Synthesis, cytotoxicity and antiplasmodial activity of novel ferrocenyl-artemisinin hybrids

C de Lange

orcid.org / 0000-0002-4252-5433

Thesis submitted in fulfilment of the requirements for the degree Doctor of Philosophy in Pharmaceutical Sciences at the North-West University

Promotor:	Prof DD N'Da
Co-Promotor:	Prof RK Haynes
Additional Co-Promotor:	Dr FJ Smit

Graduation: October 2018

Student number: 20256353

NORTH-WEST UNIVERSITY
NOORDWES-UNIVERSITEIT
YUNIBESITI YA BOKONE-BOPHIRIMA

Higher Degrees Administration

SOLEMN DECLARATION AND PERMISSION TO SUBMIT

1. Solemn declaration by student

I, **Christo de Lange**

declare herewith that the thesis/dissertation/mini-dissertation/article entitled (**exactly as registered/approved title**),

Synthesis, cytotoxicity and antiplasmodial activity of novel ferrocenyl-artemisinin hybrids

which I herewith submit to the North-West University is in compliance/partial compliance with the requirements set for the degree:

PhD

is my own work, has been text-edited in accordance with the requirements and has not already been submitted to any other university.

LATE SUBMISSION: If a thesis/dissertation/mini-dissertation/article of a student is submitted after the deadline for submission, the period available for examination is limited. No guarantee can therefore be given that (should the examiner reports be positive) the degree will be conferred at the next applicable graduation ceremony. It may also imply that the student would have to re-register for the following academic year.

My Ethics number is: NA

My ORCiD is: 0000-0002-4252-5433

Signature of Student: Christo de Lange — Digitally signed by Christo de Lange Date: 2018.10.25 10:56:16 +02'00'

University Number: 20256353

Signed on this 25 day of October of 20 18

2. Permission to submit and solemn declaration by supervisor/promoter

The undersigned declares that the thesis/dissertation/mini-dissertation complies with the specifications set out by the NWU and that:

- the student is hereby granted permission to submit his/her mini-dissertation/ dissertation/thesis:
  (●) Yes ( ) No
- that the student's work has been checked by me for plagiarism (by making use of TurnItIn software for example) and a satisfactory report has been obtained:
  (●) Yes ( ) No

Signature of Supervisor/Promoter: Prof. David D. N'Da — Digitally signed by Prof. David D. N'Da Date: 2018.10.25 10:56:00 +02'00'

Date: 25 October 2018

Preface

This thesis is submitted in an article format in accordance with the General Academic Rules (A) of the North-West University. Three articles, two of which have been published, are included in this thesis:

Chapter 3: Article 1

Non-acetal artemisinin derivative – Worth the fuss? A mini-review. *Intended to be submitted to Current Medicinal Chemistry Journal.*

Chapter 4: Article 2

Synthesis, in vitro antimalarial activities and cytotoxicities of amino-artemisinin-ferrocene derivatives. This article was published in Bioorganic & Medicinal Chemistry Letters, Volume 28, Issue 3, 1 February 2018, Pages 289-292 (https://doi.org/10.1016/j.bmcl.2017.12.057).

Chapter 5: Article 3

Synthesis, in vitro antimalarial activities and cytotoxicity of amino-artemisinin-1, 2-disubstituted ferrocene derivatives. The article was published in Bioorganic and Medicinal Chemistry Letters, Volume 28, Issue 19, 15 October 2018, Pages 3161-3163 (https://doi.org/10.1016/j.bmcl.2018.08.037).

LETTER OF AGREEMENT

Potchefstroom, 28 January 2019

TO WHOM IT MAY CONCERN

Dear Sir/Madam,

CO-AUTHORSHIP ON RESEARCH PAPER

The undersigned as co-authors of the research articles listed below, hereby give permission to Mr. Christo De Lange to submit them as part of his PhD degree thesis in Pharmaceutical Chemistry at the North-West University.

1. C. de Lange, F.J. Smit, R.K. Haynes, D.D. N'Da. ***Non-acetal artemisinin derivative – Worth the fuss?*** - *Awaiting submission.*

2. C. de Lange, D. Coertzen, F.J. Smit, J.F. Wentzel, H.N. Wong, L.-M. Birkholtz, R.K. Haynes, D.D. N'Da. "**Synthesis, in vitro antimalarial activities and cytotoxicities of amino-artemisinin-ferrocene derivatives**", *Bioorg. Med. Chem. Lett.* 2018, 28, 289-292.

3. C. de Lange, D. Coertzen, F.J. Smit, J.F. Wentzel, H.N. Wong, L.-M. Birkholtz, R.K. Haynes, D.D. N'Da. "**Synthesis, antimalarial activities and cytotoxicities of amino-artemisinin-1,2-disubstituted ferrocene hybrids**", *Bioorg. Med. Chem. Lett.* 2018, 28, 3161-3163.

Yours Sincerely,

Prof D.D. N'Da

Prof R.K. Haynes

Prof L-M Birkholtz

Acknowledgements

I hereby wish to express my appreciation to the following individuals:

- Prof DD N'Da, my Promoter, for his insight, encouragement and support throughout the study.
- Prof RK Haynes, my Co-Promoter, for his insights and ideas throughout the study.
- Dr FJ Smit, my additional Assistant Promoter, for his assistance in the laboratory and the countless hours spend revising my work.
- Dr JHL Jordaan, for his consistent support and for the collection of MS data.
- Mr A Joubert, for the collection of the NMR data.
- Dr HN Wong, for her assistance in the laboratory.
- Prof L Birkholtz and Dr D Coertzen from UP, for the antimalarial screening of the derivatives.
- Dr JF Wentzel, for the cytotoxicity and anticancer screening of the derivatives.
- The NRF, MRC and the North-West University, for financial support.
- Prof S van Dyk, Director of the School of Pharmacy, for her support.
- Prof A Wessels and Prof G Terre' Blanche for allowing me to be a demonstrator for them.
- All of my friends that encouraged and kept me going when times got tough.
- My family for their support and believe in me throughout the whole process.
- My loving wife for her constant encouragement and love.

I give thanks to my Heavenly Farther for His inspiration, grace and mercy.

Abstract

Malaria is caused by a parasite of the genus *Plasmodium*. Of the five species that infect humans, *Plasmodium falciparum* is the most dangerous. This disease caused 435 thousand deaths in 2017. It is estimated that 266 thousand of these deaths were children, under the age of five. With the reports of malaria resistance towards artemisnin, there is a desperate need for new and effective antimalarial drugs. In the search for these new antimalarial drugs, two series of artemisinin-ferrocene derivatives were prepared and investigated during this study.

A mini-review (Chapter 3) was written in order to compare the most potent non-acetal artemisinin derivatives. In order to compare these derivatives, relative activity was used. Due to the large variety of *Plasmodium falciparum* strains used it is difficult to truly compare these derivatives. The general lack of toxicity data for these derivatives makes it difficult to establish whether the activity is due to toxicity. The logP value was calculated for these derivatives to be able to estimate toxicity. It was shown that there is some connection between lipophilicity and toxicity.

The first series (Chapter 4) of amino-ferrocene-artemisinin derivatives was synthesized by the coupling of various mono-substituted ferrocene derivatives to 10α-(1′-piperazino)-10-deoxo-10-dihydroartemisinin (DHA-pip) through condensation and reductive amination. These derivatives were screened against the chloroquine-sensitive (CQ-sensitive) NF54 and chloroquine-resistant (CQ-resistant) K1 and W2 *P. falciparum* strains. Cytotoxicity was assessed against the Hek293 cell line while anticancer activity was assessed against the A375 cell line. The derivatives retained good antimalarial activity while being very selective towards parasitized cells in the presence of mammalian cells. Additionally these derivatives were in general more selective towards cancer cells in the presence of mammalian cells.

The second series (Chapter 5) of amino-artemisinin-1, 2-disubstituted ferrocene derivatives was synthesized through reductive amination of aminoferrocenealdehydes to DHA-pip. These derivatives were screened against the CQ-sensitive NF54 and CQ-resistant K1 and W2 *P. falciparum* strains. Cytotoxicity was assessed against the Hek293 cell line while anticancer activity was assessed against the A375 cell line. These derivatives were also screened against *P falciparum* NF54 gametocytes. Two of these derivatives were more active than DHA while the activity of one of these derivatives might be attributed to toxicity.

The least antimalarial active derivative was more active and selective towards cancer cells in the presence of mammalian cells.

This study resulted in a number of derivatives with different antiplasmodial activities. The derivatives of series 2 were the most active due to the single ring disubstituted ferrocene derivatives. The derivatives that were synthesized during the study illustrate a low potential for resistance and addresses the problem of P. falciparum. These derivatives could potentially serve as lead compounds for future antimalarial drugs.

Keywords: Malaria, artemisinin, ferrocene, hybrids, gametocytes

Opsomming

Malaria word deur ’n parasiet uit die genus *Plasmodium* veroorsaak. Uit die vyf spesies wat mense kan besmet is *Plasmodium falciparum* die gevaarlikste. Hierdie siekte het omtrent 435 duisend sterftes veroorsaak in 2017. Dit word beraam dat 266 duisend van hierdie sterftes was kinders onder die ouderdom van vyf jaar. Met verslae van weerstandbiedigheid teen artemisiniene, is daar ’n noodsaaklikheid vir nuwe en effektiewe anti-malariamiddels. In die soektog na hierdie anti-malariamiddels, is twee artemisinien-ferroseen reekse verbindings tydens hierdie studie gesintetiseer en ondersoek.

’n Mini-oorsig (Hoofstuk 3) was geskryf om die mees kragtigste nie-asetaal artemisinien-verbindings te vergelyk. Ten einde hierdie afgeleides te vergelyk, is relatiewe aktiwiteit gebruik. As gevolg van die groot verskeidenheid *Plasmodium falciparum* stamme, is dit moeilik om hierdie verbindings werklik te vergelyk. Die algemene tekort aan toksisiteitsdata vir hierdie verbindings maak dit moeilik om vas te stel of hierdie aktiwiteit as gevolg van toksisiteit is. Die logP waarde is bereken vir hierdie verbindings om die toksisiteit te skat. Daar was getoon dat daar ’n verband bestaan tussen lipofilisiteit en toksisiteit is. Daar is bevind dat die gebrek aan vergelykbaarheid en toksisteit die ideale teen-malariamiddel weerhou om ooit gesintetiseer te word.

Die eerste reeks verbindings (Hoofstuk 4) het aminoferroseniel-artemisiniene behels, wat deur middle van kondensasie en reduktiewe aminering van verskeie ferrosiniel intermediêre met 10α-(1′-piperasienniel)-10-deoksie -10-dihdroartemisinien (DHA-pip) gesintetiseer is. Hierdie verbindings is teen die CQ-sensitiewe NF54 en die CQ-weerstandige K1 en W2 *P. falciparum* stamme getoets. Sitotoksisiteit is geassesseer teen die Hek293 sellyn terwyl die teen-kankeraktiwiteit teen die A375 sellyn geassesseer is. Die verbindings het goeie teen-malaria aktiwiteit behou terwyl hulle baie selektief was teenoor parasiete in die teenwoordigheid van soodier selle. Daarbenewens was hierdie verbindings meer selektief teenoor kankerselle in die teenwoordigheid van soogdier selle.

Die tweede reeks (Hoofstuk 5) het amino-artemisinien 1,2-digesubstitueerde ferroseniel verbindings behels, wat deur reduktiewe aminering van aminoferrosenielalhiede en DHA-pip gesintetiseer was. Hierdie verbindings is teen die CQ-sensitiewe NF54 en die CQ-weerstandige K1 en W2 *P. falciparum* stamme getoets. Sitotoksisiteit is geassesseer teen die Hek293 sellyn terwyl die teen-kankeraktiwiteit teen die A375 sellyn geassesseer is.

Hierdie verbindings is ook teen *P. falciparum* NF54 gametosiete getoets. Twee van hierdie verbindings was meer aktief as dihidroartemisiniel alhoewel een van hierdie verbindings se aktiwiteit aan toksisiteit toegeskryf kan word. Die verbinding wat die minste aktief was teenoor malaria was weer meer aktief en selektief teenoor kankerselle in die teenwoordigheid van soogdierselle.

Hierdie studie het gelei tot ’n aantal verbindings met verskillende antiplasmodiale aktiwiteite. Die verbindings van reeks 2 was die mees aktiefste weens die enkelring digesubstitueerde ferroseniel verbindings. Die verbindings wat tydens hierdie studie gesintetiseer was toon ’n lae potensiaal vir weerstand vorming en spreek die probleem van weerstandige *P. falciparum* aan. Hierdie verbindings is potensiële leidingverbindings om as toekomstige antimalariamiddels te dien.

List of Contents

List of figures

List of tables and schemes

List of abbreviations

°C	degrees Celsius
µL	microlitre
ACT	artemisinin combination therapy
AM	artemether
ART	artemisinin
AS	artesunate
ATP	Adenosine triphosphate
CNS	Central nervous system
Cp	cyclopentadiene
CQ	chloroquine
DDT	Dichlorodiphenyltrichloroethane
DHA	dihydroartemisinin
DHF	dihydrofolate
DHFR	dihydrofolate reductase
dhfr-ts	Dihydrofolate Reductase-Thymidylate. Synthase
DHFS	dihydrofolate synthase
DHP	dihydropteroate
DHPP	dihydropteridine phosphate
DHPS	dihydropteroate synthase
DIAD	Diisopropyl azodiformate
DNA	Deoxyribonucleic acid
FAD	flavin adenine dinucleotide
$FADH_2$	reduced flavin adenine dinucleotide
fc	ferrocene
FMN	flavin mononucleotide
FQ	ferroquine
G6PD	glucose-6-phosphate dehydrogenase
GR	glutathione reductase
GSH	glutathione
GSH-Px	glutathione-dependent peroxidise
GSSG	glutathione disulfide
h	hour
HOMO	Highest occupied molecular orbital

HRMS	high resolution mass spectroscopy
i-BuLi	*iso*-butyllithium
IC_{50}	half maximal inhibitory concentration
IR	infrared
IRS	indoor residual spraying
ITN	insecticide treated nets
K_d	dissociation constant
logP	a measure of lipophilicity
LUMO	Lowest unoccupied molecular orbital
$NADP^+$	oxidised NADPH
NADPH	Nicotinamide adenine dinucleotide phosphate
n-BuLi	*normal*-butyllithium
nM	nanomolar
NMR	nuclear magnetic resonance
*p*ABA	*para*-amino benzoic acid
Pf	*Plasmodium faciparum*
*Pf*ATP6	Plasmodium falciparum Ca(2+)-ATPase
PfCRT	Plasmodium falciparum chloroquine resistance transporter
*Pf*MDR1	Plasmodium falciparum multidrug-resistance gene 1
*Pf*NHE1	Plasmodium falciparum Na+/H+ Exchanger (Pfnhe-1) Gene
PPh_3	Triphenylphosphine
PVC	polyvinyl chloride
RBC	red blood cell
RDT	rapid diagnostic test
ROS	reactive oxygen species
SERCA	sarco-endoplasmic reticulum membrane calcium ATPase
SOD	superoxide dismutase
t-BuLi	*tert*-Butyllithium
THF	tetrahydrofuran
$Trx(S)_2$	thioredoxin
$Trx(SH)_2$	Reduced thioredoxin
Trx-Px	thioredoxin-dependent peroxidise
UV	ultra violet
WHO	World Health Organization

Chapter 1:
Introduction and Problem Statement

1.1 Background

According to the World Health Organization (WHO), there were approximately 435 000 deaths in 2017. Of these deaths an astounding 92 % occurred in Africa, of which 61 % were children under the age of five (WHO, 2018). Malaria is a disease caused by an intercellular parasite of the genus *Plasmodium*. Humans can be infected with malaria by the following species: *P. falciparum*, *P. knowlesi*, *P. malariae*, *P. ovale* and *P. vivax* (Cox *et al.*, 2008). Malaria cannot be transmitted at temperatures below 20°C or above 35°C and as water is needed for mosquitoes to breed it makes sense that malaria is predominant in tropical and subtropical regions (Wernsdorfer, 2012). Additionally some of the poorest and least–developed countries fall in these regions making the availability of resources and logistics needed for malaria prevention and cure cumbersome.

The geographical distribution of *Plasmodia* species varies. *P. falciparum* is the dominant species in sub-Sahara Africa and warmer regions of Asia and South America (Gething *et al.*, 2011). *P. vivax* on the other hand is not as sensitive to cool temperatures and is dominant in India and South America. The low prevalence of *P. vivax* and *P. knowlesi* in the African continent is due to the lack of the Duffy antigen in the black African community (Cutbush *et al.*, 1950; Miller *et al.*, 1976; Neote *et al.*, 1994). This antigen is located on the surface of the red blood cells and the lack of this antigen leads to a natural resistance towards *P. vivax* and *P. knowlesi* to the individuals. Other erythrocyte disorders that grant the individual malaria immunity is Sickle cell anaemia and glucose-6-phosphate dehydrogenase deficiency (Ayi *et al.*, 2004; Williams, 2006). *P. knowlesi* is a malaria parasite found in long-tailed macaque monkeys and is transmitted to humans, mainly distributed through South East Asia (Cox-Singh *et al.*, 2008; Singh *et al.*, 2004). *P. malariae and P. ovale* is found in sub-Sahara Africa, Papua New Guinea and in South East Asia (Boutin *et al.*, 2005; Mehlotra *et al.*, 2000; Win *et al.*, 2002).

As a person is infected with malaria there is an incubation period before the onset of symptoms. Initially the symptoms of malaria manifest itself as headache, slight fever, muscle

pain and nausea similar to flu symptoms (Schlagenhauf & Steffen, 1994). As the infection progresses, it is followed by fevers due to the rupturing of eurythrocytes (James *et al.*, 1936). It is known that *P. vivax* causes a number of deaths but *P. falciparum* is the leading cause of malaria related deaths (Bartoloni & Zammarchi, 2012). The rapid reproduction of *P. falciparum* leads to high levels of parasitemia in a short amount of time (Newby *et al.*, 2008). In the majority of the cases it leads to severe malaria (Jakeman *et al.*, 1999). The most dangerous complications that can develop are cerebral malaria and severe anaemia (Goldsmith, 1997). Although this is the standard route of the manifestation of malaria symptoms there are some variations between species. It was found that *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* are all capable of asymptomatic infections (Alves *et al.*, 2002; Rojo-Marcos *et al.*, 2011; Vinetz *et al.*, 1998). Moreover some of the *P. vivax* and *P. ovale* parasites are able to become dormant and cause a relapse of malaria months or years after the initial infection (Cogswell, 1992). These two species are at greater risk of developing resistance due to relapse of malaria (Farooq & Mahajan, 2004).

It was reported by the WHO that the frequency of malaria infections dropped by 21% between 2010 and 2015. During this time the fatalities also decreased globally by 29%. These statistics were obtained by the increased efforts of the WHO to distribute insecticide treated bed nets and applying indoor residual spraying. Further to this the increased use of rapid diagnostic testing enabled physicians to rapidly distinguish between malaria and non-malaria fevers. The most effective treatment of *P. falciparum* is artemisinin based combinational therapy. This strategy was formulated by the WHO in order to protect the artemisinin class preventing it from falling victim to *P. falciparum* resistance. But alas, despite all of these efforts, there is clear evidence of artemisinin resistance.

P. falciparum has grown resistant towards chloroquine, sulfadoxine and pyrimetamine, mefloquine, atovaquone and proguanil, artemether and lumefantrine (Dondorp *et al.*, 2009; Fivelman *et al.*, 2002; Gregson & Plowe, 2005; Payne, 1987; Price *et al.*, 2004). The most effective treatment left for *P. falciparum* is the artemisinin class (Figure 1.1) (Graham *et al.*, 2010). Artemisinin (qinghaosu) is a sesquiterpene lactone extracted from sweet wormwood (Artemisia annua) (Klayman, 1985). Uncomplicated cases of malaria should be treated with artemisinin combination therapies (ACTs) while severe malaria should be treated with artesunate (Dondorp *et al.*, 2010).

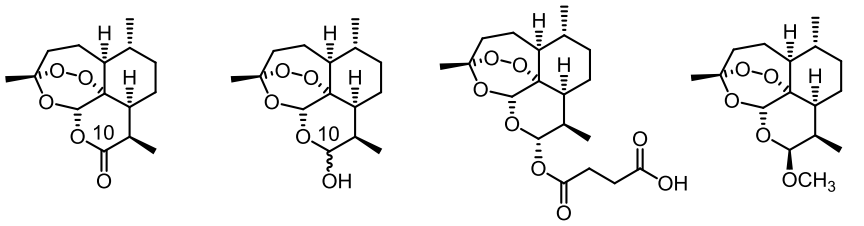


Figure 1.1: Clinically used artemisinins.

The mechanism of action of artemisinins is widely debated but the co-factor theory seems to be unifying (Haynes *et al.*, 2012). As the parasite exists in an oxidative–stressed environment it is crucial to maintain the redox homeostasis. This is achieved by the glutathione:glutathione disulfide (GSH:GSSG) ratio (Ursini *et al.*, 2016). GSH is needed by enzymes to convert damaging substances such as peroxides to water and oxygen, GSH in turn is reduced to GSSG. To convert GSSG back to GSH the flavoenzyme, glutathione reductase (GR) catalyses the reaction using nicotinamide adenine dinucleotide phosphate (NADPH) (Färber *et al.*, 1998). It was found that when yeast GR was treated with artemisinin, an increased consumption of NADPH and decreased the GSSG reduction was observed (Haynes *et al.*, 2010). Additionally GSSG can also be converted to GSH by thioredoxin which is flavin adenine dinucleotide ($FADH_2$) dependent (Jortzik & Becker, 2012). It was found by Haynes and co-workers that artemisinins oxidize reduced $FADH_2$, reduced flavin mononucleotide ($FMNH_2$), reduced riboflavin and model reduced flavins (Haynes *et al.*, 2010). The $FADH_2$ required by the parasites' redox system to function optimally are consumed by artemisinin. This leads to enhanced turnover of NADPH - eventually a choke point is reached wherein the requirements by the enzyme for NADPH exceeds the supply.

In 2002 the first sign of ACT resistance was observed with a decline in efficacy for artemisinin-mefloquine treatment (Denis *et al.*, 2002). Fourteen years later the ACT of dihydroartemisinin-piperaquine did not cure half of the patients treated (Fairhurst & Dondorp, 2016). This observation was made in Western-Cambodia, an area known for the formation of resistance towards previously used antimalarials. Moreover, the WHO identified several sites with suspected or confirmed artemisinin resistance. On the Cambodian border, the Vietnamese province of Binh Phuoc reported a higher than 10% ACT failure rate (WHO, 2016). The common metabolite of the clinically used artemisinin, which might be implicated in artemisinin resistance, is dihydroartemisinin (Davis *et al.*, 2005; Mbengue *et al.*, 2015; Paloque *et al.*, 2016). Dihydroartemisinin has a short elimination half–life of 1.9 hrs and is

stable in simulated stomach acid for 17 hrs (Jung & Lee, 1998; Teja-isavadharm *et al.*, 1996). The elimination half–life can be addressed through hybridisation with a longer acting pharmacophore while the metabolite dihydroartemisinin can be avoided through non-acetal derivatives.

A novel approach to address resistance is by developing hybrid drugs (Walsh & Bell, 2009). This is achieved by combining two pharmacophores *via* a chemical bond (Meunier & Vásquez, 2008). These pharmacophores should have different biological functions and by combining them into one hybrid drug the activity should be better than the individual components. Hybrid drugs can interact with a target in one of three different ways. Firstly the two targets are related to one another and the hybrid drug interacts with both of them simultaneously. Secondly targets are in different organelles and the hybrid drug interacts independently. Lastly both the pharmacophores of the hybrid drug has the same target.

One of the leading examples of a hybrid drug that overcame resistance is ferroquine; ferrocene incorporated into the structure of chloroquine (Figure 1.2) (Biot *et al.*, 1997). The position of ferrocene within chloroquine is important as ferroquine was the most active of the more than 50 chloroquine-ferrocene compounds screened (Dive & Biot, 2008). A contributing factor to the activity of ferroquine is that ferrocene has the ability to undergo redox reactions and generate reactive oxygen species (ROS) whereas chloroquine cannot (Dubar *et al.*, 2008).

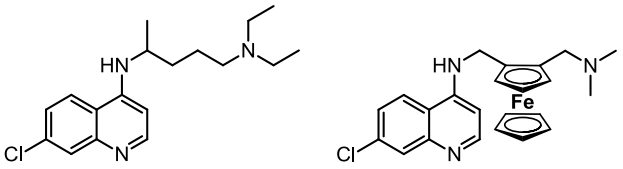
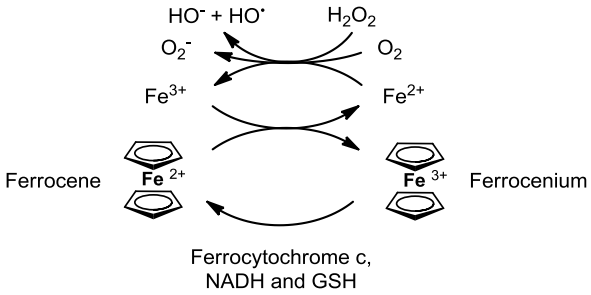


Figure 1.2: Structures of chloroquine and ferroquine.

Upon metabolism the ferrocenes' Fe^{2+} centre is capable of acting as a redox centre that undergoes redox cycling (Scheme 1.1). Ferrocene could be oxidized by free or labile Fe^{3+} to form ferrocenium (ferrocene-Fe^{3+}) (Dubar *et al.*, 2011; Kitaguchi & Yoshimura, 2010; Pladziewicz & Espenson, 1973). The newly generated free or labile Fe^{2+} is oxidized by oxygen to form superoxide which could then form hydroxyl radicals *via* the Fenton pathway. This increases the ROS which interrupts the redox homeostasis of the parasite. Ferrocenium in turn is reduced to ferrocene by metalloproteins (ferrocytochrome c), NADH and thiols such

as GSH (Carlson & Miller, 1983; Matsue *et al.*, 1987; Pladziewicz *et al.*, 1985; Pladziewicz & Carney, 1982). Unfortunately, these enzymes are only present *in vivo* and the full potential of the compounds will only become apparent in animal studies.



Scheme 1.1: Redox cycling reactions of ferrocene.

Although the mechanisms by which artemisinins exert their antimalarial activity involve ROS, thus this feature will be enhanced by linking ferrocene to the artemisinin structure. Various groups studied the effect of incorporating a ferrocene moiety onto the artemisinin structure with only one derivative showing promise. The group of Delheas synthesized an amine-containing ferrocenyl artemisinin derivative (Figure 1.3) and was the most potent towards chloroquine resistant *P. falciparum (*Delhaes *et al., 2000)*. An IC_{50} value of 14 nM was obtained against the Dd2 strain while artemisinin had a value of 13 nM. Unfortunately as many other derivatives it lacks toxicity data.

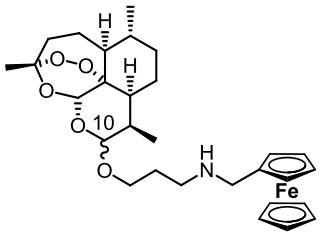


Figure 1.3: Ferrocenyl artemisinin derivative.

The metabolite of all the clinically used artemisinins is dihydroartemisinin (Figure 1.1) which is linked to both neurotoxicity (Brewer *et al.*, 1994; Schmuck *et al.*, 2002) and resistance (Mbengue *et al.*, 2015; Paloque *et al.*, 2016). This metabolite can be avoided by replacing the C10 oxygen with either a carbon or nitrogen functionality. The group of Jung was the first to synthesize and report the characteristics of such a derivative known as deoxoartemisinin (Figure 1.4) (Jung *et al.*, 1990). It was first synthesized in 1989 and was eight times more active than artemisinin. By removing the unstable acetal functionality, deoxoartemisinin had

a half-life of 231.36 hrs in simulated stomach acid compared to artemisinin that only had a half-life of 23.50 hrs (Jung & Lee, 1998). Unfortunately, as with many other derivatives, deoxoartemisinin lacks any toxicity data.

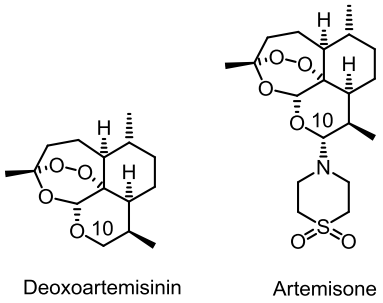


Figure 1.4: Non-acetal derivatives.

Artemisone a C-10 non-acetal alkylamino-artemisinin is a very attractive compound because it avoids the dihydroartemisinin metabolite and lacks neurotoxicity (Figure 1.4) (Haynes *et al.*, 2006; Schmuck *et al.*, 2003). It enjoys a favourable logP value of 2.49, an extended half-life compared to the commercially available artemisinins, enhanced anti-plasmodial activity and thermal stability (Haynes *et al.*, 2006; Nagelschmitz *et al.*, 2008). The elimination half-live of artemisone is 5 hours and reaches maximum blood concentrations within 1.5 hours comparable to the clinically used artemisinins (Vivas *et al.*, 2007). Artemisone was 10 times more potent than artesunate (Figure 1.1) against 12 different *P. falciparum* strains and also 4–10 times more potent than artesunate in rodent models (Vivas *et al.*, 2007). It was found that artemisone was more effective in treating cerebral malaria than artesunate. It was for these reasons that an alkylamino-artemisinin scaffold was used in this study to investigate alkylamino-ferrocene-artemisinin hybrids.

1.2 Aim and objectives

With confirmed resistance towards the artemisinin class there is a need for new artemisinin derivatives to which there is no resistance. To explore new and dual functional hybrid drugs artemisinin-ferrocene hybrids will be investigated. The proposed action of these hybrids would be that after the peroxide functionality artemisinin is destroyed the ferrocene moiety would continue to act as an ROS generator, causing additional damage to the parasite leading to its death. With the ferrocene moiety working independently, it will be a more active drug. Additionally, these derivatives will be coupled together by means of a piperazine linker with the hope that the additional amine functionalities will aid in an improved

pharmacokinetic profile for these derivative. Also these non-acetal derivatives will not be metabolised to dihydroartemisinin, eliminating the possible cause of resistance and associated toxicity.

In order to achieve the aim of this study, the following objectives had to be achieved:

- Synthesis of new amino-artemisinin-ferrocene hybrid derivatives.
- The characterisation of these hybrids by means of nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS) and infrared spectroscopy (IR).
- Determination of the *in vitro* antiplasmodial activity of all targeted hybrid derivatives.
- Determination of the *in vitro* cytotoxicity of synthesised hybrid derivatives.

1.3 References

Alves, F.P., Durlacher, R.R., Menezes, M.J., Krieger, H., Silva, L.H.P. & Camargo, E.P. 2002. High prevalence of asymptomatic Plasmodium vivax and Plasmodium falciparum infections in native Amazonian populations. *The American Journal of Tropical Medicine and Hygiene*, 66(6):641-648.

Ayi, K., Turrini, F., Piga, A. & Arese, P. 2004. Enhanced phagocytosis of ring-parasitized mutant erythrocytes: a common mechanism that may explain protection against falciparum malaria in sickle trait and beta-thalassemia trait. *Blood*, 104(10):3364-3371.

Bartoloni, A. & Zammarchi, L. 2012. Clinical aspects of uncomplicated and severe malaria. *Mediterranean Journal of Hematology and Infectious Diseases*, 4(1).

Biot, C., Glorian, G., Maciejewski, L.A., Brocard, J.S., Domarle, O., Blampain, G., Millet, P., Georges, A.J., Abessolo, H. & Dive, D. 1997. Synthesis and antimalarial activity in vitro and in vivo of a new ferrocene− chloroquine analogue. *Journal of Medicinal Chemistry*, 40(23):3715-3718.

Boutin, J., Pradines, B., Legros, F., Rogier, C. & Migliani, R. 2005. Epidemiology of malaria. *La Revue du Praticien*, 55(8):833-840.

Brewer, T.G., Peggins, J.O., Grate, S.J., Petras, J., Levine, B.S., Weina, P.J., Swearengen, J., Heiffer, M.H. & Schuster, B.G. 1994. Neurotoxicity in animals due to arteether and artemether. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 88:33-36.

Carlson, B.W. & Miller, L.L. 1983. Oxidation of NADH by ferrocenium salts. Rate-limiting one-electron transfer. *Journal of the American Chemical Society*, 105(25):7453-7454.

Cogswell, F.B. 1992. The hypnozoite and relapse in primate malaria. *Clinical Microbiology Reviews*, 5(1):26-35.

Cox-Singh, J., Davis, T.M., Lee, K.-S., Shamsul, S.S., Matusop, A., Ratnam, S., Rahman, H.A., Conway, D.J. & Singh, B. 2008. Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening. *Clinical Infectious Diseases*, 46(2):165-171.

Cutbush, M., Mollison, P. & Parkin, D.M. 1950. A new human blood group. *Nature*, 165(4188):188-189.

Davis, T., Karunajeewa, H. & Ilett, K. 2005. Artemisinin-based combination therapies for uncomplicated malaria. *The Medical Journal of Australia*, 182(4):181-185.

Delhaes, L., Biot, C., Berry, L., Maciejewski, L., Camus, D., Brocard, J. & Dive, D. 2000. Novel ferrocenic artemisinin derivatives: synthesis, in vitro antimalarial activity and affinity of binding with ferroprotoporphyrin IX. *Bioorganic & Medicinal Chemistry*, 8(12):2739-2745.

Denis, M.B., Davis, T.M., Hewitt, S., Incardona, S., Nimol, K., Fandeur, T., Poravuth, Y., Lim, C. & Socheat, D. 2002. Efficacy and safety of dihydroartemisinin-piperaquine (Artekin) in Cambodian children and adults with uncomplicated falciparum malaria. *Clinical Infectious Diseases*, 35(12):1469-1476.

Dive, D. & Biot, C. 2008. Ferrocene conjugates of chloroquine and other antimalarials: the development of ferroquine, a new antimalarial. *ChemMedChem*, 3(3):383-391.

Dondorp, A.M., Nosten, F., Yi, P., Das, D., Phyo, A.P., Tarning, J., Lwin, K.M., Ariey, F., Hanpithakpong, W. & Lee, S.J. 2009. Artemisinin resistance in Plasmodium falciparum malaria. *New England Journal of Medicine*, 361(5):455-467.

Dondorp, A.M., Yeung, S., White, L., Nguon, C., Day, N.P., Socheat, D. & Von Seidlein, L. 2010. Artemisinin resistance: current status and scenarios for containment. *Nature Reviews Microbiology*, 8(4):272-280.

Dubar, F., Egan, T., Pradines, B., Kuter, D., Ncokazi, K., Forge, D., Paul, J., Pierrot, C., Kalamou, H. & Khalife, J. 2011. The antimalarial ferroquine: role of the metal and intramolecular hydrogen bond in activity and resistance. *ACS Chemical Biology*, 6:275-287.

Dubar, F., Khalife, J., Brocard, J., Dive, D. & Biot, C. 2008. Ferroquine, an ingenious antimalarial drug–thoughts on the mechanism of action. *Molecules*, 13(11):2900-2907.

Fairhurst, R.M. & Dondorp, A.M. 2016. Artemisinin-resistant Plasmodium falciparum malaria. *Microbiology Spectrum*, 4(3).

Färber, P., Arscott, L., Williams, C., Becker, K. & Schirmer, R. 1998. Recombinant Plasmodium falciparum glutathione reductase is inhibited by the antimalarial dye methylene blue. *FEBS Letters*, 422(3):311-314.

Farooq, U. & Mahajan, R. 2004. Drug resistance in malaria. *Journal of Vector Borne Diseases*, 41(3/4):45.

Fivelman, Q.L., Butcher, G.A., Adagu, I.S., Warhurst, D.C. & Pasvol, G. 2002. Malarone treatment failure and in vitro confirmation of resistance of Plasmodium falciparum isolate from Lagos, Nigeria. *Malaria Journal*, 1(1):1.

Gething, P.W., Van Boeckel, T.P., Smith, D.L., Guerra, C.A., Patil, A.P., Snow, R.W. & Hay, S.I. 2011. Modelling the global constraints of temperature on transmission of Plasmodium falciparum and P. vivax. *Parasites and Vectors*, 4(1):92.

Goldsmith, R.S. 1997. Infectious diseases: protozoal & helminthic. *Current Medical Diagnosis and Treatment*:1294-1355.

Graham, I.A., Besser, K., Blumer, S., Branigan, C.A., Czechowski, T., Elias, L., Guterman, I., Harvey, D., Isaac, P.G. & Khan, A.M. 2010. The genetic map of Artemisia annua L. identifies loci affecting yield of the antimalarial drug artemisinin. *Science*, 327(5963):328-331.

Gregson, A. & Plowe, C.V. 2005. Mechanisms of resistance of malaria parasites to antifolates. *Pharmacological Reviews*, 57(1):117-145.

Haynes, R.K., Chan, W.C., Wong, H.N., Li, K.Y., Wu, W.K., Fan, K.M., Sung, H.H., Williams, I.D., Prosperi, D. & Melato, S. 2010. Facile oxidation of leucomethylene blue and dihydroflavins by artemisinins: relationship with flavoenzyme function and antimalarial mechanism of action. *ChemMedChem*, 5(8):1282-1299.

Haynes, R.K., Cheu, K.W., Chan, H.W., Wong, H.N., Li, K.Y., Tang, M.M.K., Chen, M.J., Guo, Z.F., Guo, Z.H. & Sinniah, K. 2012. Interactions between artemisinins and other antimalarial drugs in relation to the cofactor model—a unifying proposal for drug action. *ChemMedChem*, 7(12):2204-2226.

Haynes, R.K., Fugmann, B., Stetter, J., Rieckmann, K., Heilmann, H.D., Chan, H.W., Cheung, M.K., Lam, W.L., Wong, H.N. & Croft, S.L. 2006. Artemisone—a highly active antimalarial drug of the artemisinin class. *Angewandte Chemie*, 118(13):2136-2142.

Jakeman, G., Saul, A., Hogarth, W. & Collins, W. 1999. Anaemia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. *Parasitology*, 119(2):127-133.

James, S., Nicol, W. & Shute, P. 1936. Clinical and parasitological observations on induced malaria: *Proceedings of the Royal Society of Medicine*, 29(8):879-894.

Jortzik, E. & Becker, K. 2012. Thioredoxin and glutathione systems in Plasmodium falciparum. *International Journal of Medical Microbiology*, 302(4):187-194.

Jung, M., Bustos, D.A., ElSohly, H.N. & McChesney, J.D. 1990. A concise and stereoselective synthesis of (+)-12-n-butyldeoxoartemisinin. *Synlett* (12):743-744.

Jung, M. & Lee, S. 1998. Stability of acetal and non acetal-type analogs of artemisinin in simulated stomach acid. *Bioorganic & Medicinal Chemistry Letters*, 8(9):1003-1006.

Kitaguchi, Y.O.H. & Yoshimura, K. 2010. Oxidation reaction of 1,1′-bis (o-substituted phenyl) ferrocenes. *Organic Communications*, 3(4):92.

Klayman, D.L. 1985. Qinghaosu (artemisinin): an antimalarial drug from China. *Science*, 228:1049-1056.

Matsue, T., Suda, M., Uchida, I., Kato, T., Akiba, U. & Osa, T. 1987. Electrocatalytic oxidation of NADH by ferrocene derivatives and the influence of cyclodextrin complexation. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, 234(1-2):163-173.

Mbengue, A., Bhattacharjee, S., Pandharkar, T., Liu, H., Estiu, G., Stahelin, R.V., Rizk, S.S., Njimoh, D.L., Ryan, Y. & Chotivanich, K. 2015. A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria. *Nature*, 520(7549):683-687.

Mehlotra, R., Lorry, K., Kastens, W., Miller, S., Alpers, M., Bockarie, M., Kazura, J. & Zimmerman, P. 2000. Random distribution of mixed species malaria infections in Papua New Guinea. *The American Journal of Tropical Medicine and Hygiene*, 62(2):225-231.

Meunier, D. & Vásquez, C. 2008. On shadowing the hybrid character of actions: A communicational approach. *Communication Methods and Measures*, 2(3):167-192.

Miller, L.H., Mason, S.J., Clyde, D.F. & McGinniss, M.H. 1976. The resistance factor to Plasmodium vivax in blacks: the Duffy-blood-group genotype, FyFy. *New England Journal of Medicine*, 295(6):302-304.

Nagelschmitz, J., Voith, B., Wensing, G., Roemer, A., Fugmann, B., Haynes, R.K., Kotecka, B.M., Rieckmann, K.H. & Edstein, M.D. 2008. First assessment in humans of the safety, tolerability, pharmacokinetics, and ex vivo pharmacodynamic antimalarial activity of the new artemisinin derivative artemisone. *Antimicrobial Agents and Chemotherapy*, 52(9):3085-3091.

Neote, K., Mak, J., Kolakowski, L.J. & Schall, T. 1994. Functional and biochemical analysis of the cloned Duffy antigen: identity with the red blood cell chemokine receptor. *Blood*, 84(1):44-52.

Newby, Z.E., O'Connell Iii, J., Robles-Colmenares, Y., Khademi, S., Miercke, L.J. & Stroud, R.M. 2008. Crystal structure of the aquaglyceroporin PfAQP from the malarial parasite Plasmodium falciparum. *Nature Structural and Molecular Biology*, 15(6):619-625.

Paloque, L., Ramadani, A.P., Mercereau-Puijalon, O., Augereau, J.-M. & Benoit-Vical, F. 2016. Plasmodium falciparum: multifaceted resistance to artemisinins. *Malaria Journal*, 15(1):149.

Payne, D. 1987. Spread of chloroquine resistance in Plasmodium falciparum. *Parasitology Today*, 3(8):241-246.

Pladziewicz, J.R., Brenner, M.S., Rodeberg, D.A. & Likar, M.D. 1985. Kinetic study of the oxidation of spinach plastocyanin by ferrocenium ion derivatives. *Inorganic Chemistry*, 24(10):1450-1453.

Pladziewicz, J.R. & Carney, M.J. 1982. Reduction of ferricenium ion by horse heart ferrocytochrome c. *Journal of the American Chemical Society*, 104(12):3544-3545.

Pladziewicz, J.R. & Espenson, J.H. 1973. Kinetics and mechanisms of some electron transfer reactions of ferrocenes. *Journal of the American Chemical Society*, 95(1):56-63.

Price, R.N., Uhlemann, A.-C., Brockman, A., McGready, R., Ashley, E., Phaipun, L., Patel, R., Laing, K., Looareesuwan, S. & White, N.J. 2004. Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. *The Lancet*, 364(9432):438-447.

Rojo-Marcos, G., Cuadros-González, J., Gete-Garcia, L., Gomez-Herruz, P., López-Rubio, M. & Esteban-Gutierrez, G. 2011. Plasmodium ovale infection: description of 16 cases and a review. *Enfermedades Infecciosas y Microbiologia Clinica*, 29(3):204-208.

Schlagenhauf, P. & Steffen, R. 1994. Stand-by treatment of malaria in travellers: a review. *The Journal of Tropical Medicine and Hygiene*, 97(3):151-160.

Schmuck, G., Roehrdanz, E., Haynes, R.K. & Kahl, R. 2002. Neurotoxic mode of action of artemisinin. *Antimicrobial Agents and Chemotherapy*, 46(3):821-827.

Schmuck, G., Temerowski, M., Haynes, R. & Fugmann, B. 2003. Identification of non-neurotoxic artemisinin derivatives in vivo and in vitro. *Research Advances in Antimicrobial Agents and Chemotherapy*, 3:35-47.

Singh, B., Sung, L.K., Matusop, A., Radhakrishnan, A., Shamsul, S.S., Cox-Singh, J., Thomas, A. & Conway, D.J. 2004. A large focus of naturally acquired Plasmodium knowlesi infections in human beings. *The Lancet*, 363(9414):1017-1024.

Teja-isavadharm, P., Nosten, F., Kyle, D., Luxemburger, C., Ter Kuile, F., Peggins, J., Brewer, T. & White, N. 1996. Comparative bioavailability of oral, rectal, and intramuscular artemether in healthy subjects: use of simultaneous measurement by high performance liquid chromatography and bioassay. *British Journal of Clinical Pharmacology*, 42(5):599-604.

Ursini, F., Maiorino, M. & Forman, H.J. 2016. Redox homeostasis: The Golden Mean of healthy living. *Redox Biology*, 8:205-215.

