound into the acidic food vacuole. The substitution of a methyl group (CH_3) did not affect the antimalarial activity to such a large degree, as seen by **Cu2MT** (IC_{50} value: 9.26 ± 0.33 μM) and the methyl substituted **CupMe2MT** (IC_{50} value: 6.02 ± 0.66 μM) (Table 4.2). This could be explained by the lack of unpaired electrons when compared to the methoxy substituted on **CupMeO2MT**, as well as stable hydrogen bonds between the carbon and hydrogen atom (Brown and Lemay Jr, 2000).

The pKa and consequent acid/base parameters would also have on effect on the difference in the activity of the two compounds against the different strains. As discussed, studies have shown a difference in the pH of the food vacuole of the HB3 chloroquine-sensitive strain, and Dd2 a chloroquine-resistant strain, with the chloroquine-resistant strain having a slightly more acidic pH. However, the same theory cannot necessarily be applied to the 3D7 and FCR-3 strain used in this study as it cannot be assumed that all chloroquine-sensitive and chloroquine-resistant strains share the same pH values. As such further studies would need to be conducted to determine the specific pH of the food vacuole of these two strains in order to determine the relation between pH, different compound substituents and drug sensitivity.

The pH parameters and pKa constants are complex and involve the interplay and interaction between various other factors which would need to also be examined to fully understand the effects the different substituents would have on the antimalarial activity of the compounds.

4.5.1.3 Transition based antimalarials

The incorporation of transition metals into antimalarials is not an entirely new avenue of research in the field of malaria, and has provided means of improving antimalarial activity (Sekhon and Bimal, 2012). As evident from the results, the use of copper improved the antimalarial activity of the compounds in each instance (Table 4.2). The use of metals incorporated into compounds for antimalarial use provides a means of

improving on physicochemical properties of the compound as well as improving on overall activity.

There are various strategies available for the use of metals in antimalarial treatment, one such example is the incorporation of a transition metal into a standard antimalarial such as chloroquine in order to improve pharmacological properties of the drug such as lipophilicity thereby enhancing the overall activity of the parent drug. The attachment of gold and ruthenium into a chloroquine scaffold was found to enhance the antimalarial activity of the parent compound against resistant strains of *Plasmodium falciparum* through an enhancement of lipophilicity and structural changes to the parent drug (Navarro *et al.*, 2011). Lipophilicity is an important is an important property required for optimal antimalarial activity as the compound needs to transverse three membranes to enter the parasite and four to enter the food vacuole where it can prevent haemoglobin digestion or haemozoin formation (Martínez *et al.*, 2009). Although **MT** and **MA** derivatives were not able to exhibit very potent activity, the increase in lipophilicity could prove advantageous with regards to increasing uptake of the compounds into the malaria parasite and if achieved assist with achieving good activity in *in vivo* studies and bioavailability of the drug.

Another strategy would be to incorporate a metal into the scaffold of a known antimalarial in order to enhance the known antimalarial activity of the parent compound. The integration of platinum (II) into 4-aminoquinoline derivatives proved to enhance the inhibition of the haem detoxification process by up to 80% during an *in vivo* study conducted using a *P. berghei* strain in rats (De Souza *et al.*, 2011). The compound, ferroquine is another example of an organometal, where ferrocene has been integrated into a chloroquine scaffold. Ferroquine displayed activity against both chloroquine-sensitive as well as chloroquine-resistant *P. falciparum* and *P. vivax* strains. The activity of ferroquine provides a unique dual mechanism of action. The first manner in which it produces its effect is through the inhibition of β-haematin formation, which is similar to its parent compound chloroquine (Biot *et al.*, 2011). The second mechanism of action is through redox activation from ferrocene to ferricinium which increases the production of radicals that increase oxidative stress (Biot *et al.*, 2011). As such, further studies could be done by incorporating transition metals other than copper in order to improve the antimalarial activity of the **MT** and **MA** derivatives.

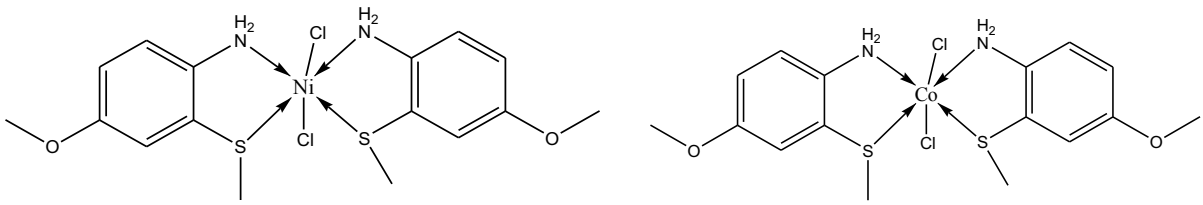
The next step in the use of metals in malaria treatment would be to explore the synthesis of metal-based parent drugs based on new targets. Although this may be a feasible alternative, careful study would need to be completed to ensure there is no disruption of the metal homeostasis as a result of metal being released form the metal chelate; as this could potentiate the already challenged metal homeostasis in the infected host (Seyrek *et al.*, 2005). As such, although the current configuration of metal binding to the **MA** and **MT** derivatives did not affect the RBC integrity of the host (Appendix C: Table C.1; Table C.2), an alteration in the transition metal of choice or binding mechanism could do so.

4.5.2 Mechanism of action: Anti-oxidant activity

4.5.2.1 Free radical scavenging activity

When comparing the anti-oxidant activity of the **MT** and **MA** derivatives using the DPPH assay (Appendix C: Table C.4) it was noted that only the compounds bound to nickel and cobalt displayed potent free radical scavenging activity, to a greater degree than Trolox™. It is significant to note that both cobalt and nickel have proven capable of producing ROS and therefore exhibit pro-oxidant activity (Valko *et al.*, 2005). Cobalt has been reported to promote the production of reactive species that are capable of degrading deoxyribose (Shi *et al.*, 1993)**.** When bound to the **MT** and **MA** derivatives the pro-oxidant effect was absent for both metals. Studies conducted in the ability of cobalt and nickel to produce peroxides revealed that thiol (-SH) chelators such as cysteine activated both nickel and cobalt's ability to produce peroxide radicals (Shi *et al.*, 1993). When examining the base structures of the MA group (Figure 4.15), it could be proposed that the presence of the thiol group could have played a role in its ability to behave as an anti-oxidant. Based on the results obtained it is evident that the methoxy substituent at the *para* position in the compounds bound to nickel and cobalt slightly improved the free radical scavenging activity when compared to the *ortho* position of the substituent. For example, the *para* substituted **Ni4MeO2MA** (IC_{50} value: 9.63 ± 0.69 μM) compared to the *ortho* substituted **Ni6MeO2MA** (IC_{50} value: 11.4 ± 1.21 μM) (Appendix C: Table C.4). When comparing the **MT** and **MA** groups it is important to note that although methylthiomethyl groups have proven free radical scavenging activity, in this

particular compound it was unable to contribute to the free radical scavenging activity of the compound (Kuhns *et al.*, 1994).



NiMeO2MA **Co4MeO2MA**

Figure 4.15: The structures of the nickel and cobalt bound MA derivatives with that displayed the most free radical scavenging activity.