Vinetz, J.M., Li, J., McCutchan, T.F. & Kaslow, D.C. 1998. Plasmodium malariae infection in an asymptomatic 74-year-old Greek woman with splenomegaly. *New England Journal of Medicine*, 338(6):367-371.

Vivas, L., Rattray, L., Stewart, L.B., Robinson, B.L., Fugmann, B., Haynes, R.K., Peters, W. & Croft, S.L. 2007. Antimalarial efficacy and drug interactions of the novel semi-synthetic endoperoxide artemisone in vitro and in vivo. *Journal of Antimicrobial Chemotherapy*, 59(4):658-665.

Walsh, J. & Bell, A. 2009. Hybrid drugs for malaria. *Current Pharmaceutical Design*, 15(25):2970-2985.

Wernsdorfer, W.H. 2012. Global challenges of changing epidemiological patterns of malaria. *Acta Tropica*, 121(3):158-165.

Williams, T.N. 2006. Human red blood cell polymorphisms and malaria. *Current Opinion in Microbiology*, 9(4):388-394.

Win, T.T., Lin, K., Mizuno, S., Zhou, M., Liu, Q., Ferreira, M., Tantular, I., Kojima, S., Ishii, A. & Kawamoto, F. 2002. Wide distribution of Plasmodium ovale in Myanmar. *Tropical Medicine & International Health*, 7(3):231-239.

WHO. 2016: Artemisinin and artemisinin-based combination therapy resistance: status report.

WHO. 2018. World malaria report 2018. *Geneva: WHO.*

Chapter 2:
Literature review

2.1 Introduction

In 500 B.C., the Romans described a fever called “bad air”, better known as malaria today (Hempelmann & Krafts, 2013). They observed that if the water of a swamp or marshland was drained, the number of fever incidences decreased. This created a belief that remained for more than 2000 years that malaria was caused by the vapours and mists from marshes and swamps. Hippocrates, a Greek physician, was able to differentiate between different types of malaria by describing the fevers as quotidian (with a periodicity of 24 hours), tertian (a periodicity of 48 hours) and quartan (a periodicity of 72 hours) (Strong, 1944).

It was not until 1880 that Charles Louise Alphonse Laveran observed parasites in the blood of a patient suffering from malaria (Cox, 2010). Laveran named his severe Summer-Autumn (malignant tertian) malaria *Laverania malariae*, which would later become known as the malaria caused by the *Plasmodium falciparum* parasite. The first step towards the differentiation of different types of malaria was made by an Italian neurophysiologist, Camillo Golgi (Golgi, 1886). He postulated that there were at least two types of malaria, namely tertian and quartan. He also observed that the fever of the patient coincided with the release of new parasites into the bloodstream. Four years later, two Italians, Giovanni Batista Grassi and Raimondo Filetti, were the first ones to name the two of the malaria types that affected humans, namely *P. vivax and P. malariae* (CDC, 2016b). Other species of human malaria are *P. knowlesi* and *P. ovale*.

On the 20th of August 1897, the landmark discovery in the field of malaria was made by Ronald Ross (Ross, 1897). The malaria parasite was found in the stomach tissue of an *Anopheline* mosquito that previously fed on a malaria patient. Two years later in India, using malaria in a bird model, he found that the malaria parasites developed inside the mosquitos’ stomach and then migrated to the salivary glands. This is the pathway that malaria uses to spread: during the blood meals of the mosquito. Thus, the myths surrounding malaria transmission were finally debunked.

In this chapter, the epidemiology of malaria, the malaria parasite life-cycle and the mechanisms of resistance are briefly discussed. Furthermore, the diagnosis, prevention and control, chemotherapy of malaria, ferrocene and ferrocene-artemisinins are addressed.

2.2 Epidemiology

According to the World Health Organization (WHO), there were approximately 435 000 malaria related deaths worldwide (WHO, 2018). Of these deaths, about 92% occurred in Africa, of which 61% were children under the age of five years old.

There are a number of factors that contribute to the high incidence of the disease especially in Africa. Climate plays an important role. For malaria to be successfully transmitted, the female *Anopheles* must live long enough to become infected with malaria. Afterwards, the malaria parasite have to undergo the sporogonic cycle, which takes 9-21 days at 25 °C and then cycle within the mosquito to be able to inject the human host with sporozoites species (Wernsdorfer, 2012). It was found that a temperature fluctuation of around < 21 °C speeds up parasite development (Paaijmans *et al.*, 2009). A warmer climate could also increase the human contact with mosquitoes since people may sleep outside or they will be spending more time outside at night. This is why malaria occurs so frequently in sub-Saharan Africa.

The second main reason for the wide occurence of malaria is the type of mosquitoes or vectors. There are mainly two predominant vectors in Africa, namely *Anopheles arabiensis* and *Anopheles coluzzi* (Killeen *et al.*, 2017) (Figure 2.1). These *Anopheleses* are anthropophilic, which means that they prefer to obtain their blood meals from humans. *A. coluzzi* are endophagic (preferring to bite indoors) and endophilic (preferring to rest indoors) while *A. arabiensis* are mainly exophagic (preferring outdoor biting) and exophilic (preferring to rest outdoors) (Meyers *et al.*, 2016). Although these are the main behaviour patterns for these species, it was found that there was a shift from endophagic to exophagic behaviour when the use of bed nets were introduced (Sougoufara *et al.*, 2014).

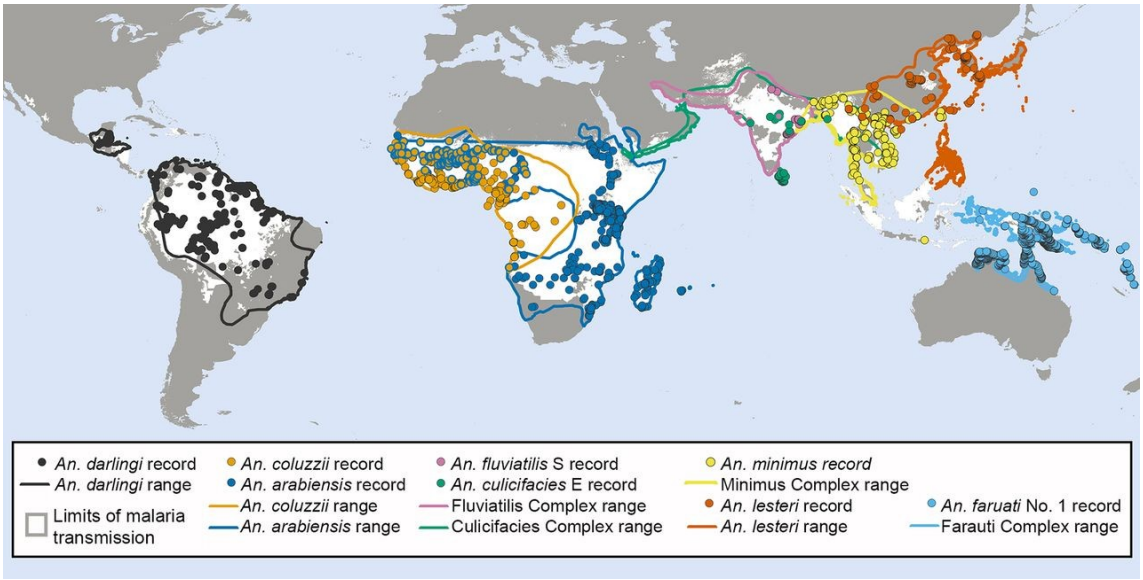


Figure 2.1: Malaria vector species feeding on humans and animals (Killeen *et al.*, 2017).

The last main reason for the large number of fatalities can be gleaned from Figure 2.2 that represents the distribution of *P. falciparum*, which is prevalent in Africa (WHO, 2010). *P. falciparum* malaria is responsible for life-threatening complications (Snow *et al.*, 2005). The most distinctive complications are cerebral malaria and severe anaemia (Pasvol, 2005). Other manifestations include respiratory distress, renal failure, hypoglycaemia, circulatory collapse, coagulation failure and impaired consciousness.

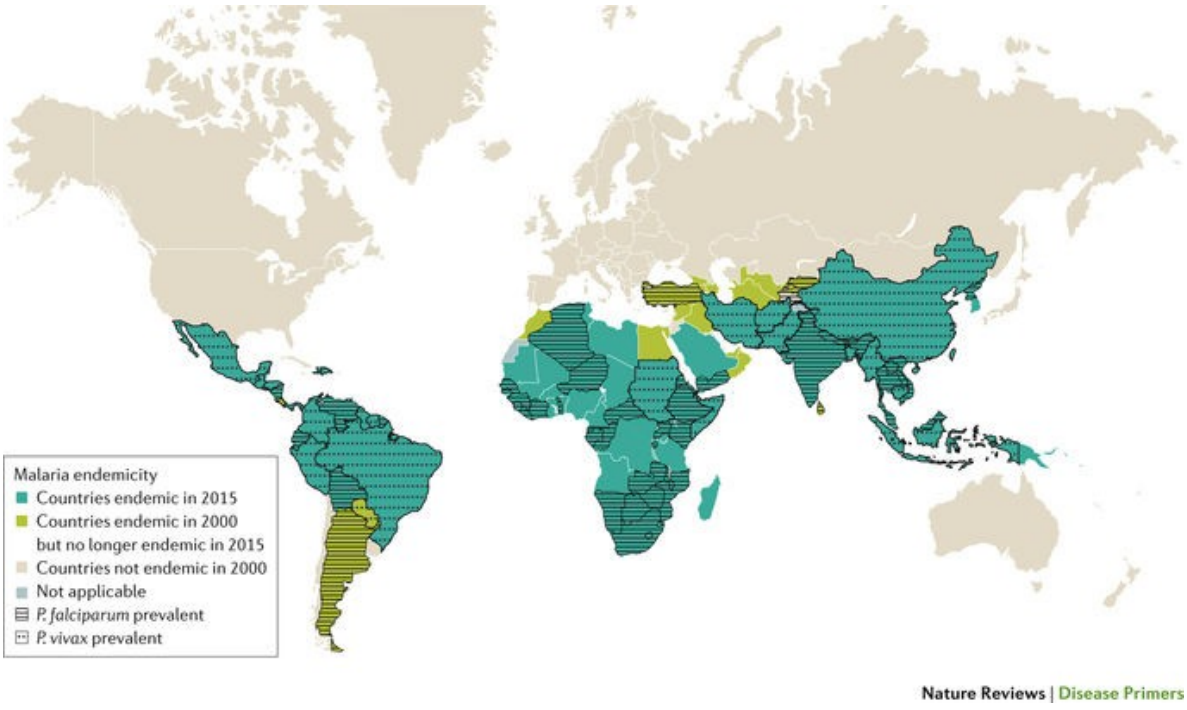


Figure 2.2: Worldwide distribution of *P. falciparum* (Phillips *et al.*, 2017).

2.3 The life-cycle of malaria

The life-cycle (**Figure 2.3**) of the malaria parasite can be divided into two main parts, namely the human cycle and the mosquito cycle. The human cycle consists of the liver A and blood stage B. As discussed in § 2.1 with Ross' discovery of the malaria sporozoites, these enter the human host through the saliva of the infected female *Anopheles* when taking a blood meal 1 (Rosenberg *et al.*, 1990). These sporozoites have a limited time (1-3 hrs) to reach the liver 2 before they are no longer motile (Ménard *et al.*, 2008). The sporozoites move through the blood capillaries and interact with the Küpffer cell to enter the liver cell or hepatocyte.

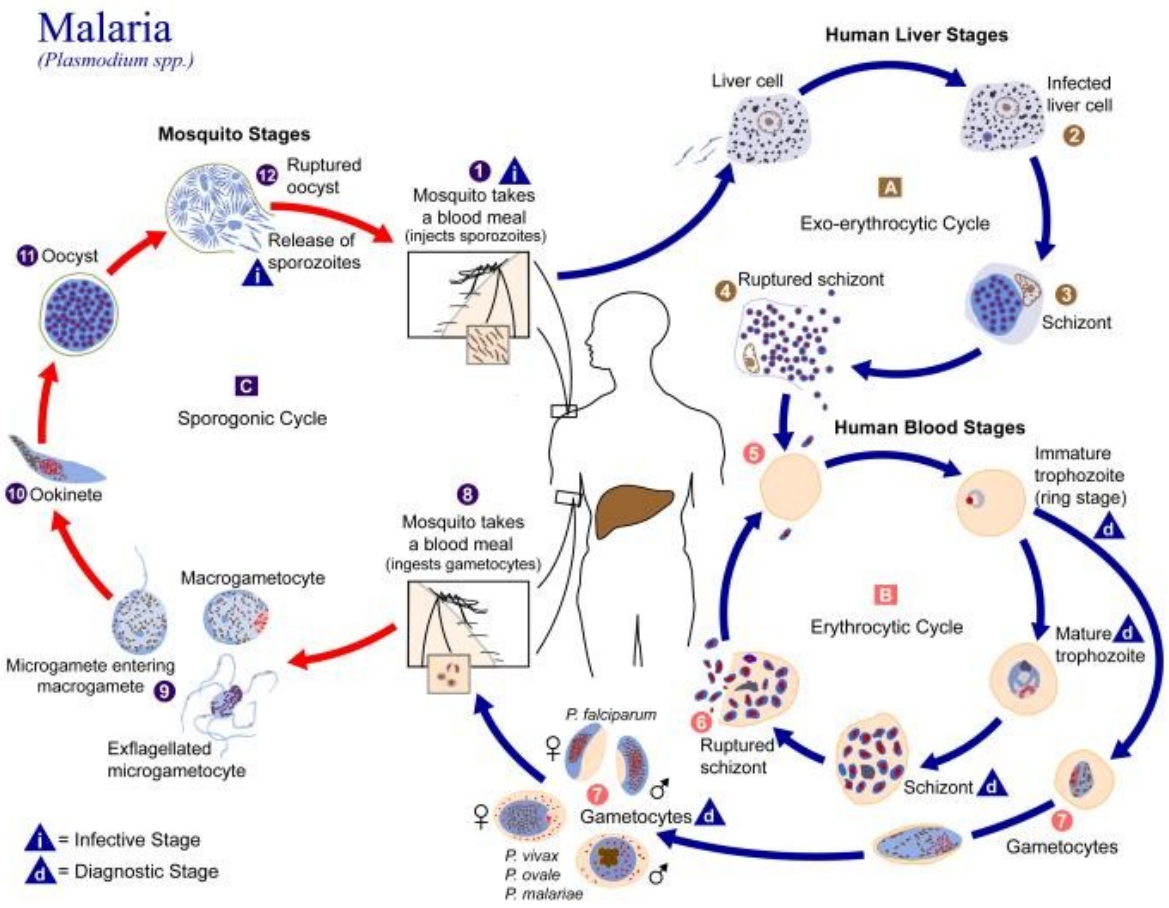


Figure 2.3: Malaria life cycle (CDC, 2016a).

2.3.1 Human liver stage

The human liver stage is also known as the exo-erythrocytic cycle A. In the hepatocytes, the sporozoites matures into forms called schizonts 3. They then undergo asexual amplification to develop into liver-stage merozoites (Doolan *et al.*, 2009). This process is called exo-erythrotic schizogony and can last for 2-10 days. After 1 to 2 weeks, a schizont can contain up to 30 000 merozoites (Cowman & Crabb, 2002).

When the liver's schizont ruptures 4, the merozoites are released into the bloodstream where they enter red blood cells and begin their erythrocytic stage of their life-cycle (Doolan *et al.*, 2009). However, not all of the *Plasmodia* follow the same route. *P. vivax* and *P. ovale* do not all form mezorites, but they form some hypnozoites. These are dormant and can remain that way for months, or even years. They can then generate merozoites, which cause a relapse of malaria (Cogswell, 1992).

2.3.2 Human blood stage

The erythrocytic stage begins when the merozoites infect red blood cells (RBCs) B. The merozoite gains entry by attaching itself to RBC 5. Reorientation follows so that the apical end can form a tight junction with the RBC. From here, it moves into the RBC and finally reseal the RBC membrane (Farrow *et al.*, 2011). Here, the merozoite flattens out into the immature trophozoite/ring stage. Its diet mainly consists of the cytosol, by endocytosis, as a source of essential amino acids and haemoglobin (Bannister *et al.*, 2000).

The ingested haemoglobin is broken down to ferriprotoporphyrin IX and is toxic due to its ability to induce redox cycling and to generate a reactive oxygen species (ROS) (Dassonville-Klimpt *et al.*, 2011; Kumar & Bandyopadhyay, 2005). The ferriprotoporphyrin IX mainly becomes detoxified inside the parasite's food vacuole where the acidic conditions, namely a pH of 5.2, promote the formation of hemozoin (malaria pigment) (Dassonville-Klimpt *et al.*, 2011; Kumar & Bandyopadhyay, 2005). The trophozoites undergo nuclear division forming schizonts and producing new merozoites in the RBC. Finally, the RBC ruptures and the merozoites are released into the bloodstream, ready to infect new RBCs (Bannister *et al.*, 2000). After a few of these cycles, some of these merozoites develop into male and female gametocytes 7. The gametocytes circulate in the peripheral circulation and are ingested by the *Anopheline* mosquito when it takes a blood meal 8 (Kuehn *et al.*, 2010).

2.3.3 Sporogonic cycle

This cycle begins when the *Anopheline* ingests blood infected with gametocytes (Kuehn *et al.*, 2010). After ingestion by the mosquito, the male and female gametocytes are released from their red blood cells in response to environmental changes, including temperature and pH changes (Sinden *et al.*, 1996). In the midgut of the mosquito, the gametocytes mature into gametes. The male gametocytes undergo division and develop flagella which turn them into motile micro gametes. These fertilise the female macro gametes to form zygotes ❾, then ookinetes ❿, and then they mature into oocysts ⓫. Within a few days, the oocysts rupture and release thousands of sporozoites ⓬ which collect within the salivary glands of the mosquito – ready to infect the next human host (Touray *et al.*, 1992).

2.4 Pathology

After a person has been infected with malaria, symptoms occur within 10–21 days. These symptoms are the result of the rupturing of the erythrocytes (Malaguarnera & Musumeci, 2002). Initially, the symptoms manifest themselves as headaches, slight fever, muscle pain and nausea – much like flu symptoms. This phase is followed by febrile attacks, also known as paroxysms (James *et al.*, 1936). The paroxysms appear in three different stages. During the cold stage, the person experiences intense feelings of cold and shivering that last between 15–50 minutes. The heat stage is characterised by feelings of intense heat, dry burning skin and a throbbing headache which lasts between 2–6 hours. Lastly, this is followed by the sweating stage where the person experiences profuse sweating, a decline in body temperature and exhaustion, leading to sleep – this stage lasts between 2–4 hours (Alvarez *et al.*, 2005). The time between these paroxysms are also indicative of the type of malaria: 24 hours for *P. knowlesi*, 48 hours for *P. falciparum*, *P. vivax* and *P. ovale* and 72 hours for *P. malariae*. The most dangerous of the *Plasmodium* species is *P. falciparum*. The main reason for this is due to the high levels of parasitaemia which lead to a higher level of destruction of the erythrocytes (Jakeman *et al.*, 1999). These levels give rise to severe malaria in 90% of cases. Complications experienced are renal failure, respiratory distress, hypoglycaemia, circulatory collapse, coagulation failure and impaired consciousness. The most dangerous complications that can occur is cerebral malaria and severe anaemia (Goldsmith, 1997).

2.5 Diagnosis

Malaria is diagnosed mainly by microscopy or by a rapid diagnostic test (RDT). Microscopy is the most widely used diagnostic method and has a detection limit of 250 parasites/μL for thin smears (Fançony *et al.*, 2013; Harchut *et al.*, 2013). The main limitation of this method is that it requires a highly trained technician which may become fatigued due to the frequent high workload associated with this profession (Ansah *et al.*, 2010; Reyburn *et al.*, 2007). This can be overcome by the use RDTs – a method that requires minimal training. RDTs work by detecting parasite specific antigens, but these may be affected by residual parasite antigens. Also, the consistency of RDTs varies between brands and batches (Alonso & Tanner, 2013; Mouatcho & Goldring, 2013).

More recently, an analytical tool called the Sight Diagnostic Parasight platform is under development and might be the future of malaria diagnosis. The detection limit for this device is currently as low as 20 parasites/μL with future updates being as low as 5 parasites/μL (Eshel *et al.*, 2017). Unfortunately, the device is currently unable to distinguish between *P. vivax* and *P. ovale* – but fortunately, the treatments for these parasites are the same. *P. falciparum* identification is, however, highly specific.

2.6 Control and prevention

The two most commonly used methods for the prevention of malaria are insecticide-treated nets (ITN) and indoor residual spraying (IRS). It is estimated that these two methods have helped to prevent 663 million cases of malaria in Africa alone (Cibulskis *et al.*, 2016). Although mosquitoes have developed some resistance to the insecticides used on ITNs, these still prevent biting during night-time use.

Currently, there are numerous new strategies formulated to combat the mosquito vector. Attractive toxic sugar baits (ATSBs) are part of the lure- and kill-strategy being evaluated. A 10% sucrose solution combined with boric acid or ivermectin is used in bait stations or is sprayed on vegetation (Barreaux *et al.*, 2017; Tenywa *et al.*, 2017). In field trials it was found that these ATSBs killed up to 90% of the mosquito population (Qualls *et al.*, 2015). Eave tubes are another part of the lure- and kill-stratagem. These tubes are simply PVC tubes covered with an insecticide-treated mesh net. As the mosquitoes try to enter the house through the tubes, they come in contact with the electrostatic insecticide on the mesh. The

insecticide is then transferred to the mosquito, which leads to its death (Andriessen *et al.*, 2015).

The behaviour of mosquitoes while mating could also be exploited. They form swarms, not higher than 3m above the ground, which could easily be sprayed with an insecticide (Diabate & Tripet, 2015). Endectocides that target mosquitoes while taking a blood meal are also being field tested, but these have short half-life times and there is a lack of understanding of the mechanism of action. Furthermore, spatial repellents ensure an environment that is free of mosquitoes – thus helping to decrease malaria transmission. One such compound is dichlorodiphenyltrichloroethane (DDT). The success of DDT is primarily attributed to it being a spatial repellent rather than being a toxic substance. This kind of substance modifies the behaviour of the mosquitoes, and currently the debate is on whether or not these should be a toxic or an irritant substance. This issue requires further investigation.

Lastly, vaccination may be an option. Over the last 10 years, there were at least 40 different vaccines that reached clinical trials. The RTS,S/AS01 vaccine, targeting the pre-erythrocytic stage, is the only vaccine that shows promise and is recommended for pilot implementation studies in Africa. During the phase 3 trials, it showed a 45.7% protection among infants over an 18 month period after 3 vaccination doses.

2.7 Chemotherapy

This chapter also introduces the main antimalarial drugs with a focus on the use of these as partner drugs in antimalarial combinational therapy (ACT). The currently used antimalarials are divided into five main pharmacological classes, namely quinoline and quinoline-based antimalarials, antifolates, antibiotics, hydroxynapthoquinones and, lastly, the artemisinin class.

2.7.1 Quinoline and quinoline based antimalarials

2.7.1.1 Aryl-amino alcohols

Quinine

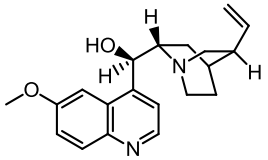


Figure 2.4: Quinine.

The bark of the fever tree (cinchora tree) has been used to treat fever since the Inca civilisation. One of the active compounds found in this bark was quinine. The bark of the cinchora tree, which was later exported to treat malaria in Europe (Bruce-Chwatt, 1988). Although the isolation of this active alkaloid is controversial it was either in Germany in 1819 or 1820 by French chemists (Meshnick & Dobson, 2001). The first successful synthesis was reported in 1944 by American chemists. This late finding is ascribed to the fact that the supply of cinchona bark to America was cut off because of the Japanese presence in the south Pacific during World War II (Schlitzer, 2007; Wacks, 2013; Woodward & Doering, 1945). Quinine is an active blood schizonticide against *P. falciparum*, *P. malaria*, *P. ovale* and *P. vivax* and it is also somewhat active gametocytocidal against *P. malaria* and *P. vivax* (Murambiwa *et al.*, 2011).

Resistance to quinine probably emerged due to the short half-life of only 8–10 hours. It is generally accepted that quinine accumulates in the parasites' acidic digestive food vacuole and inhibits haemozoin biomineralisation (Fitch, 2004). A decrease in sensitivity towards quinine was reported in Brazil in 1910 (Björkman & Phillips-Howard, 1990; Meshnick, 1997), and in some parts of Asia its efficacy has fallen below 50% (Giboda & Denis, 1988). It was found that the main 3 genes responsible for quinine resistance are *Pf* chloroquine resistance transporter (*Pf*CRT), Plasmodium falciparum multidrug-resistance gene 1 (*Pf*MDR1) and Plasmodium falciparum Na+/H+ Exchanger Gene (*Pf*NHE1) (Cooper *et al.*, 2002; Cooper *et al.*, 2007; Nkrumah *et al.*, 2009; Sidhu *et al.*, 2002).

Quinine is still widely used as a monotherapeutic drug in Africa due to its affordability (Watsierah & Ouma, 2014). The quinoline-based antimalarials are mefloquine, amodiaquine, primaquine, halofentrine and lumefantrine.

Mefloquine

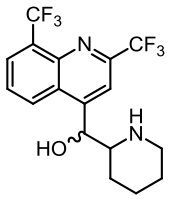


Figure 2.5: Mefloquine.

Mefloquine was discovered between 1963–1976 when the USA army launched a drug discovery programme during the Vietnam War (White, 1992). Mefloquine is a 4-methanolquinoline, with blood schizonticidal activity against the asexual stages of *P. falciparum* and *P. vivax*. Mefloquine reaches peak concentrations within 24 hours. and has an elimination half-life of 2–3 weeks (Stepniewska & White, 2008). It can be used as a prophylactic drug, but it has neuropsychiatric side effects such as psychosis, seizures, hallucinations and vertigo (Weinke *et al.*, 1991).

Resistance to mefloquine was first noted in Thailand in 1982 (Boudreau *et al.*, 1982). Resistance have also been reported in Africa, and this might be due to quinine resistance (Oduola *et al.*, 1988; White, 1994). The *P. falciparum* develops resistance to mefloquine by the amplification of the *Pf*MDR1 gene and the over-expression of its protein product Pgh-1 (Cowman & Crabb, 2002; Peel *et al.*, 1993; Wilson *et al.*, 1993).

Halofantrine

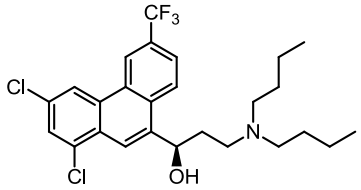


Figure 2.6: Halofantrine.

As is the case with mefloquine, halofantrine was discovered in the 1960's during the Vietnam War by the Walter Reed Army Institute of Research (Ugochukwu *et al.*, 2008). It reaches peak plasma concentrations within 4–8 hrs, and has an elimination half-life of 3–7 days for the active metabolite (de Villiers *et al.*, 2008). The use of halofantrine has been withdrawn due to the significant risk of death resulting from ventricular tachyarrhythmia. As with the previous drugs, mutations of the *Pf*MDR1 gene are responsible for causing resistance. It has

been reported that the mutations of this gene modified the transport of halofantrine, and this might be indicative of being a mechanism of resistance to this drug (Sanchez *et al.*, 2008).

Lumefantrine

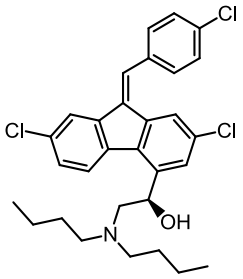


Figure 2.7: Lumefantrine.

Lumefantrine is structurally related to quinine and mefloquine, and therefore it is believed that the mechanism of action should be similar to these (Alin *et al.*, 1999). Although lumefantrine induces fewer cardio-cytotoxic side-effects, it is not without its problems (van Agtmael *et al.*, 1999). Slow absorption, low bioavailability, and having weaker antimalarial activity than halofantrine indicate that lumefantrine cannot be used as monotherapy (White *et al.*, 1999). However, the bioavailability can be increased by as much as a factor of 16 when taken with a fatty meal. The terminal elimination half-life varies between 30–107 hours. Mutations in the *Pf*MDR1 gene are associated with lumefantrine resistance (Nzila *et al.*, 2012).

2.7.1.2 4-Aminoquinolines

Chloroquine

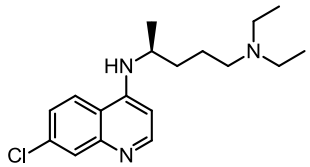


Figure 2.8: Chloroquine (CQ).

Chloroquine is a derivative of quinine and is the most widely used antimalarial drug. It was first synthesised in 1934 by Hans Andersag and his co-workers at Bayer laboratories under the trade name Resochin (Savarino *et al.*, 2003). Chloroquine is a schizonticide against chloroquine-sensitive *P. falciparum* and is used in areas with predominant *P. vivax*

transmission. It is also active towards asexual erythrocytic *P. malariae*, *P. ovale* and *P. vivax* (Murambiwa et al., 2011). It has a long half-life of approximately 60 days.

A number of the reasons for its widespread use include affordability, efficacy and low toxicity (Fidock *et al.*, 2004; Plowe, 2005). It was once so popular that it was added to table salt in an effort to eradicate malaria, but by the end of the 1950's resistance appeared at the Thai-Cambodian border and South America (Contacos *et al.*, 1963; Eyles *et al.*, 1963; Winstanley *et al.*, 2002). In the late 1970's, the first reports of resistance in East Africa emerged (Peters, 1970). Chloroquine might increase the infectivity of gametocytes *in vivo* (Ramkaran & Peters, 1969). Polymorphisms in *Pf*CRT are the main reason for chloroquine resistance (Sidhu *et al.*, 2002).

Amodiaquine

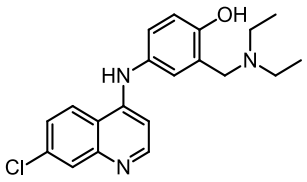


Figure 2.9: Amodiaquine.

Amodiaquine is structurally related to chloroquine, but differs in the side chain – which gives it efficacy towards some of the chloroquine-resistant strains of *P. falciparum*. Amodiaquine is not currently used as prophylaxis due to rare hepatoxicity and agranulocytosis after long-term prophylaxis. Its use was discouraged since the 1980's (Taylor & White, 2004). During the 1990's, amodiaquine was reconsidered for use after the wide-spread chloroquine resistance emerged. This drug is currently used in West Africa for the treatment of uncomplicated malaria (Gil & Berglund, 2007; Kremsner & Krishna, 2004). Amodiaquine has a short half-life of 4–12 hours, but is rapidly metabolised to N-desethylamodiaquine which has a long half-life of 9–18 days (Holmgren *et al.*, 2006; Li *et al.*, 2002). It was also found that amodiaquine has an enhanced affinity for an intraparasitic binding site over chloroquine, which explains the increased level of accumulation (Hawley *et al.*, 1996). Resistance has been reported in South America, Asia and East Africa (Khaliq *et al.*, 1987; Kremsner *et al.*, 1988; Mutabingwa *et al.*, 2005). *Pf*CRT and *Pf*MDR1 mutations are linked to a decreased susceptibility.

Piperaquine

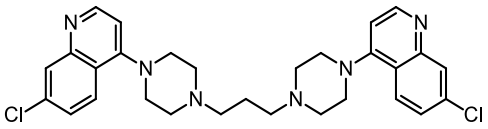


Figure 2.10: Piperaquine.

Piperaquine is a bisquinoline, that was synthesised by both the Shanghai Pharmaceutical Industry Research Institute in China and Rhone Poulenc in France during the 1960's (Chen *et al.*, 1982). The use of piperaquine declined in the 1980's with the development of resistance, because it was the primary anti-malarial in China and was used on large scale (Davis *et al.*, 2005). The bulkiness of piperaquine inhibits the transporters that efflux compounds from the parasites food vacuole (O'Neill *et al.*, 1998; Vennerstrom *et al.*, 1992). Evidence also suggests that the haeme-digestion pathway in the parasite is inhibited (Davis *et al.*, 2005). It is currently used in conjunction with dihydroartemisinin in the ACT drug regime (O'Neill *et al.*, 2011).

2.7.1.3 8-Aminoquinolines

Primaquine

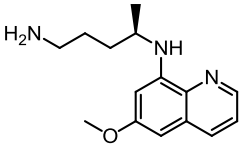


Figure 2.11: Primaquine.

As with many of the other antimalarials, primaquine emerged due to war-driven research. This drug was researched during the War of the Pacific and became available to American troops during the Korean War (Baird & Hoffman, 2004). Primaquine is effective against all *Plasmodia* gametocytes as well as hypnozoites of *P. vivax* and *P. ovale* (Baird *et al.*, 1995). It has a short half-life of only 6 hours, which might contribute to the absence of resistance after 50 years (Arnold *et al.*, 1961). However, primaquine is dangerous to patients with a glucose-6-phosphate dehydrogenase (G6PD) defiency and may lead to haemolytic anaemia (Burgoine *et al.*, 2010). Although the mechanism of action is unknown, studies showed that primaquine accumulates in the mitochondria and results in structural changes to the inner membranes, thus destroying the parasites' mitochondrial function (Krungkrai *et al.*, 1999;

Lanners, 1991; Peters *et al.*, 1984; Rotman, 1975). Other studies suggest that primaquine binds with *Pf*CRT and inhibits CQ transport, this could lead to synergism between the two antimalarials and reverse CQ resistance (Bray *et al.*, 2005; Egan, 2008; Sanchez *et al.*, 2004).

2.7.2 Hydroxynaphthoquinones

Atovaquone

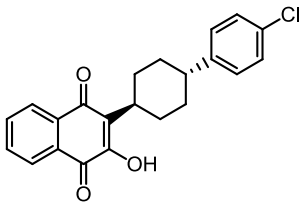
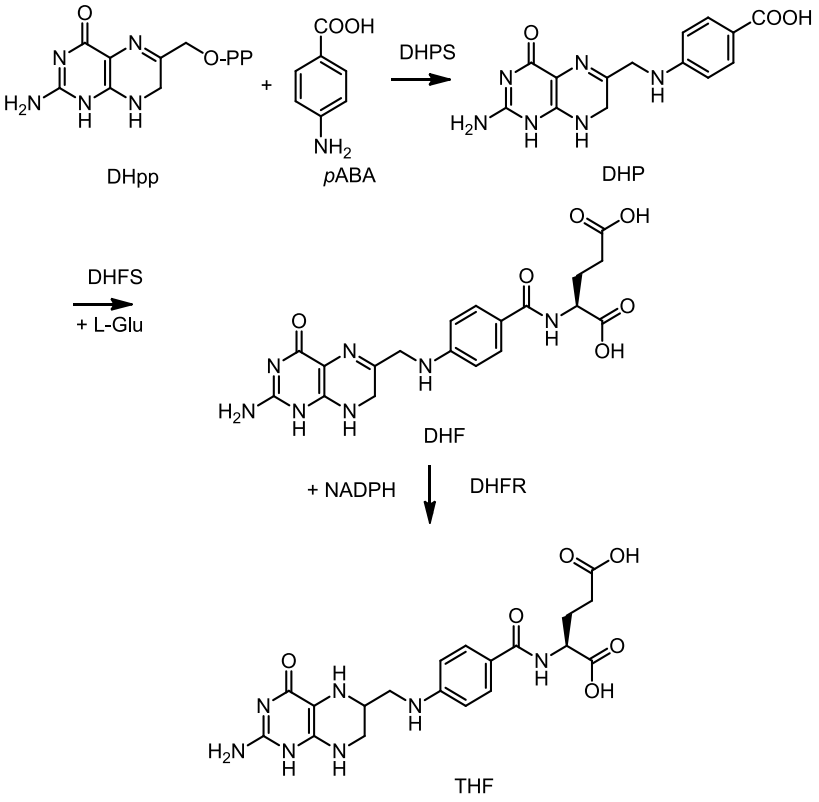


Figure 2.12: Atovaquone.

Atovaquone was introduced in 1996, with the first reports of resistance in Thailand emerged later that year (Wongsrichanalai *et al.*, 2002). Being effective against chloroquine-resistant *P. falciparum*, it is used in conjunction with proguanil (see, Antifolates) (Looareesuwan *et al.*, 1999). The main mechanism of action of atovaquone is that it interferes with the mitochondrial electron transport (Fry & Pudney, 1992; Painter *et al.*, 2007; Srivastava *et al.*, 1997). Because the asexual blood stage of the parasite relies on glycolysis for an energy rather than ATP, a disruption of the pyrimidine biosynthesis might explain the activity of antovaquone (Waters & Edstein, 2011). Resistance is due to single-point mutations in a gene encoding cytochrome *b* in the parasite (Korsinczky *et al.*, 2000).

2.7.3 Antifolates

Antifolates disrupt the folic acid synthesis pathway that supplies cofactors which are essential for the synthesis of amino acids and DNA (Babiker & Mackinnon, 2005). This pathway (**Scheme 2.1**) begins by the condensation of dihydropteridine phosphate (DHPP) with *para*-amino benzoic acid (*p*ABA) by dihydropteroate synthase (DHPS) to dihydropteroate (DHP). Glutamate is added to DHP by dihydrofolate synthase (DHFS) forming dihydrofolate (DHF). DHF is reduced to form tetrahydrofolate (THF) and is catalysed by dihydrofolate reductase (DHFR) (Djapa *et al.*, 2006).



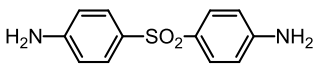
Scheme 2.1: Folic acid synthesis pathway.

Antifolate agents are divided into two classes: class I inhibits DHPS and class II inhibits DHFR.

Class I DHPS inhibitors:

These antifolates mimic *para*-aminobenzoic acid (*p*ABA) and inhibit the formation of DHP (Hawser *et al.*, 2006; Olliaro, 2001). The sulfadrugs are divided into two groups, namely sulphonamides and sulphones (Michel, 1968).

Dapsone



27

Figure 2.13: Dapsone.

Dapsone was first synthesised in 1908 and is the most potent DHPS inhibitor of malaria (Ford, 2000). Due to the high toxicity and limited efficacy, development of this drug has since

come to a halt (Rieckmann *et al.*, 1968; Sheehy *et al.*, 1967). Maloprim® is a combination of dapsone and pyrimethamine and is used in combination with chloroproguanil for treating malaria.

Sulfalene

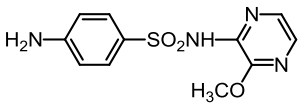


Figure 2.14: Sulfalene.

The first published report of sulfalene was in 1960 and was synthesised at Farmitalia (Anand, 1983; Baruffa, 1966). Sulfalene is used in combination with pyrimethamine under the trade name of Metakelfin®.

Sulfadoxine

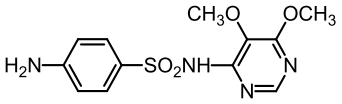


Figure 2.15: Sulfadoxine (**29**).

Sulfudoxine is combined with pyrimethamine under the trade name Fansidar®.

Class II DHFR inhibitors:

These antifolates inhibit DHFR and prevent the NADPH-dependent reduction of DHF to THF (Olliaro, 2001). Class II antifolates are progaunil, chloroprogaunil and pyrimethamine.

Proguanil

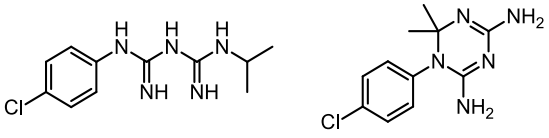


Figure 2.16: Proguanil and metabolite cycloguanil.

This drug was discovered in 1945 through a British research programme by Imperial Chemical Industries during the Second World War (Curd *et al.*, 1945). Progaunil is a prodrug that metabolises into cycloguanil, which is the inhibitor of DHFR (Carrington *et al.*, 1951).

Malarone® is a combination of proguanil and atovaquone that acts synergistically, although the mechanism for this synergy is not yet understood (Kain, 2003).

Chlorproguanil

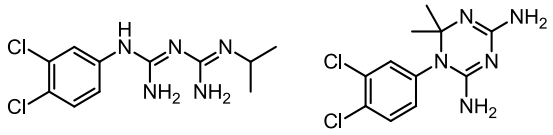


Figure 2.17: Chloroproguanil and metabolite chlorocycloguanil.

Chlorproguanil is synthesised when proguanil is chlorinated. This is also a pro drug where upon metabolism the active metabolite chlorocycloguanil is generated (Watkins *et al.*, 1988).

Pyrimethamine

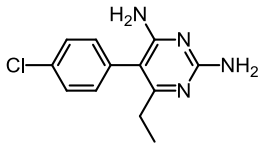


Figure 2.18: Pyrimethamine.

Pyrimethamine was synthesised in the late 1940s and belongs to the 2, 4-diaminopyrimidine family. It was noted by researchers that pyrimethamine is the most widely used antimalarial antifolate (Hitchings *et al.*, 1950). Development of pyrimethamine resistance is associated with point mutations in the *dhfr* domain of the *dhfr-ts* gene (Foote *et al.*, 1990; Peterson *et al.*, 1990; Peterson *et al.*, 1988).

2.7.4 Antibiotics

After the discovery of antibiotics, these were tested on animal malaria models. In 1952, a number of antibiotics were tested of which 8 showed activity towards malaria (Coatney & Greenberg, 1952). Unfortunately, these drugs were too slow-acting to be of clinical use (Coatney & Greenberg, 1952). This might have been the first observation of the delayed-death effect by antibiotics. Several groups described this effect with relation to various antibiotics (Dahl & Rosenthal, 2007; Dahl *et al.*, 2006; Goodman *et al.*, 2007; Ramya *et al.*, 2007; Sidhu *et al.*, 2007). When the *P. falciparum* parasites are treated with antibiotics, the parasites proceed through the erythrocytic life-cycle and are released as merozoites. These merozoites invade new erythrocytes to form schizonts. The schizonts were found to be

unable to form functional merozoites (Dahl *et al.*, 2006). There are three antibiotics used in the treatment of malaria are tetracycline, doxycycline and clindamycin. Antibiotics are used for the treatment of uncomplicated malaria in combination with quinine or artesunate.

Tetracycline

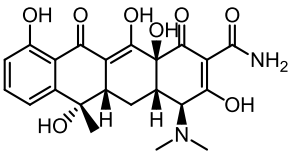


Figure 2.19: Tetracycline.

Tetracycline was synthesised in 1952 by Pfizer chemist Lloyd Conover (Conover, 1984). The bacteria *Streptomyces* naturally produce cycline from which the synthetic antibiotic tertracycline is derived (Tan *et al.*, 2011). It was indicated by the group of Dahl that the site of action of tetracycline is the apicoplast of the parasite (Dahl & Rosenthal, 2007; Dahl *et al.*, 2006).

Doxycycline

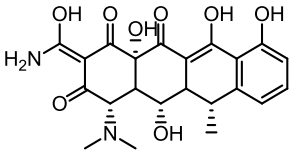


Figure 2.20: Doxycycline.

Doxycycline is a semi-synthetic derived from oxytetracycline and was discovered in the early 1960's. Pfizer patented the synthesis under U.S. Pat. No. 3200149 (1965) (Beereboom *et al.*, 1965). The mode of action is similar to that of tetracycline. Doxycycline is used as prophylaxis by aircrews and divers where mefloquine cannot be used due to its neuropsychiatric side-effects (Ashley & White, 2005). Doxycycline has a half-life of 15 – 25 hours (Tan *et al.*, 2011)

Clindamycin

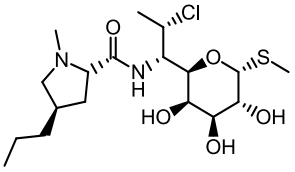


Figure 2.21: Clindamycin.

Clindamycin was first synthesised in 1966 (Magerlein *et al.*, 1966). Clindamycin is derived from lincomycin produced by *Streptomyces lincolnensis* (Mason *et al.*, 1963). As with tetracyclines, clindamycin targets the apicoplast (Goodman *et al.*, 2007). Clindamycin has an elimination half-life of 2–3 hours and is safe for use by children and pregnant women (Lell & Kremsner, 2002).

2.7.5 Artemisinins

2.7.5.1 Introduction

In China, an infused tea of qinghao (sweet wormwood, *Artemisia annua*) was used to treat fever and chills. It was later discovered that its active ingredient is artemisinin (Tu, 2016). Artemisinin is a sesquiterpene lactone and derives its antimalarial activity from the peroxide bridge. It targets the asexual stage of the infection. It is predominantly active against the ring and trophozoite stages of infection. Artemisinins are currently used in combination therapies in order to protect the artemisinin from development of resistance as has been the case with most previous antimalarials.