4.5.2.2 Redox activity of nickel and cobalt

Nickel is found in several oxidative states, and although its therapeutic use is relatively unexplored it most notably plays a significant role in plant systems (Zamponi *et al.*, 1996). Nickel has been proposed to initiate lipid peroxidation through the production of hydroxyl radicals due to the interaction with hydrogen peroxide and the superoxide radical (Chen *et al.*, 2002). Cobalt is considered an essential trace element, as the body is unable to synthesize it and as such it must be obtained from external sources (Roth *et al.*, 1996). The most significant use of cobalt is as an integral part of vitamin B12. Cobalt has proven to induce a carcinogenic effect through the production of free-radicals in reaction to hydrogen peroxide. (Beyersmann and Hartwig, 1992). Although both nickel and cobalt have proven to display a pro-oxidant effect, when bound to the **MA** derivatives it has the opposite effect and was able contribute to the compounds ability to scavenge the DPPH radical (Appendix C: Table C.4) (Figure 4.8). Studies have shown that acyclic ligands bound to nickel display moderate anti-oxidant activity when compared to compounds bound to copper and cobalt (Rathi and Singh, 2015). When cobalt was complexed to quercetin, it was revealed that the integration of cobalt into the complex enhanced the anti-oxidant activity of the parent compounds, through a decrease in oxidation potential. As such the complexed quercetin was more readily oxidized by the free radical when compared to the uncomplexed quercetin (Bukhari *et al.*, 2008).

The use of the DPPH free radical scavenging assay to determine the anti-oxidant behaviour of the novel compound allows a very conservative indication of the anti-oxidant activity. This assay is based on the ability of the compound to bind to the unpaired electron found on the DPPH radical, as such determines the ability of the compound to act as a proton donor. In the case of **Ni4MeO2MA, Co4MeO2MA, Ni6MeO2MA** and **Co6MeO2MA** (Appendix C: Table C.4) (Figure 4.8), it could be assumed that the cationic nature of the transition metals contributed to the ability of the compound to form a stable bond with the DPPH radical. As such although these four compounds revealed free radical scavenging activity, the compounds are known contributors to the production of ROS and further studies would need to be conducted to determine whether they would contribute to the generation of ROS in a biological system (Molyneux, 2004).

4.5.2.2 Inhibition of lipid peroxidation

In contrast to the results obtained from the free radical scavenging assay, measuring the anti-oxidant activity using the lipid peroxidation inhibition assay revealed the compounds bound to copper produced an inhibitory effect on lipid peroxidation, thereby exhibiting anti-oxidant behaviour (Appendix C: Table C.5) (Figure 4.9). The integration of copper into the **MT** and **MA** derivatives had a very potent effect on the ability of the compound to inhibit lipid peroxidation, where the % lipid peroxidation inhibition activity at 50 µM of the base compound **2MT** increased from 2.69% to 78.08% for **Cu2MT** when copper was integrated into the compound (Appendix C: Table C.5)

When comparing the lipid peroxidation inhibition activity between the **MT** and **MA** groups the activity was similar for most of the compounds with a few exceptions. The *para* and *ortho* methoxy substituted **MA** derivatives displayed inhibition activity as opposed to their **MT** counterparts that displayed negligible activity when screened at 50 µM. Where, **oMeO2MT** displayed inhibition activity of 6.16% at 50 µM compared to 79.94% for **6MeO2MA** (Appendix C: Table C.5). When copper was integrated into the compound, the **MT** derivative, **CupMeO2MT** (IC_{50} value: 5.67 ± 0.41 µM) displayed a more potent inhibition activity on lipid peroxidation than its **MA** counterpart **Cu4MeO2MA** (IC_{50} value: 18.1 ± 1.51 µM) (Figure 4.9). However, when comparing the inhibitory activity of **Cu2MT** (IC_{50} value: 6.1 ± 2.0 µM) to **CupMeO2MT**

(IC_{50} value: 5.67 ± 0.41 µM), the difference was negligible and one could assume the effect of the methoxy group alone on the anti-oxidant activity is very small.

The integration of halides such as Br and Cl into the copper bound compounds maintained the inhibition activity, and in the case of the **Cu2MA**, the inhibitory activity was slightly improved with the substitution of Cl at the *para* position. Studies have shown that the substitution of halides such as Br and Cl with copper showed a slightly improved activity of the complex when compared to the unbound parent ligands. This was as a result of the anion or negative charge on the halide affecting the reduction potential of copper, thereby increasing its ability to act as an anti-oxidant. This could be applied to **Cu4Cl2MA** (IC_{50} value: 6.57 ± 1.71 µM) (Figure 4.9) (Bandy *et al.*, 2001).

4.5.2.3 Anti-oxidant activity of copper

Copper is found in different redox states, as such it has the ability to participate in redox reactions (Seyrek *et al.*, 2005). With regards to antimalarial activity, it is proposed to act through the generation of ROS within the parasite (Gokhale *et al.*, 2006); although if this is so, its cationic nature or positive redox state allows it to behave as a free radical scavenger with anti-oxidant activity (Wu *et al.*, 2011). Several studies conducted on various compounds to determine the effect integrating copper into a compound would have on anti-oxidant activity. One such study compared the anti-oxidant activity of cobalt and copper complexed to a benzimidazole ligand. The study revealed that the compound containing copper displayed more effective hydroxyl radical scavenging activity than the compound complexed to cobalt, as well as ascorbic acid (Wu *et al.*, 2011). A more complex study investigated the use of mixed ligand copper complexes to mimic superoxide dismutase activity. Superoxide dismutase is an anti-oxidant enzyme that catalyses the conversion of a superoxide anion radical, $O_2^{\bullet-}$ to H_2O_2 and O_2 (Figrue 4.16). The study revealed that the Cu(II) arrangement in the compounds enabled the interaction with the radical and their dissociation to oxygen and hydrogen peroxide (Mehta *et al.*, 2015). The use of copper bound to acyclic ligands revealed moderate anti-oxidant activity in a concentration dependant manner (Rathi and Singh, 2015), which was the case with the copper bound MT and MA derivative used in this study (Appendix C: Table C.5).

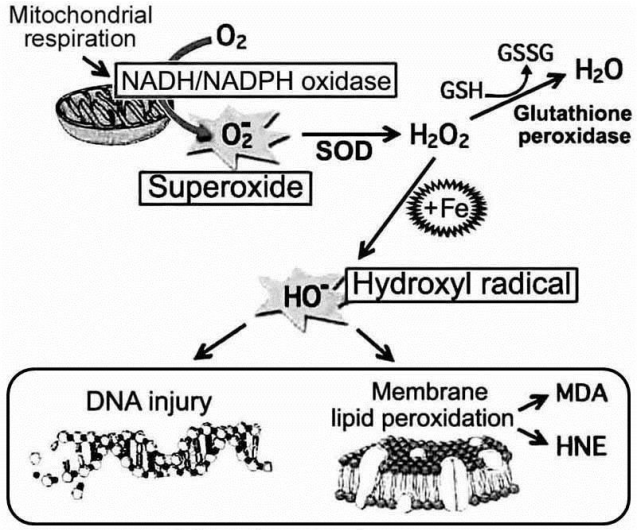


Figure 4.16 Diagram illustrating the contribution of superoxide to oxidative stress, and the production of hydroxyl radicals (Moghadam *et al.*, 2015)

4.5.2.4 Transition metal: Anti-oxidant and pro-oxidant activity

The proposed theory in which the copper bound MA and MT derivatives produced their antimalarial effect is through their ability to act as pro-oxidants, with **CupMeO2MT** (IC_{50} value: 2.66 ± 0.12 μM) (Figure 4.13). When tested for anti-oxidant activity **CupMeO2MT** (IC_{50} value: 5.67 ± 0.41 μM) (Appendix C: Table C.5) displayed potent inhibition of lipid peroxidation comparable to that of Trolox™. As such copper displayed the ability to act as a pro-oxidant, as well as an anti-oxidant. Although **CupMeO2MT** was unable to scavenge DPPH free radicals as a measure of anti-oxidant activity (Appendix C: Table C.4). The ability of copper to act as an anti-oxidant *in vitro* is greatly influenced by its molecular structure. The flexibility of the Cu(II) arrangement determines the possibility of electron transfer which results in reduction to Cu(I). Limited steric hindrance between copper and the oxygen radical *in vitro* allows it to exchange molecules rapidly, and the stabilizing effect of the π-electrons on the complex formed between copper and the oxygen radical all contribute to its anti-oxidant activity (Mehta *et al.*, 2015). Therefore the molecular structure of the copper bound to the **MT** and **MA** derivatives would have an effect on the speed as well as tendency for electron donation and bond formation to occur between radicals and copper.

Several factors contribute to whether a compound would act as a pro-oxidant or an anti-oxidant and the degree to which it would produce an effect. Ascorbate, or vitamin C is a well known anti-oxidant, but has the ability to act as a pro-oxidant based on its concentration. At high concentrations it acts as a pro-oxidant, and at low concentrations it tends to act as an anti-oxidant (Buettner and Jurkiewicz, 1996). In this study screening was conducted at the same concentration for all the assays, as such further dose dependant studies would have to be performed to verify if for example **CupMeO2MT** acted as a pro-oxidant or anti-oxidant.

The rate at which a Fenton reaction takes place is significantly affected by pH. When measuring the rate at which transition metals would initiate the Fenton reaction in biological systems would be dependant on the type of buffer chosen to complete the reaction in, as buffers could affect the metal binding capabilities (HEPES > TRIS > phosphate buffer). It has been reported that changes in pH play a role in the ability of the transition metals to participate in Fenton-like reactions to produce oxidizing species. At increased pH levels, iron was inactivated, and unable to produce oxidizing species; whilst copper was able to still produce an effect. Cobalt displayed an increase in activity as the pH increased, but at very high pH levels (>8.9) it was deactivated (Strlič *et al.*, 2003). Nickel's activity increased with an increase in pH, specifically at 7.8. As such there was a general increase in the rate of production of oxidizing species as the pH was increased. At a neutral pH the decreasing rates of production of oxidizing species was as follows: Cu(II) > Co(II) > Ni(II) (Strlič *et al.*, 2003). Therefore one could propose that pH could play a role in the **MA** or **MT** compounds ability to display pro-oxidant or anti-oxidant behaviour.

Oxidative damage to lipids result in changes to cellular membranes which have a direct result on cellular function contributing to various pathological conditions such as cancer, heart disease and diabetes (Kohen and Nyska, 2002). The degree of efficacy of anti-oxidants to protect against oxidative damage to lipids would rely on the compounds lipophilicity. The compounds ability to be incorporated into a lipid bilayer directly effects the even distribution of the compound into a lipid bilayer. The more evenly distributed it is the more accessible the protons are to interact with free radicals found on the surface (Zhang *et al.*, 2006). The copper bound **MA** and **MT** derivatives have displayed lipid peroxidation activity comparable to that of Trolox™

and additional understanding is required on their lipophilic nature, in order to determine their ability to act as efficient anti-oxidants *in vivo*.

4.5.3 Toxicity evaluation

The antimalarial activity of the **MT** and **MA** derivatives were directed against the intra-erythrocytic parasite rather than affecting the integrity of the host red blood cell as the compounds displayed negligible haemolytic activity (Appendix C: Table C.1; Table C.2). When considering the safety index calculated for the compounds by comparing the antimalarial effect to the cytotoxic effect on human kidney epithelial cells, it was noted that although **CupMeO2MT** and **Cu4MeO2MA** displayed antimalarial activity through a proposed pro-oxidant effect, this was not observed in the human kidney epithelial cells, indicated by a safety index >10 (Table 4.3). This is a notable finding if the proposed pro-oxidant mechanism of action is proven correct in future studies. The specificity of the compounds to have limited pro-oxidant effects on normal human cells would prove advantageous.

Of the compounds tested for antimalarial activity, most of the compounds were relatively safe with less than 50% cytotoxic to the non-cancerous kidney cells, and no effect on the integrity of the RBCs by all the compounds (Appendix C: Table C.1; Table C.2). Although these results demonstrate a positive result in terms of initial safety studies, it has been shown that anti-oxidant compounds have the ability to interfere with cell proliferation results when using the MTT-based cellular viability assay (Bruggisser *et al.*, 2002). Therefore, further studies using other cellular viability methodologies (such as tritiated thymidine incorporation) should be conducted in order fully understand the toxicity profile of the **MT** and **MA** derivatives which displayed anti-oxidant activity (Ducklow and Hill, 1985).

4.5.3.1 Cytotoxic and anti-proliferative activity

When considering the cytotoxic properties of the compounds on the leukemic cell line compared to the non-cancerous human kidney epithelium cell line, the compounds were observed to be more selective to the human kidney cell line, as indicated by the small SI_{nc} values (Table 4.4) (Figure 4.6). However there was no correlation between the activity of the compounds on the two different cell lines

(Figure 4.7). The integration of copper into the **MT** and **MA** derivatives increased the cytotoxic activity on both cell lines. When comparing the % cell viability of the base ligand **2MT** (105%) and the copper bound **Cu2MT** (4.65%) at 50 μM, it was noted that the introduction of copper played a substantial role in the anti-proliferative activity of the compounds (Appendix C: Table C.3). **CupMeO2MT** (Figure 4.6) displayed greater cytotoxic activity on the leukemic cell line when compared to the non-cancerous kidney epithelium. This is in contrast to **CupMe2MT** (Figure 4.6), which displayed greater cytotoxic activity on the non-cancerous kidney epithelium compared to the leukemic cell line. However in contrast to camptothecin the compounds inhibitory activity was negligible, and further studies would need to confirm on additional cell lines and an additional cellular viability assay. Studies have shown that compounds bound to nickel and cobalt have the ability to produce a carcinogenic effect (Valko *et al.*, 2006), but the binding of Co and Ni to the **MA** and **MT** derivatives did not effect its ability to do so. As evident from the K562 cell proliferation at 50 μM for **CooMe2MT** (83.54%) and **Ni6MeO2MA** (75.64%) (Appendix C: Table C.3).