The mechanism of action of artemisinin is not fully understood and a range of theories has been put forward. The theories that are explored here are the inhibition of *Pf*ATP6, haeme pathway, protein alkylation, mitochondrial function, parasite membrane and co-factor.

2.7.5.2 Mechanisms of action

Inhibition of PfATP6

It was first hypothesised that the mechanism of action of artemisinin is the inhibition of the enzyme *Pf*ATP6. This enzyme is important for the oxidative metabolism within the parasite (Eckstein-Ludwig *et al.*, 2003). This theory developed after the discovery that thapsigargin

inhibits the mammalian sarco-endoplasmic reticulum membrane calcium ATPase (SERCA) (Karunaweera *et al.*, 1992). Thapsigargin is a sesquirterpene lactone, and artemisinin showed similar activity on mammalian SERCA (Eckstein-Ludwig *et al.*, 2003). The connection is that *Pf*ATP6 is the only enzyme that is similar to these mammalian SERCA. The group of Eckstein-Ludwig conducted experiments and presented three findings:

1- Artemisinin and thapsigargin inhibits *Pf*ATP6
2- There were no effects on non SERCA Ca^{2+} ATPase or other malaria transporters
3- When the parasitized erythrocytes were incubated with an iron chelator, artemisinin had no effect.

Unfortunately, this theory was discredited when it was shown that artemisinin did not bind to *Pf*ATP6 (Abiodun *et al.*, 2013; Arnou *et al.*, 2011). It is speculated that this inhibition of *Pf*ATP6 rather involves the regulation of calcium due to elevated oxidative stress caused by artemisinin (Haynes *et al.*, 2012).

Haeme pathway

Haeme consists of a Fe^{2+} centre within porphyrin. Haeme proposed to activate the peroxide group of artemesinin, which is then held to generate carbon-centered free radicals (Woodrow *et al.*, 2005; Zhang & Gerhard, 2008). These radicals are supposed to alkylate heme (Berman & Adams, 1997). The alkylated heme then is unable to be transformed into non-toxic hemozoin (Karunajeewa, 2011). The group of Meshnick were the first to identify haeme-drug adducts (Meshnick *et al.*, 1994; Meshnick *et al.*, 1991). On the other hand, it was demonstrated that artemisinin was able to inhibit the formation of β-haematin, but C10-deoxyartemisinin, lacking oxygen at the C10 position was unable to inhibit this formation (Haynes *et al.*, 2003). This illustrates that the reactivity was not related to antimalarial action (Haynes, 2005). Additionally, the observation was made that there is a scarcity of haeme-artemisinin adducts in rodent malaria models (Krishna *et al.*, 2006).

Protein alkylation

The group of Ying-Zi found that artemisinin was able to form covalent adducts with protein (Ying-Zi *et al.*, 1993; Ying-Zi *et al.*, 1994). It was found that when dihydroartemisinin reacts with haemoglobin, 80% of the dihydroartemisinin was attached to the globin part. This was illustrated by radioactively labelling six malarial proteins using artemisinins (Asawamahasakda *et al.*, 1994). These proteins were not strain or stage-specific. These

proteins appeared to be associated with the parasite membranes. Finally, it was concluded that the radicals generated by artemisinins would not be sufficient to act as alkylating agents (Haynes *et al.*, 2013).

Mitochondrial function hypothesis

This hypothesis was presented when it was found that there was a selective toxicity within both the mitochondria of yeast and malaria parasites, but not on the mitochondria of mammalian cells (Wang *et al.*, 2010). It was observed that when the mitochondria of yeast and malaria parasites were treated with artemisinin, there was an increase in the production of reactive oxygen species (ROS). When deoxyartemisinin was used (lacking the endoperoxide bridge), there was no production of ROS or mitochondria toxicity. This theory was discredited after treating infected RBCs with 40 times the IC_{50} value of artemisinin for 4 hours, with no obvious effect on the morphology of the mitochondria (del Pilar Crespo *et al.*, 2008).

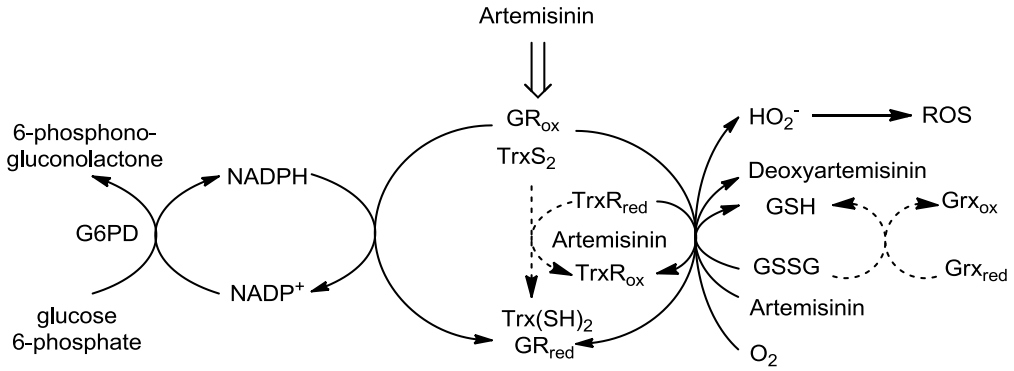
Parasite membrane

The parasite membrane was identified as a possible target after it was observed that artemisinin causes early effects on the morphology of the digestive vacuole (del Pilar Crespo *et al.*, 2008). After 4 hours of treating infected RBCs with artemisinin, punctured structures were observed in either the DV or the cytoplasm of the parasite. It was established that artemisinin caused membrane damage by accumulating within the neutral lipids (Hartwig *et al.*, 2009). However, it was found that artemisinin did not cause the same extent of oxidative degradation of the phospholipids as tetraoxanes did (Kumura *et al.*, 2009).

Antioxidant system: Co factors

The antioxidant system is needed to maintain a redox environment for normal cell function. This redox state is regulated by the ratios of reduced and oxidized nicotinamide adenine dinucleotide phosphate ($NADPH:NADP^+$), glutathione (GSH:GSSG) and thioredoxin ($Trx(SH)_2:Trx(S)_2$) (**Scheme 2.2**). In order to protect the cell from ROS damage, superoxide dismutase (SOD) converts the ROS species to hydrogen peroxide and oxygen (Fridovich, 1972). Hydrogen peroxide is then reduced to water and oxygen by haem-peroxidase catalase, glutathione-dependent peroxidise (GSH-Px) and thioredoxin-dependent peroxidise (Trx-Px) (Sies, 1997; Wood *et al.*, 2003). GSH-Px and Trx-Px obtain their reducing

equivalents from the glutathione and the thioredoxin redox systems (Arnér & Holmgren, 2000; Becker *et al.*, 2000; Filomeni *et al.*, 2002; Yodoi *et al.*, 2001). In order to convert peroxide to less damaging substances, 2 GSH molecules are needed and are converted to a glutathione disulfide (GSSG). Glutathione reductase (GR) is the enzyme that is needed to convert GSSG back to GSH (Ulusu *et al.*, 2000). GR is a flavin adenine dinucleotide ($FADH_2$)-dependent enzyme. Additionally, NADPH is needed in these reactions and is supplied by the rate-limiting step in the hexose monophosphate shunt.



Scheme 2.2: Antioxidant system (Haynes *et al*., 2012).

During the erythrocytic cycle of the *P. falciparum*, it finds itself in a highly oxidative-stressed environment. Oxidative stress is caused by ferriprotoporhyrin IX leaking into the cytosol, the release of superoxide due to the oxidation of haemoglobin iron and from the mitochondrial electron transport chain (Jortzik & Becker, 2012). The redox homeostasis of the parasite is dependent on the GSH:GSSG ratio. GSSG can be recycled by GR but also by thioredoxin, plasmoredoxin and dihydrolipoamide-dependent reactions (Becker *et al.*, 2003a; Becker *et al.*, 2003b; Kanzok *et al.*, 2000). The *P. falciparum* GR specifically reduces GSSG and is heavily FAD and NADPH dependent.

When yeast GR was treated with artemisinin, an increased consumption of NADPH and a decrease of GSSG reduction were observed. It was found by Haynes and co-workers that artemisinins rapidly oxidize reduced flavin cofactors such as reduced flavin adenine dinucleotide ($FADH_2$), reduced flavin mononucleotide (FMN), reduced riboflavin and model reduced flavins (Haynes *et al.*, 2010). The $FADH_2$ required by GR and or TrxR for the production of GSH is consumed by artemisinin. It is postulated that the decrease in GSH leads to a burst of ROS as associated with artemisinin. Additionally, Fe^{3+} can oxidize the reduced flavin co-factor of GR after artemisinin exposure. A decrease in GSH could also lead to cell death (Seiler *et al.*, 2008; Yang & Stockwell, 2016). This mechanism of action does not support the idea of artemisinins reacting with Fe^{II} in order to exert their antimalarial

activity (O'Neill *et al.*, 2010). It is well known that Fe^{II} participates in Fenton reactions generating ROS prior to the addition of artemisinins (Goldstein & Meyerstein, 1999; MacFaul *et al.*, 1998; Walling, 1998). The group of Haynes concluded that the clean production of carbon radicals from oxygen radicals from artemisinins is highly unlikely due to the complexity of the reaction (Haynes *et al.*, 2007).

2.7.5.3 Artemisinin and its first generation semisynthetic peroxides

Artemisinin is a sesquiterpene lactone and derives its antimalarial quality from the endoperoxide incorparated within the trioxane moiety (Haynes, 2001). Klayman described the following properties of artemisinin; it is poorly soluble in water or oil, it decomposes in protic solvents, and is soluble in most aprotic solvents (Klayman, 1985). Other derivatives of artemisinin that is used in treating malaria include dihydroartemisinin (DHA), artesunate and artemether (Figure 2.22). The ester derivative artesunate metabolises to dihydroartemisinin while clinically used artemisinin artemether undergo oxidative dealkylation and revert to dihydroartemisinin (Haynes, 2006).

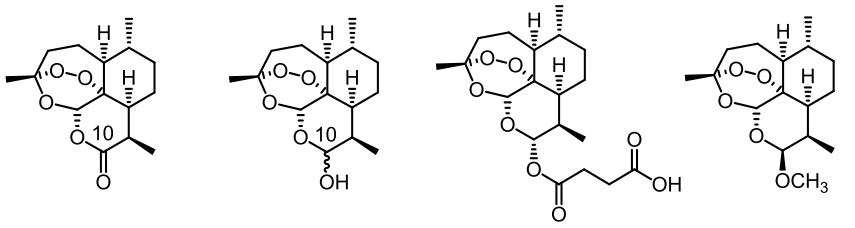


Figure 2.22: Artemisinin and the clinically used artemisinins.

DHA is the active metabolite of these artemisinins and has a half-life of 45 minutes (Batty *et al.*, 1998; Ilett *et al.*, 2002). Due to the short half-life of these artemisinin derivatives there is a minimal period of time available for the selection of resistant strains (Stepniewska & White, 2008). White reported that if artemisinin was used as monotherapy, 10% of the patients failed treatment (White, 2008). Recrudescence and a reduced efficacy would eventually lead to parasites developing resistance.

In order to be able to use artemisinin for treatment, the solubility had to be improved. The first route that was followed was by making these derivatives more oil-soluble. This was done by the China Cooperative Research Group in 1982. Artemisinin was reduced by sodium borohydride to give dihydroartemisinin which was converted to artemether and arteether (China Cooperative Research, 1982). In order to make artemisinin more water-soluble, artesunate was synthesised from dihydroartemisinin (Ploypradith, 2004).

Although the solubility problem was addressed by the above derivatives, new problems arose. Artemether and arteether suffer from poor and erratic absorption after intramuscular injection (Kager *et al.*, 1994; Teja-isavadharm *et al.*, 1996). These derivatives appear to be neurotoxic and might be due to the metabolism of these derivatives to dihydroartemisinin (Brewer *et al.*, 1994; Maggs *et al.*, 1997). It was found that when artemether and arteether in oil formulations have a slow release which translates into longer exposure times injection (Li *et al.*, 1999). Artesunate is unstable in the formulation medium for intravenous administration (Barradell & Fitton, 1995).

2.7.6 Artemisinin combinational therapy (ACT)

Although the artemsinin class is effective for treating malaria, when used as monotherapy there is a high rate of recrudescence. A 5-day treatment regime had a 25% recrudescence rate (Hien & White, 1993; McIntosh & Olliaro, 1999). Because of poor patient compliance, a 7-day regime was found to be impractical.

With ACT treatment, the artemisinin component rapidly reduces the parasite numbers 100 to 1000 fold per cycle rapidly (White *et al.*, 2008). This treatment provides rapid relief of symptoms, but artemisinin derivatives are also eliminated quickly. The partner drug has a longer half-life that provides parasite clearance after the last dose is taken (Karunajeewa, 2011). With the combination of different drugs with different mechanisms of action, the chances of resistance towards the artemisinin class are decreased. The five approved ACTs are artesunate-mefloquine, artemether-lumefantrine, artesunate-amodiaquine, artesunate-sulfadoxine-pyrimethamine and dihydroartemisinin-piperaquine.

2.7.7 Resistance towards artemisinin

Suspected resistance occurs when there is an increased parasite clearance time in more than 10% of cases with detectable parasites on day 3 of treatment with an ACT. Confirmed resistance occurs when there is treatment failure with an oral artemisinin-based monotherapy with adequate antimalarial blood concentration, as evidenced by the persistence of parasites for 7 days, or the presence of parasites on day 3 and recrudescence within 28 or 48 days.

The emergence of resistance towards the ACT treatment was first reported in 2002 with a decrease in efficacy. Studies on the Cambodia-Thailand border over several years revealed the following artesunate-mefloquine cure rates: in 2002 it was 85.7%, in 2003 it was 78.6% and in 2004 it was 79.3% (Denis *et al.*, 2006; Vijaykadga *et al.*, 2006). Artesunate-mefloquine was first introduced in Cambodia in 2000 (Wongsrichanalai & Meshnick, 2008). It was later decided to change the drug policy in areas with a higher than 10% treatment failure rate to artesunate-mefloquine. The efficacy of dihydroartemisinin-piperaquine was evaluated in Cambodia and it was found that the 28-day cure rate was 96.6% (Denis *et al.*, 2002). Unfortunately it was later reported that in some areas of Western Cambodia, dihydroartemisinin-piperaquine failed to cure half of all patients treated (Fairhurst & Dondorp, 2016). Even more worrisome is the emergency response to artemisinin resistance in the greater Mekong sub-region report, by the WHO, that identified at least 17 sites (from 2013 to 2015) where there is suspected or confirmed artemisinin resistance (WHO, 2013). In the "Artemisinin and ACT resistance October 2016" report the failure rate in the Binh Phuoc province is still higher than 10% (WHO, 2016b).

When artemisinin is used as monotherapy, there is recrudescence even though it has the ability to reduce parasites 10 000 fold per cycle (White, 1997). It was originally thought that the recrudescence was due to the short half-life of artemisinins that results in inadequate drug concentrations for a sufficiently long period of time to kill the parasites (Giao *et al.*, 2001). It was observed that treating patients with artemisinin for 3 hours a day for a week was more effective than a continuous treatment for 72 hours (Bwijo *et al.*, 1997a; Bwijo *et al.*, 1997b). It was proposed that the recrudescence is due to a survival mechanism of the parasite (Hoshen *et al.*, 2000; Kyle & Webster, 1996). When the early ring-stage intra-erythrocytic cycle of the parasite is exposed to the artemisinin class a portion of the parasites becomes dormant (Cheng *et al.*, 2012; Teuscher *et al.*, 2012; Teuscher *et al.*, 2010). When the artemisinin concentration drops below the therapeutic level, the dormant

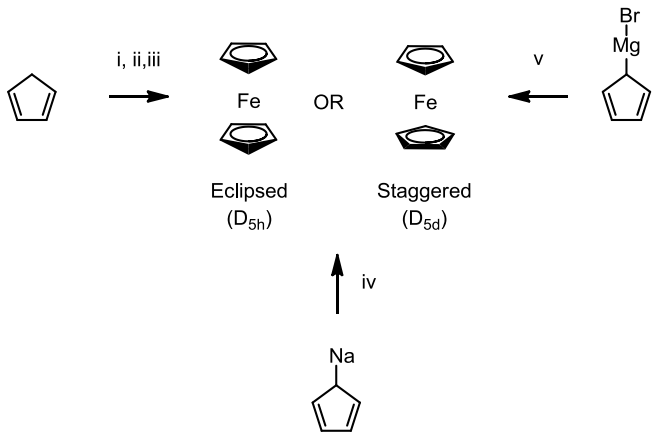
parasites continue with the rest of their life-cycle. This ultimately leads to treatment failure and resistance.

In search for a molecular marker to identify artemisinin resistance, an artemisinin-resistant parasite line was genome-sequenced (Ariey *et al.*, 2014). The F32-Tanzania clone (artemisinin-sensitive) was exposed to artemisinin for a period of 5 years to obtain the artemisinin-resistant F32-ART5 parasite line. The F32-ART5 was sequenced together with the F32-TEM clone that was not exposed to artemisinin. Mutations in the propeller domain of the Kelch 13 (K13) gene destabilise the domain scaffold and alter its function. It is speculated that the function of these mutations provide a cytoprotective response to the pro-oxidant activity of artemisinins (Ariey *et al.*, 2014). After this discovery, many other groups have verified that K13 mutations are indicative of artemisinin resistance. There were 26 different mutations recorded in early 2015 which changed to 54 later the same year (Huang *et al.*, 2015; Tun *et al.*, 2015).

2.8 Ferrocene

2.8.1 Introduction

Ferrocene was almost simultaneously discovered by two different research groups using two very different methods (Kealy & Pauson, 1951; Miller *et al.*, 1952; Rausch *et al.*, 1957). Kealy and Pauson oxidised cyclopentadienylmagnesium bromide with ferric chloride while Miller, Tebboth and Tremaine reacted cyclopentadiene (Cp) vapour with reduced iron (**Scheme 2.3**).



Scheme 2.3: Preparation of ferrocene. i. ferrous chloride and an organic base (Birmingham *et al.*, 1954). ii. iron pentacarbonyl mixing vapours to 200°C (Wilkinson *et al.*, 1954). iii. Sodium acetate, $HgCl_2$ in methanol stirred with the reacted with iron powder (Issleib & Brack, 1956). iv. Ferrous chloride (Wilkinson *et al.*, 1956). v. ferrous acetylacetonate-pyridine complex in benzene (Wilkinson *et al.*, 1954).

Ferrocene has a number of unique properties. It is more lipophilic than benzene and is also more electron-donating than benzene (Hansch & Leo, 1979). Ferrocene obeys the 18 electron rule which consists of six d-electrons (supplied by Fe^{2+}) and 12-π electrons (from the two cyclopentadinyl anions). The HOMO and LUMO energy diagram of ferrocene is depicted in Figure 2.23. The HOMO of ferrocene can be considered as the a'_{1g} (d_z^2) or the e_{2g} ($d_{x^2-y^2}$, d_{xy}) (Barlow & Marder, 2000; Lin *et al.*, 1998). This is important, because on substitution, the perturbation is not the a'_{1g} but the e_{2g} MO. The presence of the electron-withdrawing acyl group, with its relatively low-lying LUMO, has the effect of lowering the energy of the ferrocene HOMO, making the molecule more difficult to oxidise (Green, 1981).

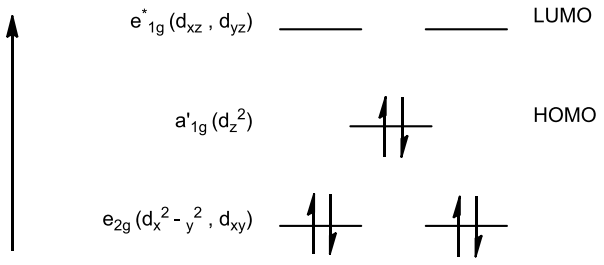


Figure 2.23: HOMO and LUMO of ferrocene.

Ferrocene can undergo electrophilic substitution via one of two mechanisms. The first mechanism is where the electrophile interacts with the iron before being transferred to the aromatic ring followed by deprotonation. The second mechanism entails the electrophile

attack of the aromatic ring followed by deprotonation (Cunningham, 1997; Sharma *et al.*, 2015). There is a requirement for these reactions, namely that the electrophile must not be oxidising, and thus direct halogenations and nitration cannot be carried out using conventional methods.

Friedel-Crafts acylation is a method with which a variety of ferrocenyl ketones has been prepared. Ferrocene undergoes Friedel-Crafts acylation 10^6 times faster than benzene. This reaction yields both mono and 1,1′-disubstituted ferrocenes. Catalysts for this reaction include aluminium chloride, hydrogen fluoride, stannic chloride and boron trifluoride. M. D. Rausch, M. Vogel and H. Rosenberg reported in unpublished results that Friedel-Crafts alkylation of ferrocene produced polyalkylated products and low yields of the desired compounds.

Lithiation of ferrocene occurs with i-BuLi, t-BuLi or n-BuLi. The protons of the aromatic rings are weakly acidic and can be deprotonated. A mono lithiated ferrocene can exclusively be formed with a stoichiometric amount of t-BuLi or i-BuLi. When n-BuLi is used a mixture of mono and 1,1'-disubstituted ferrocenes are synthesised.

2.8.2 Ferrocene pharmacophore

Twelve years after the discovery of ferrocene, the first chronic toxicity studies were conducted. It was found that there is a distinct increase in the liver iron concentration (Yeary, 1969). The urinary excretion from these experiments contained neither ferrocene nor inorganic iron. The group of Hanzlik were among the first groups who started to investigate the metabolism of ferrocene (Hanzlik & Soine, 1978). This *in vitro* metabolism study consisted of viable liver microsomes, NADPH and molecular oxygen. It was suggested that cytochrome P-450 hydroxylated ferrocene, and is then responsible for iron release – or it is conjugated and excreted. Later on, enriching a diet of rats with (3,5,5-trimethylhexanoyl)ferrocene (TMH-ferrocene) (patented as fertiliser in 1979) was used as an animal model in order to investigate hereditary haemochromatosis (Longueville & Crichton, 1986; Ward *et al.*, 1991).

A ^{59}Fe labelled metabolism study with ferrocene, TMH-ferrocene, TMH_2-ferrocene and $FeSO_4$ was conducted in 1993 (Nielsen & Heinrich, 1993). 61% of the iron of ferrocene was excreted through urine compared to 2.51% of TMH-ferrocene. 90% of the iron of TMH_2-

ferrocene and $FeSO_4$ was mainly in excreted faecal matter with 46.9% for TMH-ferrocene and 10.2% for ferrocene. This is indicative that the metabolism is different for each derivative. It was also found that the absorbability of iron runs parallel with the lipophilic character of the derivative with ferrocene at 88% and TMH-ferrocene 50%. The first clinically approved drug containing ferrocene was Ferrocerone; it is used in the Russia for treating anaemia iron-deficiency (Nesmeyanov *et al.*, 1971).

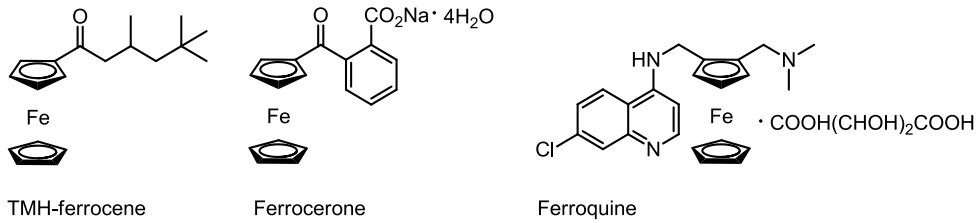


Figure 2.24: Ferrocene derivatives.

Ferrocene salts proved to have an antiproliferative effect on various cancer cells (Köpf‐Maier *et al.*, 1984). The group of Osella illustrated that the ferrocene moiety has the potential to generate hydroxyl radicals under physiological conditions through Fenton-type reactions (Osella *et al.*, 2000). Even though ferrocene compounds show great promise as anticancer agents, they are overshadowed by well-established derivatives such as cisplatin (Martins *et al.*, 2014). CNS-active drugs, anti-inflammatory and analgesic drugs showed no improvement with the incorporation of ferrocene into their structures.

The most promising ferrocene derivative was synthesised in 1994 and was active against chloroquine parasites (Dive & Biot, 2008). It was found that the location of the ferrocene in the chloroquine nucleus affected the activity - if the ferrocene was attached to the quinoline rings of CQ, activity was not improved. The first derivatives that the group of Biot synthesised, the ferrocene was attached onto the main rings of chloroquine without any improvement to the activity (Biot, 1998).

Various derivatives have been synthesised with a view to explore chain length, different positions of ferrocene within the chain, both rings of ferrocene being substituted and various substitutions on the ring. However, ferroquine was still found to be the most favourable. The IC_{50} activity of chloroquine on the W2 *P. falciparum* strain is 138.9 nM, while for ferroquine it is 9.7 nM (Wani *et al.*, 2015). Ferroquine was subjected to *in vitro* human and animal hepatic models in order to identify major metabolites (Daher *et al.*, 2006). It was found that the major metabolites had intact ferrocene moieties.

When CQ is compared to FQ, the differences are mainly in the shape, volume, lipophilicity, basicity and electronic profile (Chavain *et al.*, 2008). It was found that ferroquine can undergo reversible 1 electron redox reactions (Dubar *et al.*, 2013). This concurs with the observation that ferroquine can produce μM amounts of hydroxyl radicals from H_2O_2 (Dubar *et al.*, 2008). While investigating the mechanism of action, another special feature of ferroquine was identified. Ferroquine, while interacting with ferroprotoporhyrin IX, assumes a flip-flop configuration leaving the ferrocene moiety exposed (Dubar *et al.*, 2008). This may aid in the transport from aqueous medium to the hydrophobic membranes (Biot *et al.*, 2009).

2.8.3 Ferrocene artemisinins

There are only 9 ferrocenyl monomer artemisinin derivatives that have been reported. The first to synthesise these derivatives were by the group of Paitayatat in 1997 with the starting material being artemisitene (Paitayatat *et al.*, 1997). The ferrocene derivatives were reacted with lithium diisopropylamide to yield the corresponding anion, in THF at -78 °C. This was added to a solution of artemisitene, in THF at -78 °C whereupon it underwent conjugate addition to the exocyclic double bond of artemisitene.

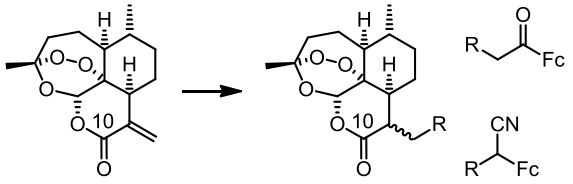


Figure 2.25: Ferrocenyl artemisinin derivatives of Paitayatat *et al*.

These ferrocene derivatives were part of a larger group of synthesised derivatives that were, used to evaluate the interaction of artemisinin derivatives with ferroprotoporphyrin IX. At the time it was hypothesised that the mechanism of action of artemisinins was to interact with haem, leading to adduct formation.

To further investigate the interaction of artemisinin-ferrocenyl derivatives and haem, the group of Delhaes synthesised four derivatives (Delhaes *et al.*, 2000). The most active of these derivatives contained a secondary amine within the chain. Unfortunately the yields for this derivative were low – ranging between 28–36%. The activity did not increase with the addition of ferrocene to artemisinin.

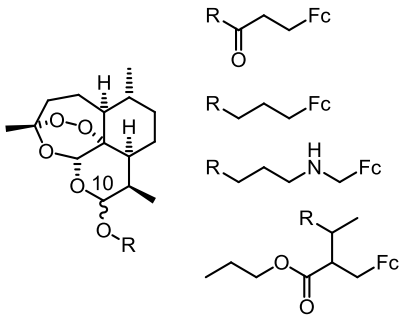


Figure 2.26: Ferrocenyl artemisinin derivatives synthesized by Delheas *et al*.

Reiter *et al*. synthesised a variety of ferrocene artemisinin monomers between 2014 and 2015 (Reiter *et al.*, 2015; Reiter *et al.*, 2014). By chlorinating ferrocenecarboxylic acid, Mitsunobu estherification was achieved by utilising DIAD and PPh_3. The C10 non-acetal derivative was chosen as an intermediate because it was shown that the intermediate is 15–22 times more stable and has a greater bioavailability than established artemether and arteether (Jung *et al.*, 2002; Jung & Lee, 1998; Jung *et al.*, 2003). The IC_{50} values of these derivatives were higher than that of dihydroartemisinin; this implies that the ferrocene does not activate the endoperoxide moiety. However, it was noted that the closer the ferrocene was to the artemisinin, the more active the derivative was. The synthesised compounds were stable after heating them at 60°C for 20 hours, less than 5% decomposition was observed. The eugonol ferrocene artemisinin hybrid was the least active antimalarial derivative synthesised, although it is also the most potent anticancer derivative.

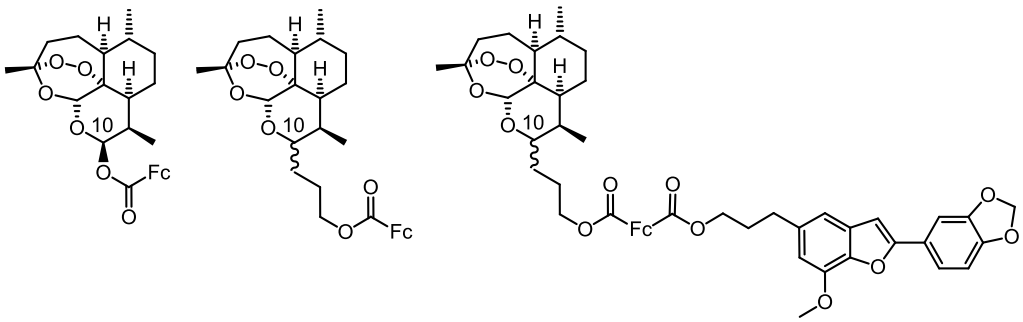
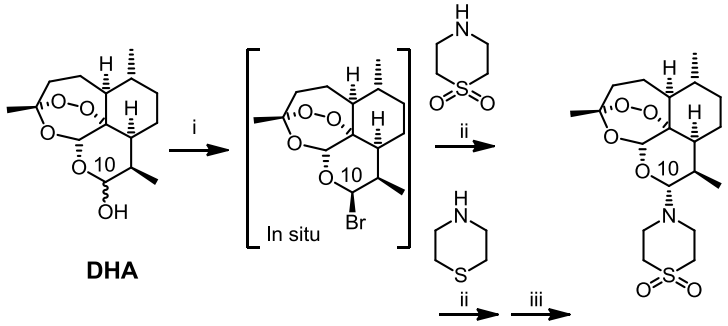


Figure 2.27: Ferrocenyl artemisinin derivatives of Reiter *et al*.

2.8.4 Other artemisinin derivatives

Artemisone

Artemisone was first synthesized by the goup of Haynes. It can be prepared by 1 of 2 synthetic routes (**Scheme 3**) (Haynes *et al.*, 2006). The first route consists of the halogenation of dihydroartemisinin and is reacted with thiomorpholine-S, S-dioxide. Alternatively, after the halogenation of sihydroartemisinin it is reacted with thiomorpholine which is oxidised in order to obtain artemisone with an overall yield of 58%. Artemisone also has a favourable logP value of 2.49 (Haynes *et al.*, 2006). The elimination half-life of this derivative is 5 hours, and like other artemisinins, it reaches maximum blood concentrations within 1.5 hours (Vivas *et al.*, 2007).



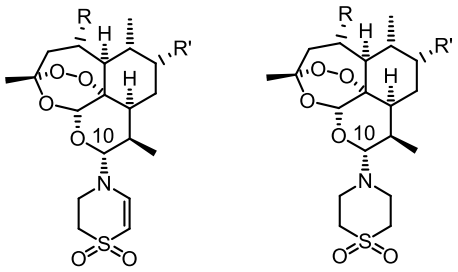
Scheme 2.4: Synthesis of artemisone i) TMSCl, NaBr, toluene 0 °C, ii and iii secondary amine, Et_3N, CH_2Cl_2, 0-20 °C, iii) dichloromethane, *N*-methylmorpholine-*N*-oxide, powdered molecular sieve, and tetrapropylammonium perruthenate.

Artemisone was 10 times more potent than artesunate against 12 different *P. falciparum* strains and also 4–10 times more potent than artesunate in rodent models (Vivas *et al.*, 2007). Other animal studies were carried out on aotus monkeys infected with *P. falciparum FVO*. The monkeys treated with artemisone had no parasites after a 24 hour period, while the artesunate-treated monkeys still had parasites present after 48 hours.

The group of Vivas examined the *in vitro* and *in vivo* drug-drug interactions of artemisone with other antimalarials(Vivas *et al.*, 2007). It was found that artemisone showed a slight degree of synergy with mefloquine, lumefantrine or quinine. *In vivo* there were various degrees of synergy between artemisone and chloroquine, clindamycin or mefloquine. This derivative showed great clinical promise in phase 1 trials since no subject developed any

serious adverse reactions. Furthermore, during phase IIb trials, it cured 5/5 patients treated for cerebral malaria with half of the dose needed in comparison to artesunate.

The major advantage of this derivative can be seen when it is subjected to the liver enzyme CYP3A4. This suggests that the common artemisinin metabolite, dihydroartemisinin, is avoided together with the accompanied neurotoxicity. Neurotoxicity is of significant concern because as the threat of resistance becomes more prominent, the strategies to maintain the efficacy of artemisinin may involve increased dosages (Das *et al.*, 2012). An added benefit of the avoidance of the dihydroartemisinin metabolite is that dihydroartemisinin might be implicated in artemisinin resistance since it is the common metabolite of clinically used artemisinins (Mbengue *et al.*, 2015; Paloque *et al.*, 2016). The main metabolites that are formed of this derivative are illustrated in Figure 2.28.



M1: R=R'=H
M4: R=OH, R'=H
M5: R=H, R'=OH

M2: R=OH,R'=H
M3: R=H, R'=OH

Figure 2.28: Artemisone metabolites.

Non-acetal derivatives

Jung was the first to synthesise a non-acetal derivative (Jung *et al.*, 1990). With its remarkable acid stability and potency, it caused a large number of these types of derivatives to be synthesised. Since this study also involves C-10 modified non-acetal derivatives, a mini-review of these follows in Chapter 3. With the threat of resistance looming ever closer, the review consists of the most potent derivatives in comparison with artemisinin, synthesis and biological evaluations.

2.9 Summary

Malaria is caused by a parasite that kills hundreds of thousands of people each year. The only effective treatment for malaria is the artemisinin family. The only problem with the artemisinin class is that it has a very short half-life and if all of the parasites were not killed while it is active there is a high possibility of these parasites gaining resistance towards artemisinin. To prevent this from happening artemisinin is used in combination with another anti-malarial, with a longer half-life, which is known as artemisinin combinational therapy. There is clear evidence that this treatment is failing and the parasites are growing resistant.

The half-life of the artemisinins could be improved if the C-10 acetal functionality was improved. In order to address the issue of resistance the focus was on another derivative that overcame resistance which is ferroquine. By imbedding the ferrocene moiety within the structure of chloroquine the resistance was overcome. Ferrocene have the ability to undergo redox reactions and generate reactive oxygen species. If the co-factor mechanism of action is considered for artemisinin this would mean that the combination of ferrocene with artemisinin would lead to a very potent pharmacophore. Artemisinin would gradually decrease the ability of the parasite to protect itself from free radicals while ferrocene would increase these radicals.

From the above literature study this was not the case. Most of the ferrocene-artemisinin hybrids that were synthesized did not indicate any increase in activity towards the malaria parasites. The only hybrid that indicated a slight increase in activity was the derivative that Delheas *et al*. synthesised and it contained an amine functionality in close proximity to ferrocene. Based on these observations the study commenced.

2.10 References

Abiodun, O.O., Brun, R. & Wittlin, S. 2013. In vitro interaction of artemisinin derivatives or the fully synthetic peroxidic anti-malarial OZ277 with thapsigargin in Plasmodium falciparum strains. *Malaria Journal*, 12(1):43.

Alin, M.H., Björkman, A. & Wernsdorfer, W. 1999. Synergism of benflumetol and artemether in Plasmodium falciparum. *The American Journal of Tropical Medicine and Hygiene*, 61(3):439-445.

Alonso, P.L. & Tanner, M. 2013. Public health challenges and prospects for malaria control and elimination. *Nature Medicine*, 19(2):150-155.

Alvarez, J.R., Al-Khan, A. & Apuzzio, J.J. 2005. Malaria in pregnancy. *Infectious Diseases in Obstetrics and Gynecology*, 13(4):229-236.

Anand, N. 1983. Sulfonamides: structure-activity relationships and mechanism of action. Inhibition of folate metabolism in chemotherapy. Springer. p. 25-54).

Andriessen, R., Snetselaar, J., Suer, R.A., Osinga, A.J., Deschietere, J., Lyimo, I.N., Mnyone, L.L., Brooke, B.D., Ranson, H. & Knols, B.G. 2015. Electrostatic coating enhances bioavailability of insecticides and breaks pyrethroid resistance in mosquitoes. *Proceedings of the National Academy of Sciences*, 112(39):12081-12086.

Ansah, E.K., Narh-Bana, S., Epokor, M., Akanpigbiam, S., Quartey, A.A., Gyapong, J. & Whitty, C.J. 2010. Rapid testing for malaria in settings where microscopy is available and peripheral clinics where only presumptive treatment is available: a randomised controlled trial in Ghana. *British Medical Journal*, 340:c930.

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A.-C., Khim, N., Kim, S., Duru, V., Bouchier, C. & Ma, L. 2014. A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. *Nature*, 505(7481):50-55.

Arnér, E.S. & Holmgren, A. 2000. Physiological functions of thioredoxin and thioredoxin reductase. *The FEBS Journal*, 267(20):6102-6109.

Arnold, J., Alving, A., Clayman, C.B. & Hochwald, R.S. 1961. Induced primaquine resistance in vivax malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 55(4):345-350.

Arnou, B., Montigny, C., Morth, J.P., Nissen, P., Jaxel, C., Møller, J.V. & le Maire, M. 2011. The Plasmodium falciparum Ca2+-ATPase PfATP6: insensitive to artemisinin, but a potential drug target: Portland Press Limited.

Asawamahasakda, W., Ittarat, I., Pu, Y.-M., Ziffer, H. & Meshnick, S.R. 1994. Reaction of antimalarial endoperoxides with specific parasite proteins. *Antimicrobial Agents and Chemotherapy*, 38(8):1854-1858.

Ashley, E.A. & White, N.J. 2005. Artemisinin-based combinations. *Current Opinion in Infectious Diseases*, 18(6):531-536.

Babiker, H.A. & Mackinnon, M.J. 2005. Drug resistance in malaria: its population biology and implications for control. *Acta Tropica*, 94(3):161-162.

Baird, J.K., Fryauff, D.J., Basri, H., Bangs, M.J., Subianto, B., Wiady, I., Leksana, B., Masbar, S., Richie, T.L. & Jones, T.R. 1995. Primaquine for prophylaxis against malaria among nonimmune transmigrants in Irian Jaya, Indonesia. *The American Journal of Tropical Medicine and Hygiene*, 52(6):479-484.

Baird, J.K. & Hoffman, S.L. 2004. Primaquine therapy for malaria. *Clinical Infectious Diseases*, 39(9):1336-1345.

Bannister, L., Hopkins, J., Fowler, R., Krishna, S. & Mitchell, G. 2000. A brief illustrated guide to the ultrastructure of Plasmodium falciparum asexual blood stages. *Parasitology Today*, 16(10):427-433.

Barlow, S. & Marder, S.R. 2000. Electronic and optical properties of conjugated group 8 metallocene derivatives. *Chemical Communications*(17):1555-1562.

Barradell, L.B. & Fitton, A. 1995. Artesunate. *Drugs*, 50(4):714-741.

Barreaux, P., Barreaux, A.M., Sternberg, E.D., Suh, E., Waite, J.L., Whitehead, S.A. & Thomas, M.B. 2017. Priorities for Broadening the Malaria Vector Control Tool Kit. *Trends in Parasitology*, 33(10):763-774.

Baruffa, G. 1966. Clinical trials in Plasmodium falciparum malaria with a long-acting sulphonamide. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 60(2):222-224.

Batty, K.T., Thu, A., Thi, L., Davis, T.M., Ilett, K.F., Xuan Mai, T., Canh Hung, N., Phuc Tien, N., Powell, S.M. & Van Thien, H. 1998. A pharmacokinetic and pharmacodynamic study of intravenous vs oral artesunate in uncomplicated falciparum malaria. *British Journal of Clinical Pharmacology*, 45(2):123-129.

Becker, K., Gromer, S., Schirmer, R.H. & Müller, S. 2000. Thioredoxin reductase as a pathophysiological factor and drug target. *The FEBS Journal*, 267(20):6118-6125.

Becker, K., Kanzok, S.M., Iozef, R., Fischer, M., Schirmer, R.H. & Rahlfs, S. 2003a. Plasmoredoxin, a novel redox-active protein unique for malarial parasites. *The FEBS Journal*, 270(6):1057-1064.

Becker, K., Rahlfs, S., Nickel, C. & Schirmer, R.H. 2003b. Glutathione–functions and metabolism in the malarial parasite Plasmodium falciparum. *Biological chemistry*, 384(4):551-566.

Beereboom, J.J., Blackwood, R.K., Rennhard, H.H. & Stephens, J.C.R. 1965. Alpha-6-deoxytetracycline derivatives and process: Google Patents.

Berman, P.A. & Adams, P.A. 1997. Artemisinin enhances heme-catalysed oxidation of lipid membranes. *Free Radical Biology and Medicine*, 22(7):1283-1288.

Biot, C. 1998. Molécules ferrocéniques antipaludiques: synthèse, caractérisation et activité. Lille 1. (Doctoral dissertation).

Biot, C., Chavain, N., Dubar, F., Pradines, B., Trivelli, X., Brocard, J., Forfar, I. & Dive, D. 2009. Structure–activity relationships of 4-N-substituted ferroquine analogues: Time to re-

evaluate the mechanism of action of ferroquine. *Journal of Organometallic Chemistry*, 694(6):845-854.

Birmingham, J., Seyferth, D. & Wilkinson, G. 1954. A new preparation of bis-cyclopentadienyl-metal compounds. *Journal of the American Chemical Society*, 76(16):4179-4179.

Björkman, A. & Phillips-Howard, P. 1990. Drug-resistant malaria: mechanisms of development and inferences for malaria control. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 84(3):323-324.

Boudreau, E., Webster, H.K., Pavanand, K. & Thosingha, L. 1982. Type II mefloquine resistance in Thailand. *The Lancet*, 320(8311):1335.

Bray, P.G., Deed, S., Fox, E., Kalkanidis, M., Mungthin, M., Deady, L.W. & Tilley, L. 2005. Primaquine synergises the activity of chloroquine against chloroquine-resistant P. falciparum. *Biochemical Pharmacology*, 70(8):1158-1166.

Brewer, T.G., Peggins, J.O., Grate, S.J., Petras, J., Levine, B.S., Weina, P.J., Swearengen, J., Heiffer, M.H. & Schuster, B.G. 1994. Neurotoxicity in animals due to arteether and artemether. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 88:33-36.

Bruce-Chwatt, L. 1988. Three hundred and fifty years of the Peruvian fever bark. *British Medical Journal (Clinical Research ed.)*, 296(6635):1486.

Burgoine, K.L., Bancone, G. & Nosten, F. 2010. The reality of using primaquine. *Malaria Journal*, 9(1):376.

Bwijo, B., Alin, M.H., Abbas, N., Eriksson, Ö. & Björkman, A. 1997a. Repetitive dosing of artemisinin and quinine against Plasmodium falciparum in vitro: a simulation of the in vivo pharmacokinetics. *Acta Tropica*, 65(1):11-22.

Bwijo, B., Alin, M.H., Abbas, N., Wernsdorfer, W. & Björkman, A. 1997b. Efficacy of artemisinin and mefloquine combinations against Plasmodium falciparum. In vitro simulation of in vivo pharmacokinetics. *Tropical Medicine & International Health*, 2(5):461-467.

Carrington, H., Crowther, A., Davey, D., Levi, A. & Rose, F. 1951. A metabolite of 'Paludrine'with high antimalarial activity. *Nature*, 168(4286):1080-1080.