Copper's ability to produce a pro-oxidant as well as anti-oxidant effect as displayed by the **MA** and **MT** derivatives could account for the lack of correlation between the activity of the copper bound **MT** and **MA** derivatives on the two different cell lines. The use of metals in cancer therapy is not a new concept, where one of the most commonly used anti-proliferative therapies is a platinum based compound, cisplatin (Kostova, 2006). Apart from this, copper homeostasis plays an integral role in carcinogenesis with studies proving that cancer cells proliferate into tumours easier under high-copper environments. As such a drug with a copper-lowering capability has been proposed for anti-proliferative activity (Valko *et al.*, 2006). The use of copper complexed to not only novel compounds, but known anti-proliferative agents have proven successful. Studies have proven the use of organic copper complexes inhibiting proteasome activity *in vivo* and *in vitro,* induce apoptosis resulting in producing an anti-proliferative effect (Daniel *et al.*, 2004). Studies conducted on the use of copper complexed to doxorubicin a known anti-proliferative agent has proven to decrease the size of tumours in mice when compared to doxorubicin administration alone (Fan *et al.*, 2009). Further studies on copper have revealed that copper complexed to a known antioxidant, pyrrolidine dithiocarbamate forms a potent proteasome inhibitor in human breast cancer cells, but not in normal cells. Therefore

the use of copper and metals in general with regards to cancer therapy would need to be carefully regulated.

4.6 Summary

- The copper bound **MT** and **MA** derivatives have shown antimalarial activity against both a 3D7 strain as well as a FCR-3 strain, with the methoxy substituted copper bound compounds, **CupMeO2MT** and **Cu4MeO2MA** displaying the most activity.
- The antimalarial activity is proposed to act through a pro-oxidant effect (Gokhale *et al.*, 2003).
- **Ni4MeO2MA, Co4MeO2MA, Ni6MeO2MA** and **Co6MeO2MA** displayed anti-oxidant activity through the ability to effectively scavenge free radicals, as well as retain their carcinogenic properties.
- The copper bound **MT** and **MA** derivatives displayed potent anti-oxidant activity through their ability to inhibit free radical scavenging activity with **CupMeO2MT** displaying the most favourable activity.
- The compounds had a fairly safe toxicity profile as they displayed no effect on the integrity of red blood cells and displayed negligible activity on the proliferation of normalised human kidney epithelium.
- The compounds displayed moderate anti-proliferative activity and the introduction of copper into the **MT** and **MA** derivatives showed a clear improvement on anti-proliferative activity.
- Further studies would need to be conducted in order to establish whether the copper bound compounds produced their antimalarial effect through the use of the production of ROS, as well as to determine their pKa values and the effect the anti-oxidant compounds would have on cell proliferation using an assay other than the MTT-based cellular viability assay.
- Further studies would need to be conducted on the anti-oxidant activity of **Ni4MeO2MA, Co4MeO2MA, Ni6MeO2MA** and **Co6MeO2MA** and the manner in which they are able to produce ROS and in this instance act as antioxidants.

CHAPTER 5

Conclusion

The spread of resistance to standard antimalarial treatment has urged researchers to explore new avenues for new drugs as well as targets. For both sets of compounds although vastly different in their chemical aspects, both were composed of two entities to compliment each other's individual activity. In the case of the aminoquinoline derivatives, it was the use of two very different compounds incorporated through the use of crystal and salt formation. These compounds were able to inhibit parasite growth through a proposed pro-oxidant effect without any effect on the host's red blood cell membrane integrity. Further research would need to be conducted to confirm this proposed mechanism of action.

The use of crystal and salt formation proved advantageous in certain aspects as various components of the parent compound were retained, such as the anti-oxidant activity of gallic acid in compound **III** and 8-hydroxyquinoline compound **V**. While the use of multicomponent systems and co-crystals provides a novel approach to the method in which drugs may be delivered. The challenge with regards to its use is the inability to predict whether a salt or co-crystal will form, as well as the inability to predict the pharmacological properties of the resultant component, and the degree to which it would retain the potency of the parent compound and efficacy in producing its desired mechanism of action (Childs *et al.*, 2007) . As such gallic acid was able to produce a proposed pro-oxidant effect in compound **I** in a salt form, and a potent anti-oxidant effect in crystal form. The formation of the salt and cocrystal from this specific group of compounds did not produce a more potent antimalarial effect than its aminoquinoline parent compounds, as such it would not prove feasible to conduct further studies on these specific compounds with regards to antimalarial activity, but perhaps other combinations could be further examined.

In the case of the **MT** and **MA** derivatives the incorporation of copper into the base ligands proved successful in improving antimalarial activity, as well as the anti-oxidant activity through the inhibition of lipid peroxidation. The proposed mechanism

of action is through a pro-oxidant effect. In this respect the incorporation of copper into the base ligands proved to display a pro-oxidant, as well as an anti-oxidant effect. The various substituents at the *ortho* and *para* positions proved to have an influence on the activity of the compounds. The binding of cobalt and nickel to the **MA** derivatives enhanced the metals' ability to effectively scavenge free radicals. The use of incorporating metals into compounds as a means of enhancing activity, or improving the physicochemical properties of a compound, could prove beneficial. Although this is true the disruption of metal homeostasis is always a possibility and care should be taken when introducing metals into the body. **CupMeO2MT** and **Cu4MeO2MA** both displayed antimalarial activity without having an effect on red blood cell membrane integrity, as well as a safety index >10. **CupMeO2MT** displayed potent inhibition of lipid peroxidation and further investigation would need to be conducted on its ability to act possibly as a pro-oxidant, as well as anti-oxidant.

While the use of a pro-oxidant as an antimalarial could prove effective in inhibiting growth of the malaria parasite the challenge with regards to its use is the lack of specificity in the manner in which these compounds produce an effect, with the risk of causing oxidative damage to biological targets. This study has investigated the possible antimalarial activity of novel compounds and with current research being aimed at the next generation of hybrid antimalarial compounds being focused as a new method of drug design, the incorporation of transition metals should be further explored.

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APPENDIX A: (Extract from article submitted for publication showing the purity and validation of the aminoquinoline derivatives)

Prithiviraj Khakhlary, Shamillah Holland, Robyn Lynne van Zyl, and Jubaraj B. Baruah.

Potent Antimalaria Salts, Cocrystals and Derivatives of Aminoquinolines with Hydroxyaromatic Acids. Submitted to Journal of Pharmacological Reports, January 2016.