CDC. 2016a. About Malaria. https://www.cdc.gov/malaria/about/biology/index.html Date of access: June 15 2017.

CDC. 2016b. The history of malaria, an ancient disease. www.cdc.gov/malaria/history/ Date of access: 13 June 2016.

Chavain, N., Vezin, H., Dive, D., Touati, N., Paul, J.-F., Buisine, E. & Biot, C. 2008. Investigation of the redox behavior of ferroquine, a new antimalarial. *Molecular Pharmaceutics*, 5(5):710-716.

Chen, L., Qu, F. & Zhou, Y. 1982. Field observations on the antimalarial piperaquine. *Chinese Medical Journal*, 95(4):281-286.

Cheng, Q., Kyle, D.E. & Gatton, M.L. 2012. Artemisinin resistance in Plasmodium falciparum: A process linked to dormancy? *International Journal for Parasitology: Drugs and Drug Resistance*, 2:249-255.

China Cooperative Research, G. 1982. The chemistry and synthesis of qinghaosu derivatives. *Journal of Traditional Chinese Medicine*, 2(1):1-16.

Cibulskis, R.E., Alonso, P., Aponte, J., Aregawi, M., Barrette, A., Bergeron, L., Fergus, C.A., Knox, T., Lynch, M. & Patouillard, E. 2016. Malaria: global progress 2000–2015 and future challenges. *Infectious Diseases of Poverty*, 5(1):61.

Coatney, G.R. & Greenberg, J. 1952. The use of antibiotics in the treatment of malaria. *Annals of the New York Academy of Sciences*, 55(1):1075-1081.

Cogswell, F.B. 1992. The hypnozoite and relapse in primate malaria. *Clinical Microbiology Reviews*, 5(1):26-35.

Conover, L.H. 1984. Discovering tetracycline. *Research Management*, 27(5):17-22.

Contacos, P.G., Lunn, J.S. & Coatney, G.R. 1963. Drug-resistant falciparum malaria from Cambodia and Malaya. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 57(6):417-424.

Cooper, R.A., Ferdig, M.T., Su, X.-Z., Ursos, L.M., Mu, J., Nomura, T., Fujioka, H., Fidock, D.A., Roepe, P.D. & Wellems, T.E. 2002. Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses inPlasmodium falciparum. *Molecular Pharmacology*, 61(1):35-42.

Cooper, R.A., Lane, K.D., Deng, B., Mu, J., Patel, J.J., Wellems, T.E., Su, X. & Ferdig, M.T. 2007. Mutations in transmembrane domains 1, 4 and 9 of the Plasmodium falciparum chloroquine resistance transporter alter susceptibility to chloroquine, quinine and quinidine. *Molecular Microbiology*, 63(1):270-282.

Cowman, A.F. & Crabb, B.S. 2002. A parasite genome sheds light on an old enemy. *Nature Biotechnology*, 20(11):1098-1099.

Cox, F.E. 2010. History of the discovery of the malaria parasites and their vectors. *Parasites & Vectors*, 3(1):5.

Cunningham, A.F. 1997. Mechanism of mercuration of ferrocene: General treatment of electrophilic substitution of ferrocene derivatives. *Organometallics*, 16(6):1114-1122.

Curd, F., Davey, D. & Rose, F. 1945. Studies on Synthetic Antimalarial Drugs: I.—Biological Methods. *Annals of Tropical Medicine & Parasitology*, 39(3-4):139-156.

Daher, W., Pelinski, L., Klieber, S., Sadoun, F., Meunier, V., Bourrié, M., Biot, C., Guillou, F., Fabre, G. & Brocard, J. 2006. In vitro metabolism of ferroquine (SSR97193) in animal and human hepatic models and antimalarial activity of major metabolites on Plasmodium falciparum. *Drug Metabolism and Disposition*, 34(4):667-682.

Dahl, E.L. & Rosenthal, P.J. 2007. Multiple antibiotics exert delayed effects against the Plasmodium falciparum apicoplast. *Antimicrobial Agents and Chemotherapy*, 51(10):3485-3490.

Dahl, E.L., Shock, J.L., Shenai, B.R., Gut, J., DeRisi, J.L. & Rosenthal, P.J. 2006. Tetracyclines specifically target the apicoplast of the malaria parasite Plasmodium falciparum. *Antimicrobial Agents and Chemotherapy*, 50(9):3124-3131.

Das, D., Tripura, R., Phyo, A.P., Lwin, K.M., Tarning, J., Lee, S.J., Hanpithakpong, W., Stepniewska, K., Menard, D. & Ringwald, P. 2012. Effect of high-dose or split-dose artesunate on parasite clearance in artemisinin-resistant falciparum malaria. *Clinical Infectious Diseases*, 56(5):e48-e58.

Dassonville-Klimpt, A., Jonet, A., Pillon, M., Mullié, C. & Sonnet, P. 2011. Mefloquine derivatives: synthesis, mechanisms of action, antimicrobial activities. *Science against Microbial Pathogens: Communicating Current Research and Technological Advances*, 3.

Davis, T.M., Hung, T.-Y., Sim, K., Karunajeewa, H.A. & Ilett, K.F. 2005. Piperaquine. *Drugs*, 65(1):75-87.

de Villiers, K.A., Marques, H.M. & Egan, T.J. 2008. The crystal structure of halofantrine–ferriprotoporphyrin IX and the mechanism of action of arylmethanol antimalarials. *Journal of Inorganic Biochemistry*, 102(8):1660-1667.

del Pilar Crespo, M., Avery, T.D., Hanssen, E., Fox, E., Robinson, T.V., Valente, P., Taylor, D.K. & Tilley, L. 2008. Artemisinin and a series of novel endoperoxide antimalarials exert early effects on digestive vacuole morphology. *Antimicrobial Agents and Chemotherapy*, 52(1):98-109.

Delhaes, L., Biot, C., Berry, L., Maciejewski, L., Camus, D., Brocard, J. & Dive, D. 2000. Novel ferrocenic artemisinin derivatives: synthesis, in vitro antimalarial activity and affinity of binding with ferroprotoporphyrin IX. *Bioorganic & Medicinal Chemistry*, 8(12):2739-2745.

Denis, M.B., Davis, T.M., Hewitt, S., Incardona, S., Nimol, K., Fandeur, T., Poravuth, Y., Lim, C. & Socheat, D. 2002. Efficacy and safety of dihydroartemisinin-piperaquine (Artekin) in Cambodian children and adults with uncomplicated falciparum malaria. *Clinical Infectious Diseases*, 35(12):1469-1476.

Denis, M.B., Tsuyuoka, R., Poravuth, Y., Narann, T.S., Seila, S., Incardona, S., Lim, P., Sem, R., Socheat, D. & Christophel, E.M. 2006. Surveillance of the efficacy of artesunate

and mefloquine combination for the treatment of uncomplicated falciparum malaria in Cambodia. *Tropical Medicine & International Health*, 11(9):1360-1366.

Diabate, A. & Tripet, F. 2015. Targeting male mosquito mating behaviour for malaria control. *Parasites & Vectors*, 8(1):347.

Dive, D. & Biot, C. 2008. Ferrocene conjugates of chloroquine and other antimalarials: the development of ferroquine, a new antimalarial. *ChemMedChem*, 3(3):383-391.

Djapa, L.Y., Zelikson, R., Delahodde, A., Bolotin-Fukuhara, M. & Mazabraud, A. 2006. Plasmodium vivax dihydrofolate reductase as a target of sulpha drugs. *FEMS Microbiology Letters*, 256(1):105-111.

Doolan, D.L., Dobaño, C. & Baird, J.K. 2009. Acquired immunity to malaria. *Clinical Microbiology Reviews*, 22(1):13-36.

Dubar, F., Khalife, J., Brocard, J., Dive, D. & Biot, C. 2008. Ferroquine, an ingenious antimalarial drug–thoughts on the mechanism of action. *Molecules*, 13(11):2900-2907.

Dubar, F., Slomianny, C., Khalife, J., Dive, D., Kalamou, H., Guérardel, Y., Grellier, P. & Biot, C. 2013. The ferroquine antimalarial conundrum: redox activation and reinvasion inhibition. *Angewandte Chemie International Edition*, 52(30):7690-7693.

Eckstein-Ludwig, U., Webb, R., Van Goethem, I., East, J., Lee, A., Kimura, M., O'neill, P., Bray, P., Ward, S. & Krishna, S. 2003. Artemisinins target the SERCA of Plasmodium falciparum. *Nature*, 424(6951):957-961.

Egan, T.J. 2008. Haemozoin formation. *Molecular and Biochemical Parasitology*, 157(2):127-136.

Eshel, Y., Houri-Yafin, A., Benkuzari, H., Lezmy, N., Soni, M., Charles, M., Swaminathan, J., Solomon, H., Sampathkumar, P. & Premji, Z. 2017. Evaluation of the Parasight Platform for Malaria Diagnosis. *Journal of Clinical Microbiology*, 55(3):768-775.

Eyles, D.E., Hoo, C., Warren, M. & Sandosham, A. 1963. Plasmodium falciparum resistant to chloroquine in Cambodia. *The American Journal of Tropical Medicine and Hygiene*, 12(6):840-843.

Fairhurst, R.M. & Dondorp, A.M. 2016. Artemisinin-resistant Plasmodium falciparum malaria. *Microbiology Spectrum*, 4(3).

Fançony, C., Sebastião, Y.V., Pires, J.E., Gamboa, D. & Nery, S.V. 2013. Performance of microscopy and RDTs in the context of a malaria prevalence survey in Angola: a comparison using PCR as the gold standard. *Malaria Journal*, 12(1):284.

Farrow, R.E., Green, J., Katsimitsoulia, Z., Taylor, W.R., Holder, A.A. & Molloy, J.E. 2011. The mechanism of erythrocyte invasion by the malarial parasite, Plasmodium falciparum. (*In*. Seminars in cell & developmental biology organised by: Elsevier. p. 953-960).

Fidock, D.A., Rosenthal, P.J., Croft, S.L., Brun, R. & Nwaka, S. 2004. Antimalarial drug discovery: efficacy models for compound screening. *Nature Reviews Drug Discovery*, 3(6):509-520.

Filomeni, G., Rotilio, G. & Ciriolo, M.R. 2002. Cell signalling and the glutathione redox system. *Biochemical Pharmacology*, 64(5):1057-1064.

Fitch, C.D. 2004. Ferriprotoporphyrin IX, phospholipids, and the antimalarial actions of quinoline drugs. *Life Sciences*, 74(16):1957-1972.

Foote, S.J., Galatis, D. & Cowman, A.F. 1990. Amino acids in the dihydrofolate reductase-thymidylate synthase gene of Plasmodium falciparum involved in cycloguanil resistance differ from those involved in pyrimethamine resistance. *Proceedings of the National Academy of Sciences*, 87(8):3014-3017.

Ford, P.G. 2000. Dapsone. *Current Problems in Dermatology*, 12(5):242-245.

Fridovich, I. 1972. Superoxide radical and superoxide dismutase. *Accounts of Chemical Research*, 5(10):321-326.

Fry, M. & Pudney, M. 1992. Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4'-chlorophenyl) cyclohexyl]-3-hydroxy-1, 4-naphthoquinone (566C80). *Biochemical Pharmacology*, 43(7):1545-1553.

Giao, P.T., Binh, T.Q., Kager, P.A., Long, H.P., Van Thang, N., Van Nam, N. & de Vries, P.J. 2001. Artemisinin for treatment of uncomplicated falciparum malaria: is there a place for monotherapy? *The American Journal of Tropical Medicine and Hygiene*, 65(6):690-695.

Giboda, M. & Denis, M. 1988. Response of Kampuchean strains of Plasmodium falciparum to antimalarials: in-vivo assessment of quinine and quinine plus tetracycline; multiple drug resistance in vitro. *The Journal of Tropical Medicine and Hygiene*, 91(4):205-211.

Gil, J. & Berglund, E.G. 2007. CYP2C8 and antimalaria drug efficacy. *Pharmacogenomics*, 8(2):187-198.

Goldsmith, R.S. 1997. Infectious diseases: protozoal & helminthic. *Current Medical Diagnosis and Treatment*:1294-1355.

Goldstein, S. & Meyerstein, D. 1999. Comments on the mechanism of the “Fenton-like” reaction. *Accounts of Chemical Research*, 32(7):547-550.

Golgi, C. 1886. Malarial infection. *Archivio per Le Scienze Mediche*, 10:109-135.

Goodman, C.D., Su, V. & McFadden, G.I. 2007. The effects of anti-bacterials on the malaria parasite Plasmodium falciparum. *Molecular and Biochemical Parasitology*, 152(2):181-191.

Green, J.C. 1981. Gas phase photoelectron spectra of d-and f-block organometallic compounds. Bonding Problems. Springer. p. 37-112).

Hansch, C. & Leo, A. 1979. Substituent constants for correlation analysis in chemistry and biology: Wiley.

Hanzlik, R.P. & Soine, W.H. 1978. Enzymic hydroxylation of ferrocene. *Journal of the American Chemical Society*, 100(4):1290-1291.

Harchut, K., Standley, C., Dobson, A., Klaassen, B., Rambaud-Althaus, C., Althaus, F. & Nowak, K. 2013. Over-diagnosis of malaria by microscopy in the Kilombero Valley, Southern Tanzania: an evaluation of the utility and cost-effectiveness of rapid diagnostic tests. *Malaria Journal*, 12(1):159.

Hartwig, C.L., Rosenthal, A.S., D'Angelo, J., Griffin, C.E., Posner, G.H. & Cooper, R.A. 2009. Accumulation of artemisinin trioxane derivatives within neutral lipids of Plasmodium falciparum malaria parasites is endoperoxide-dependent. *Biochemical Pharmacology*, 77(3):322-336.

Hawley, S.R., Bray, P.G., Park, B.K. & Ward, S.A. 1996. Amodiaquine accumulation in Plasmodium falciparum as a possible explanation for its superior antimalarial activity over chloroquine. *Molecular and Biochemical Parasitology*, 80(1):15-25.

Hawser, S., Lociuro, S. & Islam, K. 2006. Dihydrofolate reductase inhibitors as antibacterial agents. *Biochemical Pharmacology*, 71(7):941-948.

Haynes, R.K. 2001. Artemisinin and derivatives: the future for malaria treatment? *Current Opinion in Infectious Diseases*, 14(6):719-726.

Haynes, R.K. 2005. Reply to Comments on "Highly Antimalaria-Active Artemisinin Derivatives: Biological Activity Does Not Correlate with Chemical Reactivity". *Angewandte Chemie International Edition*, 44(14):2064-2065.

Haynes, R.K. 2006. From artemisinin to new artemisinin antimalarials: biosynthesis, extraction, old and new derivatives, stereochemistry and medicinal chemistry requirements. *Current Topics in Medicinal Chemistry*, 6(5):509-537.

Haynes, R.K., Chan, W.C., Lung, C.M., Uhlemann, A.C., Eckstein, U., Taramelli, D., Parapini, S., Monti, D. & Krishna, S. 2007. The Fe2+-Mediated decomposition, PfATP6 binding, and antimalarial activities of artemisone and other artemisinins: The unlikelihood of C-Centered radicals as bioactive intermediates. *ChemMedChem*, 2(10):1480-1497.

Haynes, R.K., Chan, W.C., Wong, H.N., Li, K.Y., Wu, W.K., Fan, K.M., Sung, H.H., Williams, I.D., Prosperi, D. & Melato, S. 2010. Facile oxidation of leucomethylene blue and dihydroflavins by artemisinins: relationship with flavoenzyme function and antimalarial mechanism of action. *ChemMedChem*, 5(8):1282-1299.

Haynes, R.K., Cheu, K.-W., N'Da, D., Coghi, P. & Monti, D. 2013. Considerations on the mechanism of action of artemisinin antimalarials: Part 1-The'carbon radical'and'heme'hypotheses. *Infectious Disorders-Drug Targets (Formerly Current Drug Targets-Infectious Disorders)*, 13(4):217-277.

Haynes, R.K., Cheu, K.W., Chan, H.W., Wong, H.N., Li, K.Y., Tang, M.M.K., Chen, M.J., Guo, Z.F., Guo, Z.H. & Sinniah, K. 2012. Interactions between artemisinins and other antimalarial drugs in relation to the cofactor model—a unifying proposal for drug action. *ChemMedChem*, 7(12):2204-2226.

Haynes, R.K., Fugmann, B., Stetter, J., Rieckmann, K., Heilmann, H.D., Chan, H.W., Cheung, M.K., Lam, W.L., Wong, H.N. & Croft, S.L. 2006. Artemisone—a highly active antimalarial drug of the artemisinin class. *Angewandte Chemie*, 118(13):2136-2142.

Haynes, R.K., Monti, D., Taramelli, D., Basilico, N., Parapini, S. & Olliaro, P. 2003. Artemisinin antimalarials do not inhibit hemozoin formation. *Antimicrobial Agents and Chemotherapy*, 47(3):1175-1175.

Hempelmann, E. & Krafts, K. 2013. Bad air, amulets and mosquitoes: 2,000 years of changing perspectives on malaria. *Malaria Journal*, 12(1):232.

Hien, T.T. & White, N.J. 1993. Qinghaosu. *The Lancet*, 341(8845):603-608.

Hitchings, G.H., Elion, G.B., Falco, E.A., Russell, P.B., Sherwood, M.B. & Vanderwerff, H. 1950. Antagonists of nucleic acid derivatives I. The Lactobacillus casei model. *Journal of Biological Chemistry*, 183(1):1-9.

Holmgren, G., Gil, J.P., Ferreira, P.M., Veiga, M.I., Obonyo, C.O. & Björkman, A. 2006. Amodiaquine resistant Plasmodium falciparum malaria in vivo is associated with selection of pfcrt 76T and pfmdr1 86Y. *Infection, Genetics and Evolution*, 6(4):309-314.

Hoshen, M., Na-Bangchang, K., Stein, W. & Ginsburg, H. 2000. Mathematical modelling of the chemotherapy of Plasmodium falciparum malaria with artesunate: postulation of 'dormancy', a partial cytostatic effect of the drug, and its implication for treatment regimens. *Parasitology*, 121(3):237-246.

Huang, F., Takala-Harrison, S., Jacob, C.G., Liu, H., Sun, X., Yang, H., Nyunt, M.M., Adams, M., Zhou, S. & Xia, Z. 2015. A single mutation in K13 predominates in Southern China and is associated with delayed clearance of Plasmodium falciparum following artemisinin treatment. *The Journal of Infectious Diseases*, 212(10):1629-1635.

Ilett, K.F., Batty, K.T., Powell, S.M., Binh, T.Q., Thu, L.T.A., Phuong, H.L., Hung, N.C. & Davis, T.M. 2002. The pharmacokinetic properties of intramuscular artesunate and rectal dihydroartemisinin in uncomplicated falciparum malaria. *British Journal of Clinical Pharmacology*, 53(1):23-30.

Issleib, K. & Brack, A. 1956. Eine neue, einfache Ferrocen-Darstellung. *Z. Naturforschg.*, 11b:2.

Jakeman, G., Saul, A., Hogarth, W. & Collins, W. 1999. Anaemia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. *Parasitology*, 119(2):127-133.

James, S., Nicol, W. & Shute, P. 1936. Clinical and parasitological observations on induced malaria: *Proceedings of the Royal Society of Medicine*, 29(8):879-894.

Jortzik, E. & Becker, K. 2012. Thioredoxin and glutathione systems in Plasmodium falciparum. *International Journal of Medical Microbiology*, 302(4):187-194.

Jung, M., Bustos, D.A., ElSohly, H.N. & McChesney, J.D. 1990. A concise and stereoselective synthesis of (+)-12-n-butyldeoxoartemisinin. *Synlett*, 1990 (12):743-744.

Jung, M., Lee, K., Kendrick, H., Robinson, B.L. & Croft, S.L. 2002. Synthesis, stability, and antimalarial activity of new hydrolytically stable and water-soluble (+)-deoxoartelinic acid. *Journal of Medicinal Chemistry*, 45(22):4940-4944.

Jung, M. & Lee, S. 1998. Stability of acetal and non acetal-type analogs of artemisinin in simulated stomach acid. *Bioorganic & Medicinal Chemistry Letters*, 8(9):1003-1006.

Jung, M., Lee, S., Ham, J., Lee, K., Kim, H. & Kim, S.K. 2003. Antitumor activity of novel deoxoartemisinin monomers, dimers, and trimer. *Journal of Medicinal Chemistry*, 46(6):987-994.

Kager, P., Schultz, M., Zijlstra, E., Van Den Berg, B. & van Boxtel, C.J. 1994. Arteether administration in humans: preliminary studies of pharmacokinetics, safety and tolerance. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 88:53-54.

Kain, K.C. 2003. Current status and replies to frequently posed questions on atovaquone plus proguanil (Malarone®) for the prevention of malaria. *BioDrugs*, 17(1):23-28.

Kanzok, S.M., Schirmer, R.H., Türbachova, I., Iozef, R. & Becker, K. 2000. The thioredoxin system of the malaria parasite Plasmodium falciparum glutathione reduction revisited. *Journal of Biological Chemistry*, 275(51):40180-40186.

Karunajeewa, H.A. 2011. Artemisinins: artemisinin, dihydroartemisinin, artemether and artesunate. Treatment and Prevention of Malaria. Springer. p. 157-190).

Karunaweera, N.D., Grau, G.E., Gamage, P., Carter, R. & Mendis, K.N. 1992. Dynamics of fever and serum levels of tumor necrosis factor are closely associated during clinical paroxysms in Plasmodium vivax malaria. *Proceedings of the National Academy of Sciences*, 89(8):3200-3203.

Kealy, T. & Pauson, P. 1951. A new type of organo-iron compound. *Nature*, 168(4285):1039-1040.

Khaliq, A.A., Fox, E., Sarwar, M. & Strickland, G.T. 1987. Amodiaquine fails to cure chloroquine resistant Plasmodium falciparum in the Punjab. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 81(1):157-159.

Killeen, G.F., Kiware, S.S., Okumu, F.O., Sinka, M.E., Moyes, C.L., Massey, N.C., Gething, P.W., Marshall, J.M., Chaccour, C.J. & Tusting, L.S. 2017. Going beyond personal protection against mosquito bites to eliminate malaria transmission: population suppression

of malaria vectors that exploit both human and animal blood. *BMJ Global Health*, 2(2):e000198.

Klayman, D.L. 1985. Qinghaosu (artemisinin): an antimalarial drug from China. *Science*, 228:1049-1056.

Köpf-Maier, P., Köpf, H. & Neuse, E.W. 1984. Ferrocenium salts—the first antineoplastic iron compounds. *Angewandte Chemie International Edition*, 23(6):456-457.

Korsinczky, M., Chen, N., Kotecka, B., Saul, A., Rieckmann, K. & Cheng, Q. 2000. Mutations in Plasmodium falciparumCytochrome b That Are Associated with Atovaquone Resistance Are Located at a Putative Drug-Binding Site. *Antimicrobial Agents and Chemotherapy*, 44(8):2100-2108.

Kremsner, P., Zotter, G., Feldmeier, H., Graninger, W., Rocha, R. & Wiedermann, G. 1988. A comparative trial of three regimens for treating uncomplicated falciparum malaria in Acre, Brazil. *The Journal of Infectious Diseases*, 158(6):1368-1371.

Kremsner, P.G. & Krishna, S. 2004. Antimalarial combinations. *The Lancet*, 364(9430):285-294.

Krishna, S., Woodrow, C.J., Staines, H.M., Haynes, R.K. & Mercereau-Puijalon, O. 2006. Re-evaluation of how artemisinins work in light of emerging evidence of in vitro resistance. *Trends in Molecular Medicine*, 12(5):200-205.

Krungkrai, J., Burat, D., Kudan, S., Krungkrai, S. & Prapunwattana, P. 1999. Mitochondrial oxygen consumption in asexual and sexual blood stages of the human malarial parasite, Plasmodium falciparum. *The Southeast Asian Journal of Tropical Medicine and Public Health*, 30(4):636-642.

Kuehn, A., Simon, N. & Pradel, G. 2010. Family members stick together: multi-protein complexes of malaria parasites. *Medical Microbiology and Immunology*, 199(3):209-226.

Kumar, S. & Bandyopadhyay, U. 2005. Free heme toxicity and its detoxification systems in human. *Toxicology Letters*, 157(3):175-188.

Kumura, N., Furukawa, H., Onyango, A.N., Izumi, M., Nakajima, S., Ito, H., Hatano, T., Kim, H.-S., Wataya, Y. & Baba, N. 2009. Different behavior of artemisinin and tetraoxane in the oxidative degradation of phospholipid. *Chemistry and Physics of Lipids*, 160(2):114-120.

Kyle, D. & Webster, H. 1996. Postantibiotic effect of quinine and dihydroartemisin on Plasmodium falciparum in vitro: implications for a mechanism of recrudescence. (*In*. XIVth International Congress for Tropical Medicine and Malaria: abstract 0-22 organised by.

Lanners, H.N. 1991. Effect of the 8-aminoquinoline primaquine on culture-derived gametocytes of the malaria parasitePlasmodium falciparum. *Parasitology Research*, 77(6):478-481.

Lell, B. & Kremsner, P.G. 2002. Clindamycin as an antimalarial drug: review of clinical trials. *Antimicrobial Agents and Chemotherapy*, 46(8):2315-2320.

Li, Q., Brueckner, R., Peggins, J., Trotman, K. & Brewer, T. 1999. Arteether toxicokinetics and pharmacokinetics in rats after 25 mg/kg/day single and multiple doses. *European Journal of Drug Metabolism and Pharmacokinetics*, 24(3):213-223.

Li, X.-Q., Björkman, A., Andersson, T.B., Ridderström, M. & Masimirembwa, C.M. 2002. Amodiaquine Clearance and Its Metabolism toN-Desethylamodiaquine Is Mediated by CYP2C8: A New High Affinity and Turnover Enzyme-Specific Probe Substrate. *Journal of Pharmacology and Experimental Therapeutics*, 300(2):399-407.

Lin, L., Berces, A. & Kraatz, H.-B. 1998. Ferrocenic acid derivatives: towards rationalizing changes in the electronic and geometric structures. *Journal of Organometallic Chemistry*, 556(1):11-20.

Longueville, A. & Crichton, R.R. 1986. An animal model of iron overload and its application to study hepatic ferritin iron mobilization by chelators. *Biochemical Pharmacology*, 35(21):3669-3678.

Looareesuwan, S., Chulay, J.D., Canfield, C.J. & Hutchinson, D. 1999. Malarone (atovaquone and proguanil hydrochloride): a review of its clinical development for treatment of malaria. Malarone Clinical Trials Study Group. *The American Journal of Tropical Medicine and Hygiene*, 60(4):533-541.

MacFaul, P.A., Wayner, D. & Ingold, K. 1998. A radical account of “oxygenated Fenton chemistry”. *Accounts of Chemical Research*, 31(4):159-162.

Magerlein, B., Birkenmeyer, R. & Kagan, F. 1966. Chemical modification of lincomycin. *Antimicrobial Agents and Chemotherapy*, 6:727.

Maggs, J.L., Madden, S., Bishop, L.P., O’Neill, P.M. & Park, B.K. 1997. The rat biliary metabolites of dihydroartemisinin, an antimalarial endoperoxide. *Drug Metabolism and Disposition*, 25(10):1200-1204.

Malaguarnera, L. & Musumeci, S. 2002. The immune response to Plasmodium falciparum malaria. *The Lancet Infectious Diseases*, 2(8):472-478.

Martins, P., Marques, M., Coito, L., JL Pombeiro, A., Viana Baptista, P. & R Fernandes, A. 2014. Organometallic compounds in cancer therapy: Past lessons and future directions. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)*, 14(9):1199-1212.

Mason, D., Lummis, W. & Dietz, A. 1963. U-22956, A NEW ANTIBIOTIC. I. DISCOVERY AND BIOLOGICAL ACTIVITY. *Antimicrobial Agents and Chemotherapy*, 10:110-113.

Mbengue, A., Bhattacharjee, S., Pandharkar, T., Liu, H., Estiu, G., Stahelin, R.V., Rizk, S.S., Njimoh, D.L., Ryan, Y. & Chotivanich, K. 2015. A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria. *Nature*, 520(7549):683-687.

McIntosh, H. & Olliaro, P. 1999. Artemisinin derivatives for treating uncomplicated malaria. *The Cochrane Library*.

Ménard, R., Heussler, V., Yuda, M. & Nussenzweig, V. 2008. Plasmodium pre-erythrocytic stages: what’s new? *Trends in Parasitology*, 24(12):564-569.

Meshnick, S. 1997. Why does quinine still work after 350 years of use? *Parasitology Today*, 13(3):89-90.

Meshnick, S., Little, B. & Yang, Y. 1994. Alkylation of proteins by artemisinin. *Biochemical Pharmacology*, 48:569-573.

Meshnick, S.R. & Dobson, M.J. 2001. The history of antimalarial drugs. *Antimalarial Chemotherapy*:15-25.

Meshnick, S.R., Thomas, A., Ranz, A., Xu, C.-M. & Pan, H.-Z. 1991. Artemisinin (qinghaosu): the role of intracellular hemin in its mechanism of antimalarial action. *Molecular and Biochemical Parasitology*, 49(2):181-189.

Meyers, J.I., Pathikonda, S., Popkin-Hall, Z.R., Medeiros, M.C., Fuseini, G., Matias, A., Garcia, G., Overgaard, H.J., Kulkarni, V. & Reddy, V.P. 2016. Increasing outdoor host-seeking in Anopheles gambiae over 6 years of vector control on Bioko Island. *Malaria Journal*, 15(1):239.

Michel, R. 1968. Comparative study of the association of sulfalene and pyrimethamine and of sulfalene alone in mass chemoprophylaxis of malaria. *Médecine tropicale: revue du Corps de santé colonial*, 28(4):488.

Miller, S.A., Tebboth, J.A. & Tremaine, J.F. 1952. 114. Di cyclo pentadienyliron. *Journal of the Chemical Society (Resumed)*:632-635.

Mouatcho, J.C. & Goldring, J.D. 2013. Malaria rapid diagnostic tests: challenges and prospects. *Journal of Medical Microbiology*, 62(10):1491-1505.

Murambiwa, P., Masola, B., Govender, T., Mukaratirwa, S. & Musabayane, C. 2011. Anti-malarial drug formulations and novel delivery systems: a review. *Acta Tropica*, 118(2):71-79.

Mutabingwa, T.K., Anthony, D., Heller, A., Hallett, R., Ahmed, J., Drakeley, C., Greenwood, B.M. & Whitty, C.J. 2005. Amodiaquine alone, amodiaquine+ sulfadoxine-pyrimethamine, amodiaquine+ artesunate, and artemether-lumefantrine for outpatient treatment of malaria in Tanzanian children: a four-arm randomised effectiveness trial. *The Lancet*, 365(9469):1474-1480.

Nesmeyanov, A., Bogomolova, L., Viltchevskaya, V., Palitsyne, N., Andrianova, I. & Belozerova, O. 1971. Ferrocerone. *US Patent*, 119356.

Nielsen, P. & Heinrich, H.C. 1993. Metabolism of iron from (3, 5, 5-trimethylhexanoyl) ferrocene in rats: A dietary model for severe iron overload. *Biochemical Pharmacology*, 45(2):385-391.

Nkrumah, L.J., Riegelhaupt, P.M., Moura, P., Johnson, D.J., Patel, J., Hayton, K., Ferdig, M.T., Wellems, T.E., Akabas, M.H. & Fidock, D.A. 2009. Probing the multifactorial basis of Plasmodium falciparum quinine resistance: evidence for a strain-specific contribution of the sodium-proton exchanger PfNHE. *Molecular and Biochemical Parasitology*, 165(2):122-131.

Nzila, A., Okombo, J., Ohuma, E. & Al-Thukair, A. 2012. Update on the in vivo tolerance and in vitro reduced susceptibility to the antimalarial lumefantrine. *Journal of Antimicrobial Chemotherapy*, 67(10):2309-2315.

O'Neill, P.M., Bray, P.G., Hawley, S.R., Ward, S.A. & Park, B.K. 1998. 4-Aminoquinolines—Past, present, and future; A chemical perspective. *Pharmacology & Therapeutics*, 77(1):29-58.

O'Neill, P.M., Barton, V.E. & Ward, S.A. 2010. The molecular mechanism of action of artemisinin—the debate continues. *Molecules*, 15(3):1705-1721.

O'Neill, P.M., Barton, V.E., Ward, S.A. & Chadwick, J. 2011. 4-Aminoquinolines: chloroquine, amodiaquine and next-generation analogues. Treatment and Prevention of Malaria. Springer. p. 19-44).

Oduola, A.M., Milhous, W., Weatherly, N., Bowdre, J. & Desjardins, R. 1988. Plasmodium falciparum: induction of resistance to mefloquine in cloned strains by continuous drug exposure in vitro. *Experimental Parasitology*, 67(2):354-360.

Olliaro, P. 2001. Mode of action and mechanisms of resistance for antimalarial drugs. *Pharmacology & Therapeutics*, 89(2):207-219.

Osella, D., Ferrali, M., Zanello, P., Laschi, F., Fontani, M., Nervi, C. & Cavigiolio, G. 2000. On the mechanism of the antitumor activity of ferrocenium derivatives. *Inorganica Chimica Acta*, 306(1):42-48.

Paaijmans, K.P., Read, A.F. & Thomas, M.B. 2009. Understanding the link between malaria risk and climate. *Proceedings of the National Academy of Sciences*, 106(33):13844-13849.

Painter, H.J., Morrisey, J.M., Mather, M.W. & Vaidya, A.B. 2007. Specific role of mitochondrial electron transport in blood-stage Plasmodium falciparum. *Nature*, 446(7131):88-91.

Paitayatat, S., Tarnchompoo, B., Thebtaranonth, Y. & Yuthavong, Y. 1997. Correlation of antimalarial activity of artemisinin derivatives with binding affinity with ferroprotoporphyrin IX. *Journal of Medicinal Chemistry*, 40(5):633-638.

Paloque, L., Ramadani, A.P., Mercereau-Puijalon, O., Augereau, J.-M. & Benoit-Vical, F. 2016. Plasmodium falciparum: multifaceted resistance to artemisinins. *Malaria Journal*, 15(1):149.

Pasvol, G. 2005. Management of severe malaria: interventions and controversies. *Infectious Disease Clinics*, 19(1):211-240.

Peel, S.A., Merritt, S.C., Handy, J. & Baric, R.S. 1993. Derivation of highly mefloquine-resistant lines from Plasmodium falciparum in vitro. *The American Journal of Tropical Medicine and Hygiene*, 48(3):385-397.

Peters, W. 1970. Chemotherapy and drug resistance in malaria. *Chemotherapy and drug resistance in malaria.*

Peters, W., Ellis, D., Boulard, Y. & Landau, I. 1984. The chemotherapy of rodent malaria XXXVI: Causal prophylaxis. Part IV. The activity of a new 8-aminoquinoline, WR 225, 448 against exo-erythrocytic schizonts of Plasmodium yoelii yoelii. *Annals of Tropical Medicine & Parasitology*, 78(5):467-478.

Peterson, D.S., Milhous, W.K. & Wellems, T.E. 1990. Molecular basis of differential resistance to cycloguanil and pyrimethamine in Plasmodium falciparum malaria. *Proceedings of the National Academy of Sciences*, 87(8):3018-3022.

Peterson, D.S., Walliker, D. & Wellems, T.E. 1988. Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. *Proceedings of the National Academy of Sciences*, 85(23):9114-9118.

Phillips, M.A., Burrows, J.N., Manyando, C., van Huijsduijnen, R.H., Van Voorhis, W.C. & Wells, T.N.C. 2017. Malaria. *Nature Reviews Disease Primers*, 3:17050.

Plowe, C. 2005. Antimalarial drug resistance in Africa: strategies for monitoring and deterrence. Malaria: Drugs, Disease and Post-genomic Biology. Springer. p. 55-79).

Ploypradith, P. 2004. Development of artemisinin and its structurally simplified trioxane derivatives as antimalarial drugs. *Acta Tropica*, 89(3):329-342.

Qualls, W.A., Müller, G.C., Traore, S.F., Traore, M.M., Arheart, K.L., Doumbia, S., Schlein, Y., Kravchenko, V.D., Xue, R.-D. & Beier, J.C. 2015. Indoor use of attractive toxic sugar bait (ATSB) to effectively control malaria vectors in Mali, West Africa. *Malaria Journal*, 14(1):301.

Ramkaran, A. & Peters, W. 1969. Infectivity of chloroquine resistant Plasmodium berghei to Anopheles stephensi enhanced by chloroquine. *Nature*, 223(5206):635-636.

Ramya, T., Mishra, S., Karmodiya, K., Surolia, N. & Surolia, A. 2007. Inhibitors of nonhousekeeping functions of the apicoplast defy delayed death in Plasmodium falciparum. *Antimicrobial Agents and Chemotherapy*, 51(1):307-316.

Rausch, M., Vogel, M. & Rosenberg, H. 1957. Ferrocene: A novel organometallic compound. *J. Chem. Educ*, 34(6):268.

Reiter, C., Fröhlich, T., Zeino, M., Marschall, M., Bahsi, H., Leidenberger, M., Friedrich, O., Kappes, B., Hampel, F. & Efferth, T. 2015. New efficient artemisinin derived agents against human leukemia cells, human cytomegalovirus and Plasmodium falciparum: 2nd generation 1, 2, 4-trioxane-ferrocene hybrids. *European Journal of Medicinal Chemistry*, 97:164-172.

Reiter, C., Karagöz, A.Ç., Fröhlich, T., Klein, V., Zeino, M., Viertel, K., Held, J., Mordmüller, B., Öztürk, S.E. & Anıl, H. 2014. Synthesis and study of cytotoxic activity of 1, 2, 4-trioxane-

and egonol-derived hybrid molecules against Plasmodium falciparum and multidrug-resistant human leukemia cells. *European Journal of Medicinal Chemistry*, 75:403-412.

Reyburn, H., Mbakilwa, H., Mwangi, R., Mwerinde, O., Olomi, R., Drakeley, C. & Whitty, C.J. 2007. Rapid diagnostic tests compared with malaria microscopy for guiding outpatient treatment of febrile illness in Tanzania: randomised trial. *British Medical Journal*, 334(7590):403.

Rieckmann, K.H., Brewer, G.J. & Powell, R.D. 1968. Effects of diaphenylsulphone (dapsone) against Plasmodium vivax of South West Pacific origin. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 62(5):649-653.

Rosenberg, R., Wirtz, R.A., Schneider, I. & Burge, R. 1990. An estimation of the number of malaria sporozoites ejected by a feeding mosquito. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 84(2):209-212.

Ross, R. 1897. On some peculiar pigmented cells found in two mosquitos fed on malarial blood. *British Medical Journal*, 2(1929):1786.

Rotman, A. 1975. Genetics of a primaquin-resistant yeast. *Journal of General Microbiology*, 89(1):1-10.

Sanchez, C.P., McLean, J.E., Stein, W. & Lanzer, M. 2004. Evidence for a substrate specific and inhibitable drug efflux system in chloroquine resistant Plasmodium falciparum strains. *Biochemistry*, 43(51):16365-16373.

Sanchez, C.P., Rotmann, A., Stein, W.D. & Lanzer, M. 2008. Polymorphisms within PfMDR1 alter the substrate specificity for anti-malarial drugs in Plasmodium falciparum. *Molecular Microbiology*, 70(4):786-798.

Savarino, A., Boelaert, J.R., Cassone, A., Majori, G. & Cauda, R. 2003. Effects of chloroquine on viral infections: an old drug against today's diseases. *The Lancet Infectious Diseases*, 3(11):722-727.

Schlitzer, M. 2007. Malaria chemotherapeutics part I: History of antimalarial drug development, currently used therapeutics, and drugs in clinical development. *ChemMedChem*, 2(7):944-986.

Seiler, A., Schneider, M., Förster, H., Roth, S., Wirth, E.K., Culmsee, C., Plesnila, N., Kremmer, E., Rådmark, O. & Wurst, W. 2008. Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent-and AIF-mediated cell death. *Cell Metabolism*, 8(3):237-248.

Sharma, N., Ajay, J.K., Venkatasubbaiah, K. & Lourderaj, U. 2015. Mechanisms and dynamics of protonation and lithiation of ferrocene. *Physical Chemistry Chemical Physics*, 17(34):22204-22209.

Sheehy, T.W., Reba, R.C., Neff, T.A., Gaintner, J.R. & Tigertt, W.D. 1967. Supplemental sulfone (dapsone) therapy: use in treatment of chloroquine-resistant falciparum malaria. *Archives of Internal Medicine*, 119(6):561-566.

Sidhu, A.B.S., Sun, Q., Nkrumah, L.J., Dunne, M.W., Sacchettini, J.C. & Fidock, D.A. 2007. In vitro efficacy, resistance selection, and structural modeling studies implicate the malarial parasite apicoplast as the target of azithromycin. *Journal of Biological Chemistry*, 282(4):2494-2504.

Sidhu, A.B.S., Verdier-Pinard, D. & Fidock, D.A. 2002. Chloroquine resistance in Plasmodium falciparum malaria parasites conferred by pfcrt mutations. *Science*, 298(5591):210-213.

Sies, H. 1997. Oxidative stress: oxidants and antioxidants. *Experimental Physiology*, 82(2):291-295.

Sinden, R., Butcher, G., Billker, O. & Fleck, S. 1996. Regulation of infectivity of Plasmodium to the mosquito vector. *Advances in Parasitology*, 38:53-117.

Snow, R.W., Guerra, C.A., Noor, A.M., Myint, H.Y. & Hay, S.I. 2005. The global distribution of clinical episodes of Plasmodium falciparum malaria. *Nature*, 434(7030):214-217.

Sougoufara, S., Diédhiou, S.M., Doucouré, S., Diagne, N., Sembène, P.M., Harry, M., Trape, J.-F., Sokhna, C. & Ndiath, M.O. 2014. Biting by Anopheles funestus in broad daylight after use of long-lasting insecticidal nets: a new challenge to malaria elimination. *Malaria Journal*, 13(1):125.

Srivastava, I.K., Rottenberg, H. & Vaidya, A.B. 1997. Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite. *Journal of Biological Chemistry*, 272(7):3961-3966.

Stepniewska, K. & White, N. 2008. Pharmacokinetic determinants of the window of selection for antimalarial drug resistance. *Antimicrobial Agents and Chemotherapy*, 52(5):1589-1596.

Strong, R.P. 1944. Stitts diagnosis, prevention and treatment of tropical diseases. Vol. 1: Blakiston Co.

Tan, K.R., Magill, A.J., Parise, M.E. & Arguin, P.M. 2011. Doxycycline for malaria chemoprophylaxis and treatment: report from the CDC expert meeting on malaria chemoprophylaxis. *The American Journal of Tropical Medicine and Hygiene*, 84(4):517-531.

Taylor, W.R.J. & White, N.J. 2004. Antimalarial drug toxicity. *Drug Safety*, 27(1):25-61.

Teja-isavadharm, P., Nosten, F., Kyle, D., Luxemburger, C., Ter Kuile, F., Peggins, J., Brewer, T. & White, N. 1996. Comparative bioavailability of oral, rectal, and intramuscular artemether in healthy subjects: use of simultaneous measurement by high performance liquid chromatography and bioassay. *British Journal of Clinical Pharmacology*, 42(5):599-604.

Tenywa, F.C., Kambagha, A., Saddler, A. & Maia, M.F. 2017. The development of an ivermectin-based attractive toxic sugar bait (ATSB) to target Anopheles arabiensis. *Malaria Journal*, 16(1):338.

Teuscher, F., Chen, N., Kyle, D.E., Gatton, M.L. & Cheng, Q. 2012. Phenotypic changes in artemisinin-resistant Plasmodium falciparum lines in vitro: evidence for decreased sensitivity to dormancy and growth inhibition. *Antimicrobial Agents and Chemotherapy*, 56(1):428-431.

Teuscher, F., Gatton, M.L., Chen, N., Peters, J., Kyle, D.E. & Cheng, Q. 2010. Artemisinin-induced dormancy in Plasmodium falciparum: duration, recovery rates, and implications in treatment failure. *The Journal of Infectious Diseases*, 202(9):1362-1368.

Touray, M.G., Warburg, A., Laughinghouse, A., Krettli, A.U. & Miller, L.H. 1992. Developmentally regulated infectivity of malaria sporozoites for mosquito salivary glands and the vertebrate host. *Journal of Experimental Medicine*, 175(6):1607-1612.