Synthesis and characterization of the Salts and Cocrystal:

Salts **I-III** and cocrystal **IV** were prepared by crystallization of solution of 1:1 mole ratio of the corresponding aminoquinoline and respective hydroxyaromatic acid. In each case, aminoquinoline and hydroxyaromatic acid was dissolved in methanol and kept undisturbed for crystallization. Spectroscopic and analytical details of the salts and cocrystal are as follows: Salt **I**: Elemental anal Calcd. For $C_{16}H_{16}N_2O_4$, C, 63.99; H, 5.37; N, 9.33; and found C, 63.97; H, 5.41, N 9.46. ^{1}H-NMR (DMSO-d_6): 8.79 (d, *J* = 4.0 Hz, 1H), 8.59 (d, *J* = 8.4 Hz, 1H), 7.79 (d, *J* = 7.6 Hz, 1H), 7.49-7.37 (m, 3H), 7.41 (q, *J* = 4.0 Hz, 1H), 7.19(d, *J* = 8.4 Hz, 1H), 6.93 (d, *J* = 8.0 Hz, 2H), 6.73(d, *J* = 8.0 Hz, 2H), 4.18 (s, 2H). IR (KBr, cm^{-1}): 3444 (s), 3321(m), 3082 (w), 1627 (s), 1589 (s), 1566 (m), 1482 (m), 1454 (m), 1432 (w), 1384 (m), 1361(m), 1330 (m), 1305 (w), 1252 (w), 1138 (w), 1028 (w), 859 (w), 787 (m), 702 (w), 664 (w). Salt **II**: Elemental anal Calcd. For $C_{16}H_{16}N_2O_6$, C, 57.83, H, 4.85; N, 8.43; found C, 57.79, H, 4.88; N, 8.45. ^{1}H-NMR (DMSO-d_6): 9.23 (s, 1H), 8.76 (d, *J* = 4.0 Hz, 1H), 8.53 (d, *J* = 8.4 Hz, 1H), 7.43 (t, *J* = 8.0 Hz, 2H), 7.36 (q, *J* = 4.0 Hz, 1H), 7.19 (d, *J* = 8.0 Hz, 1H), 6.94 (s, 3H), 6.72 (d, *J* = 7.6 Hz, 2H), 5.9 (s, 2H). IR (KBr, cm^{-1}): 3458 (s), 3348 (s), 3241 (m), 3076 (w), 2671 (w), 1644 (w), 1599 (w), 1526 (m), 1471 (w), 1360 (s), 1307 (m), 1228 (m), 1179 (w), 1102 (w), 1035 (m), 886 (w), 786 (m), 746 (w), 703 (w), 575 (w). Salt **III**: Elemental anal Calcd. for $C_{20}H_{16}N_2O_3$, C, 72.28; H, 4.85; N, 8.43; found C, 72.33; H, 4.86; N,8.69. ^{1}HNMR (DMSO-d^6, 400MHz): 8.72 (d, J = 8Hz, 1H), 8.30 (d, J = 8Hz, 1H), 8.17 (d, J = 8.4 Hz, 1H), 7.90 (d, J = 8 Hz, 1H), 7.76 (d, J = 8.8 Hz, 1H),

7.67 (t, J = 1.2, 1H), 7.59 (t, J = 8.4 Hz, 1H), 7.46-7.39 (m, 1H), 7.39 (d, J = 8.4 Hz, 1H), 7.31 (t, J = 7.6 Hz, 1H), 7.07 (d, J = 8Hz, 1H), 6.89 (d, J = 7.6 Hz, 1 H). IR (KBr, cm^{-1}): 3424 (m), 3210 (m), 2923 (w), 1631 (m), 1582 (s), 1504 (m), 1467 (m), 1405 (s), 1377 (m), 1328 (m), 1309 (m), 1213 (w), 1017 (w), 863 (w), 815 (m), 795 (m), 771 (m), 693 (m). Cocrystal **IV**: Elemental anal Calcd. for $C_{16}H_{14}N_2O_5$; C, 61.14; H, 4.49; N, 8.91; found C, 61.20; H, 4.47; N, 9.10. ^{1}H-NMR (DMSO-d_6): 9.23 (s, 1H),8.87(s, 1H), 8.71 (dd, *J* = 1.6 Hz, *J* = 2.4 Hz,1H), 8.17 (dd, *J* = 1.6 Hz, *J* = 6.8 Hz, 1H), 7.46 (q, *J* = 4.0 Hz 1H), 7.30 (t, *J* = 8.0 Hz, 1H), 7.06 (dd, *J* = 0. 8 Hz, 1H), 6.91 (s, 2H), 6.86 (dd, *J* = 1.2 Hz, 1H), 5.90 (s, 1H), 4.09 (s, 3H). IR (KBr, cm^{-1}): 3516 (m), 3400 (m), 3309 (m), 1686 (s), 1620 (m), 1519 (w), 1425 (m), 1374 (w), 1324 (s), 1272 (w), 1188 (m), 1032 (m), 900 (w), 857 (w), 825 (w), 787 (w), 766 (w), 718 (w).

Derivatives of aminoquinolines:

Compounds **V-VI** were prepared by reported procedure [7]. Compound **VII** was prepared by published procedure [5]. Synthesis of ethyl-4-(2-oxo-2-(quinolin-8-ylamino)ethoxy)benzoate (**VIII**): To a solution of 8-aminoquinoline (0.72 g, 5 mmol) in dry dichloromethane (20 ml), triethylamine (0.71 ml, 5 mmol) was added. Solution was stirred at 0ºC for 15 mins, followed by which bromoacetyl bromide (0.52 ml, 6 mmol) was added. Reaction mixture was then stirred overnight and filtered. Filtrate was washed with water (10 ml), dried over anhydrous sodium sulphate and then the solvent was removed under reduced pressure. Product was obtained as a brown solid that was purified by recrystallisation from dichloromethane. Amide obtained from above reaction (0.53 g, 2 mmol), ethyl 4-hydroxybenzoate (1.66 g, 10 mmol) and potassium carbonate (1.07 g, 7 mmol) were added to dry acetone (20 ml) under nitrogen and the reaction mixture was stirred at 60 ºC for 9 hrs. After completion of reaction solvent was removed under reduced pressure to obtain a pale yellow solid which was recrystallized from methanol. IR (KBr, cm^{-1}): 3430 (m), 3323 (m) 2977 (w), 1701(s), 1691(s), 1607 (s), 1544 (s), 1507 (m), 1485 (m), 1427 (w), 1388 (w), 1367 (w), 1330 (w), 1318 (w), 1301 (w), 1279 (m), 1248(s), 1167 (m), 1107 (m), 1055 (m), 841(w), 823 (w), 791(m), 764 (w), 755 (w), 686 (w), 638 (w), 567 (w), 500 (w). ^{1}H-NMR ($CDCl_3$, 400MHz): 10.88 (s, 1H), 8.82 (q, *J* = 1.6 Hz, 1H), 8.78 (t, *J* = 4.4 Hz, 1H), 8.14 (dd, *J* = 1.6 Hz, 1H), 8.05 (dd, *J* = 2.0 Hz, 2H), 7.52 (d, *J* = 4.0 Hz, 2H),

7.44 (q, *J* = 4.0 Hz, 1H), 7.11 (d, *J* = 8.8 Hz, 2H), 4.76 (s, 2H), 4.35 (q, *J* = 7.2 Hz, 2H), 1.37 (t, *J* = 7.2 Hz, 3H). ESI mass: 351.0266. Calcd. Mass for [M + 1]: $C_{20}H_{19}N_2O_4$, 351.1345.