Tu, Y. 2016. Artemisinin—A Gift from Traditional Chinese Medicine to the World (Nobel Lecture). *Angewandte Chemie International Edition*.

Tun, K.M., Imwong, M., Lwin, K.M., Win, A.A., Hlaing, T.M., Hlaing, T., Lin, K., Kyaw, M.P., Plewes, K. & Faiz, M.A. 2015. Spread of artemisinin-resistant Plasmodium falciparum in Myanmar: a cross-sectional survey of the K13 molecular marker. *The Lancet Infectious Diseases*, 15(4):415-421.

Ugochukwu, C., Ebong, P. & Eyong, E. 2008. Biochemical implication of long term administration of Halofantrine hydrochloride (Halfan) on estradiol levels of female Wistar rats. *Pakistan Journal of Nutrition*, 7(2):227-230.

Ulusu, N., Acan, N., Turan, B. & Tezcan, E. 2000. The effect of selenium on glutathione redox cycle enzymes of some rabbit tissues. *Trace Elements and Electrolytes*, 17(1):25-29.

van Agtmael, M., Bouchaud, O., Malvy, D., Delmont, J., Danis, M., Barette, S., Gras, C., Bernard, J., Touze, J.-E. & Gathmann, I. 1999. The comparative efficacy and tolerability of CGP 56697 (artemether+ lumefantrine) versus halofantrine in the treatment of uncomplicated falciparum malaria in travellers returning from the Tropics to The Netherlands and France. *International Journal of Antimicrobial Agents*, 12(2):159-169.

Vennerstrom, J.L., Ellis, W.Y., Ager Jr, A.L., Andersen, S.L., Gerena, L. & Milhous, W.K. 1992. Bisquinolines. 1. N, N-bis (7-chloroquinolin-4-yl) alkanediamines with potential against chloroquine-resistant malaria. *Journal of Medicinal Chemistry*, 35(11):2129-2134.

Vijaykadga, S., Rojanawatsirivej, C., Cholpol, S., Phoungmanee, D., Nakavej, A. & Wongsrichanalai, C. 2006. In vivo sensitivity monitoring of mefloquine monotherapy and

artesunate–mefloquine combinations for the treatment of uncomplicated falciparum malaria in Thailand in 2003. *Tropical Medicine & International Health*, 11(2):211-219.

Vivas, L., Rattray, L., Stewart, L., Robinson, B., Fugmann, B., Haynes, R., Peters, W. & Croft, S. 2007. Antimalarial efficacy and drug interactions of the novel semi-synthetic endoperoxide artemisone in vitro and in vivo. *Journal of Antimicrobial Chemotherapy*, 59(4):658-665.

Wacks, R.E. 2013. " Don't Strip-Tease for Anopheles": A history of malaria protocols during World War II. The Florida State University.

Walling, C. 1998. Intermediates in the reactions of Fenton type reagents. *Accounts of Chemical Research*, 31(4):155-157.

Wang, J., Huang, L., Li, J., Fan, Q., Long, Y., Li, Y. & Zhou, B. 2010. Artemisinin directly targets malarial mitochondria through its specific mitochondrial activation. *PLOS ONE*, 5(3):e9582.

Wani, W.A., Jameel, E., Baig, U., Mumtazuddin, S. & Hun, L.T. 2015. Ferroquine and its derivatives: new generation of antimalarial agents. *European Journal of Medicinal Chemistry*, 101:534-551.

Ward, R., Florence, A., Baldwin, D., Abiaka, C., Roland, F., Ramsey, M., Dickson, D., Peters, T. & Crichton, R. 1991. Biochemical and biophysical investigations of the ferrocene-iron-loaded rat. An animal model of primary haemochromatosis. *European Journal of Biochemistry*, 202(2):405.

Waters, N.C. & Edstein, M.D. 2011. 8-Aminoquinolines: primaquine and tafenoquine. Treatment and Prevention of Malaria. Springer. p. 69-94).

Watkins, W., Brandling-Bennett, A., Nevill, C., Carter, J., Boriga, D., Howells, R. & Koech, D. 1988. Chlorproguanil/dapsone for the treatment of non-severe Plasmodium falciparum malaria in Kenya: a pilot study. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 82(3):398-403.

Watsierah, C.A. & Ouma, C. 2014. Access to artemisinin-based combination therapy (ACT) and quinine in malaria holoendemic regions of western Kenya. *Malaria Journal*, 13(1):290.

Weinke, T., Trautmann, M., Held, T., Weber, G., Eichenlaub, D., Fleischer, K., Kern, W. & Pohle, H.D. 1991. Neuropsychiatric side effects after the use of mefloquine. *The American Journal of Tropical Medicine and Hygiene*, 45(1):86-91.

Wernsdorfer, W.H. 2012. Global challenges of changing epidemiological patterns of malaria. *Acta Tropica*, 121(3):158-165.

White, N. 1992. Antimalarial pharmacokinetics and treatment regimens. *British Journal of Clinical Pharmacology*, 34(1):1-10.

White, N. 1994. Mefloquine. *British Medical Journal*, 308(6924):286.

White, N. 1997. Assessment of the pharmacodynamic properties of antimalarial drugs in vivo. *Antimicrobial Agents and Chemotherapy*, 41(7):1413.

White, N.J. 2008. Qinghaosu (artemisinin): the price of success. *Science*, 320(5874):330-334.

White, N.J., Stepniewska, K., Barnes, K., Price, R.N. & Simpson, J. 2008. Simplified antimalarial therapeutic monitoring: using the day-7 drug level? *Trends in Parasitology*, 24(4):159-163.

White, N.J., van Vugt, M. & Ezzet, F.D. 1999. Clinical pharmacokinetics and pharmacodynamics of artemether-lumefantrine. *Clinical Pharmacokinetics*, 37(2):105-125.

WHO. 2010. Malaria. WHO Fact Sheet No. 94, WHO Media centre, Geneva.

WHO. 2013. Emergency response to artemisinin resistance in the Greater Mekong subregion: regional framework for action 2013-2015: World Health Organization.

WHO. 2016. Artemisinin and artemisinin-based combination therapy resistance: status report.

WHO. 2018. World Malaria Report 2018. Geneva: WHO; 2018. Licence: CC BY-NC-SA 3.0 IGO.

Wilkinson, G., Cotton, F. & Birmingham, J. 1956. On manganese cyclopentadienide and some chemical reactions of neutral bis-cyclopentadienyl metal compounds. *Journal of Inorganic and Nuclear Chemistry*, 2(2):95-113.

Wilkinson, G., Pauson, P. & Cotton, F. 1954. Bis-cyclopentadienyl Compounds of Nickel and Cobalt. *Journal of the American Chemical Society*, 76(7):1970-1974.

Wilson, C.M., Volkman, S.K., Thaithong, S., Martin, R.K., Kyle, D.E., Milhous, W.K. & Wirth, D.F. 1993. Amplification of pfmdr1 associated with mefloquine and halofantrine resistance in Plasmodium falciparum from Thailand. *Molecular and Biochemical Parasitology*, 57(1):151-160.

Winstanley, P.A., Ward, S.A. & Snow, R.W. 2002. Clinical status and implications of antimalarial drug resistance. *Microbes and Infection*, 4(2):157-164.

Wongsrichanalai, C. & Meshnick, S.R. 2008. Declining artesunate-mefloquine efficacy against falciparum malaria on the Cambodia–Thailand border. *Emerging Infectious Diseases*, 14(5):716.

Wongsrichanalai, C., Pickard, A.L., Wernsdorfer, W.H. & Meshnick, S.R. 2002. Epidemiology of drug-resistant malaria. *The Lancet Infectious Diseases*, 2(4):209-218.

Wood, Z.A., Schröder, E., Harris, J.R. & Poole, L.B. 2003. Structure, mechanism and regulation of peroxiredoxins. *Trends in Biochemical Sciences*, 28(1):32-40.

Woodrow, C., Haynes, R. & Krishna, S. 2005. Artemisinins. *Postgraduate Medical Journal*, 81(952):71-78.

Woodward, R.B. & Doering, W.v.E. 1945. The total synthesis of quinine. *Journal of the American Chemical Society*, 67(5):860-874.

Yang, W.S. & Stockwell, B.R. 2016. Ferroptosis: death by lipid peroxidation. *Trends in Cell Biology*, 26(3):165-176.

Yeary, R.A. 1969. Chronic toxicity of dicyclopentadienyliron (ferrocene) in dogs. *Toxicology and Applied Pharmacology*, 15(3):666-676.

Ying-Zi, Y., Asawamahasakda, W. & Meshnick, S.R. 1993. Alkylation of human albumin by the antimalarial artemisinin. *Biochemical Pharmacology*, 46(2):336-339.

Ying-Zi, Y., Little, B. & Meshnick, S.R. 1994. Alkylation of proteins by artemisinin: Effects of heme, pH, and drug structure. *Biochemical Pharmacology*, 48(3):569-573.

Yodoi, J., Masutani, H. & Nakamura, H. 2001. Redox regulation by the human thioredoxin system. *BioFactors*, 15(2-4):107-111.

Zhang, S. & Gerhard, G.S. 2008. Heme activates artemisinin more efficiently than hemin, inorganic iron, or hemoglobin. *Bioorganic & Medicinal Chemistry*, 16(16):7853-7861.

Chapter 3:
Mini-Review: Non-acetal artemisinin derivatives – Worth the fuss?

This chapter contains a mini-review article to be submitted to Malaria journal. This mini-review focuses on the most potent non-acetal monomer artemisinin derivatives with respect to artemisinin. These derivatives are compared by relative activity and the overall yield.

Non-acetal artemisinin derivatives – Worth the fuss? A mini-review

Christo de Lange[a], Frans J. Smit[b], Richard K. Haynes*[b], David, D. N'Da[b*]

[a] *Pharmaceutical Chemistry, School of Pharmacy, North-West University, Potchefstroom 2520, South Africa*

[b] *Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom 2520, South Africa*

*email: david.nda@nwu.ac.za, Tel: +2718 299 2256, Fax: +2718 299 4243

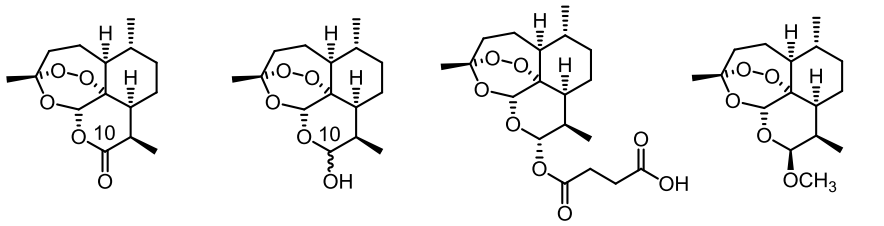
Abstract

Artemisinins are still the only effective treatment available for malaria. Artemisinin itself is also the starting material for most of the semi-synthetic derivatives. In this mini-review the most potent non-acetal monomer artemisinin derivatives with respect to artemisinin are considered. Unfortunately there is a general lack of cytotoxicity data and comparison between common malaria strains (CQS and CQR) which makes it hard to establish the true value added of these derivatives.

Keywords: *Non-acetal, antimalarial, artemisinin, malaria, deoxyartemisinins*

3.1 Introduction

The World Health Organization (WHO) reported that there were about 219 million cases of malaria in 2017 with a death rate of 0.2% (435 000) (WHO, 2018). Due to the ability of the malaria parasite to develop resistance, many clinical used drugs have become less effective in treating the disease (Fivelman *et al.*, 2002; Gregson & Plowe, 2005; Payne, 1987; Price *et al.*, 2004). This ability to develop resistance poses a major threat to the artemisinin class (**Figure 3.1**); the only effective chemotherapeutic agent left (WHO, 2010). In an attempt to prevent resistance of the parasite to the artemisinin class, the WHO recommended the use of artemisinins in combination with other classes of drugs known as artemisinin-based combination therapy (ACT) as the first line treatment for uncomplicated malaria (WHO, 2010).



Artemisinin (**1**) | Dihydro-artemisinin (**2**) | Artesunate (**3**) | Artemether (**4**)

Figure 3.1: Artemisinin derivatives

In some areas of Western Cambodia, the artemisinin combinational drug (ACT) dihydroartemisinin-piperaquine failed to cure half of all patients treated (Fairhurst & Dondorp, 2016). The WHO identified at least 17 sites (from 2013 to 2015) where there is suspected or confirmed artemisinin resistance (WHO, 2016). In a recent report issued by WHO, there is a higher than 10% failure rate of ACT in the Binh Phuoc province (Vietnam) (WHO, 2016).

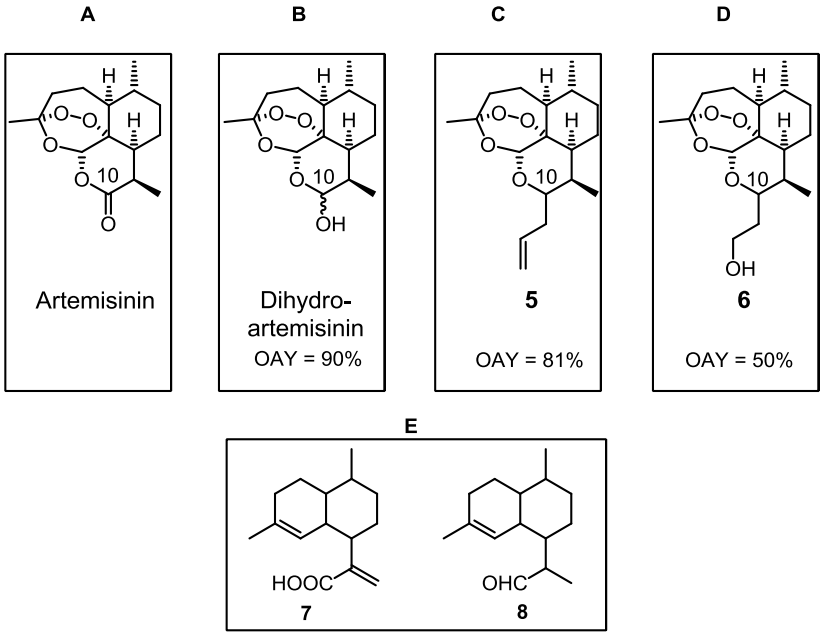
The metabolite of these artemisinins is dihydroartemisinin, which is neurotoxic and might also be implicated in artemisinin resistance (Mbengue *et al.*, 2015; Paloque *et al.*, 2016; Schmuck *et al.*, 2002). With a short elimination half-life of 3-4 hours for artemisinin and 40-60 min for dihydroartemisinin, resistance poses a major threat for these derivatives (Davis *et al.*, 2005). Thus, a subsequent important factor to consider for future artemisinin derivatives is a longer half-life. Since the life-cycle of the *Plasmodium falciparum* is 48 hours, this can serve as a guide for the minimum half-life of artemisinin derivatives. Aqueous solubility also plays an important role (Medhi *et al.*, 2009). This was improved for artemisinin when **3** was synthesised. By rendering the artemisinin-derivative more aqueous soluble, it would act faster – hence the importance of this consideration.

Jung postulated that if the stability of the derivative increased, so would the half-life within the body (Jung, 1994). Although this was never proven, another discovery was made; namely, by removing the oxygen at C-10, deoxyartemisinin (**9**) was obtained - this derivative displayed greatly enhanced remarkable acid stability. The main idea was that if it was more acid-stable, it would have more time to be absorbed and have a greater potency. This was also proved by comparing the stability of acetal and non-acetal artemisinin derivatives in simulated stomach acid (Jung & Lee, 1998). It was found that the C10 deoxyartemisinin derivatives were 15-22 times more acid-stable, than the acetal artemisinins. This discovery led to further investigations such as increasing the potency by adding different functional groups at the C-10 position.

Artemisinin is also used to synthesise the other clinically used artemisinins dihydroartemisinin (**2**, 84-90% yield), which is then used to synthesise artemether (**3**, 87% yield from DHA) (Stringham & Teager, 2012). However, this process increases the cost of the derivative due to the use of additional chemicals, purification and loss of starting material. The treatment cost for *P. falciparum* and *P. vivax* were estimated to vary from US$ 3.39 (dihydroartemisinin-piperaquine) to US$ 5.19 (artemether-lumefantrine) (Davis *et al.*, 2011).

In order to be able to compare these derivatives to some extent, the IC_{50} values were standardised to relative activity, or in terms of the greater levels of activity of this derivative compared to the parent drug artemisinin.

The derivatives in this review can be grouped into five groups, depending on the starting material (**Scheme 3.1**). For group A, the starting material is artemisinin (**1**) and the overall reaction yields for these deirivatives are some of the highest. For group B, the starting material is dihydroartemisinin. This is synthesised from **1** with a yield of 90%. 10-β-Allyldeoxoartemisinin (**5**) is used for group C derivatives, synthesised from dihydroartemisinin in high yields (90%) from **1**. For group D derivatives, 10β-(2-hydroxyethyl)-deoxoartemisinin (**6**) is used as starting material with an overall yield of 50% starting from **1**. Finally, the derivativxes in group E could possibly satisfy the future demand for cheap and eco-friendly derivatives since it is synthesised from dihydroartemisinicacid (**7**) or dihydroartemisinyl-aldehyde (**8**).



Scheme 3.1: Grouping of the non-acetal derivatives based on starting material with the yield from artemisinin in brackets

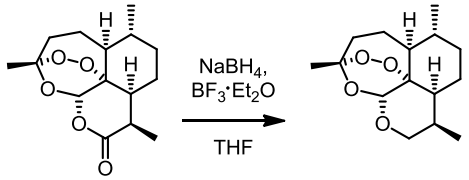
3.2 Synthesis and antimalarial activity

3.2.1 Group A derivatives

Artemisinin is extracted from *Artemisia annua* in low yields of 0.4 – 1.2 %. This has a bearing on the commercialisation and distribution of this antimalarial (Gilmore *et al.*, 2014). Artemisinic acid (AA) and dihydroartemisinic acid (DAA) (**7**), also extracted from *Artemisisia annua*, can be converted into artemisinin. The group of Haynes was the first to report the direct conversion of DAA to artemisinin and variations of this method are still in use today (Haynes & Vonwiller, 1990). This conversion was accomplished by photooxygenation of DAA in the presence of Rose Bengal and $Cu(OSO_2CF_3)_2$ as catalyst with an overall yield 29%. In a recent review, it was made clear that the main limitation of these conversion reactions of AA and DAA is that the yields never exceed 57% for artemisinin (Vil *et al.*, 2017). Additionally, many of these reactions require expensive UV-irradiation equipment.

Deoxoartemisinin (**9**) was the first non-acetal artemisinin derivative that was synthesised from artemisinin in 1989 (**Scheme 3.2**) (Jung *et al.*, 1990b). Applying a one-step reduction of artemisinin with $NaBH_4$ in the presence of BF_3•Et_2O in THF, it delivered a yield of 71%. This derivative was tested on the Indochina (W2) strain, which is chloroquine quinine, pyrimethamine, cycloguanil, and sulfadoxine-resistant and mefloquine-sensitive, as well as

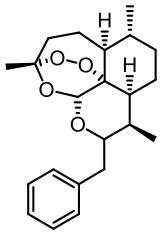
on the Sierra Leone (D6), (chloroquine-sensitive and mefloquine-resistant), of intra-erythrocytic *P. falciparum* parasites. **9** was eight times more active than artemisinin with a half-life of over 213 hours in acidic media. Despite enjoying good antimalarial activity as well as increased half-life, **9** lacks any toxicity data.



1 **9 (71%)**

Scheme 3.2: Synthesis of Deoxyartemisinin (**9**) from **1** (Jung *et al.*, 1990b)

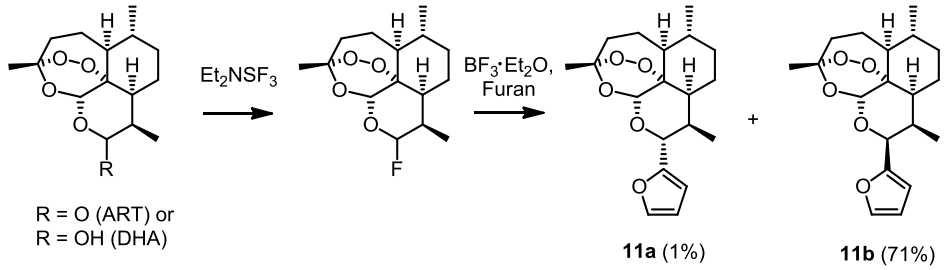
The benzyldeoxoartemisinin (**10**) had an IC_{50} value of less than 0.17 ng/mL against the W2 strain, which is seven times more active than artemisinin (Jung & Lee, 1998). It also had a remarkable half-life of 285.6 hours in an acidic solution (1mg/mL, 0.01 N HCl) with a pH of 2 at 37 °C. Lee and Oh reported in 2002, the synthesis of this product with an overall yield reported of 32.5 % starting from **1** (Lee & Oh, 2002).



10 (32.5%)

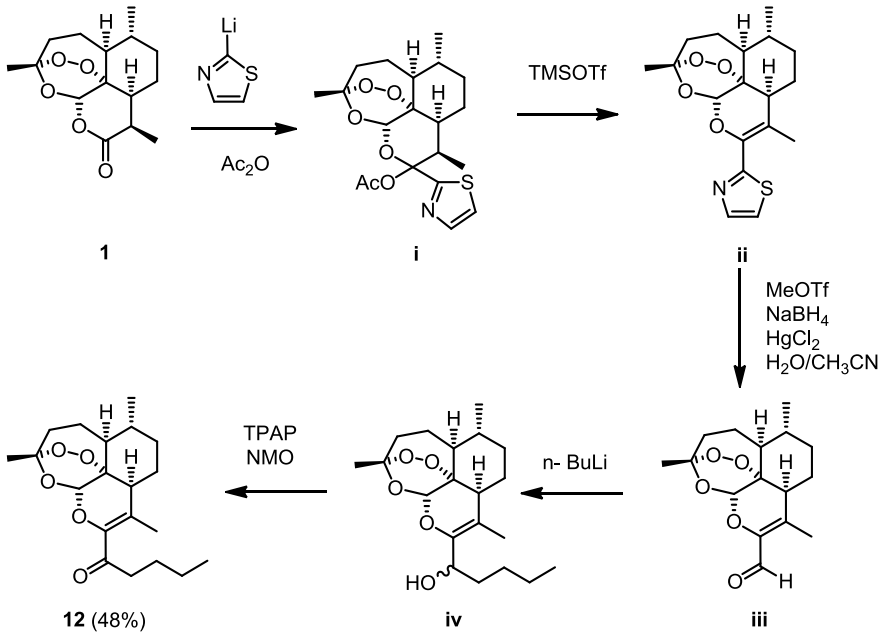
Figure 3.2: Benzyldeoxoartemisinin (**10**)

(**Scheme 3.4**) (O'Dowd *et al.*, 1999). This was reacted with triethylsilane and trimethylsilyl A series of furan derivatives was reported in 1999, of which the furan derivative (**11**) was the most potent against the NF54 strain of *P. falciparum* (**Scheme 3.3**) (Posner *et al.*, 1999). Starting from artemisinin or dihydroartemisinin, **11** was synthesised with a 72% yield starting from **1**. It was noted that two diastereomeres were synthesised and both had very different antimalarial activities. The C-9β (**11b**) methyl diastereomer had a 35-fold higher *in vitro* antimalarial activity than the C-9α (**11a**) methyl diastereomer. Fortunately, **11b** was the main product with a yield of 71 %.



Scheme 3.3: Furan deoxoartemisinin derivative, **11** (Posner *et al.*, 1999)

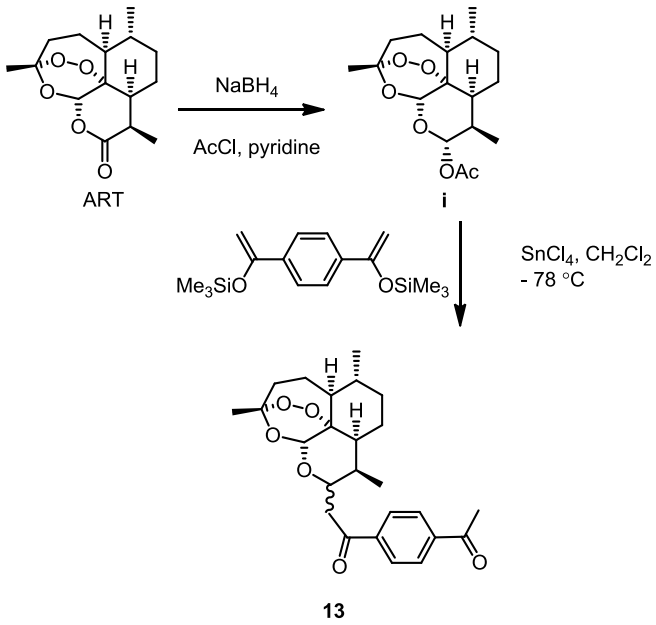
Derivative **12** is a derivative in its own class with an alkene bond adjacent to the C-10 carbon within the artemisinin structure. Artemisinin was reacted with 2-lithiothiazole at -65 °C and was acetalyted *in situ* in order to obtain the thiazole carbonyl adduct, **i**, in high yield triflate to obtain the 9,10 alkene; **ii**. This alkene thiazole was N-methylated, reduced and hydrolyzed to form the 9, 10-unsaturated C-10 aldehyde **iii**. Further reactions with *n*-Butyl lithium, tetrapropylammonium perruthenate and N-methylmorpholine N-oxide delivered **12** with an overall yield of 48% from **1**. This derivative was two times more active towards the *NF54* strain than artemisinin *in vitro*.



Scheme 3.4: Synthesis of **12**

On the other hand, the group Posner synthesised C-10 non-acetal dimers with a *p*-diacetylbenzene linker (**13**). **13** was obtained in both the *α* and *β* monomers in 3 % and 14 % yields respectively from artemisinin (**Scheme 3.5**) (Posner *et al.*, 2002). The approach was

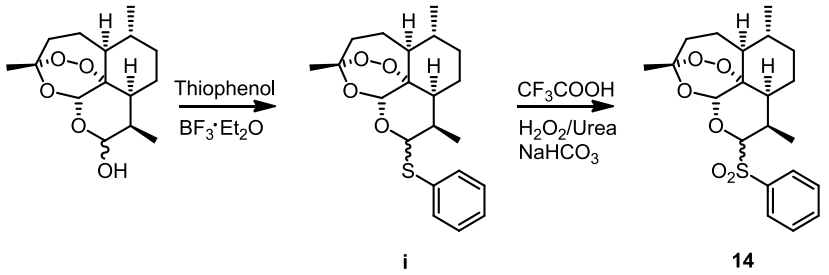
to start from artemisinin and to synthesise the dihydroartemisinin *α*-acetate **i**. Tin tetrachloride was then used to promote the double coupling with the disenol trimethylsilyl ether of *p*-diacetylbenzene (through Stille coupling). It was found that the *β* derivative was slightly more active than the *α* derivative, with IC_{50} values 3.0 and 4.4 respectively. These two derivatives were also more potent than artemisinin, which had an IC_{50} value of 7.6 towards the NF54 *P. falciparum* strain. Although the aim was to synthesise di-substituted derivatives, the overall yield for mono-substituted derivatives was 15.3% (from **1**) and for the di-substituted derivatives was 54% (from **1**).



Scheme 3.5: Synthesis of **13**

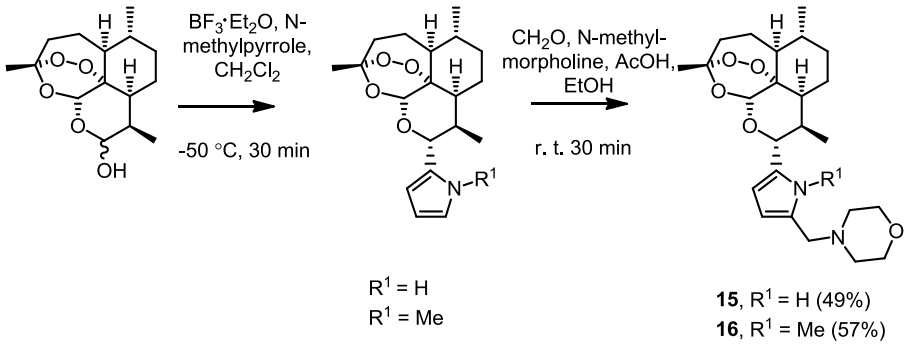
3.2.2 Group B derivatives

Dihydroartemisinin is synthesised from artemisinin using $NaBH_4$ in methanol or ethanol at low temperatures with high yields (Shrimali *et al.*, 1998; Singh *et al.*, 2001; Sy & Brown, 2001). Derivative **14** is yet another illustration of the α and β isomers showing different activity levels (**Scheme 3.6**) (Lee *et al.*, 2005). Dihydroartemisinin was reacted with thiophenol and BF_3•Et_2O was used as a catalyst to obtain **i**. Oxidation was then conducted with a H_2O_2/urea complex, trifluoroacetic acid and $NaHCO_3$ to obtain the final benzenesulfonoyl artemisinins. It was found that the β isomer was ten times more active than the α isomer. The yield for this derivative was only reported as high.



Scheme 3.6: Synthesis of **14**

The group of Pacorel incorporated a C-10 pyrrole mannich-base derivative into dihydroartemisinin (**Scheme 3.7**) (Pacorel *et al.*, 2009). One of the main reasons for this was to explore the possibility of formulating these derivatives as water-soluble salts. The most active derivatives towards the K1 chloroquine-resistant strain incorporated the morpholine functionality where either hydrogen (**15**) or methyl group (**16**) at R^1 was the most active. The calculated logP values were 3.01 for **15** and 3.33 for **16**. Another C-10 non-acetal derivative functionalised with morpholine had a logP value of 3.05, and it was very cyto- and neurotoxic (Haynes *et al.*, 2007). The cause of the toxicity is yet to be determined – whether or not it is the logP or the morpholine functionality.

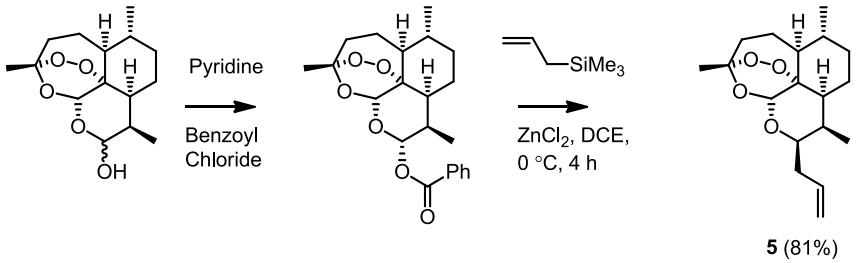


Scheme 3.7: Pyrrole Mannich base derivative **15** and **16** (Pacorel *et al.*, 2009)

3.2.3 Group C derivatives:

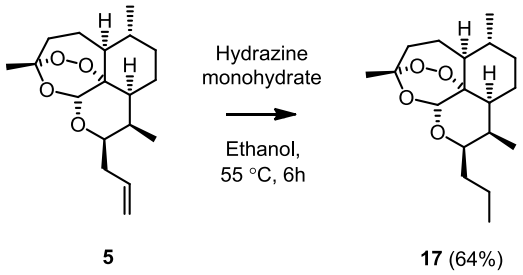
10-β-Allyldeoxoartemisinin (**5**) was first reported in 1992 by the group of Haynes (Haynes & Vonwiller, 1992). Starting from DAA and following a complex synthesis route, **5** was obtained with an overall yield of 25%. The following year this derivative was synthesised by adding allyltrimethylsilane and BF_3•Et_2O to a -55 °C solution of dihydroartemisinin under argon, giving rise to **5** with an overall yield of 50% from artemisinin (Pu *et al.*, 1993). A higher

yielding reaction is depicted in **Scheme 3.8** (Jeyadevan *et al.*, 2004). It had IC_{50} values of 0.59 and 1.07 nM towards the W2 and D6 strains, respectively.



Scheme 3.8: Synthesis of **5** (Jeyadevan *et al*., 2004)

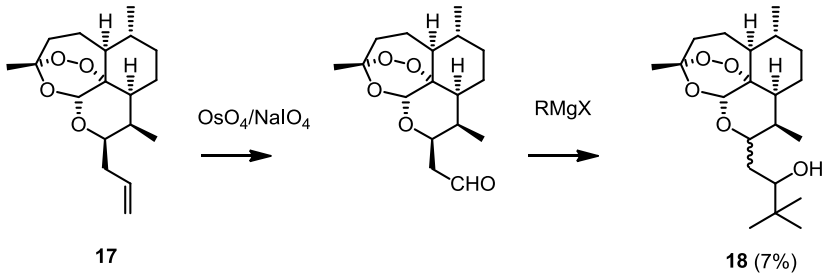
In 1995 a one-pot synthesis reaction was conducted with 12β-allyldeoxoartemisinin (**5**), hydrazine monohydrate and ethanol at 55°C to synthesise the 12β-*n*-propyldeoxoartemisinin (**17**) derivative from **5** (**Scheme 3.9**) (Pu & Ziffer, 1995).



Scheme 3.9: Synthesis of **17**

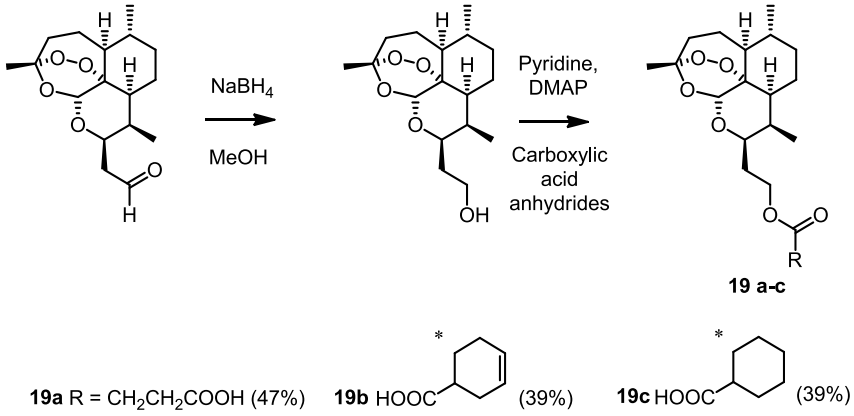
An interesting observation was made when two 10-(2-hydroxy-1-naphthyl) deoxoartemisinin isomers had very different ED_{50} and ED_{90} values (Wang *et al.*, 1999). From this stemmed a range of synthesised *α* and *β* deoxoartemisinins of which 3, 3-dimethyl-2-butanol (**18**) was the most active (**Scheme 3.10**) (Ma *et al.*, 2000). In general, the *β* derivatives were more active than the *α* derivatives, this statement was also the case with the synthesised isomers of **18**. The *β* isomer was at least five times more active than the corresponding *α* isomer measured in relative activity. Out of the five different isomers that synthesised, three *β* isomers where more active than their *α* analogues against both the W2 and D6 strains. For the other two isomers, the *β* isomers were found to be more active against the W2 strain and the *α* isomers were more active against the D6 strain. This phenomena were also noted for different isomers of **10,** which showed different activities (Avery *et al.*, 1996). When the α and β isomers of **10** were treated with $FeBr_2$ in order to promote the rearrangement of

bioactive analogues, it was found that each isomer had a unique decomposition pathway which provided a possible explanation for the activities.



Scheme 3.10: 3,3-dimethyl-2-butanol derivative **18** from **17** (Ma *et al.*, 2000)

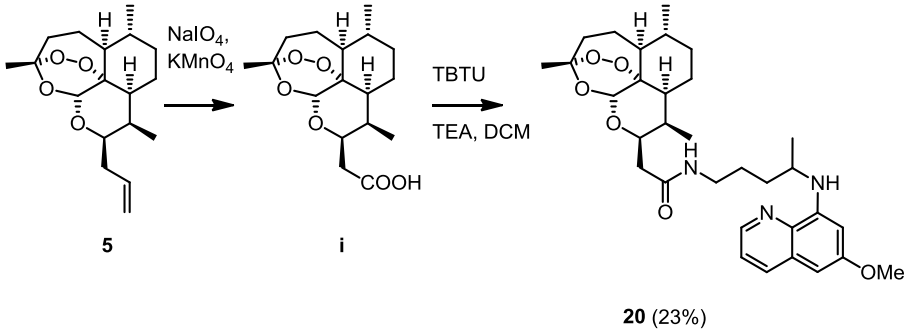
In an attempt to increase the bioavailability of these derivatives, a hydrophilic carboxylic acid functionality (**19 a-c**) was incorporated into the deoxoartemisinin structure (Khac *et al.*, 2005). Although the solubility of the compounds was not reported, the potency was very good – being more than twenty five times more active against the W2 strain, and twenty times more active against the Ghana (RO-33) strain than artemisinin. Artemisinin aldehyde (**Scheme 3.11**) was reduced to an alcohol by using sodium borohydride in methanol. This derivative was then reacted with different carboxylic acid anhydrides in the presence of pyridine and 4-(dimethylamino) pyridine.



Scheme 3.11: Synthesis of **19 a-c** by Khac *et al*. (Khac *et al.*, 2005)

A hybrid between artemisinin and primaquine, **20**, was synthesised in 2011 (**Scheme 3.12**) (Capela *et al.*, 2011; Smithuis *et al.*, 2010). With an IC_{50} value slightly lower than artemisinin, this could potentially be a good compound but a lower bioavailability and metabolic stability was reported for **20**. The calculated logP value for this derivative was 5.25 and 2.90 for artemisinin. 10β-(2-carboxyethyl) deoxoartemisinin (**i** in **Scheme 3.12**) was reacted with

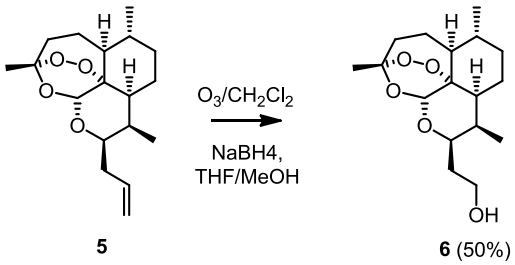
primaquine in the presence of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate (TBTU) and TEA in DCM. The overall yield for this compound was 23% from **1**.



Scheme 3.12: Artemisinin and primaquine hybrid **20** (Capela *et al.*, 2011)

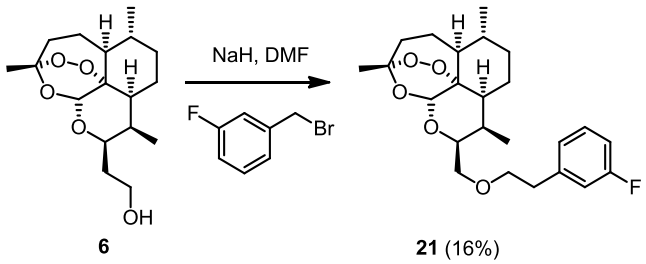
3.2.4 Group D derivatives:

The synthesis of 12β-(2-hydroxyethyl)deoxoartemisinin, **6**, was first reported in 1996 (Pu & Ziffer, 1995). **5** was dissolved in CH_2Cl_2 and subjected to a stream of O_3 at -78 °C for 30 min (**Scheme 3.13**). The solvent was removed under pressure and a 9:1 solvent mixture of methanol-THF was added at 0 °C, it was treated with an excess $NaBH_4$ and left to stir for several hours. There is a difference in the yields reported for this compound. In 2004, a yield of 55% was reported for this derivative when the reaction was scaled up (Jeyadevan *et al.*, 2004). Later, in 2009, a yield of 62% was reported for **6**, but there was no mention of the scale that was used (Araújo *et al.*, 2009). Being the latest reported yield, this will be used for further calculations of the yield from **1**. Derivative **6** is less active than artemisinin but serves as an intermediate for the majority of the derivatives for this review.



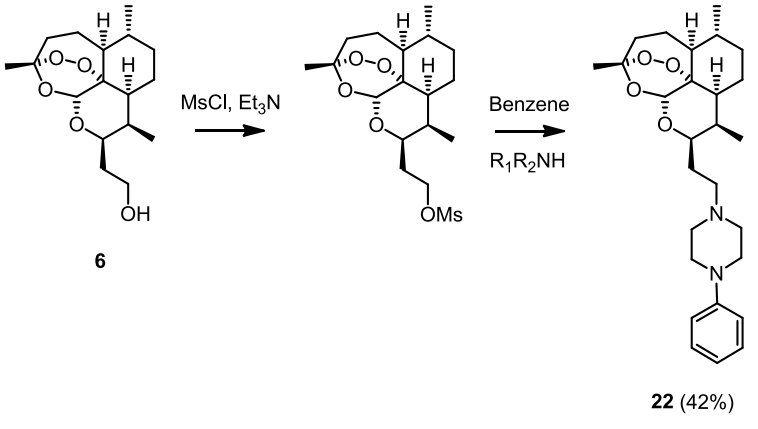
Scheme 3.13: Synthesis of 12β-(2-hydroxyethyl)deoxoartemisinin (**6**) by Araújo *et al*. (Araújo *et al.*, 2009)

It was illustrated previously that the antimalarial potency of a simplified trioxane alcohol could be improved by converting it to the benzyl fluoro-ether derivative (Posner *et al.*, 1995). This stimulated the synthesis of a range of derivatives by attaching a lipophillic fluorine-containing aromatic group to the intermediate **6** (O'Neill *et al.*, 1999). The most active of these was the 10*β*-[2-(2-Fluorobenzyloxy) ethyl] deoxoartemisinin (**21**). The synthesis was conducted beginning with DHA with a final yield of 32% from **6** (**Scheme 3.14**).



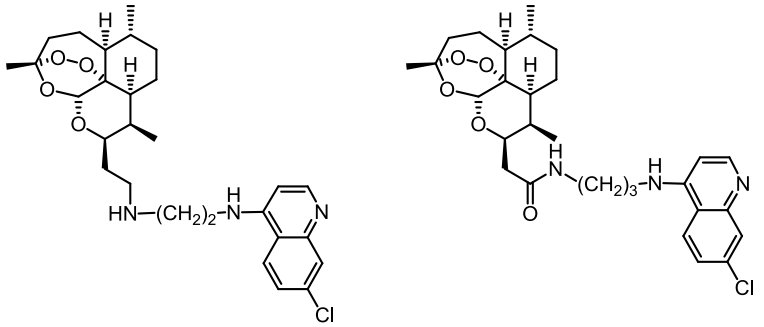
Scheme 3.14: 10*β*-[2-(2-Fluorobenzyloxy)ethyl]deoxoartemisinin (**21**) with a 16 %yield from **1** (O'Neill *et al.*, 1999)

Derivative **22** has a piperazino functionality incorporated into the 10β-(2-hydroxyethyl)-deoxoartemisinin, reported in 2002 (**Scheme 3.15**) (Hindley *et al.*, 2002). The rationale behind the design was to mimic chloroquine (containing two basic amino groups) so that the artemisinin derivative would also accumulate in the acidic food vacuole of the parasite. Even though this was not the case, it was demonstrated that a diamino functionality, **22**, was more active than a monoamine functionality. **22** was four times more active against the K1 strain than artemisinin *in vitro*.



Scheme 3.15: Synthesis of **22** (Hindley *et al.*, 2002)

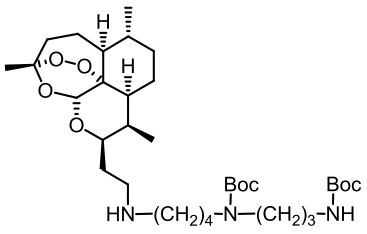
The group of Araújo incorporated 4-amino-7-chloroquinoline linkers into the 10β-(2-hydroxyethyl)-deoxoartemisinin (**Figure 3.2**) (Araújo *et al.*, 2009). This would allow the final hybrid drug to accumulate in the acidic food vacuole. Both of these derivatives were more active than artemisinin. 10β-(2-hydroxyethyl)-deoxoartemisinin was oxidised to its corresponding aldehyde, which then undergoes reductive amination in the presence of sodium triacetoxyborohydride to give **23**. In order to synthesise **24**, it was necessary to oxidise the alcohol to a carboxylic acid and then to convert it to the acid chloride with oxalyl chloride.