Methyl-3-(2-oxo-2-(quinolin-8-ylamino)ethoxy)benzoate (**IX**): Similar procedure was followed for the synthesis of **IX** as for **VIII** except methyl-3-hydroxybenzoate (0.45 g, 3 mmol) was used instead of ethyl-4-hydroxybenzoate. IR (KBr, cm^{-1}): 3410 (m), 3332 (m) 2990 (w), 2926 (w), 1708 (s), 1683 (s), 1585 (w), 1541 (s), 1485 (m), 1458 (m), 1435 (m), 1377 (w), 1372 (w), 1313 (w), 1296 (s), 1283 (m), 1222 (w), 1187 (w), 1103 (m), 1075 (m), 988 (w), 889 (w), 827 (w), 793 (w), 758 (m), 733 (m), 678 (w), 600 (w), 576 (w), 550 (w). ^{1}H-NMR ($CDCl_3$, 400MHz): 10.91(s, 1H), 8.84 (q, *J* = 1.2 Hz, 1H), 8.79 (t, *J* = 4.4 Hz, 1H), 8.14 (dd, *J* = 1.6 Hz, 1H), 7.74 (dd, *J* =1.2 Hz, 2H), 7.52 (d, *J* = 4.4 Hz, 2H), 7.45 (m, 1H), 7.32 (dd, *J* = 2.4 Hz, 2H), 4.76 (s, 2H), 1.21(s, 3H). ESI mass: 337.0094.Calcd. Mass for [M + 1]: $C_{19}H_{17}N_2O_4$, 337.1188.

APPENDIX B1: ETHICS DOCUMENTS

R14/49 Dr Robyn L van Zyl

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140669

NAME: (Principal Investigator)	Dr Robyn L van Zyl
DEPARTMENT:	Pharmacy & Pharmacology Medical School
PROJECT TITLE:	The Chemotherapeutic Properties of Novel Synthetic and Natural Compounds (Renewal previously M090532)
DATE CONSIDERED:	29/05/2009 (Initial Approval) 26/06/2014 (Renewal)
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	26/06/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature

Date 21-07-2014

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX B2

Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE
(R 14/16)

CLEARANCE CERTIFICATE - RENEWAL **PROTOCOL NUMBER: 20090503**

BRIEF DESCRIPTION OF APPLICATION:

Chemotherapeutic properties of novel synthetic and natural compounds

APPLICANT: Dr R van Zyl

SCHOOL/DEPARTMENT : Pharmacy/Pharmacology

DATE CONSIDERED: 20090528 and 20140327

DECISION OF COMMITTEE:

Approved unconditionally

1. This clearance certificate expires on 20190402 and may be renewed on application
2. An annual report must be provided on the anniversary date of this certificate, for as long as the project continues
3. Notification of any proposed modifications must be submitted on the attached form

DATE: 20140403 **CHAIRPERSON** _____
(Professor A Capovilla)

DECLARATION BY APPLICANT:

To be completed in duplicate and **one copy** returned to the Secretary, Room 10004, 10th floor, Senate House, University.

1. I have read, understood and accepted the approval conditions above
2. I agree to submit a yearly progress report to the Committee and to submit an interim report on the form provided, in the event of any significant unforeseen event, *e.g.* suspension of a drug trial, temporary closure or relocation of my laboratory, etc
3. I note that the University Safety Officer, or his/her representative, may at any reasonable time inspect my laboratory or trial site to ensure compliance with current Health and Safety legislation. I undertake to offer my full co-operation in any such inspection.
4. I have read, understood and will comply with the *recommended standard operating procedures for the handling of biohazardous materials* posted at http://web.wits.ac.za/Academic/Research/Biosafety.htm
5. I declare (delete as appropriate) that:
    a. I have all the approvals required by statute or regulation and by the funding agencies supporting this work, or
    b. that I will not begin work until such approvals are obtained

Signed: _____ **Date:** _____

MSWorks2000/Iain0015/IBCClearRenew.wps

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX B3

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

Ref: W-CJ-130301-4

01/03/2013

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Prof R van Zyl, Miss S Holland (student no 0601805N).**

Project title: **The anti-malarial properties of novel metal chelating agents.**

Reason: This is a laboratory study using cell lines including Graham and K562. No humans are involved.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON - JONES
HREC (MEDICAL)
2013 -03- 01
JOHANNESBURG

Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Research Office, Senate House, Wits

APPENDIX C: TABLES FOR CHAPTER 4

Table: C.1: The Haemolytic and cytotoxic activity of the of the MT derivatives using the HEK- 293 cell line.

Compound	% Haemolytic activity (at 50 µM % ± s.d)	% Cell viability (at 50 µM % ± s.d)	% Cell viability (IC_{50} ± s.d µM)
2MT	4.06 ± 2.88	56.25 ± 9.56	n.d
Cu2MT	15.2 ± 2.47	6.35 ± 20.67	15.35 ± 4.22
Co2MT	0.35 ± 0.09	70.41 ± 16.35	n.d
pCl2MT	3.08 ± 2.32	62.09 ± 12.09	n.d
oCl2MT	3.97 ± 2.76	54.18 ± 6.04	n.d
CupCl2MT	16.73 ± 0.71	52.0 ± 7.08	n.d
CuoCl2MT	16.65 ± 0.78	57.2 ± 11.13	n.d
CopCl2MT	1.24 ± 0.35	56.28 ± 7.07	n.d
pBr2MT	1.9 ± 1.39	57.26 ± 10.73	n.d
oBr2MT	0.1 ± 0.01	69.53 ± 15.61	n.d
CupBr2MT	n.d	56.15 ± 7.38	n.d
CuoBr2MT	17.19 ± 1.25	53.14 ± 6.17	n.d
CopBr2MT	1.27 ± 0.76	61.77 ± 17.5	n.d
CooBr2MT	0.02 ± 0.95	78.7 ± 20.5	n.d
oNO_2MT	2.25 ± 1.39	54.05 ± 5.58	n.d
Cup$NO_2$2MT	22.59 ± 2.02	54.29 ± 5.49	n.d
Cop$NO_2$2MT	6.49 ± 1.44	64.85 ± 5.75	n.d
pMe2MT	1.22 ± 1.54	56.02 ± 11.84	n.d
oMe2MT	3.54 ± 2.85	57.53 ± 13.29	n.d
CopMe2MT	0.36 ± 0.13	66.15 ± 9.45	n.d
CooMe2MT	0.98 ± 0.24	52.9 ± 8.02	n.d
CupMe2MT	14.47 ± 0.91	20.24 ± 8.8	**11.23 ± 2.86**
CuoMe2MT	21.7 ± 0.97	5.81 ± 1.28	20.48 ± 2.84
pMeO2MT	2.98 ± 1.61	60.1 ± 11.13	n.d
oMeO2MT	2.37 ± 1.74	65.21 ± 17.61	n.d
CopMeO2MT	n.d	62.5 ± 4.88	n.d
CooMeO2MT	1.98 ± 1.48	63.46 ± 3.45	n.d
CupMeO2MT	0.1 ± 0.01	4.51 ± 1.0	29.03 ± 6.89
CuoMeO2MT	3.27 ± 0.55	16.33 ± 9.34	41.85 ± 8.56
NipMeO2MT	1.98 ± 0.53	59.69 ± 5.23	n.d
NioMeO2MT	1.06 ± 0.69	59.98 ± 9.87	n.d
Chloroquine	**2.09 ± 0.59**	n.d	n.d
Quinine	**8.22 ± 2.17**	n.d	n.d
Camptothecin	n.d	**88.1 ± 10.8**	**0.11 ± 0.01**

n.d: not done

Table: C.2: The Haemolytic and cytotoxic activity of the of the MA derivatives using the HEK- 293 cell line.

Compound	**% Haemolytic activity** (at 50 µM % ± s.d)	**% Cell viability** (at 50 µM % ± s.d)	**% Cell viability** (IC_{50} ± s.d µM)
2MA	0.86 ± 2.31	57.13 ± 6.06	n.d
Cu2MA	13.04 ± 16.86	25.22 ± 11.91	71.92 ± 7.63
Co2MA	5.47 ± 4.22	79.7 ± 15.95	n.d
4Cl2MA	10.62 ± 13.3	66.37 ± 7.54	n.d
6Cl2MA	9.17 ± 12.38	55.63± 5.15	n.d
Cu4Cl2MA	**22.75 ± 1.08**	50.37± 5.52	n.d
Cu6Cl2MA	15.46 ± 23.73	50.4 ± 6.72	n.d
Co4Cl2MA	1.66 ± 0.77	54.28 ± 4.51	n.d
4Br2MA	5.95 ± 4.97	52.16 ± 2.12	n.d
Cu4Br2MA	17.42 ± 25	52.34 ± 2.98	n.d
Cu6Br2MA	15.04 ± 23.57	53.02 ± 1.62	n.d
4Me2MA	1.69 ± 1.83	57.98 ± 14.04	n.d
6Me2MA	1.24 ± 2.15	55.97 ± 7.02	n.d
Co4Me2MA	2.63 ± 0.62	72.89 ± 16.83	n.d
Cu4Me2MA	21.94 ± 2.80	52.55 ± 3.52	n.d
Cu6Me2MA	8.83 ± 4.17	21.51 ± 5.23	66.99 ± 3.74
4MeO2MA	2.76 ± 2.79	66.3 ± 11.2	n.d
6MeO2MA	2.73 ± 2.87	64.06 ± 10.42	n.d
Co4MeO2MA	6.37 ± 1.6	58.84 ± 9.46	n.d
Co6MeO2MA	n.d	68.89 2.73	n.d
Cu4MeO2MA	3.23 ± 1.19	9.41 ± 8.33	**46.97 ± 10.32**
Cu6MeO2MA	7.43 ± 2.11	27.48 ± 11.0	75.78 ± 2.8
Ni4MeO2MA	4.66 ± 1.35	51.95 ± 13.05	n.d
Ni6MeO2MA	2.22 ± 0.46	65.74 ± 7.7	n.d
Chloroquine	**2.09 ± 0.59**	n.d	n.d
Quinine	**8.22 ± 2.17**	n.d	n.d
Camptothecin	n.d	**88.1 ± 10.8**	**0.11 ± 0.01**

n.d: not done

Table C.3: Anti-cancer activity of the MT and MA derivatives on the K562 cancer cell line.

Compound	**% Cell viability** (at 50 µM % ± s.d)	**Cell viability** (IC_{50} ± s.d µM)	**Compound**	**% Cell viability** (at 50 µM % ± s.d)	**Cell viability** (IC_{50} ± s.d µM)
2MT	105 ± 5.88	n.d	2MA	**103.83** ± 12.64	n.d
Cu2MT	4.65 ± 1.98	33.5 ± 6.1	Cu2MA	9.5 ± 2.7	24.07 ± 5.56
Co2MT	91.88 ± 13.66	n.d	Co2MA	80.8 ± 21.7	n.d
pCl2MT	113.8 ± 10.33	n.d	4Cl2MA	87.52 ± 6.4	n.d
oCl2MT	139.03 ± 34.33	n.d	6Cl2MA	101 ± 6.7	n.d
CupCl2MT	61.24 ± 2.79	n.d	Cu4Cl2MA	88.6 ± 8.2	n.d
CuoCl2MT	63.44 ± 13.18	n.d	Cu6Cl2MA	73.59 ± 7.5	n.d
CopCl2MT	72.83 ± 18.82	n.d	Co4Cl2MA	82.74 ± 21.73	n.d
pBr2MT	113.44 ± 15.26	n.d	4Br2MA	92.87 ± 15.34	n.d
oBr2MT	132.32 ± 28.2	n.d			
CupBr2MT	67.45 ± 9.55	n.d	Cu4Br2MA	96.46 ± 3.29	n.d
CuoBr2MT	70.41 ± 17.39	n.d	Cu6Br2MA	65.44 ± 8.95	n.d
CopBr2MT	68.38 ± 16.83	n.d			
CooBr2MT	79.86 ± 16.43	n.d			
oNO_2MT	64.74 ± 6.37	n.d			
Cup$NO_2$2MT	61.32 ± 6.3	n.d			
Cop$NO_2$2MT	75.5 ± 12.83	n.d			
pMe2MT	104.86 ± 6.5	n.d	4Me2MA	103.07 ± 7.25	n.d
oMe2MT	112.58 ± 4.32	n.d	6Me2MA	93.7 ± 18.8	n.d
CopMe2MT	72.53 ± 20.14	n.d	Co4Me2MA	64.65 ± 10.96	n.d
CooMe2MT	83.54 ± 12.97	n.d			
CupMe2MT	4.2 ± 3.02	24.83 ± 1.27	Cu4Me2MA	75.4 ± 8.1	n.d
CuoMe2MT	3.73 ± 9.23	22.9 ± 0.4	Cu6Me2MA	65.94 ± 6.92	n.d
pMeO2MT	110.01 ± 12.22	n.d	4MeO2MA	86.23 ± 6.8	n.d
oMeO2MT	118.21 ± 2.31	n.d	6MeO2MA	110.28 ± 8.59	n.d
CopMeO2MT	76.23 ± 17.43	n.d	Co4MeO2MA	74.12 ± 8.36	n.d
CooMeO2MT	74.78 ± 14.79	n.d	Co6MeO2MA	65.84 ± 11.08	n.d
CupMeO2MT	**0.1 ± 0.01**	**12.25 ± 1.97**	Cu4MeO2MA	0.1 ± 0.01	25.06 ± 3.65
CuoMeO2MT	11.58 ± 1.82	14.66 ± 2.32	Cu6MeO2MA	59.05 ± 9.11	n.d
NipMeO2MT	66.79 ± 10.47	n.d	Ni4MeO2MA	77.89 ± 19.38	n.d
NioMeO2MT	64.59 ± 5.65	n.d	Ni6MeO2MA	75.64 ± 19.05	n.d
Camptothecin	**22.5 ± 3.28**	**4.80 ± 0.30**			

n.d: not done

Table C.4: Free radical scavenging activity of the MT and MA derivatives.