23 (7%) **24** (33%)

Figure 3.3: Chloroquine linkers **23** and **24**

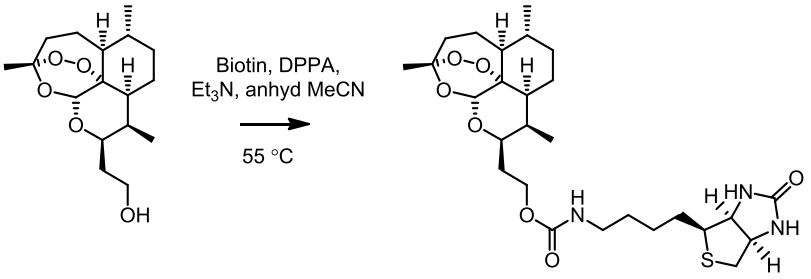
An artemisinin-spermidine derivative (**25**) was synthesised in 2010 (**Figure 3.3**) (Chadwick *et al.*, 2010). By incorporating a naturally occurring polyamine into the artemisinin structure, the aim was to use this derivative to fight tumour cells. The most active derivative towards the 3D7 *P. falciparum* strain was the 10β-[2-(N^1,N^4-di-tert-butoxycarbonylspermidine)ethyl] deoxoartemisinin. (4-Amino-butyl)-(3-tert-butoxycarbonylamino-propyl)- carbamic acid tert-butyl ester was reacted with 10-(2-methanesulfonylethyl)deoxoartemisinin in benzene and was stirred at 75 °C for 72 hours. **25** was 43 times more active than artemisinin, although when the Boc protecting groups were removed, the activity dropped to 28 times less than that of artemisinin.



25 (25%)

Figure 3.4: Derivative **25** synthesised by Chadwick *et al*. (Chadwick *et al.*, 2010)

In 2010, an interesting derivative was synthesised using biotin (**26**) (**Scheme 3.16**) as a starting reagent (Barton *et al.*, 2010). The rationale behind the biotin functionality was to synthesise an antimalarial probe for protein labelling. The isolation of the proteins was conducted by means of biotin/streptavidin binding. Although this derivative was not specifically designed for curing malaria, it did show good antimalarial activity.



6 **26** (34%)

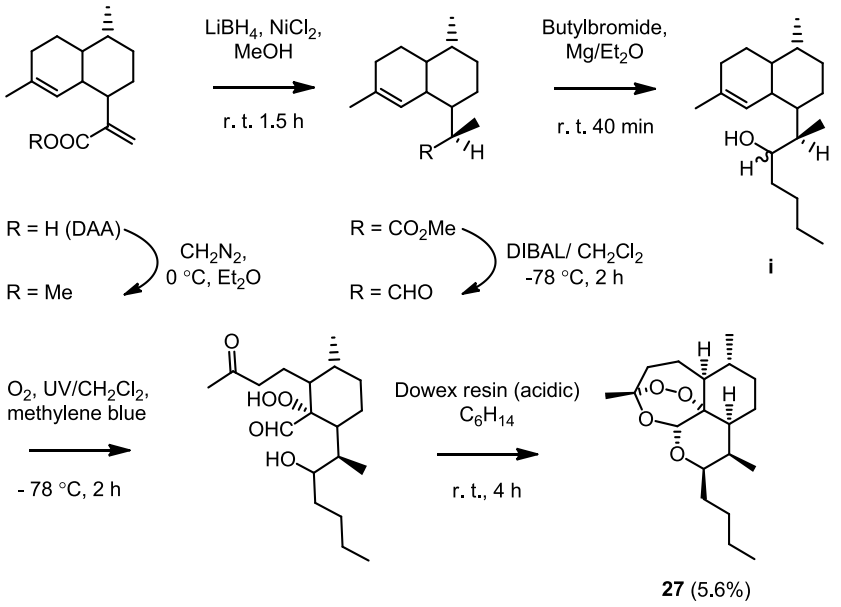
Scheme 3.16: Biotin derivative **26** (Barton *et al.*, 2010)

3.2.5 Group E derivatives:

Artemisinin production is mainly achieved by isolation from *Artemisia annua* that influences its availability and high costs (Enserink, 2005; Haynes, 2006; Laughlin, 1994; Wallaart *et al.*, 1999; White, 2008). To aid in the production of artemisinin, the waste product artemisinic acid (AA) is converted to dihydroartemisinic acid (DAA) (**7**) by means of genetically modified yeast (Gilmore *et al.*, 2014). DAA is then transformed into artemisinin through photochemistry and acid-induced rearrangement with a yield of 69% (Kopetzki *et al.*, 2013; Lévesque & Seeberger, 2012). Unfortunately, these types of reactions are plagued by low yields and require expensive equipment.

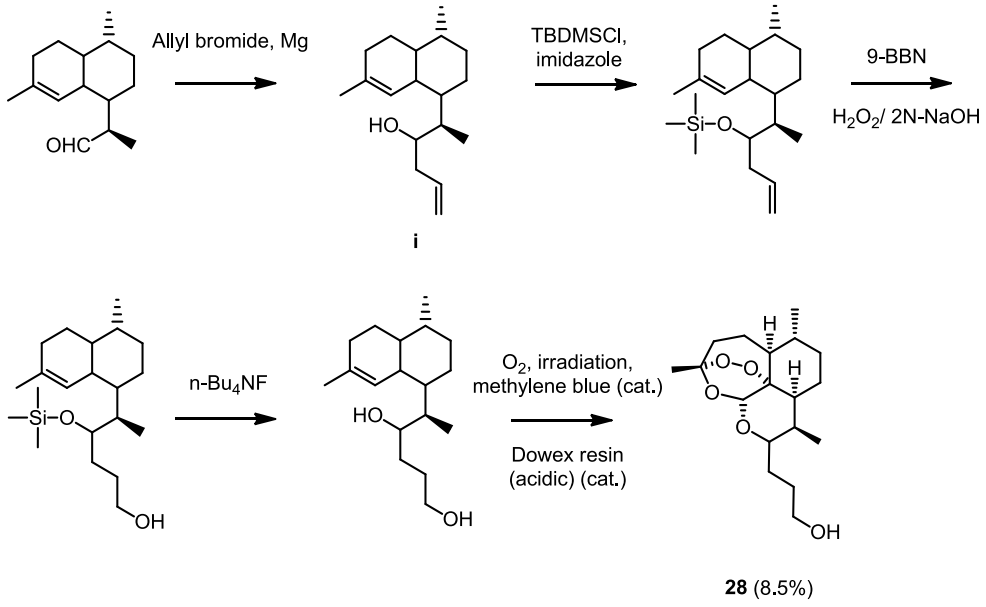
Some of the first deoxoartemisinin derivatives that were synthesised were produced by transforming the DAA to dihydroartemisinylaldehyde (**8**) (overall yield from this transformation 62%). The aldehyde is then reacted with Grignard reagents that deliver intermediates (**i**) in high yields (95% for **27** and 62% for **28**). This is followed by UV radiation and, lastly, acidic ring closure giving **27** in 12% (DAA 5.6% yield from DAA) and **28** in 23% yields, respectively (7% yield from DAA). The overall synthesis for **27** is illustrated in **Scheme 3.17** (Jung *et al.*, 1990c). This derivative have an equal activity against a chloroquine resistant (K1) strain as artemisinin it therefore showed good potential. **27** was

five times more active than artemisinin against chloroquine-resistant malaria, and the solubility was also improved by the addition of the alcohol functionality (Jung *et al.*, 1991).



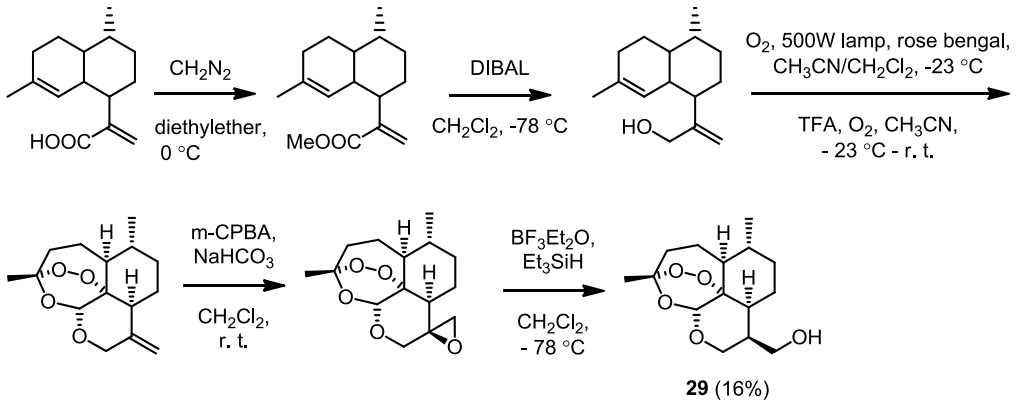
Scheme 3.17: 12-n-Butyldeoxoartemisinin (**27**) synthesis (Jung *et al.*, 1990a)

Later, in 1991, the group of Jung also synthesised the 12-(3'-hydroxy-n-propyl)-deoxoartemisinin (**28**) as described in the paragraph above (**Scheme 3.18**) (Jung et al., 1991). Being five times more active than artemisinin against chloroquine-resistant malaria, the solubility was also improved by adding the alcohol functionality.



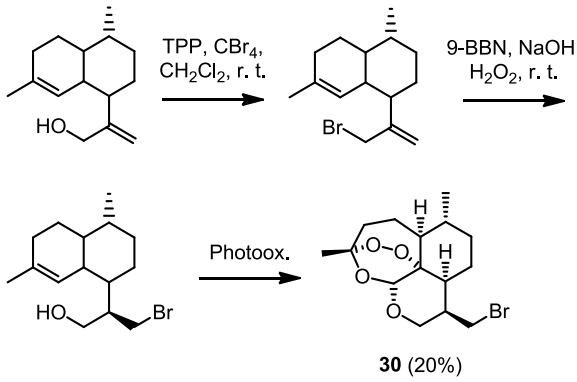
Scheme 3.18: Synthesis of **28**

In 2001, derivative **29** was synthesised using a complex synthetic route from artemisinic acid (Jung *et al.*, 2001). The artemisinic acid is converted to deoxoartemisitene, which is then epoxidized and reduced with DIBAL and BF_3•Et_2O to synthesise **29** (**Scheme 3.19**). It was found that **28** was four times more water-soluble than artemisinin and had a half-life of fifteen times longer in an acidic media at a pH 2 at a temperature of 37 °C than artemisinin. An IC_{50} value of 0.1 ng/mL was obtained when screened against both the 3D7 and K1 strains of *P. falciparum*.



Scheme 3.19: Synthesis of derivative **29**

It was later reported by Jung *et al.* that the methylbromide derivative (**30**) was more active against the K1 strain than artemisinin (**Scheme 3.20**) (Jung *et al.*, 2004). Additionally, it was found that when incorporating electron withdrawing groups to the C9 position, a decrease in antimalarial activity was observed. In simulated stomach acidic conditions (pH 2.0, 37 °C) this derivative's half-life was fifteen times longer than that of artemisinin.



Scheme 3.20: Synthesis of derivative **30**

3.3 *In vivo* activity and cytotoxicity

Of the twenty-five derivatives discussed in this review only seven were tested *in vivo*. Of these only one derivative underwent cytotoxic evaluation towards mammalian cells. *In vitro* cytotoxic studies are crucial for predicting the potential *in vivo* toxicity. When the cytotoxic IC_{50} value is compared to the anti-malarial IC_{50} value, an *in vitro* therapeutic index is obtained (McKim & James, 2010). This value can then be used to compare derivatives and to determine which functionalities will decrease or contribute to potential toxicity.

Derivative **10** was given in a single dose of 640 mg/kg and cured all of the *P. bergei* mice, as did artemisinin. In a 320 mg/kg dose, **10** cured 2/5 mice whereas artemisinin cured 0/5 mice. These antimalarials were dissolved in peanut oil and administered subcutaneously. The mice were considered cured if they survived for 60 days. Derivative **11** outshined artemisinin obtaining ED_{50} and ED_{90} in mg/kg values of 1.2 and 2.0 respectively, as opposed to artemisinin that obtained 3.0 and 8.5 respectively. Acute *in vivo* toxicity studies were conducted on male CD-1 mice intraperitoneally, and it was found to be comparable to artemether.

Derivatives **15** and **16** underwent cytotoxic screening on the KB mammalian cell line, and it was found that these derivatives were more toxic than artemisinin. The results of the Peter's 4-day suppressive test indicated that these derivatives at doses of 30 mg/kg were 100% curative. *In vivo*, these derivatives were highly active towards the *P. berghei*, obtaining ED_{50} and ED_{90} values of 2.1 and 4.3 for **15** and 1.7 and 5.2 for **16** respectively. Artesunate was only able to obtain values of 3.2 and over 10.

Derivative **17** was tested on mice infected with *P. berghei*, and in comparison with artemether it was slightly less active. Although it was found to be the most active, the trade-off was serious toxicity. The toxicity was determined using a mouse model. It was found that **17** had an identical toxicity to artemether. It was also reported that a dose of 400 mg/kg resulted in paralysis and death.

The hybrid of artemisinin and primaquine, derivative **20**, underwent extensive biological evaluation. Firstly, the ability of this derivative to inhibit *P. berghei* within human hepatoma cells. **20** displayed IC_{50} values 18 times lower than a 1:1 artemisinin-primaquine fixed combination. This indicated that **20** can prevent intracellular parasite replication. When the derivative was tested on mice with a *P. berghei* liver infection, **20** had less of an effect than

that of primaquine. This derivative was evaluated *in vivo* on a murine blood infection, in order to establish the efficacy of **20**. The amount of parasitaemia within the mice reached zero on day nine without any recurrence of the parasitaemia. More importantly, the mice had a 100% survival rate. It was concluded that *in vivo*, this derivative had enhanced pharmacokinetic properties as well treating blood stage malaria infection with greater efficacy than those of artemisinin or artemisinin-primaquine. Lastly, derivative **21** was found to be less active than artemether *in vivo* on mice infected with *P. berghei* by obtaining an ED_{50} value of 5.08 mg/kg compared to 3.15 mg/kg obtained by artemehter.

3.4 Conclusion

The main focus of this article was to explore the most potent non-acetal derivatives with at least IC_{50} values being in either in nM (together with the IC_{50} value of artemisinin) or expressed as relative activity. This ensured a degree of comparison since the activities of artemisinin vary between different strains and in different laboratories.

Table 3.1: Relative activities and ClogP values of derivatives

Derivatives	**ClogP** [a]	**Relative activities** [b]					
		D6	*NF54*	*3D7*	*HB3*	*W2*	*K1*
5	4.35	0.8				0.5	
6	2.48				0.9		
9	3.16	4.0				8.1	
10	5.23					7.1	
11a α	4.38		7.1				
11b β			0.2				
12	4.64		2.3				
13a β	4.29		1.7				
13b α			2.5				
14[c]	4.01						
15	2.72			5.1			2.6
16	3.18			3.7			4.3
17	4.72	2.1				2.0	
18 α	4.05	1.6				1.0	
18 β		6.8				5.4	
19 a	2.99						
19 b	4.12					25	
19 c	4.29						
20	5.20					0.9	
21	5.20				13.8		3.5
22	5.00						4.0
23	5.27			2.1			1.1
24	4.70			1.2			1.2
25	6.40			44.0			
26	3.03			4.6			

27	5.25		1	
28	2.88		5.3	
29	1.62	100.0		3.3
30	3.34	50.0		20.0

[a] Advanced Chemistry Inc. ACD/ChemSketch, **2012**, version 14.01. http://www.acdlabs.com
[b] The relative activities was calculated by IC_{50} (Artemisinin)/ IC_{50} (derivative)
[c] Relative activity towards FCR-3 = 2.5 FCR-8 = 1.7

Comparing these derivatives, only nine are within the acceptable calculated logP value range of 3. Only three of these derivatives have any toxicity data. **9** was the highest-yielding reaction, and it was more active and acid-stable than artemisinin. It is an easily synthesised derivative from either artemisinin or dihydroartemisinin in a single step procedure with a high yield of 71%. Because of its high crystallinity, it can easily be formulated. Like artemisinin, it also cured five out five mice infected with *P. Berghei* with a single dose of 640 mg/kg. While it showed good activity (being eight times more active than artemisinin) it unfortunately it lacks any toxicity data.

The alcohol derivative **6** with a carbon chain length of two had a high overall yield, but was not as active as artemisinin. Extending this chain length to three resulted in a very active derivative, but because of the route chosen, there was only a 7% overall yield of **28**. With an alternative synthesis route, these yields could be greatly increased if it were a lead compound.

19a and **26** will suffer similar fates by being metabolised to **7**. This indicates the importance of the toxicity data enabling one to compare it with dihydroartemisinin. **19a** is twenty five times more potent than artemisinin and has a similar structure to that of artesunate. It can also be formulated into a salt. It could potentially be a lead compound. **26** also has a carboxylic acid functionality and is five times more active than artemisinin.

Derivatives **29** and **30** are unique in the sense that they are modified at C-9 rather than at C-10. The logP value of **29** indicates that these will be very soluble in water. **29** was a 100 times more potent than artemisinin towards the *P. falciparum* 3D7 strain but was only three times more active towards the *P. falciparum* K1 strain. **30** was the most active towards the *P. falciparum* K1 and could serve as a valuable intermediate for future derivatives. Since it is synthesised from what can be considered waste material with an overall yield of 20%, and already containing a good leaving group, it might serve as a valuable platform for future synthesis. The question remains, however: what about toxicity?

All of these derivatives avoid being metabolised to dihydroartemisinin and could potentially have increased stability and diminished neurotoxicities as is the case of dihydroartemisinin. Of the nine derivatives that have a favourable logP value, of which three have additional amine functionalities. It is known that amines are necessary for cells to grow and differentiate (Thomas & Thomas, 2001). Including amines into the structure may aid in the absorption of these derivatives, as well as being protonated within the acidic food vacuole of the parasite. Additionally, the overall yields of these derivatives are important as this information may serve as a guide for future starting material and/or reaction to be used.

In a field that attempts to cure a disease that destroys a total 429 000 lives a year, more needs to be done to ensure that the common goal is attained. The research that is conducted in this field will be worth more if these derivatives were comparable with one another. Using a standard such as artemisinin will enable researchers from all over the world to compare their derivatives. This will, additionally, aid in the search for new lead derivatives. Another difficulty that has been identified is the lack of common *P. falciparum* strains. During phase 1 screening of the derivatives, there should a common chloroquine-sensitive and chloroquine-resistant *P. falciparum* strain. Together with this, there is a need evaluate the toxicity of these compounds. Cytotoxicity screening may guide the research towards a universal drive for the improvement of the pharmacokenetics of derivatives. The cell line to be used would ideally consist of primary hepatocytes as these have both phase I and phase II drug metabolising capabilities (McKim & James, 2010). The major drawbacks of these cells is the need for animals, time needed for the isolation of cells, and cost. Until there is a universal: screening standard, malaria strains and cytotoxicity evaluation studies the common goal of an effective antimalarial treatment will not be reached. These derivatives are worth the fuss!

Abbreviations

CQS : Chloroquine sensitive; CQR : Chloroquine resistant; WHO : World Health Organisation; ACT : Artemisinin combinational therapy; $t_{1/2}$: Half-life; OAY : Overall yield; US$: United States dollar; IC_{50} : Half maximal inhibitory concentration; AA : Artemisinic acid; DAA : Dihydroartemisinic acid; UV : Ultra violet; ng/mL : Nanogram per mililiter; ˚C : Degrees Celcius; nM : Nanomolar; ED_{50} : Effective dose for 50% of the population; ED_{90} : Effective dose for 90% of the population; logP : Partition coefficient; min : Minutes; pH : Concentration of hydrogen ions in solution; mg / kg : Miligram per kilogram; ClogP : Calculated partition coefficient.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CdL drafted this manuscript, and FJS, DDN and RKH critically revised it. All authors read and approved the final manuscript.

Acknowledgements

The authors wish to thank the North-West University for financial support. This research project was funded by the South African Medical Research Council (MRC) with funds from the National Treasury under its Economic Competitiveness and Support Package.

3.5 References

Araújo, N.C., Barton, V., Jones, M., Stocks, P.A., Ward, S.A., Davies, J., Bray, P.G., Shone, A.E., Cristiano, M.L. & O'Neill, P.M. 2009. Semi-synthetic and synthetic 1, 2, 4-trioxaquines and 1, 2, 4-trioxolaquines: synthesis, preliminary SAR and comparison with acridine endoperoxide conjugates. *Bioorganic and Medicinal Chemistry Letters*, 19(7):2038-2043.

Avery, M.A., Fan, P., Karle, J.M., Bonk, J.D., Miller, R. & Goins, D.K. 1996. Structure− activity relationships of the antimalarial agent artemisinin. 3. Total synthesis of (+)-13-carbaartemisinin and related tetra-and tricyclic structures. *Journal of Medicinal Chemistry*, 39(9):1885-1897.

Barton, V., Ward, S.A., Chadwick, J., Hill, A. & O'Neill, P.M. 2010. Rationale design of biotinylated antimalarial endoperoxide carbon centered radical prodrugs for applications in proteomics. *Journal of Medicinal Chemistry*, 53(11):4555-4559.

Capela, R., Cabal, G.G., Rosenthal, P.J., Gut, J., Mota, M.M., Moreira, R., Lopes, F. & Prudêncio, M. 2011. Design and evaluation of primaquine-artemisinin hybrids as a multistage antimalarial strategy. *Antimicrobial Agents and Chemotherapy*, 55(10):4698-4706.

Chadwick, J., Jones, M., Mercer, A.E., Stocks, P.A., Ward, S.A., Park, B.K. & O'Neill, P.M. 2010. Design, synthesis and antimalarial/anticancer evaluation of spermidine linked

artemisinin conjugates designed to exploit polyamine transporters in Plasmodium falciparum and HL-60 cancer cell lines. *Bioorganic and Medicinal Chemistry*, 18(7):2586-2597.

Davis, T., Karunajeewa, H.A. & Ilett, K.F. 2005. Artemisinin-based combination therapies for uncomplicated malaria. *The Medical Journal of Australia*, 182(4):181-185.

Davis, W.A., Clarke, P.M., Siba, P.M., Karunajeewa, H.A., Davy, C., Mueller, I. & Davis, T.M. 2011. Cost-effectiveness of artemisinin combination therapy for uncomplicated malaria in children: data from Papua New Guinea. *Bulletin of the World Health Organization*, 89(3):211-220.

Enserink, M. 2005. Source of new hope against malaria is in short supply. *Science*, 307(5706):33.

Fairhurst, R.M. & Dondorp, A.M. 2016. Artemisinin-resistant Plasmodium falciparum malaria. *Microbiology Spectrum*, 4(3).

Fivelman, Q.L., Butcher, G.A., Adagu, I.S., Warhurst, D.C. & Pasvol, G. 2002. Malarone treatment failure and in vitro confirmation of resistance of Plasmodium falciparum isolate from Lagos, Nigeria. *Malaria Journal*, 1(1):1.

Gilmore, K., Kopetzki, D., Lee, J.W., Horváth, Z., McQuade, D.T., Seidel-Morgenstern, A. & Seeberger, P.H. 2014. Continuous synthesis of artemisinin-derived medicines. *Chemical Communications*, 50(84):12652-12655.

Gregson, A. & Plowe, C.V. 2005. Mechanisms of resistance of malaria parasites to antifolates. *Pharmacological Reviews*, 57(1):117-145.

Haynes, R.K. 2006. From artemisinin to new artemisinin antimalarials: biosynthesis, extraction, old and new derivatives, stereochemistry and medicinal chemistry requirements. *Current Topics in Medicinal Chemistry*, 6(5):509-537.

Haynes, R.K. & Vonwiller, S.C. 1990. Catalysed oxygenation of allylic hydroperoxides derived from qinghao (artemisinic) acid. Conversion of qinghao acid into dehydroginghaosu (artemisitene) and qinghaosu (artemisinin). *Journal of the Chemical Society, Chemical Communications*(6):451-453.

Haynes, R.K. & Vonwiller, S.C. 1992. Efficient Preparation of Novel Qinghaosu (Artemisinin) Derivatives: Conversion of Qinghao (Artemisinic) Acid into Deoxoqinghaosu Derivatives and 5-Carba-4-deoxoartesunic Acid1. *Synlett*, 1992(06):481-483.

Haynes, R.K., Wong, H.N., Lee, K.W., Lung, C.M., Shek, L.Y., Williams, I.D., Croft, S.L., Vivas, L., Rattray, L. & Stewart, L. 2007. Preparation of N-Sulfonyl-and N-Carbonyl-11-Azaartemisinins with Greatly Enhanced Thermal Stabilities: in vitro Antimalarial Activities. *ChemMedChem*, 2(10):1464-1479.

Hindley, S., Ward, S.A., Storr, R.C., Searle, N.L., Bray, P.G., Park, B.K., Davies, J. & O'Neill, P.M. 2002. Mechanism-based design of parasite-targeted artemisinin derivatives: synthesis and antimalarial activity of new diamine containing analogues. *Journal of Medicinal Chemistry*, 45(5):1052-1063.

Jeyadevan, J.P., Bray, P.G., Chadwick, J., Mercer, A.E., Byrne, A., Ward, S.A., Park, B.K., Williams, D.P., Cosstick, R. & Davies, J. 2004. Antimalarial and antitumor evaluation of novel C-10 non-acetal dimers of 10β-(2-hydroxyethyl) deoxoartemisinin. *Journal of Medicinal Chemistry*, 47(5):1290-1298.

Jung, M. 1994. Current developments in the chemistry of artemisinin and related compounds. *Current Medicinal Chemistry*, 1(1):35-49.

Jung, M., Bustos, D.A., ElSohly, H.N. & McChesney, J.D. 1990a. A concise and stereoselective synthesis of (+)-12-n-butyldeoxoartemisinin. *Synlett*(12):743-744.

Jung, M., Bustos, D.A., ElSohly, H.N. & McChesney, J.D. 1990b. A concise and stereoselective synthesis of (+)-12-n-butyldeoxoartemisinin. *Synlett*, 1990(12):743-744.

Jung, M., Lee, K. & Jung, H. 2001. First synthesis of (+)-deoxoartemisitene and its novel C-11 derivatives. *Tetrahedron Letters*, 42(24):3997-4000.

Jung, M., Lee, K., Jung, H., Kendrick, H., Yardley, V. & Croft, S.L. 2004. Antimalarial activities of (+)-deoxoartemisitene and its novel C-11, 13 derivatives. *Bioorganic and Medicinal Chemistry Letters*, 14(8):2001-2003.

Jung, M. & Lee, S. 1998. Stability of acetal and non acetal-type analogs of artemisinin in simulated stomach acid. *Bioorganic and Medicinal Chemistry Letters*, 8(9):1003-1006.

Jung, M., Li, X., Bustos, D.A., ElSohly, H.N., McChesney, J.D. & Milhous, W.K. 1990c. Synthesis and antimalarial activity of (+)-deoxoartemisinin. *Journal of Medicinal Chemistry*, 33(5):1516-1518.

Jung, M., Yu, D., Bustos, D., ElSohly, H.N. & McChesney, J.D. 1991. A concise synthesis of 12-(3'-hydroxy-n-propyl)-deoxoartemisinin. *Bioorganic and Medicinal Chemistry Letters*, 1(12):741-744.

Khac, V.T., Van, T.N. & Van, S.T. 2005. Synthesis of novel 10-deoxoartemisinins. *Bioorganic and Medicinal Chemistry Letters*, 15(10):2629-2631.

Kopetzki, D., Lévesque, F. & Seeberger, P.H. 2013. A Continuous-Flow Process for the Synthesis of Artemisinin. *Chemistry-a European Journal*, 19(17):5450-5456.

Laughlin, J. 1994. Agricultural production of artemisinin—a review. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 88:21-22.

Lee, S. & Oh, S. 2002. A simple synthesis of C-10 substituted deoxoartemisinin and 9-epi-deoxoartemisinin with various organozinc reagents. *Tetrahedron Letters*, 43(16):2891-2894.

Lee, S., Oh, S., Park, G.-M., Kim, T.-S., Ryu, J.-S. & Choi, H.-K. 2005. Antimalarial activity of thiophenyl-and benzenesulfonyl-dihydroartemisinin. *The Korean Journal of Parasitology*, 43(3):123.

Lévesque, F. & Seeberger, P.H. 2012. Continuous-Flow Synthesis of the Anti-Malaria Drug Artemisinin. *Angewandte Chemie International Edition*, 51(7):1706-1709.

Ma, J., Katz, E., Kyle, D.E. & Ziffer, H. 2000. Syntheses and antimalarial activities of 10-substituted deoxoartemisinins. *Journal of Medicinal Chemistry*, 43(22):4228-4232.

Mbengue, A., Bhattacharjee, S., Pandharkar, T., Liu, H., Estiu, G., Stahelin, R.V., Rizk, S.S., Njimoh, D.L., Ryan, Y. & Chotivanich, K. 2015. A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria. *Nature*, 520(7549):683-687.

McKim, J. & James, M. 2010. Building a tiered approach to in vitro predictive toxicity screening: a focus on assays with in vivo relevance. *Combinatorial Chemistry and High Throughput Screening*, 13(2):188-206.

Medhi, B., Patyar, S., Rao, R.S., Ds, P.B. & Prakash, A. 2009. Pharmacokinetic and toxicological profile of artemisinin compounds: an update. *Pharmacology*, 84(6):323-332.

O'Dowd, H., Ploypradith, P., Xie, S., Shapiro, T.A. & Posner, G.H. 1999. Antimalarial artemisinin analogs. Synthesis via chemoselective C・ C bond formation and preliminary biological evaluation. *Tetrahedron*, 55(12):3625-3636.

O'Neill, P.M., Searle, N.L., Kan, K.-W., Storr, R.C., Maggs, J.L., Ward, S.A., Raynes, K. & Park, B.K. 1999. Novel, potent, semisynthetic antimalarial carba analogues of the first-generation 1, 2, 4-trioxane artemether. *Journal of Medicinal Chemistry*, 42(26):5487-5493.

Pacorel, B., Leung, S.C., Stachulski, A.V., Davies, J., Vivas, L., Lander, H., Ward, S.A., Kaiser, M., Brun, R. & O'Neill, P.M. 2009. Modular synthesis and in vitro and in vivo antimalarial assessment of C-10 pyrrole mannich base derivatives of artemisinin. *Journal of Medicinal Chemistry*, 53(2):633-640.

Paloque, L., Ramadani, A.P., Mercereau-Puijalon, O., Augereau, J.-M. & Benoit-Vical, F. 2016. Plasmodium falciparum: multifaceted resistance to artemisinins. *Malaria Journal*, 15(1):149.

Payne, D. 1987. Spread of chloroquine resistance in Plasmodium falciparum. *Parasitology Today*, 3(8):241-246.

Posner, G.H., McGarvey, D.J., Oh, C.H., Kumar, N., Meshnick, S.R. & Asawamahasadka, W. 1995. Structure-activity relationships of lactone ring-opened analogs of the antimalarial 1, 2, 4-trioxane artemisinin. *Journal of Medicinal Chemistry*, 38(4):607-612.

Posner, G.H., Northrop, J., Paik, I.-H., Borstnik, K., Dolan, P., Kensler, T.W., Xie, S. & Shapiro, T.A. 2002. New chemical and biological aspects of artemisinin-derived trioxane dimers. *Bioorganic and Medicinal Chemistry*, 10(1):227-232.

Posner, G.H., Parker, M.H., Northrop, J., Elias, J.S., Ploypradith, P., Xie, S. & Shapiro, T.A. 1999. Orally active, hydrolytically stable, semisynthetic, antimalarial trioxanes in the artemisinin family. *Journal of Medicinal Chemistry*, 42(2):300-304.

Price, R.N., Uhlemann, A.-C., Brockman, A., McGready, R., Ashley, E., Phaipun, L., Patel, R., Laing, K., Looareesuwan, S. & White, N.J. 2004. Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. *The Lancet*, 364(9432):438-447.

Pu, Y.-M., Yeh, H. & Ziffer, H. 1993. An unusual acid-catalyzed rearrangement of 1, 2, 4-trioxanes. *Heterocycles*, 36(9):2099-2107.

Pu, Y.M. & Ziffer, H. 1995. Synthesis and Antimalarial Activities of 12. beta.-Allyldeoxoartemisinin and Its Derivatives. *Journal of Medicinal Chemistry*, 38(4):613-616.

Schmuck, G., Roehrdanz, E., Haynes, R.K. & Kahl, R. 2002. Neurotoxic mode of action of artemisinin. *Antimicrobial Agents and Chemotherapy*, 46(3):821-827.

Shrimali, M., Bhattacharya, A.K., Jain, D.C., Bhakuni, R.S. & Sharma, R.P. 1998. Sodium artelinate: a potential antimalarial. *Indian Journal of Chemistry. Sect. B*, 37(11):1161-1163.

Singh, B., Singh, D., Verma, R., Gupta, N., Jain, D. & Kumar, S. 2001. Simultaneous determination of antimalarial artemisinin, dihydroartemisinin and arteether using reversed phase high performance liquid chromatography. *Journal of the Indian Chemical Society*, 78(9):489-491.

Smithuis, F., Kyaw, M.K., Phe, O., Win, T., Aung, P.P., Oo, A.P.P., Naing, A.L., Nyo, M.Y., Myint, N.Z.H. & Imwong, M. 2010. Effectiveness of five artemisinin combination regimens with or without primaquine in uncomplicated falciparum malaria: an open-label randomised trial. *The Lancet Infectious Diseases*, 10(10):673-681.

Stringham, R.W. & Teager, D.S. 2012. Streamlined process for the conversion of artemisinin to artemether. *Organic Process Research & Development*, 16(5):764-768.

Sy, L.-K. & Brown, G.D. 2001. Synthesis of 6, 7-dehydroartemisinic acid. *Journal of the Chemical Society, Perkin Transactions 1*(19):2421-2429.

Thomas, T. & Thomas, T. 2001. Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. *Cellular and Molecular Life Sciences*, 58(2):244-258.

Vil, V.A., Yaremenko, I.A., Ilovaisky, A.I. & Terent'ev, A.O. 2017. Synthetic Strategies for Peroxide Ring Construction in Artemisinin. *Molecules*, 22(1):117.

Wallaart, T.E., van Uden, W., Lubberink, H.G., Woerdenbag, H.J., Pras, N. & Quax, W.J. 1999. Isolation and identification of dihydroartemisinic acid from Artemisia annua and its possible role in the biosynthesis of artemisinin. *Journal of Natural Products*, 62(3):430-433.

Wang, D.-Y., Wu, Y., Wu, Y.-L., Li, Y. & Shan, F. 1999. Synthesis, iron (II)-induced cleavage and in vivo antimalarial efficacy of 10-(2-hydroxy-1-naphthyl)-deoxoqinghaosu (-deoxoartemisinin). *Journal of the Chemical Society, Perkin Transactions 1*(13):1827-1832.

White, N.J. 2008. Qinghaosu (artemisinin): the price of success. *Science*, 320(5874):330-334.

WHO. 2016. Artemisinin and artemisinin-based combination therapy resistance: status report.

WHO. 2018. World Malaria Report 2018. Geneva: WHO; 2018. Licence: CC BY-NC-SA 3.0 IGO.

Chapter 4:

Synthesis, *in vitro* antimalarial activities and cytotoxicities of amino-artemisinin-ferrocene derivatives

This chapter contains a research article published in Bioorganic and Medicinal Chemistry Letters. It presents syntheses, anti-malarial activities and cytotoxicities of a series of Amino-arteminsinin-ferrocene derivatives.

Bioorganic & Medicinal Chemistry Letters 28 (2018) 289–292

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis, *in vitro* antimalarial activities and cytotoxicities of amino-artemisinin-ferrocene derivatives

Christo de Lange [a], Dina Coertzen [b], Frans J. Smit [a], Johannes F. Wentzel [a], Ho Ning Wong [a], Lyn-Marie Birkholtz [b], Richard K. Haynes [a,*], David D. N'Da [a,*]

[a] Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom 2520, South Africa
[b] Department of Biochemistry, Institute for Sustainable Malaria Control, University of Pretoria, Private Bag X20, Hatfield 028, South Africa

ARTICLE INFO

Article history:
Received 7 October 2017
Revised 20 December 2017
Accepted 23 December 2017
Available online 26 December 2017

Keywords:
Amino-artemisinin
Ferrocene
Redox
Hybrid
Malaria

ABSTRACT

Novel derivatives bearing a ferrocene attached via a piperazine linker to C-10 of the artemisinin nucleus were prepared from dihydroartemisinin and screened against chloroquine (CQ) sensitive NF54 and CQ resistant K1 and W2 strains of *Plasmodium falciparum* (*Pf*) parasites. The overall aim is to imprint oxidant (from the artemisinin) and redox (from the ferrocene) activities. In a preliminary assessment, these compounds were shown to possess activities in the low nM range with the most active being compound **6** with IC_{50} values of 2.79 nM against *Pf* K1 and 3.2 nM against *Pf* W2. Overall the resistance indices indicate that the compounds have a low potential for cross resistance. Cytotoxicities were determined with Hek293 human embryonic kidney cells and activities against proliferating cells were assessed against A375 human malignant melanoma cells. The selectivity indices of the amino-artemisinin ferrocene derivatives indicate there is overall an appreciably higher selectivity towards the malaria parasite than mammalian cells.

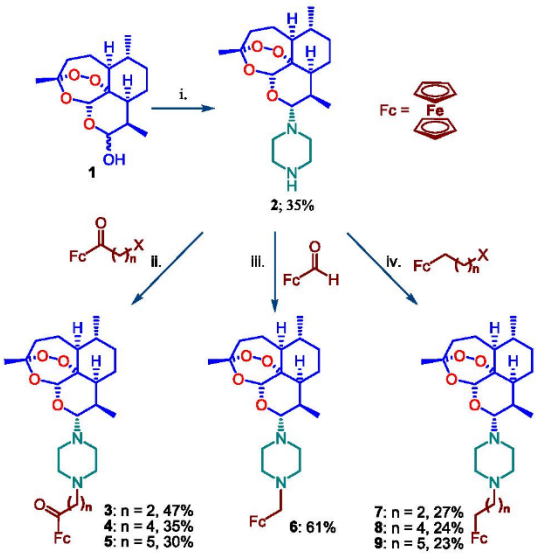
According to the World Health Organization (WHO), there were about 212 million cases of malaria amounting to approximately 429,000 deaths in 2015, of which the majority of deaths were recorded in Africa.[1] The most important parasite, *Plasmodium falciparum* (*Pf*) has acquired resistance to most drugs, including most recently the clinically-used artemisinins.[2–6] As an example, in some areas of Western Cambodia the ACT comprising dihydroartemisinin (DHA)-piperaquine failed to cure half of all patients treated.[2,3] In general, DHA **1** is either used as such, or is the active metabolite of the other clinically used artemisinins artemether and artesunate due to the metabolism of the C-10 methyl ether or facile hydrolysis of the succinate ester respectively.[4] It appears that DHA is implicated in artemisinin resistance.[5,6] Thus, it is necessary to avoid the formation of this metabolite, and so it is best to consider new derivatives not bearing an oxygen atom attached to C-10. We have shown elsewhere that artemisone, a derivative bearing an amino group at C-10 is not metabolized to DHA.[7] Further, in terms of their *in vitro* activities, C-10 substituted amino-artemisinins in general appear to be optimal substrates in direct comparison with C-10 O- and C-substituted counterparts.[8] We have shown that artemisinins act as *oxidant* drugs through the ability of the peroxide group to rapidly oxidize intracellular components such as reduced flavins of flavin disulfide reductases, and thereby perturb redox homeostasis in the malaria parasite.[9,10] The peroxide is reduced irreversibly through accepting electrons from the reduced flavin. Thus, a possible further means to address resistance is to seek additional modes of action by modifying the structure of the artemisinin through attachment of groups that may act as pharmacophores in their own right. The ferrocene pharmacophore acts as a redox centre that undergoes redox cycling. The ferrocene-Fe^{2+} may be oxidized by free or labile Fe^{3+} to form ferrocenium (ferrocene-Fe^{3+}).[11–13] In the last case, the labile Fe^{2+} thereby generated is oxidized by oxygen to form superoxide; subsequent reaction with Fe^{2+} via the Fenton pathway generates hydroxyl radicals. Thus, the increased production of reactive oxygen species (ROS) leads to perturbation of parasite redox homeostasis. Importantly, ferrocenium is reduced to ferrocene by metalloproteins (ferrocytochrome c), NADH and thiols such as glutathione (GSH).[14–17] Given that thiols are capable reductants, ferrocenium very likely is reduced also by reduced flavins (that like thiols rapidly reduce labile Fe^{3+}), although evidently this has yet to be demonstrated.[18–20] Thus, by attaching the redox active moiety to the oxidant artemisinin, the overall ability of the ensemble of oxidant and redox centres to enhance oxidative stress is greatly increased; that is, once the

* Corresponding authors.
E-mail addresses: richard.haynes@nwu.ac.za (R.K. Haynes), david.nda@nwu.ac.za (D.D. N'Da).

https://doi.org/10.1016/j.bmcl.2017.12.057
0960-894X/

peroxide pharmacophore is reduced, the ferrocene is able to continue the cycle of oxidation and reduction thereby maintaining oxidative stress in the parasite. It is noted that artemisinin-ferrocene derivatives have been prepared previously and their antimalarial activities have been assessed essentially in terms of their binding to heme.[21,22] The most noteworthy aspect of these hybrids is the evidently mutual compatibility of the peroxide with the ferrocene ferrous iron, even though free ferrous iron, with its admittedly lower oxidation potential than that of ferrous iron in ferrocene or ferrous iron in heme, are popularly ascribed to 'activating' the peroxide to induce formation of 'toxic' free radicals from the artemisinin peroxide.[23] This toxic radical concept has been thoroughly dissected on the basis of the well-established chemistry of carbon-centred free radicals and is difficult to reconcile with the behaviour of artemisinins in presence of free or hemeligated ferrous iron.[24] Be that as it may, as an exploratory venture into probing efficacies of artemisinin-ferrocene hybrids in terms of the oxidant-redox activity concept, we sought here to exploit the unique activities of C-10 amino-artemisinins through attachment of the redox-active ferrocene pharmacophore to the amino group.

DHA **1** in toluene in the presence of catalytic dimethyl sulfoxide was quantitatively converted by oxalyl chloride into the 10β-chloride and the latter treated *in situ* with piperazine to provide 10α-(1′-piperazino)-10-deoxo-10-dihydroartemisinin **2** (Scheme 1).[25] That this and the final derivatives possessed the 10α-stereochemistry was confirmed by the coupling constant of *J* 10.2 Hz between H-10 and H-9 in **2**, which is consistent with an *anti*-periplanar (*trans*-diaxial) arrangement of these protons in a chair pyranose ring.[26] This intermediate proved to be sufficiently stable for attachment of the ferrocene moiety. Friedel-Crafts acylation of ferrocene with the corresponding acyl halides gave 1-ferrocenyl-3-chloropropan-1-one (27%), 1-ferrocenyl-5-bromopentan-1-one (79%) and 1-ferrocenyl-6-bromohexan-1-one (83%) according to literature methods.[27] These intermediates were condensed with the piperazine derivative **2** in the presence of DBU



Scheme 1. Synthesis routes for amino-artemisinin-ferrocene derivatives and yields i. **1** (1 eq.), toluene, dimethyl sulfoxide (0.1 eq.), N_2, oxalyl chloride (1.1 eq.), room temperature, 2 h; piperazine (5 eq.), dichloromethane, N_2, room temperature, 16 h, 35%. ii. **2** (1 eq.), acetonitrile, ferrocenoyl halide (X = Cl, Br, see text) (1 eq.), DBU (0.225 eq.), room temperature, 24 h. iii. Ferrocenecarboxaldehyde (1 eq.), **2** (3.1 eq.), THF, N_2, sodium triacetoxyborohydride (2.5 eq), room temperature, overnight. iv. **2** (1 eq.), acetonitrile, ferrocene alkyl halide (1 eq.), DBU (2 eq.), 0.1 mL DMF, microwave.

to give the derivatives **3**–**5**. 1-Ferrocenyl-2-chloroethan-1-one and 1-ferrocenyl-4-chlorobutan-1-one were also prepared, but although the final piperazine derivatives were obtained after condensation with **2**, these could not be purified, thus precluding their further examination. For preparation of the ferrocene methyl derivative **6**, the piperazine intermediate **2** was submitted to reductive alkylation with ferrocene-carboxaldehyde and sodium triacetoxyborohydride to cleanly deliver this product (61%). In order to prepare the other alkylation products **7**–**9**, the ferrocenoyl halides were reduced with borane *tert*-butylamine and aluminium chloride to give the ferrocenyl alkyl halides (84–98%).[28] However, alkylation of the piperazine intermediate **2** with these alkyl halides was not straightforward, and microwave radiation had to be used to drive the reactions so as to provide the final derivatives **7**–**9** in approximately 20% yields.