Compound	Free radical scavenging activity (at 50 µM % ± s.d)	Free radical scavenging activity (IC_{50} ± s.d µM)	Compound	Free radical scavenging activity (at 50 µM % ± s.d)	Free radical scavenging activity (IC_{50} ± s.d µM)
2MT	0.10 ± 0.01	n.d	2MA	0.10 ± 0.01	n.d
Cu2MT	4.51 ± 1.21	n.d	Cu2MA	6.07 ± 1.12	n.d
Co2MT	0.10 ± 0.01	n.d	Co2MA	0.10 ± 0.01	n.d
pCl2MT	0.10 ± 0.01	n.d	4Cl2MA	0.10 ± 0.01	n.d
oCl2MT	0.10 ± 0.01	n.d	6Cl2MA	0.10 ± 0.01	n.d
CupCl2MT	2.86 ± 0.41	n.d	Cu4Cl2MA	0.10 ± 0.01	n.d
CuoCl2MT	5.12 ± 0.48	n.d	Cu6Cl2MA	6.59 ± 0.37	n.d
CopCl2MT	0.10 ± 0.01	n.d	Co4Cl2MA	0.10 ± 0.01	n.d
pBr2MT	0.10 ± 0.01	n.d	4Br2MA	0.10 ± 0.01	n.d
CupBr2MT	n.d	n.d	Cu4Br2MA	8.01 ± 1.37	n.d
CuoBr2MT	4.36 ± 0.87	n.d	Cu6Br2MA	0.42 ± 0.36	n.d
CopBr2MT	0.10 ± 0.01	n.d			
CooBr2MT	0.10 ± 0.01	n.d			
oNO_2MT	0.10 ± 0.01	n.d			
Cup$NO_2$2MT	2.34 ± 0.75	n.d			
Cop$NO_2$2MT	0.10 ± 0.01	n.d			
pMe2MT	0.10 ± 0.01	n.d	4Me2MA	0.10 ± 0.01	n.d
oMe2MT	0.10 ± 0.01	n.d	6Me2MA	0.10 ± 0.01	n.d
CopMe2MT	0.10 ± 0.01	n.d	Co4Me2MA	0.10 ± 0.01	n.d
CooMe2MT	0.10 ± 0.01	n.d			
CupMe2MT	6.31 ± 0.66	n.d	Cu4Me2MA	11.56 ± 2.83	n.d
CuoMe2MT	5.56 ± 1.24	n.d	Cu6Me2MA	5.00 ± 0.95	n.d
pMeO2MT	13.34 ± 2.18	n.d	4MeO2MA	37.56 ± 5.49	n.d
oMeO2MT	0.10 ± 0.01	n.d	6MeO2MA	52.54 ± 3.42	n.d
CopMeO2MT	n.d	n.d	Co4MeO2MA	62.65 ± 3.20	9.86 ± 1.53
CooMeO2MT	1.77 ± 0.58	n.d	Co6MeO2MA	62.00 ± 2.35	13.3 ± 1.15
CupMeO2MT	11.82 ± 0.75	n.d	Cu4MeO2MA	11.77 ± 1.81	
CuoMeO2MT	5.92 ± 1.14	n.d	Cu6MeO2MA	2.65 ± 0.78	
NipMeO2MT	32.25 ± 4.47	n.d	**Ni4MeO2MA**	68.14 ± 1.16	**9.63 ± 0.69**
NioMeO2MT	3.06 ± 1.53	n.d	Ni6MeO2MA	71.06 ± 0.78	11.4 ± 1.21
Trolox™	**88.1± 10.8**	**19.15 ± 1.14**			

n.d: not done

Table C.5 % Inhibition of Lipid peroxidation inhibition activity of the MT and MA derivatives.

Compound	Inhibition of lipid peroxidation inhibition activity (at 50 µM % ± s.d)	Inhibition of lipid peroxidation inhibition activity (IC_{50} ± s.d µM)	Compound	Inhibition of lipid peroxidation inhibition activity (at 50 µM % ± s.d)	Inhibition of lipid peroxidation inhibition activity (IC_{50} ± s.d µM)
2MT	2.69 ± 0.92	n.d	2MA	0.10 ± 0.01	n.d
Cu2MT	78.08 ± 1.21	6.1 ± 2.0	Cu2MA	83.44 ± 11.23	7.51 ± 1.95
Co2MT	0.10 ± 0.01	n.d	Co2MA	2.82 ±7.49	n.d
pCl2MT	0.10 ± 0.01	n.d	4Cl2MA	0.10 ± 0.01	n.d
oCl2MT	4.64 ± 1.82	n.d	6Cl2MA	0.10 ± 0.01	n.d
CupCl2MT	75.81 ± 11.45	8.33 ± 2.09	**Cu4Cl2MA**	72.25 ± 8.09	**6.57 ± 1.71**
CuoCl2MT	73.69 ± 22.35	19.24 ± 1.26	Cu6Cl2MA	81.63 ± 8.0	17.2 ± 2.96
CopCl2MT	0.10 ± 0.01	n.d	Co4Cl2MA	0.10 ± 0.01	n.d
pBr2MT	0.10 ± 0.01	n.d	4Br2MA	5.39 ± 2.52	n.d
CupBr2MT	n.d	n.d	Cu4Br2MA	8.01 ± 1.37	n.d
CuoBr2MT	78.44 ± 15.55	9.26 ± 1.32	Cu6Br2MA	81.48 ± 3.47	17.91 ± 4.21
CopBr2MT	0.10 ± 0.01	n.d			
CooBr2MT	0.10 ± 0.01	n.d			
oNO_2MT	0.10 ± 0.01	n.d			
Cup$NO_2$2MT	43.87 ± 15.04	n.d			
Cop$NO_2$2MT	0.10 ± 0.01	n.d			
pMe2MT	1.85 ± 0.16	n.d	4Me2MA	6.85 ± 2.56	n.d
oMe2MT	0.10 ± 0.01	n.d	6Me2MA	24.43 ± 11.33	n.d
CopMe2MT	0.10 ± 0.01	n.d	Co4Me2MA	0.10 ± 0.01	n.d
CooMe2MT	0.10 ± 0.01	n.d			
CupMe2MT	76.03 ± 18.18	12.99 ± 3.52	Cu4Me2MA	71.59 ± 7.68	13.0 ± 2.42
CuoMe2MT	73.47 ± 13.29	13.42 ± 3.06	Cu6Me2MA	75.68 ± 14.32	11.41 ± 1.77
pMeO2MT	27.47 ± 8.66	n.d	4MeO2MA	68.84 ± 6.95	27.77 ± 7.19
oMeO2MT	6.16 ± 2.77	n.d	6MeO2MA	79.94 ± 4.92	15.29 ± 1.14
CopMeO2MT	n.d	n.d	Co4MeO2MA	n.d	n.d
CooMeO2MT	4.06 ± 8.79	n.d	Co6MeO2MA	n.d	n.d
CupMeO2MT	73.92 ± 19.77	**5.67 ± 0.41**	Cu4MeO2MA	79.82 ± 10.6	18.1 ± 1.51
CuoMeO2MT	79.9 ± 18.13	20.42 ± 3.73	Cu6MeO2MA	87.7 ± 4.35	27.25 ± 2.99
NipMeO2MT	59.76 ± 12.48	n.d	Ni4MeO2MA	n.d	n.d
NioMeO2MT	9.1 ± 7.0	n.d	Ni6MeO2MA	n.d	n.d
Trolox™	**70.35 ± 2.84**	**5.53 ± 0.54**			

n.d: not done