In vitro antiplasmodial activity was determined against the chloroquine sensitive (CQS) NF54 strain and chloroquine resistant (CQR) K1 and W2 strains of *P. falciparum* (*Pf*) using the SYBR Green I based fluorescence assay to measure parasite proliferation.[29] The resistance index (RI) for each drug resistant strain (ratio of the IC_{50} values of the resistant to sensitive strains IC_{50} K1/ IC_{50} NF54 and IC_{50} W2/ IC_{50} NF54) was calculated as an indication of potential cross-resistance formation. The *in vitro* cytotoxicity assay was performed on human embryonic kidney cells Hek293 and anti-tumour screening was carried out on the human malignant melanoma cell line A375 as described previously.[30] The selectivity indices (SI) indicate the selectivity of the compounds towards *Pf* or cancer cells compared to mammalian cells *in vitro*. The amino-artemisinin-ferrocene derivatives showed good activity on asexual parasites with IC_{50} values in the low nM range (Table 1). The ferrocene derivatives in general tend to be less active than the comparator drugs dihydroartemisinin and artesunate (Table 1). The RI values of all of the amino-artemisinin-ferrocene derivatives were smaller than 1, which indicates a low potential for cross resistance and similar to the indices for the artemisinin reference compounds (Table 1). With the exception of compound **5**, most compounds showed good selectivity for *Pf* parasites, with SI indices >9000. Compound **5**, the least active compound towards *Pf*, was more cytotoxic towards mammalian cells than cancer cells. It is noted that compounds **4**, **8** and **9** were relatively poorly soluble in the culture medium, and meaningful data could not be obtained for compound **4**. The solubility issues aside, the three most active amino-artemisinin-ferrocene derivatives were **3**, **6** and **7**. Not unexpectedly, electron withdrawing substituents attached to the ferrocene decrease the ease of oxidation of the Fe^{2+} centre and electron donating groups have the reverse effect.[31–34] However, even though the atom adjacent to the ferrocene ring comprises different functional groups, namely electron withdrawing carbonyl for compound **3**, and electron-donating amino-methylene for compound **6** and methylene for compound **7**, activities do not vary significantly, indicating that such effects are insignificant in these screens. Conversion of DHA **1**, that has IC_{50} values of 4 ± 1 μM on the Hek293 cell line and 1 ± 0.1 μM on the A375 cell line, via the amino-artemisinin **2** into the derivatives **3**, **6** and **7** results in greatly enhanced selectivities with respect to the malaria parasites (Table 1).[35,36] In terms of overall accessibility and activities, compound **6** is identified as a hit compound.

Thus, in summary, we have demonstrated that the amino-artemisinin ferrocene derivatives retain good antimalarial activities and display good selectivities *in vitro* towards *Pf*. Clearly, in order to evaluate any role played by the ferrocene pharmacophore, it is necessary to conduct *in vivo* assays to establish if there are indeed any differences between the parent amino-artemisinin and the amino-artemisinin-ferrocene conjugates wherein redox cycling of the ferrocene moiety should continue once the peroxide of the artemisinin is reduced *in vivo*. Overall, the work provides a useful

Table 1
Biological activities of amino-artemisinin-ferrocene derivatives.

Compd	Antimalarial activity IC_{50} (± SEM) nM					Cytotoxicity IC_{50} (μM)		Antitumour IC_{50} (μM)	
	NF54	K1	RI[a]	W2	RI[b]	Hek293	SI[c]	A375	SI[d]
CQ	10.0 (3.0)	154.0 (14.0)	15.4	233.0 (49.0)	23.3	nd	nd	nd	nd
DHA **1**	2.5 (0.1)	1.5 (0.3)	0.6	1.7 (0.2)	0.6	4.0[e]	1593	1.0[e]	0.3
Ars	3.0 (0.2)	4.0 (1.0)	1.3	2.4 (0.4)	0.8	nd	nd	nd	nd
Arm	1.8 (0.1)	9.0 (2.0)	4.8	7.0 (1.0)	3.8	nd	nd	nd	nd
2	3.0 (0.3)	2.1 (0.4)	0.7	2.9 (0.6)	0.8	30.0	9780	76.0	0.3
3	9.0 (4.7)	5.0 (2.6)	0.5	6.5 (3.9)	0.7	192.0	21309	61.0	3.1
5	24.1 (6.0)	16.8 (1.5)	0.7	19.8 (4.1)	0.8	17.0	703	54.0	0.3
6	4.5 (0.6)	2.7 (0.7)	0.6	3.2 (1.0)	0.7	53.0	11597	19.0	2.7
7	7.7 (3.0)	4.6 (1.9)	0.6	5.9 (3.2)	0.7	266.0	34456	111.0	1.6
8	nd	nd	nd	nd	nd	13.0	nd	8.0	1.6
9	nd	nd	nd	nd	nd	134.0	nd	8.0	16.7

CQ chloroquine; DHA dihydroartemisinin; Ars artesunate; Arm artemether; nd not determined; Hek293 human embryonic kidney cells, A375 human malignant melanoma cells; [a]Resistance Index = IC_{50} K1/IC_{50} NF54; [b]Resistance Index = IC_{50} W2/IC_{50} NF54; [c]Selectivity Index = IC_{50} Hek293/ IC_{50} NF54; [d]Selectivity Index = IC_{50} Hek293/ IC_{50} A375; [e]historical values.[35,36]

incipient structure-activity profile for the preparation of newer compounds that together with **6** will be screened both against artemisinin resistant parasites, and *in vivo* involving a murine model to assess effects of the ferrocene moiety on activities.

Disclaimer

Any opinions, findings and conclusions, or recommendations expressed in this material are those of the authors and therefore the NRF does not accept any liability in regard thereto.

Acknowledgments

This work was funded by the South African Medical Research Council (MRC) Flagship Project MALTB-Redox with funds from National Treasury under its Economic Competitiveness and Support Package and South African National Research Foundation Grants to R. K. Haynes (UID 90682 and UID 98934) and to D. D. N'Da (UID 76443). The authors thank Mr. A Joubert for NMR analysis and Dr. JHL Jordaan for MS analysis.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2017.12.057.

References

1. World Health Orginization. World Malaria Report 2016 summary. http://apps.who.int/iris/bitstream/10665/252038/1/9789241511711-eng.pdf.
2. Fairhurst RM, Dondorp AM. Artemisinin-resistant *Plasmodium falciparum* malaria. *Microbiol Spectr*. 2016;4:10.
3. World Health Organization. Emergency response to artemisinin resistance in the Greater Mekong subregion: regional framework for action, 2013–2015. http://www.who.int/malaria/publications/atoz/9789241505321/en/.
4. Haynes RK. From artemisinin to new artemisinin antimalarials: biosynthesis, extraction, old and new derivatives, stereochemistry and medicinal chemistry requirements. *Curr Top Med Chem*. 2006;6:509–537.
5. Mbengue A, Bhattacharjee S, Pandharkar T, et al. A molecular mechanism of artemisinin resistance in *Plasmodium falciparum* malaria. *Nature*. 2015;520:683–687.
6. Paloque L, Ramadani AP, Mercereau-Puijalon O, Augereau JM, Benoit-Vical F. *Plasmodium falciparum*: multifaceted resistance to artemisinins. *Malaria J*. 2016;15:149.
7. Haynes RK, Fugmann B, Stetter J, et al. Artemisone—a highly active antimalarial drug of the artemisinin class. *Angew Chem Int Ed*. 2006;45:2082–2088.
8. Wu Y, Wu RWK, Cheu KW, et al. Methylene homologues of artemisone: an unexpected structure–activity relationship and a possible implication for the design of C10-substituted artemisinins. *ChemMedChem*. 2016;11:1469–1479.
9. Haynes RK, Cheu KW, Chan HW, et al. Interactions between artemisinins and other antimalarial drugs in relation to the cofactor model—a unifying proposal for drug action. *ChemMedChem*. 2012;7:2204–2226.
10. Haynes RK, Cheu KW, Tang MMK, et al. Reactions of antimalarial peroxides with each of leucomethylene blue and dihydroflavins: flavin reductase and the cofactor model exemplified. *ChemMedChem*. 2011;6:279–291.
11. Okada H, Kitaguchi YOH, Yoshimura K. Oxidation reaction of 1, 1'-bis (o-substituted phenyl) ferrocenes. *Org Commun*. 2010;3:92.
12. Dubar F, Egan TJ, Pradines B, et al. The antimalarial ferroquine: role of the metal and intramolecular hydrogen bond in activity and resistance. *ACS Chem Biol*. 2011;6:275–287.
13. Pladziewicz JR, Espenson JH. Kinetics and mechanisms of some electron transfer reactions of ferrocenes. *J Am Chem Soc*. 1973;95:56–63.
14. Pladziewicz JR, Carney MJ. Reduction of ferricenium ion by horse heart ferrocytochrome c. *J Am Chem Soc*. 1982;104:3544–3545.
15. Pladziewicz JR, Brenner MS, Rodeberg DA, Likar MD. Kinetic study of the oxidation of spinach plastocyanin by ferrocenium ion derivatives. *Inorg Chem*. 1985;24:1450–1453.
16. Carlson BW, Miller LL. Oxidation of NADH by ferrocenium salts. Rate-limiting one-electron transfer. *J Am Chem Soc*. 1983;105:7453–7454.
17. Matsue T, Suda M, Uchida I, Kato T, Akiba U, Osa T. Electrocatalytic oxidation of NADH by ferrocene derivatives and the influence of cyclodextrin complexation. *J Electroanal Chem Interfac*. 1987;234:163–173.
18. Pierre JL, Fontecave M, Crichton RR. Chemistry for an essential biological process: the reduction of ferric iron. *Biometals*. 2002;15:341–346.
19. Woodmansee AN, Imlay JA. Reduced flavins promote oxidative DNA damage in non-respiring escherichia coli by delivering electrons to intracellular free iron. *J Biol Chem*. 2002;277:34055–34066.
20. Takeda K, Iizuka M, Watanabe T, Nakagawa J, Kawasaki S, Niimura Y. Synechocystis DrgA protein functioning as nitroreductase and ferric reductase is capable of catalyzing the Fenton reaction. *FEBS J*. 2007;274:1318–1327.
21. Delhaes L, Biot C, Berry L, et al. Novel ferrocenic artemisinin derivatives: synthesis, in vitro antimalarial activity and affinity of binding with ferroprotoporphyrin IX. *Bioorg Med Chem*. 2000;8:2739–2745.
22. Paitayatat S, Tarnchompoo B, Thebtaranonth Y, Yuthavong Y. Correlation of antimalarial activity of artemisinin derivatives with binding affinity with ferroprotoporphyrin IX. *J Med Chem*. 1997;40:633–638.
23. Fomin V, Markin A. Oxidation mechanism of ferrocene with molecular oxygen: kinetic and thermodynamic aspects. *J Therm Anal Calorim*. 2008;92:985–987.
24. Haynes RK, Cheu K, N'Da DD, Coghi P, Monti D. Considerations on the mechanism of action of artemisinin antimalarials: Part 1-The 'carbon radical' and 'heme' hypotheses. *Infect Disord Drug Targets*. 2013;13:217–277.
25. Haynes RK. Strategies in the development and chemical modification of the new artemisinin antimalarial artemisone. *Synform*. 2008;3:34–36.
26. Lin AJ, Zikry AB, Kyle DE. Antimalarial activity of new dihydroartemisinin derivatives. 7. 4-(p-Substituted phenyl)-4 (R or S)-[10 (α or β)-dihydroartemisininoxy] butyric Acids1-6. *J Med Chem*. 1997;40:1396–1400.
27. Vogel M, Rausch M, Rosenberg H. Derivatives of Ferrocene. III. The preparation of some acylferrocenes and alkylferrocenes. *J Org Chem*. 1957;22:1016–1018.
28. Lau CK, Tardif S, Dufresne C, Scheigetz J. Reductive deoxygenation of aryl aldehydes and ketones by tert-butylamine-borane and aluminum chloride. *J Org Chem*. 1989;54:491–494.
29. Verlinden BK, Niemand J, Snyman J, et al. Discovery of novel alkylated (bis) urea and (bis) thiourea polyamine analogues with potent antimalarial activities. *J Med Chem*. 2011;54:6624–6633.
30. Wentzel JF, Lombard MJ, Du Plessis LH, Zandberg L. Evaluation of the cytotoxic properties, gene expression profiles and secondary signalling responses of cultured cells exposed to fumonisin B1, deoxynivalenol and zearalenone mycotoxins. *Arch Toxicol*. 2017;91:2265–2282.
31. Batterjee SM, Marzouk MI, Aazab ME, El-Hashash MA. The electrochemistry of some ferrocene derivatives: redox potential and substituent effects. *Appl Organomet Chem*. 2003;17:291–297.

32. Shago RF, Swarts JC, Kreft E, van Rensgurg CEJ. Antineoplastic activity of a series of ferrocene-containing alcohols. *Anticancer Res*. 2007;27:3431–3433.
33. Kuwana T, Bublitz DE, Hoh G. Chronopotentiometric studies on the oxidation of ferrocene, ruthenocene, osmocene and some of their derivatives. *J Am Chem Soc*. 1960;82:5811–5817.
34. Little WF, Eisenthal R. Interannular electronic effects in ferrocene: kinetics of reaction of substituted ferrocenoic acids with diphenyldiazomethane. *J Am Chem Soc*. 1961;83:4936–4939.
35. Birrell GW, Chavchich M, Ager AL, et al. JPC-2997, a new aminomethylphenol with high in vitro and in vivo antimalarial activities against blood stages of Plasmodium. *Antimicrob Agents Chemother*. 2015;59:170–177.
36. Lu JJ, Meng LH, Cai YJ, et al. Dihydroartemisinin induces apoptosis in HL-60 leukemia cells dependent of iron and p38 mitogen activated protein kinase activation but independent of reactive oxygen species. *Cancer Biol Ther*. 2008;7:1017–1023.

Chapter 5:

Synthesis, *in vitro* antimalarial activities and cytotoxicities of amino-artemisinin-1,2-disubstituted ferrocene derivatives

This chapter contains a research article published in Bioorganic and Medicinal Chemistry Letters. It presents syntheses, anti-malarial activities and cytotoxicities of a series of amino-arteminsinin-1,2-disubstituted ferrocene derivatives.

Bioorganic & Medicinal Chemistry Letters 28 (2018) 3161–3163

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis, antimalarial activities and cytotoxicities of amino-artemisinin-1, 2-disubstituted ferrocene hybrids

Christo de Lange[a], Dina Coertzen[b], Frans J. Smit[a], Johannes F. Wentzel[a], Ho Ning Wong[a], Lyn-Marie Birkholtz[b], Richard K. Haynes[a,*], David D. N'Da[a,*]

[a] Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom 2520, South Africa
[b] Department of Biochemistry, Genetics and Microbiology, Institute for Sustainable Malaria Control, University of Pretoria, Pretoria 0002, South Africa

ARTICLE INFO

Keywords:
Amino-artemisinin
Ferrocene
Hybrid drug
Malaria
Cytotoxicity

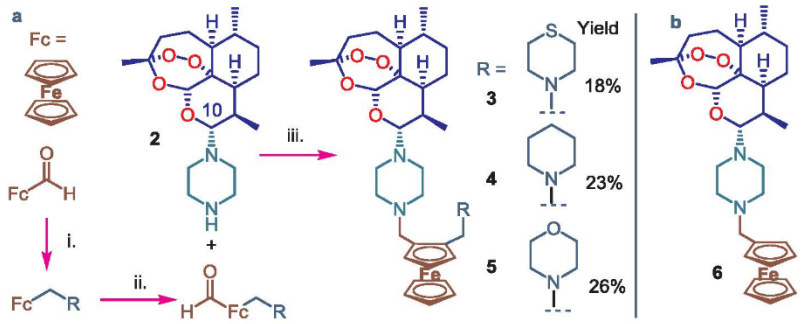
ABSTRACT

Artemisinin-ferrocene conjugates incorporating a 1,2-disubstituted ferrocene analogous to that embedded in ferroquine but attached *via* a piperazine linker to C10 of the artemisinin were prepared from the piperazine artemisinin derivative, and activities were evaluated against asexual blood stages of chloroquine (CQ) sensitive NF54 and CQ resistant K1 and W2 strains of *Plasmodium falciparum* (*Pf*). The most active was the morpholino derivative **5** with IC_{50} of 0.86 nM against *Pf* K1 and 1.4 nM against *Pf* W2. The resistance indices were superior to those of current clinical artemisinins. Notably, the compounds were active against *Pf* NF54 early and late blood stage gametocytes – these exerted > 86% inhibition at 1 μM against both stages; they are thus appreciably more active than methylene blue (~57% inhibition at 1 μM) against late stage gametocytes. The data portends transmission blocking activity. Cytotoxicity was determined against human embryonic kidney cells (Hek293), while human malignant melanoma cells (A375) were used to assess their antitumor activity.

The use of artemisinin based combination therapies (ACTs), currently the most effective for treatment of malaria, is under threat. ACTs are becoming less effective with resistance being reported towards both the artemisinin and non-artemisinin components of ACTs.[1,2] This emphasizes the need for new artemisinin derivatives that cannot be metabolized to the common artemisinin metabolite dihydroartemisinin implicated in artemisinin resistance.[3–5] This metabolite can be avoided by replacing the oxygen atom attached to C10 of the current clinical artemisinins with an amino group.[6,7] In addition, by incorporating the ferrocene pharmacophore, a mode of action complementary to that of the artemisinin comes into play. Ferrocene (in the Fe^{3+} state) undergoes facile oxidation to ferrocenium (Fe^{3+}), for example by hydrogen peroxide that is thereby reduced to hydroxyl radical in the Fenton reaction.[8] The hydroxyl radical is potently bioactive.[9] In turn, ferrocenium is reduced by NADH and glutathione (GSH) to ferrocene.[10–15] The ensuing redox cycling involving ferrocene and ferrocenium will greatly enhance hydroxyl radical flux. The most successful ferrocene-containing antimalarial drug is ferroquine, based on the chloroquine (CQ) template. The 1,2-disubstituted ferrocene is embedded within the side chain of chloroquine in close proximity to the two amino groups that allows the ferrocene to adopt a uniquely exposed configuration.[16] Ferroquine is able to generate micromolar amounts of hydroxyl radicals from H_2O_2.[17] The ability of ferrocene to generate hydroxyl radical in principle can be exploited further through conjugation to an artemisinin derivative, wherein the latter is able to induce oxidative stress by oxidizing reduced flavin cofactors that normally modulate levels of endogenous thiols required for expunging reactive oxygen species (ROS).[7,18] If redox cycling of the embedded ferrocene in the artemisinin-ferrocene hybrid can indeed maintain the reactive oxygen source, the additional oxidative stress would greatly amplify intracellular damage. Artemisinin-ferrocene hybrids were first prepared some time ago,[19,20] although the original rationalization of their antimalarial activities in terms of binding to ferroprotoporphyrin IX is open to question.[21,22] More recently, artemisinin-acyl ferrocene hybrids prepared from DHA were reported to display antimalarial activities against CQ-sensitive *Pf* 3D7 ranging from 7.2 to 30.2 nM that were inferior to those of the parent artemisinin; however, the compounds were notably cytotoxic towards multidrug-resistant leukemia cell lines.[23] Similarly an artemisinin acyl ferrocene hybrid intriguingly incorporating the redox-active thymoquinone unit was active against leukemia cell lines, but less active than artemisinin control compounds against malaria.[24] As aminoartemisinins appear to display optimal antimalarial activities,[7] we used the C10 piperazino artemisinin derivative **2**[25] to prepare hybrids bearing a terminal acyl ferrocene or alkyl ferrocene that elicited

* Corresponding authors.
E-mail addresses: haynes@ust.hk, richard.haynes@nwu.ac.za (R.K. Haynes), david.nda@nwu.ac.za (D.D. N'Da).

https://doi.org/10.1016/j.bmcl.2018.08.037
Received 28 June 2018; Received in revised form 20 August 2018; Accepted 27 August 2018
Available online 28 August 2018



Scheme 1. a. Preparation of the amino-artemisinin-1,2-disubstituted ferrocene derivatives i. Ferrocene carboxaldehyde (1.1 eq.), secondary amine (1.2 eq.), sodium triacetoxyborohydride (2 eq.), dichloromethane, N_2, room temperature, 4 h. ii. Aminoferrocene (1 eq.), potassium *tert*-butoxide (0.1 eq.), *n*-BuLi (1.1 eq.), Et_2O, Ar, room temperature, 16 h, then addition of DMF (3 eq.), 4 h. iii. **2** (3 eq.), aminoferrocenealdehyde (1.0 eq.), sodium triacetoxyborohydride (3 eq.), THF, N_2, room temperature, overnight; **b**. Compound **6** prepared from **2** according to the previously published procedure (Ref. 22).

IC_{50} activities against CQ-sensitive and -resistant *Pf* of 2.9–24.1 nM.[22] We now describe the use of the artemisinin **2** for preparation of new hybrids incorporating the 1,2-disubstituted ferrocene moiety according to the precept for ferroquine outlined above. The methods are presented in Scheme 1a. The terminal alkyl ferrocene hybrid **6** (Scheme 1b) prepared as previously described[22] is included here for comparative purposes.

The synthesis of the ferrocene derivatives was carried out in two steps. Ferrocene carboxaldehyde was submitted to reductive amination with sodium triacetoxyborohydride[26] in the presence of the secondary cyclic amine (thiomorpholine, piperidine and morpholine) to give the corresponding aminoferrocenes in yields above 80%. The aminoferrocene derivatives were then treated with *n*-butyllithium-potassium *tert*-butoxide to give the lithiated intermediate.[27,28] Treatment of the lithiated intermediate with *N,N*-dimethyl formamide (DMF) provided the corresponding amino-ferrocenealdehydes in yields after purification of 30%. The foregoing products were then coupled through reductive amination with the 10α-piperazino artemisinin **2** by using sodium triacetoxyborohydride to deliver the amino-artemisinin-1,2-disubstituted ferrocene derivatives (Scheme 1a).

Biological activities for the artemisinin-ferrocene conjugates are given in Tables 1 and 2. *In vitro* antimalarial activities were determined against the asexual blood stages of three *Pf* strains – the drug sensitive NF54, and drug-resistant K1 and W2 strains.[29] The resistance index RI is the ratio of the IC_{50} values of the resistant to sensitive strains IC_{50} K1/IC_{50} NF54 and IC_{50} W2/IC_{50} NF54, and was used as an indication of potential for cross resistance formation for each drug resistant strain (Table 1). The gametocytocidal activities were determined with *Pf* NF54 early and late stage gametocytes at two concentrations, 1 μM and 100 nM (Table 2).[30] The cytotoxicities of the derivatives were evaluated *in vitro* with human embryonic kidney cells Hek293 while anti-tumor screening was carried out with the human malignant melanoma cell line (A375) (Table 1).[31] The selectivity indexes (SI) indicate the selectivity of the compounds towards parasitized cells or cancer cells with respect to the non-proliferating mammalian cell line. Details are given in the Supplementary Material.

The activities of derivatives **4** and **5** against asexual blood stage parasites were better than those of dihydroartemisinin (DHA), artesunate (AS) and artemether (AM) towards the resistant K1 and W2 strains but were less active towards the sensitive NF54 (Table 1). In general, however, activities of the 1,2-disubstituted ferrocene hybrids here are superior to those described previously for the acyl and alkyl ferrocene hybrids[22]; activities of the best of the latter, namely compound **6**, are included for comparison in Table 1. Although the SI value of the morpholino ferrocene derivative **5** indicates that it is more selective towards parasites than mammalian cells, this SI value is lower than that of DHA, possibly indicative of generalized toxicity. In this respect, it is intriguing that the amino artemisinin derivative bearing the morpholino group attached directly to the C10 position (*cf.* compound **2**) exhibited acute toxicity.[6] While compound **3** did not have the same antimalarial potency as the other derivatives, it was relatively quite active towards the A375 melanoma cell line with respect to non-proliferating mammalian cells. The RI values of ferrocene hybrids **3–5** indicate a lower potential for resistance formation than compound **6** and the clinically used DHA, AS and AM.

The activities of the ferrocene derivatives against early (stages I-III) and late stage gametocytes (IV-V) are noteworthy. When each were applied at a concentration of 1 μM, they were approximately equipotent with methylene blue and DHA against early stage, but were appreciably more active against late stage gametocytes (Table 2). This is the first time gametocytocidal activity is reported for ferrocene-artemisinin hybrids; this is significant, as activity against late-stage gametocytes in particular portends transmission-blocking capability. For any new drug development programme, it is important that drugs have the ability to

Table 1
In vitro anti-malarial activities against *Pf* asexual blood stage parasites determined by SYBR Green I fluorescence proliferation readout, cytotoxicities and selectivity indices of amino-artemisinin ferrocene derivatives.[a]

Compd.	Antimalarial activity IC_{50} (± SEM) nM					Cytotoxicity IC_{50} (μM)		Antitumour IC_{50} (μM)	
	NF54	K1	RI[b]	W2	RI[c]	Hek293	SI[d]	A375	SI[e]
CQ	10.0 (3.0)	154.0 (14.0)	15.4	233.0 (49.0)	23.3	nd	nd	nd	nd
DHA	2.5 (0.1)	1.5 (0.3)	0.6	1.7 (0.2)	0.6	4.0[f]	1,593	1.0[f]	0.3
AS	3.0 (0.2)	4.0 (1.0)	1.3	2.4 (0.4)	0.8	nd	nd	nd	nd
AM	1.8 (0.1)	9.0 (2.0)	4.8	7.0 (1.0)	3.8	nd	nd	nd	nd
3	7.5 (2.5)	2.9 (0.3)	0.3	3.4 (1.1)	0.4	43.0	5,733	11.0	3.9
4	3.8 (1.4)	1.1 (1.1)	0.2	1.7 (0.6)	0.4	60.0	15,424	65.0	0.9
5	3.3 (1.3)	0.8 (0.2)	0.2	1.4 (0.7)	0.4	1.0	300	1.0	1.0
6[g]	4.5 (0.6)	2.7 (0.7)	0.6	3.2 (1.0)	0.7	53.0	11,597	19.0	2.7

[a]Data are from at least three independent biological replicates, n = 3, each performed in technical triplicates; CQ chloroquine; DHA dihydroartemisinin; AS artesunate; AM artemether; nd not determined; Hek293 human embryonic kidney cells, A375 human malignant melanoma cells; [b]Resistance Index = IC_{50} K1/IC_{50} NF54; [c]Resistance Index = IC_{50} W2/IC_{50} NF54; [d]Selectivity Index = IC_{50} Hek293/IC_{50} NF54; [e]Selectivity Index = IC_{50} Hek293/IC_{50} A375; [f]historical cytotoxicity and antitumour values for DHA (Refs. 32,33); [g]historical values for compound **6** (Ref. 22).

Table 2
% Inhibition *in vitro* of *Pf* NF54 gametocytes by amino-artemisinin ferrocene derivatives at 1 μM and 100 nM against early (I-III) and late stage (IV-V) gametocytes as determined with the luciferase reporter gene assay.[a]

Compound	Early stage (I-III) gametocytes		Late stage (IV-V) gametocytes	
concentration	1 μM	100 nM	1 μM	100 nM
MB	95.0 ± 1.7	nd	57.3 ± 3.96	nd
DHA	97.1 ± 0.5	nd	72.0 ± 6.7	nd
3	95.7 ± 0.33	93.8 ± 0.7	86.5 ± 3.56	84.8 ± 0.5
4	95.8 ± 0.21	95.9 ± 0.3	88.7 ± 2.04	87.0 ± 0.4
5	96.1 ± 0.37	99.1 ± 0.2	88.4 ± 0.96	87.6 ± 0.8

[a]See Ref. 30; MB Methylene Blue, DHA dihydroartemisinin; data are from a single biological replicate (n = 1) performed in technical triplicates, ± SD.

block transmission to the mosquito, in particular of resistant parasites.

Overall, the data obtained for these derivatives strongly encourages further investigation of these ferrocene-artemisinin linked derivatives, including the accessible derivative **6** described earlier,[22] with attention to be focussed on conducting assays *in vivo* so as to establish the role of the ferrocene group in carrying cytotoxic mode of action, on improving the synthetic routes, and on generating related derivatives wherein polarity of the amino group attached to the ferrocene is modulated so as to enhance drug uptake.

Acknowledgments

This work was funded by the South African Medical Research Council (MRC) Flagship Project MALTB-Redox with funds from National Treasury under its Economic Competitiveness and Support Package and a South African National Research Foundation (NRF) Grant to R. K. Haynes (UID 90682). The authors thank the following: Mr. A Joubert for NMR analysis and Dr. JHL Jordaan for MS analysis.

Disclaimer: Any opinions, findings and conclusions, or recommendations expressed in this material are those of the authors and therefore the NRF does not accept any liability in regard thereto.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.08.037.

References

1. World Health Organization. Emergency response to artemisinin resistance in the Greater Mekong subregion: regional framework for action, 2013–2015. http://www.who.int/malaria/publications/atoz/9789241505321/en/.
2. World Health Orginization. Status report on artemisinin and ACT resistance (October 2016). http://www.who.int/malaria/publications/atoz/update-artemisinin-resistance-october2016/en/.
3. Haynes RK. From artemisinin to new artemisinin antimalarials: biosynthesis, extraction, old and new derivatives, stereochemistry and medicinal chemistry requirements. *Curr Top Med Chem.* 2006;6:509–537.
4. Mbengue A, Bhattacharjee S, Pandharkar T, et al. A molecular mechanism of artemisinin resistance in *Plasmodium falciparum* malaria. *Nature.* 2015;520:683–687.
5. Paloque L, Ramadani AP, Mercereau-Puijalon O, Augereau JM, Benoit-Vical F. *Plasmodium falciparum*: multifaceted resistance to artemisinins. *Malar J.* 2016;15:149.
6. Haynes RK, Fugmann B, Stetter J, et al. Artemisone—a highly active antimalarial drug of the artemisinin class. *Angew Chem Int Ed.* 2006;45:2082–2088.
7. Wu Y, Wu RWK, Cheu KW, et al. Methylene homologues of artemisone: an unexpected structure–activity relationship and a possible implication for the design of C10-substituted artemisinins. *ChemMedChem.* 2016;11:1469–1479.
8. Huang G, Chen H, Dong Y, et al. Superparamagnetic iron oxide nanoparticles: amplifying ROS stress to improve anticancer drug efficacy. *Theranostics.* 2013;3:116–126.
9. Chavain N, Vezin H, Dive D, et al. Investigation of the redox behavior of ferroquine, a new antimalarial. *Mol Pharm.* 2008;5:710–716.
10. McDowell MS, Espenson JH, Bakac A. Kinetics of aqueous outer-sphere electron-transfer reactions of superoxide ion. Implications concerning the O_2/O_2^- self-exchange rate constant. *Inorg Chem.* 1984;23:2232–2236.
11. Pladziewicz JR, Carney MJ. Reduction of ferricenium ion by horse heart Ferrocytochrome c. *J Am Chem Soc.* 1982;104:3544–3545.
12. Pladziewicz JR, Brenner MS, Rodeberg DA, Likar MD. Kinetic study of the oxidation of spinach plastocyanin by ferrocenium ion derivatives. *Inorg Chem.* 1985;24:1450–1453.
13. Carlson BW, Miller LL. Oxidation of NADH by ferrocenium salts. Rate-limiting one-electron transfer. *J Am Chem Soc.* 1983;105:7453–7454.
14. Matsue T, Suda M, Uchida I, Kato T, Akiba U, Osa T. Electrocatalytic oxidation of NADH by ferrocene derivatives and the influence of cyclodextrin complexation. *J Electroanal Chem Interface.* 1987;234:163–173.
15. Joy AM, Goodgame DML, Stratford IJ. High efficiency of ferricenium salts as radiosensitizers of V79 cells *in vitro* and the KHT tumor *in vivo*. *Int J Radiat Oncol Biol Phys.* 1989;16:1053–1056.
16. Biot C, Taramelli D, Forfar-Bares I, et al. Insights into the mechanism of action of ferroquine. Relationship between physicochemical properties and antiplasmodial activity. *Mol Pharm.* 2005;2:185–193.
17. Dubar F, Khalife J, Brocard J, Dive D, Biot C. Ferroquine, an ingenious antimalarial drug–thoughts on the mechanism of action. *Molecules.* 2008;13:2900–2907.
18. Haynes RK, Cheu KW, Chan HW, et al. Interactions between artemisinins and other antimalarial drugs in relation to the cofactor model—a unifying proposal for drug action. *ChemMedChem.* 2012;7:2204–2226.
19. Delhaes L, Biot C, Berry L, et al. Novel ferrocenic artemisinin derivatives: synthesis, in vitro antimalarial activity and affinity of binding with ferroprotoporphyrin IX. *Bioorg Med Chem.* 2000;8:2739–2745.
20. Paitayatat S, Tarnchompoo B, Thebtaranonth Y, Yuthavong Y. Correlation of antimalarial activity of artemisinin derivatives with binding affinity with ferroprotoporphyrin IX. *J Med Chem.* 1997;40:633–638.
21. Haynes RK, Cheu K, N'Da DD, Coghi P, Monti D. Considerations on the mechanism of action of artemisinin antimalarials: Part 1-The 'carbon radical' and 'heme' hypotheses. *Infect Disord Drug Targets.* 2013;13:217–277.
22. de Lange C, Coertzen D, Smit FJ, et al. Synthesis, in vitro antimalarial activities and cytotoxicities of amino-artemisinin-ferrocene derivatives. *Bioorg Med Chem Lett.* 2018;28:289–292.
23. Reiter C, Fröhlich T, Zeino M, et al. New efficient artemisinin derived agents against human leukemia cells, human cytomegalovirus and *Plasmodium falciparum*: 2nd generation 1,2,4-trioxane-ferrocene hybrids. *Eur J Med Chem.* 2015;97:164–172.
24. Karagöz AC, Reiter C, Seo E-J, et al. Access to new highly potent antileukemia, antiviral and antimalarial agents via hybridization of natural products (homo)egonol, thymoquinone and artemisinin. *Bioorg Med Chem.* 2018;26:3610–3618.
25. Wu Y, Parapini S, Williams ID, et al. Facile preparation of *N*-glycosylated 10-piperazinyl artemisinin derivatives and evaluation of their antimalarial and cytotoxic activities. *Molecules.* 2018;23:1713. https://doi.org/10.3390/molecules23071713.
26. Abdel-Magid AF, Mehrman SJ. A review on the use of sodium triacetoxyborohydride in the reductive amination of ketones and aldehydes. *Org Process Res Dev.* 2006;5:971–1031.
27. Slocum D, Rockett B, Hauser C. Ring metalation of dimethylaminomethylferrocene with butyllithium and condensations with electrophilic compounds. Synthesis of 1, 2-disubstituted ferrocenes. *J Am Chem Soc.* 1965;87:1241–1246.
28. Nishibayashi Y, Arikawa Y, Ohe K, Uemura S. Enantioselective *ortho*-lithiation of substituted ferrocenes. *J Org Chem.* 1996;61:1172–1174.
29. Verlinden BK, Niemand J, Snyman J, et al. Discovery of novel alkylated (bis) urea and (bis) thiourea polyamine analogues with potent antimalarial activities. *J Med Chem.* 2011;54:6624–6633.
30. Reader J, Botha M, Theron A, et al. Nowhere to hide: interrogating different metabolic parameters of *Plasmodium falciparum* gametocytes in a transmission blocking drug discovery pipeline towards malaria elimination. *Mal J.* 2015;14:213.
31. Wentzel JF, Lombard MJ, Du Plessis LH, Zandberg L. Evaluation of the cytotoxic properties, gene expression profiles and secondary signalling responses of cultured cells exposed to fumonisin B1, deoxynivalenol and zearalenone mycotoxins. *Arch Toxicol.* 2017;91:2265–2282.
32. Birrell GW, Chavchich M, Ager AL, et al. JPC-2997, a new aminomethylphenol with high in vitro and in vivo antimalarial activities against blood stages of Plasmodium. *Antimicrob Agents Chemother.* 2015;59:170–177.
33. Lu JJ, Meng LH, Cai YJ, et al. Dihydroartemisinin induces apoptosis in HL-60 leukemia cells dependent of iron and p38 mitogen activated protein kinase activation but independent of reactive oxygen species. *Cancer Biol Ther.* 2008;7:1017–1023.

Chapter 6:
Summary and Conclusion

Malaria is a disease caused by an intercellular parasite of the genus *Plasmodium*. The World Health Organization (WHO), reported that there were nearly 216 million cases of malaria in 2016 with approximately 445 000 deaths. Of these deaths an astounding 91 % occurred in Africa of which it was estimated that 407 000 deaths (WHO, 2017).

The protozoan responsible for malaria belongs to the genus *Plasmodium*. The species that infect humans with malaria are *P. ovale*, *P. malariae*, *P. knowlesi*, *P. vivax* and *P. falciparum* (Cox-Singh et al., 2008). Of these species *P. vivax* is known to cause deaths but *P. falciparum* is the leading cause of malaria related deaths.

The only effective treatment of malaria is by means of chemotherapy. The treatment evolved through the decades as these parasites became resistant towards the used antimalarials. Chloroquine, mefloquine, sulfadoxine and pyrimethamine, atovaquone and progaunil all fell victim to resistance (Dondorp *et al.*, 2009; Fivelman *et al.*, 2002; Gregson & Plowe, 2005; Payne, 1987; Price *et al.*, 2004). The last truly effective treatment left was the artemisinin class. In order to protect this class it was decided by the WHO that artemisinin combinational therapy, or ACT, will be the new standard treatment of uncomplicated cases of malaria. This treatment was successful until recent reports that *P. falciparum* has developed resistance towards ACTs treated (WHO 2016). As there is currently no alternative for artemisinins there is a clear need for new antimalarials.

The simplest approach to discover new drugs is through hybridisation. This consists of combining two pharmacophores, having different modes of action, through a chemical bond with the aim to synthesise a drug with enhanced activities (Meunier, 2007; Walsh & Bell, 2009). This was illustrated by the group of Biot that incorporated ferrocene into the chloroquine which overcame chloroquine resistance (Biot *et al.*, 1997). Initially incorporation of ferrocene into the chloroquine did not deliver the desired effect, only when the ferrocene was embedded into the side chain a very active derivative was synthesised (Dive & Biot, 2008). Ferrocene was also incorporated into the artemisinin structure with some of these derivatives having equipotent activities as the artemisinin class (Delhaes *et al.*, 2000;

Paitayatat *et al.*, 1997; Reiter *et al.*, 2015; Reiter *et al.*, 2014). Although these results indicate some promise there is no indication of the potential toxicity of these derivatives.

This study was aimed at synthesising oxidant-redox hybrids that will continue in its antimalarial after the peroxide moiety is reduced. To achieve this several non-acetal amino–ferrocenyl–artemisinin derivatives was synthesized. 10α-(1'-piperazino)-10-deoxo-10-dihydroartemisinin (DHA-pip) was used as a scaffold that provided efficient access to a secondary amine for derivatisation. DHA-pip yields were influenced by the rate at which the oxalylchloride was added. The slow the addition of oxalylchlorde led to a higher the yield of the DHA-pip.

Ferrocenoyl halides and ferrocenyl alkyl halides that was synthesized was obtained in high yields. Ferrocenoyl halides were synthesised by using the Friedel acylation and these derivatives were obtained in high yields. The ferrocenoyl halides were reduced with *tert*-butylamine and aluminium chloride to give the ferrocenyl alkyl halides in high yields. The final hybrids of these ferrocene derivatives were reacted with DHA-pip in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in order to generate the amino-ferrocenyl-artemisnin derivatives. Again these derivatives were obtained in low yields. DHA-pip was submitted to reductive alkylation with ferrocene-carboxaldehyde and triacetoxyborohydride a final hybrid was obtained in high yield. This compound showed similar activities to the other compounds and due to the high yield it was identified as a hit compound that could possibly undergo *in vivo* screening.

The 1,2-disubstituted ferrocene-amino-aldehydes was synthesized in two steps. The selected secondary amine was submitted to reductive alkylation with ferrocene-carboxaldehyde and triacetoxyborohydride in order to obtain the ferrocene-amino derivatives in high yields. To obtain the ferrocene-amino-aldehydes *n*-butyllithium and DMF was used. The synthesised compounds were only obtained in low yields due to the sensitivity to light. Additionally it is believed that higher yields might have been obtained if *tert*-butyllithium was used but due to the dangers involved in handling this chemical it was not considered. Purification required that columns had to be covered with foil and multiple columns had to be run inorder to obtain the purified compound. These 1,2-disubstituted ferrocene-amino-aldehydes was then coupled to DHA-pip using triacetoxyborohydride. These final hybrids also had to be purified multiple times which eventually led to low yields. Because of the low yields of these compounds it will be advised to research alternative methods of synthesis

first before further biological investigation as it is believed that these compounds are worth while investing in.

All of these derivatives were screened *in vitro* against the NF54, K1 and W2 strains in order to establish their antimalarial activity. Cytotoxicity was evaluated on the HEK293 cell line while anticancer activity was evaluated against the A375 cell line.

By analysing the biological data that was produced during this study the following conclusions were made:

- The 1,2-disubstituted ferrocene-amino-artemisinin derivatives are active against strains *Pf.* resistant to CQ .
- Two of the 1,2-disubstituted ferrocene-amino-artemisinin derivatives was more active than clinically used artemisinins.
- Of the seven derivatives that were stable enough to undergo antimalarial evaluation five derivatives was more selective towards parasitized cells in the presence of mammalian cells than dihyroartemisinin.
- The 1,2-disubstituted ferrocene-amino-artemisinin derivatives' gametocidal activity was superior to that of dihydroartemisinin and was equipotent to the activity of methylene blue.

In summary, this project delivered two derivatives with good *in vitro* antimalarial activity: 1,2-disubstituted ferrocene-amino-artemisinin bearing the piperidine moeity (IC_{50}: NF54 3.8 nM; K1 1.1 nM, W2 1.7 nM), 1,2-disubstituted ferrocene-amino-artemisinin bearing the morpholine moeity (IC_{50}: NF54 3.3 nM; K1 0.8 nM; W2 1.4 nM), artesunate (IC_{50}: NF54 3.0 nM; K1 4.0 nM; W2 2.4 nM), artemether (IC_{50}: NF54 1.8 nM; K1 9 nM; W2 7.0 nM) and dihydroartemisinin (IC_{50}: NF54 2.5 nM; K1 1.5 nM; W2 1.7 nM). These two synthesised derivatives prove that further investigation of ferrocene-artemisinin hybrid derivatives will prove vital to overcome artemisinin resistance.

6.1 References

Biot, C., Glorian, G., Maciejewski, L.A., Brocard, J.S., Domarle, O., Blampain, G., Millet, P., Georges, A.J., Abessolo, H. & Dive, D. 1997. Synthesis and antimalarial activity in vitro and in vivo of a new ferrocene− chloroquine analogue. *Journal of Medicinal Chemistry*, 40(23):3715-3718.

Cox-Singh, J., Davis, T.M., Lee, K.-S., Shamsul, S.S., Matusop, A., Ratnam, S., Rahman, H.A., Conway, D.J. & Singh, B. 2008. Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening. *Clinical Infectious Diseases*, 46(2):165-171.

Delhaes, L., Biot, C., Berry, L., Maciejewski, L., Camus, D., Brocard, J. & Dive, D. 2000. Novel ferrocenic artemisinin derivatives: synthesis, in vitro antimalarial activity and affinity of binding with ferroprotoporphyrin IX. *Bioorganic & Medicinal Chemistry*, 8(12):2739-2745.

Dive, D. & Biot, C. 2008. Ferrocene conjugates of chloroquine and other antimalarials: the development of ferroquine, a new antimalarial. *ChemMedChem*, 3(3):383-391.

Dondorp, A.M., Nosten, F., Yi, P., Das, D., Phyo, A.P., Tarning, J., Lwin, K.M., Ariey, F., Hanpithakpong, W. & Lee, S.J. 2009. Artemisinin resistance in Plasmodium falciparum malaria. *New England Journal of Medicine*, 361(5):455-467.

Fivelman, Q.L., Butcher, G.A., Adagu, I.S., Warhurst, D.C. & Pasvol, G. 2002. Malarone treatment failure and in vitro confirmation of resistance of Plasmodium falciparum isolate from Lagos, Nigeria. *Malaria Journal*, 1(1):1.

Gregson, A. & Plowe, C.V. 2005. Mechanisms of resistance of malaria parasites to antifolates. *Pharmacological Reviews*, 57(1):117-145.

Meunier, B. 2007. Hybrid molecules with a dual mode of action: dream or reality? *Accounts of Chemical Research*, 41(1):69-77.

Paitayatat, S., Tarnchompoo, B., Thebtaranonth, Y. & Yuthavong, Y. 1997. Correlation of antimalarial activity of artemisinin derivatives with binding affinity with ferroprotoporphyrin IX. *Journal of Medicinal Chemistry*, 40(5):633-638.

Payne, D. 1987. Spread of chloroquine resistance in Plasmodium falciparum. *Parasitology Today*, 3(8):241-246.

Price, R.N., Uhlemann, A.-C., Brockman, A., McGready, R., Ashley, E., Phaipun, L., Patel, R., Laing, K., Looareesuwan, S. & White, N.J. 2004. Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. *The Lancet*, 364(9432):438-447.

Reiter, C., Fröhlich, T., Zeino, M., Marschall, M., Bahsi, H., Leidenberger, M., Friedrich, O., Kappes, B., Hampel, F. & Efferth, T. 2015. New efficient artemisinin derived agents against human leukemia cells, human cytomegalovirus and Plasmodium falciparum: 2nd generation 1, 2, 4-trioxane-ferrocene hybrids. *European Journal of Medicinal Chemistry*, 97:164-172.

Reiter, C., Karagöz, A.Ç., Fröhlich, T., Klein, V., Zeino, M., Viertel, K., Held, J., Mordmüller, B., Öztürk, S.E. & Anıl, H. 2014. Synthesis and study of cytotoxic activity of 1, 2, 4-trioxane- and egonol-derived hybrid molecules against Plasmodium falciparum and multidrug-resistant human leukemia cells. *European Journal of Medicinal Chemistry*, 75:403-412.

Walsh, J. & Bell, A. 2009. Hybrid drugs for malaria. *Current Pharmaceutical Design*, 15(25):2970-2985.

WHO. 2016. Artemisinin and artemisinin-based combination therapy resistance: status report. http://apps.who.int/iris/handle/10665/208820 [Accessed 23 November 2017].

WHO. 2018. World Malaria Report 2018. Geneva: WHO; 2018. Licence: CC BY-NC-SA 3.0 IGO.

Addendum A:
Supporting data for Chapter 4

Synthesis, in vitro antimalarial activities and cytotoxicities of amino-artemisinin-ferrocene derivatives

Christo de Lange[a], Dina Coertzen[b], Frans J. Smit[a], Johannes F. Wentzel[a], Ho Ning Wong[a], Lyn-Marie Birkholtz[b], Richard K. Haynes[a*] and David D. N'Da[a*]

[a]*Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom 2520, South Africa*
[b]*Department of Biochemistry, Institute for Sustainable Malaria Control, University of Pretoria, Private Bag X20, Hatfield 028, South Africa*

List of contents

1 General procedures

Dihydroartemisinin (DHA) (a mixture of 10-α and 10-β epimers) was purchased from Changzhou Kaixuan Chemical Co (Chunjiang,China). Ferrocene, aluminium trichloride ($AlCl_3$), piperazine, oxalyl chloride, 3-chloropropanoyl chloride, 5-bromopentanoyl chloride, 6-bromohexanoyl chloride, borane *tert*-butylamine, ferrocenecarboxaldehyde, triethylamine, sodium triacetoxyborohydride and sodium chloride were purchased from Sigma-Aldrich (Johannesburg, South Africa). Methanol, ammonium chloride, sodium bicarbonate, magnesium sulfate, diethyl ether, dichloromethane, toluene and ethyl acetate were purchased from ACE chemicals (Johannesburg, South Africa). Sodium hydroxide was purchased from Saarchem (Krugersdorp, South Africa). 1,8-Diazabicyclo(5.4.0)undec-7-ene (DBU) was purchased from Merck (Johannesburg, South Africa). All the chemicals and reagents were of analytical grade. Diethyl ether and tetrahydrofuran were dried and distilled from sodium-benzophenone, dichloromethane and toluene were dried and distilled from calcium hydride. Chemicals were used without further purification. All products from the reactions below, except where indicated, were purified by chromatography using high-purity MN silica gel 60, 70-230 mesh ASTM, supplied by Macherey-Nagel (Germany). When the relevant fractions were collected after purification by column chromatography, they were examined by Thin Layer Chromatography (TLC) on silica gel plates ($60F_{254}$) from Merck, and were single fractions that were used as such. Thus, the final derivatives were single fractions that were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance spectra recorded on the samples (below).

Microwave radiation was carried out using a CEM Discover™ focused closed vessel microwave synthesis system. The machine consists of a continuous focused microwave power delivery system with operator selectable power output from 0 to 300 W, a maximum current of 6.3 amps and a frequency of 50/60Hz. The temperature of the contents of the vessel was monitored using an IR sensor located underneath the reaction vessel. The contents of the vessel are stirred by means of a rotating magnetic plate located below the floor of the microwave cavity and a Teflon coated magnetic stir bar in the vessel.

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Advance™ III 600 spectrometer at a frequency of 600 MHz and 150 MHz, respectively, in $CDCl_3$. Chemical shifts are reported on the δ scale (ppm) downfield from tetramethylsilane (δ=0.0 ppm) using the residual solvent signal at δ 7.26 ppm (^{1}H) or δ 77.00 ppm (^{13}C) as internal standard. High resolution mass spectrometry (HRMS) was recorded on a Bruker MicroTOF Q II mass spectrometer, equipped with an atmospheric pressure chemical ionisation (APCI) or an electrospray ionisation (ESI) source, set at 200 °C or 180 °C,

respectively, using Bruker Compass DataAnalysis 4.0 software. A full scan from 50 to 1500 *m*/*z* was performed at a capillary voltage of 4500 V, an end plate offset voltage of −500 V, with the nebulizer set at 1.6 Bar and 0.4 Bar, respectively, and a collision cell RF voltage of 100 Vpp. Mass spectra (MS) were recorded in positive mode on a Thermo Electron LXQ™ ion trap mass spectrometer, equipped with Xcalibur 2.2 data acquisition and analysis software. The MS had an APCI source set at 300 °C, and was direct infusion with a Harvard syringe pump utilized at a flow rate of 10 µL/min. A full scan from 100 to 1200 amu was achieved in 1 s, with a capillary voltage of 7 V, while the corona discharge was 10 µA. Infrared (IR) spectra were recorded on a Bruker Alpha-P FTIR instrument using the Attenuated Total Reflectance (ATR) technique.

2 *In vitro* anticancer and cytotoxicity screening derivative [1]

A375 (ATCC® CRL-1619™ Human malignant melanoma) and Hek293 cells (ATCC® CRL-1573™ Human embryonic kidney cells) were cultured in Dulbecco's modified essential medium (DMEM; Hyclone, GE healthcare, South Logan, UT, USA) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% 200 mM L-Glutamine and 1% non-essential amino acids (Lonza, Basel, Switzerland). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. For compound treatment, cells were seeded in a 96 well plate and cultured until 80-90% confluent. Stock solutions for compounds were prepared in DMSO preheated to 40°C. All subsequent dilutions were prepared in serum free DMEM and vehicle controls were included in all experiments.

3-(4',5'-Dimethylthiazol-2'-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [2]

The MTT assay was used to determine *in vitro* cell viability. A375 and Hek293 cells were seeded in a 96-well plate and incubated until cells were ~90% confluent. After 24 hours exposure to the compounds (12-1800 µM), growth medium was removed, cells rinsed twice with 1 x phosphate buffered saline (PBS) and 100 µL fresh serum free medium containing 5 mg/ml MTT solution was added. Cells were then incubated for 4 hours at 37 °C, after which the MTT was carefully removed and replaced with 100 µL dimethyl sulfoxide (DMSO). After 1 hour of incubation at 37 °C, cell viability was determined using a microplate reader (SpextraMac Paradigm) at an absorbance wavelength of 550 nm and background wavelength of 630 nm with DMSO measured as a blank. Cell viability is expressed as a percentage relative to the untreated control, which is assumed to be 100 % viable. As a positive control, cells were treated with 0.01% Triton-X 100 (Sigma-Aldrich, St Louis, MO,

USA) for 4 hours. Using the MTT assay data, IC_{50} values was calculated using GraphPad Prism 5. In brief, data was normalized to the negative controls (presumed to be 100% viable), followed by the log-transformation of the concentration values. The curve was fitted using the log (inhibitor) vs. response function and the IC_{50} values calculated. Experiments were at least done in triplicate.

3 *In vitro* antimalarial assay

The *P. falciparum* parasites were maintained at 37 °C in human erythrocytes (O+) suspended in complete culture medium [RPMI 1640 medium (Sigma-Aldrich) supplemented with 25 mM HEPES (Sigma-Aldrich), 20 mM D-glucose (Sigma-Aldrich), 200 µM hypoxanthine (Sigma-Aldrich), 0.2% sodium bicarbonate, 24 µg/ml Gentamycin (Sigma-Aldrich) and 0.5% AlbuMAX II] in a gaseous environment of 90% N_2, 5% O_2, and 5% CO_2 as described. [3] *In vitro* ring-stage intra-erythrocytic *P. falciparum* NF54 parasite cultures (genotyped drug sensitive) (200 µl at 1% haematocrit, 1% parasitaemia) were treated with compounds at 5 and 1 µM. The controls for this assay included chloroquine disulfate (1 µM, as positive control) and complete RPMI media (as negative control) and grown for 96 h at 37 °C under the 90% N_2, 5% O_2, and 5% CO_2 gas mixture in 96-well plates. At the conclusion of the 96 h growth period, equal volumes (100 µl each) of the *P. falciparum* parasite cultures were combined with SYBR Green I lysis buffer (0.2 µl/ml 10 000xSYBR Green I, Invitrogen; 20 mM Tris, pH 7.5; 5 mM EDTA; 0.008% (w/v) saponin; 0.08% (v/v) Triton X-100). The samples were incubated at 37°C for 1 h after which the fluorescence was measured using a Fluoroskan Ascent FL microplate fluorometer (Thermo Scientific, excitation at 485 nm and emission at 538 nm). The 'background' fluorescence (i.e. that measured in the samples derived from chloroquine-treated iRBC samples in which parasite proliferation was completely inhibited) was subtracted from the total fluorescence measured for each sample to provide a measure of parasite proliferation. Data obtained were analyzed in Excel and sigmoidal dose-response curves were plotted using GraphPad 5.0. Experiments are always performed in technical triplicates for at least three independent biological replicates (n=3).

4 General experimental procedures

1. Ferrocene acetylation

The acid halides that were used are 3-chloropropanoyl chloride, 5-bromopentanoyl chloride and 6-bromohexanoyl chloride. The corresponding acid halide (26.9 mmol, 1 equiv) was slowly added to a stirred solution of $AlCl_3$ (26.9 mmol, 1 equiv) in 15 mL of distilled

dichloromethane under N_2 at room temperature. The reaction mixture was stirred for 2 hours after which it was cooled on an ice-salt bath. A second 2-necked flask containing a stirred solution of ferrocene (26.9 mmol, 1 equiv) in 60 mL of distilled dichloromethane under N_2 was also cooled on an ice-salt bath. The first reaction mixture was slowly added to the ferrocene solution in the second flask. This reaction mixture was left to warm to room temperature while being stirred for 16 hours. The reaction mixture was quenched over ice and extracted with dichloromethane until the dichloromethane extract was colourless. The combined extracts were dried over magnesium sulfate and the solvent was removed *in vacuo*. Purification was performed with column chromatography; eluting with dichloromethane afforded the derivatives.

2. Deoxygenation of ferrocenoyl halides [4]

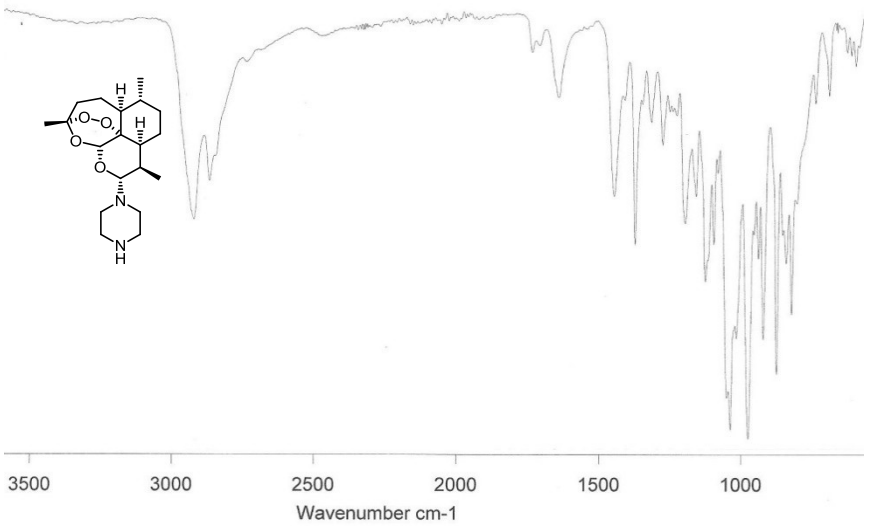
$AlCl_3$ (10.8 mmol, 3 equiv) and borane *tert*-butylamine (10.8 mmol, 3 equiv) were dissolved in 10 mL of anhydrous Et_2O and stirred at room temperature under N_2. The ferrocenoyl halide (3.6 mmol, 1 equiv) was dissolved in 10 mL of anhydrous Et_2O and was added slowly to the first reaction mixture. The reaction mixture was left to stir for 2 hours. The reaction mixture was quenched with water (20 mL) and extracted with Et_2O (3 x 40 mL). The extracts were combined, washed with brine (2 x 25 mL), dried over magnesium sulfate and the solvent was removed *in vacuo*. Purification of the residue by column chromatography and eluting with dichloromethane afforded the ferrocenyl alkyl halides.

3. Synthesis of 10α-(1'-piperazino)-10-deoxo-10-dihydroartemisinin **2**

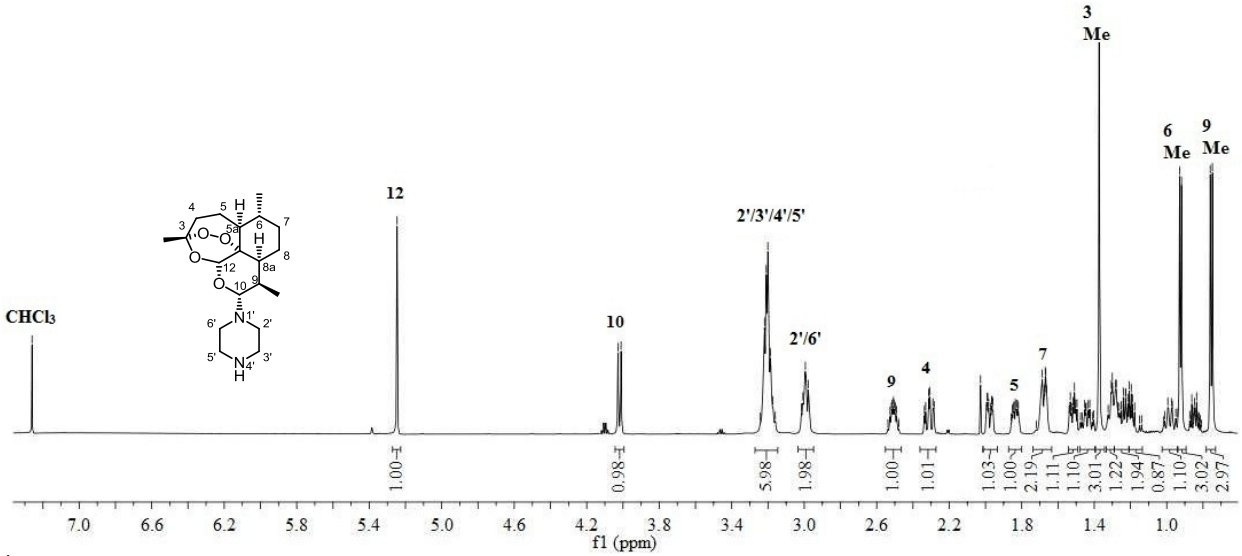
Dihydroartemisinin (14 mmol, 1 equiv) was suspended in a stirred mixture of 40 mL of anhydrous toluene and DMSO (1.4 mmol, 0.1 equiv) under N_2 at room temperature. Oxalyl chloride (15.4 mmol, 1.15 equiv) was slowly added to the reaction mixture and the final reaction mixture was left to stir for 2 hours. In a separate vessel, piperazine (70 mmol, 5 equiv) was dissolved in 50 mL of anhydrous dichloromethane, stirred under N_2 at room temperature. The first reaction mixture was slowly added to the piperazine solution and the resulting reaction mixture was allowed to stir for 16 hours. The reaction was quenched with 20 mL of saturated $NaHCO_3$ and extracted with ethyl acetate (3 x 40 mL). The combined extracts were washed with brine (2 x 25 mL) and dried over magnesium sulfate. After the solvent was removed *in vacuo*, the residue was purified by column chromatography; eluting with dichloromethane-MeOH-triethylamine (10:1:0.1) afforded the derivative, shown by TLC analysis to be a single compound.

*10α-(1'-Piperazino)-10-deoxo-10-dihydroartemisinin **2***

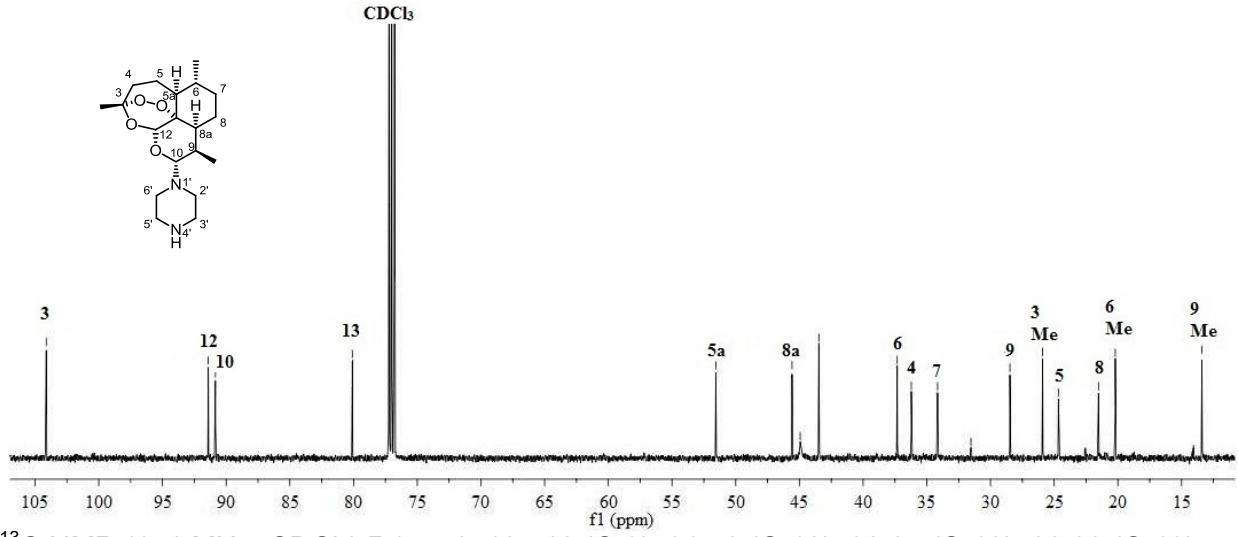
Pale yellow gum, 1.72 g (35%), R_f 0.43 (dichloromethane-MeOH 9:1).



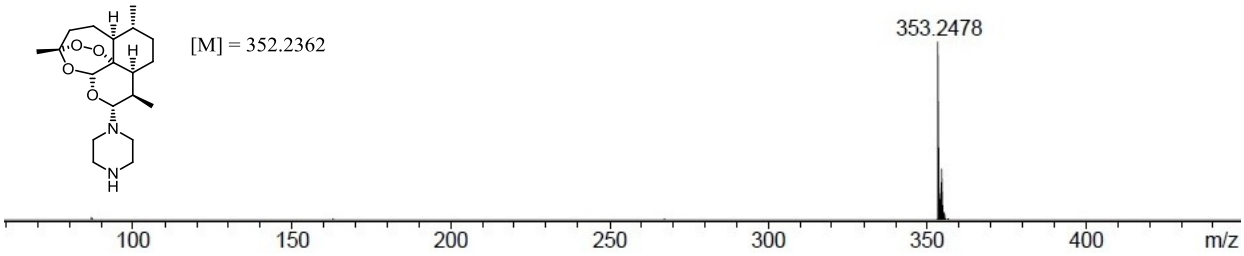
IR (ATR) ν_{max}/cm^{-1}: 2924, 2869, 1736, 1642, 1448, 1350, 1128, 1052, 978, 925, 878, 844, 825.



^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.25 (s, 1H, H-12), 4.03-4.01 (d, *J* = 10.2 Hz, 1H, H-10), 3.13-3.23 (m, 6H, H-2', H-3', H-4', H-5'), 3.02-2.98 (m, 2H, H-3',H-6'), 2.54-2.48 (m, 1H, H-9), 2.34-2.28 (td, *J* = 13.9, 3.4 Hz, 1H, H-4), 2.00-1.96 (m, 1H, H-4), 1.86-1.82 (m, 2H, H-5), 1.72-1.66 (m, 2H, H-7), 1.54-1.50 (m, 1H, H-8a), 1.48-1.40 (m, 1H, H-5), 1.37 (s, 3H, H-3 Me), 1.30-1.32 (m, 1H, H-6), 1.28-1.21 (m, 2H, H-8), 1.21-1.14 (m, 1H, H-5a), 1.02-0.95 (m, 1H, H-7), 0.93-0.92 (d, *J* = 6.3 Hz, 3H, H-6 Me), 0.76-0.75 (d, *J* = 7.1 Hz, 3H, H-9 Me).



^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 104.12 (C-3), 91.40 (C-12), 90.85 (C-10), 80.09 (C-13), 51.56 (C-5a), 45.58 (C-8a), 44.94 (C-1'/C-2'), 43.47 (C-1'/C-2'), 37.32 (C-6), 36.21 (C-4), 34.15 (C-7), 31.53, 28.47 (C-9), 25.91 (C-3 Me), 24.67 (C-5), 22.53, 21.52 (C-8), 20.20 (C-6 Me), 14.06, 13.40 (C-9 Me).



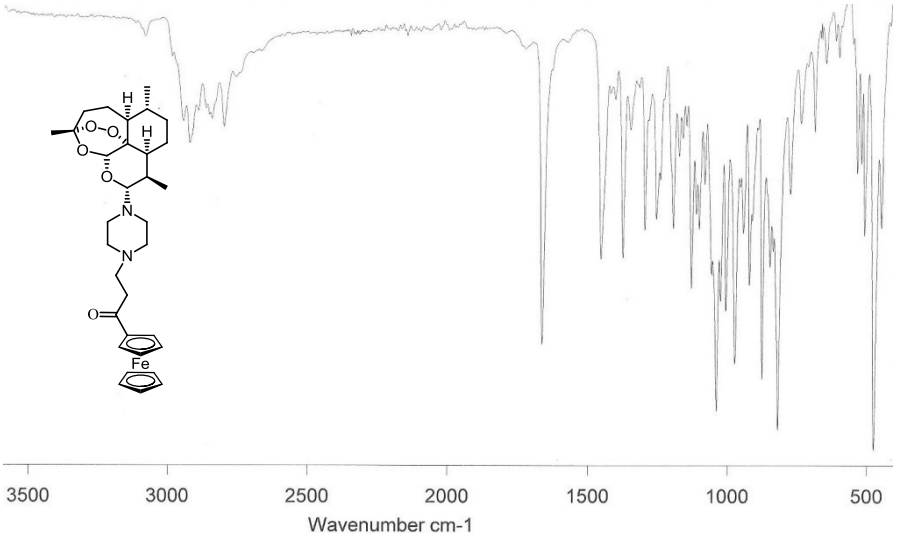
HRMS ESI: m/z $[M+H]^+$ 353.2478 (calculated for $C_{19}H_{33}N_2O_4^+$: 353.2440).

4. General procedure for the synthesis of derivatives **3** – **5**

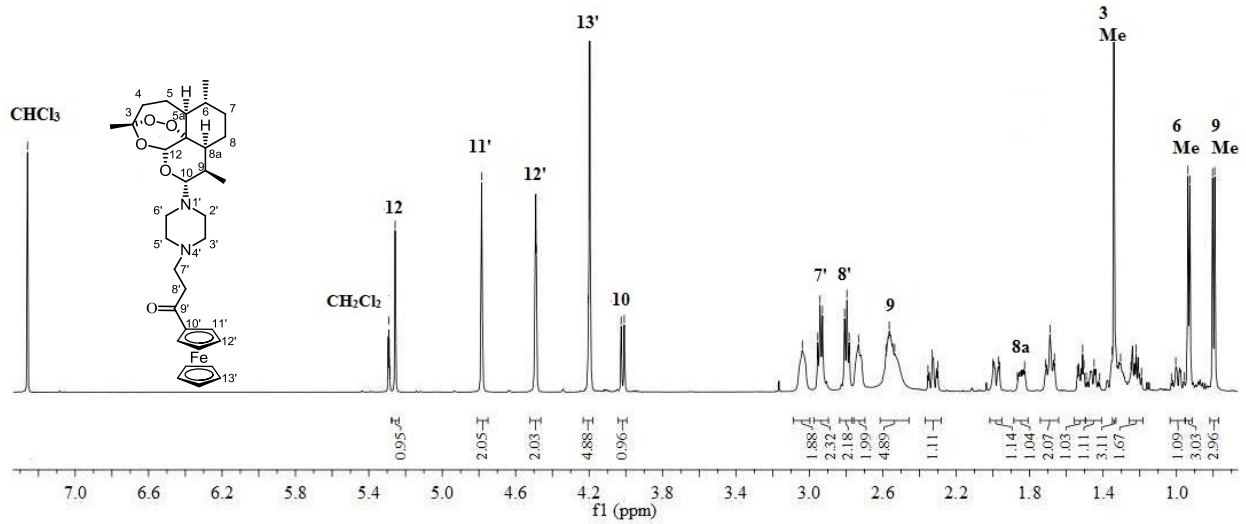
The artemisinin-piperazine derivative **2** (2.8 mmol, 1 equiv) was dissolved in a stirred solution of 20 mL anhydrous acetonitrile and DBU (5.6 mmol, 2 equiv) under N_2 at room temperature for 1 h. The ferrocenoyl halide (2.8 mmol, 1 equiv) was added to the reaction mixture and was left to stir for 24 h. The reaction was quenched with saturated NH_4Cl (20 mL) and extracted with ethyl acetate (3 x 25 mL). The combined extracts were washed with brine (25 mL) and dried over magnesium sulfate. After the solvent was removed *in vacuo*, the residue was purified by column chromatography; eluting with dichloromethane-MeOH (9:1) afforded the derivatives. All compounds after purification by column chromatography as assessed by TLC analyses were single fractions that were used as such.

10α-[1'-Piperazino-4'-(ferrocene-propan-1-one)]-10-deoxo-10-dihydroartemisinin ***3***

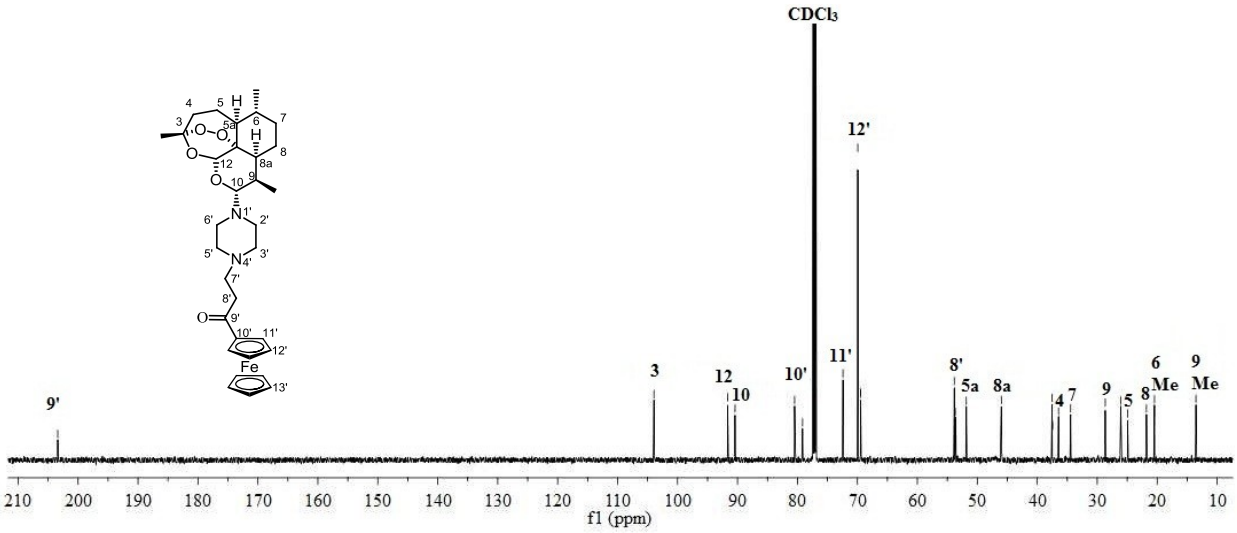
Yellow gum, 0.51 g (31%), R_f 0.48 (dichloromethane-MeOH 9:1).



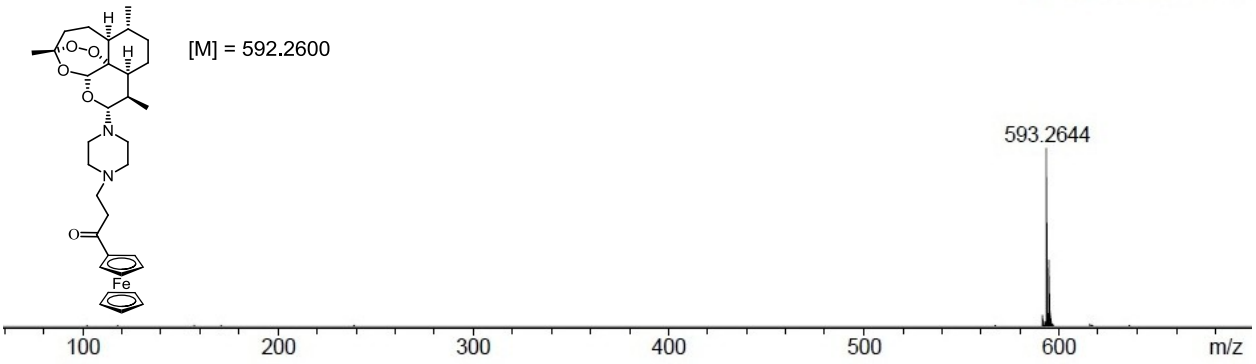
IR (ATR) ν_{max}/cm^{-1}: 3085 (aromatic C-H), 2925, 2801, 1664 (C=O), 1454, 1376, 1298, 1130, 957, 878, 821, 512, 478.



^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.26 (s, 1H, H-12), 4.79 (s, 2H, H-11'), 4.49 (s, 2H, H-12'), 4.20 (s, 5H, H-13'), 4.03-4.01 (d, *J* = 10.1 Hz, 1H, H-10), 3.04, 2.95-2.93 (t, *J* = 7.1 Hz, 2H, H-7'), 2.81-2.78 (t, *J* = 7.7 Hz, 2H, H-8'), 2.58-2.53 (s, 5H, H-9), 2.35-2.30 (td, *J* = 14.0, 3.6 Hz, 1H, H-4), 2.00-1.96 (dt, *J* = 14.5, 3.2 Hz, 1H, H-4), 1.87-1.83 (m, 1H, H-5/H-8), 1.71-1.66 (m, 2H, H-4), 1.54-1.50 (dt, *J* = 13.4, 4.2 Hz, 1H, H-8a), 1.49-1.42 (qd, *J* = 13.0, 4.7 Hz, 1H, H-5), 1.34 (s, 3H, H-3 Me), 1.24-1.19 (m, 1H, H-5a), 1.03-0.96 (qd, *J* = 13.2, 2.5, 1H, H-7), 0.94-0.93 (d, *J* = 6.3 Hz, 3H, H-6 Me), 0.80-0.79 (d, *J* = 7.1 Hz, 3H, H-9 Me).



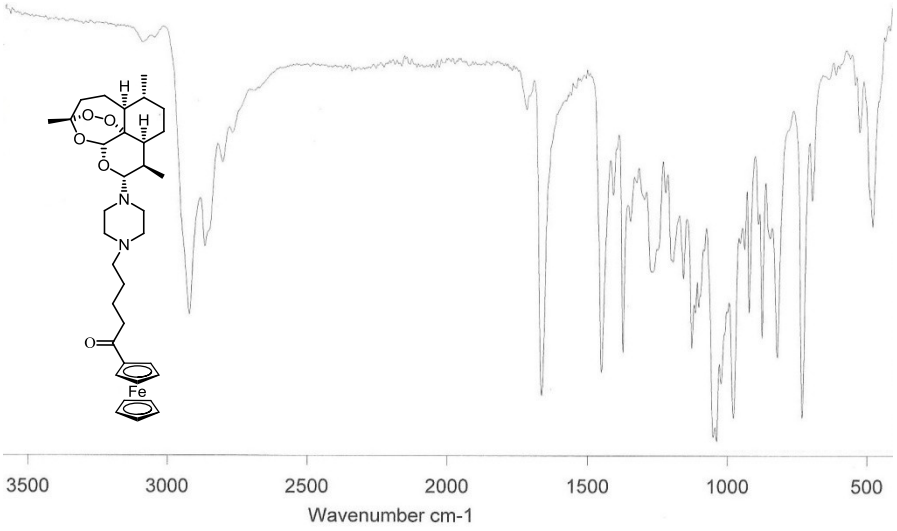
^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 203.39 (C=O), 103.94 (C-3), 91.65 (C-12), 90.41 (C-10), 80.47, 79.15 (C-10'), 72.41 (C-11'), 69.94 (C-12'), 69.47 (C-13'), 69.45, 53.83, 53.67 (C-7'), 53.57 (C-8'), 51.83 (C-5a), 45.99 (C-8a), 37.54 (C-6), 37.42, 36.45 (C-4), 34.43 (C-7), 28.65 (C-9), 26.07 (C-3 Me), 24.93 (C-5), 21.78 (C-8), 20.44 (C-6 Me), 13.53 (C-9 Me).



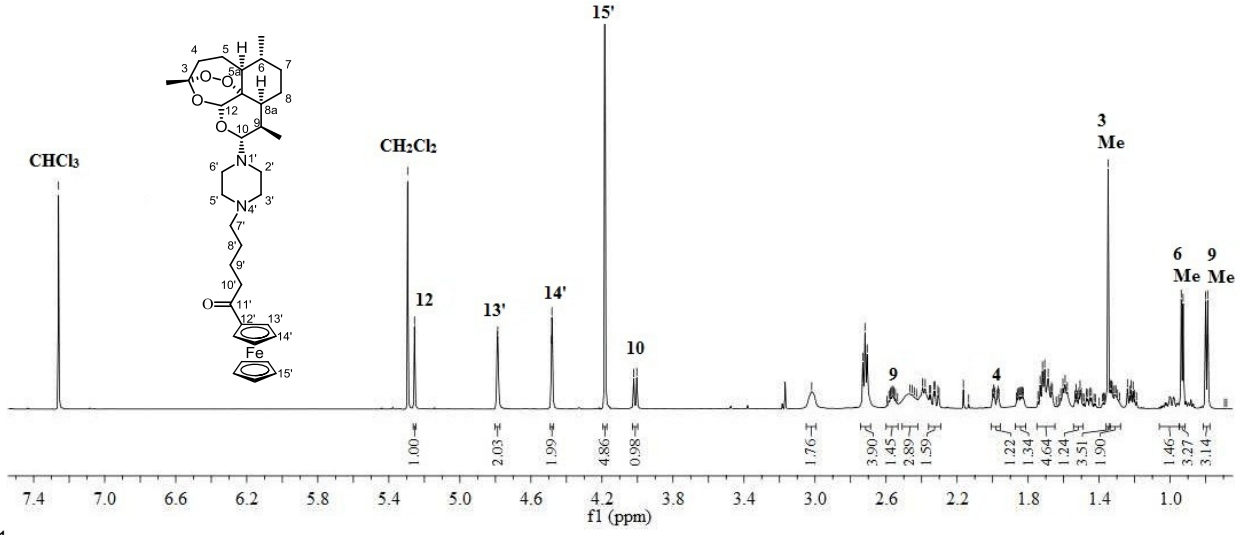
HRMS ESI: m/z $[M+H]^+$ 593.2644 (calculated for $C_{32}H_{45}N_2O_5^+$: 593.2678).

10α-[1'-Piperazino-4'-(ferrocenepentan-1-one)]-10-deoxo-10-dihydroartemisinin ***4***

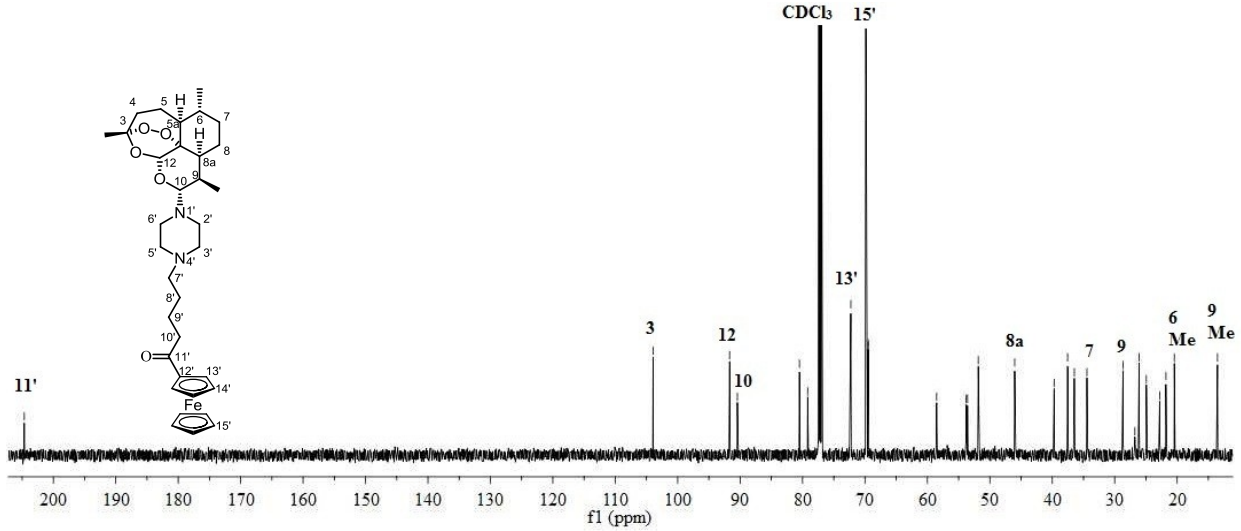
Dark orange gum, 0.71 g (41 %), R_f 0.44 (dichloromethane-MeOH 9:1).



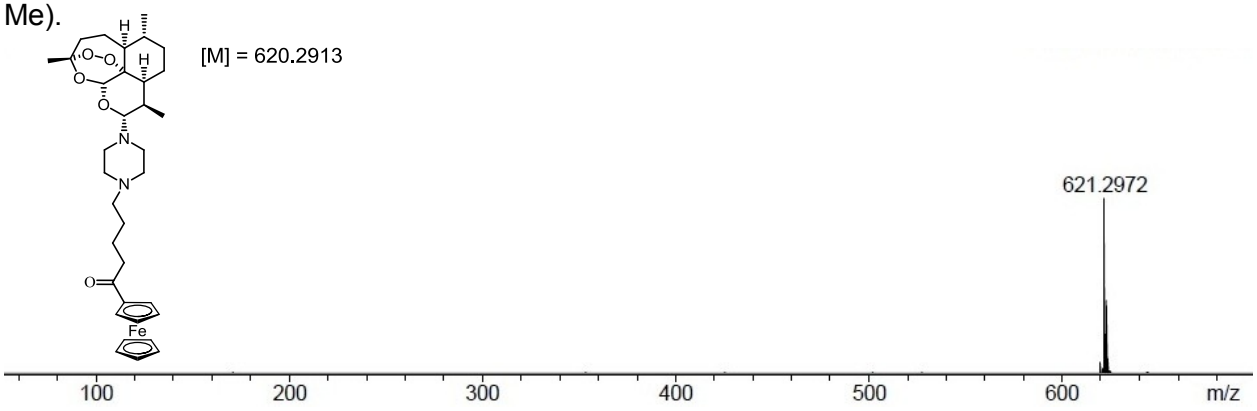
IR (ATR) ν_{max}/cm^{-1}: 3095 (aromatic C-H), 2926, 2869, 1665 (C=O), 1452, 1375, 1301, 1198, 1160, 1105, 1040, 925, 893, 851, 733.



^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.26 (s, 1H, H-12), 4.78 (d, *J* = 1.8 Hz, 2H, H-13'), 4.48 (t, *J* = 1.8 Hz, 2H, H-14'), 4.18 (s, 5H, H-15'), 4.00-4.01 (d, *J* = 10.1 Hz, 2H, H-10), 2.73-2.71 (t, *J* = 7.2 Hz, 4H, H-4'/H-5'), 2.60-2.56 (m, 1H, H-9), 2.35-2.30 (td, *J* = 13.8, 4.0 Hz, 2H, H-4), 2.00-1.96 (dq, *J* = 14.3, 3.7 Hz, 1H, H-4), 1.87-1.83 (m, 1H, H-5), 1.74-1.66 (m, 5H, H-7/H-8), 1.66-1.58 (m, 2H, H-7/H-8), 1.54-1.49 (dt, *J* = 13.6, 4.2 Hz, 1H, H-8), 1.35 (s, 3H, H-3 Me), 1.34-1.29 (m, 2H, H6/H8), 1.03-0.95 (qd, *J* = 13.1, 3.2 Hz, 1H, H-7), 0.94-0.93 (d, *J* = 6.3 Hz, 3H, H-6 Me), 0.80-0.79 (d, *J* = 7.1 Hz, 3H, H-9 Me).



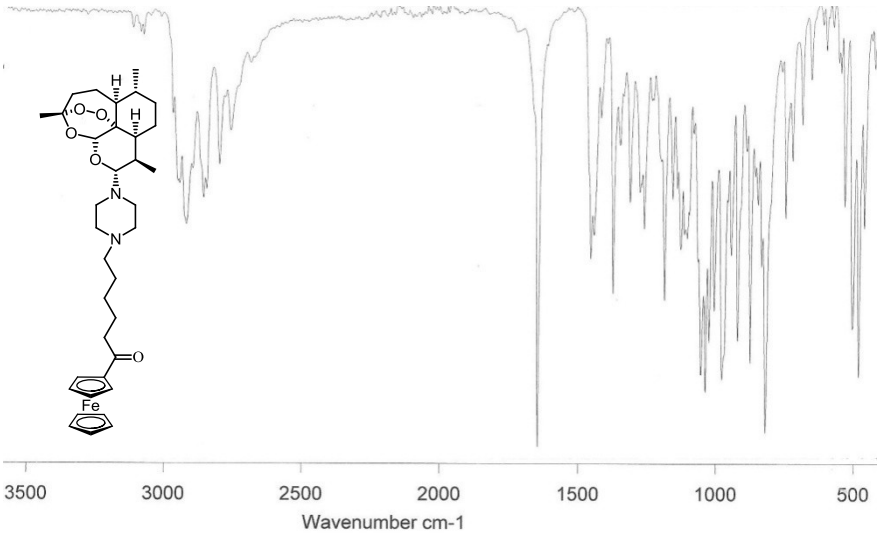
^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 204.66 (C=O), 103.94 (C-3), 91.68 (C-12), 90.44 (C-10), 80.49 (C-13), 79.18 (C-12'), 72.27 (C-13'), 69.88 (C-15'), 69.50-69.47 (C-14'), 58.53, 53.78, 53.57, 26.77, 46.02 (C-8a), 39.71 (C-2'/C-3'), 37.54 (C-6), 36.47 (C-4), 34.45 (C-7), 28.67 (C-9), 26.09 (C-3 Me), 24.93 (C-5), 22.80, 21.80 (C-8), 20.45 (C-6 Me), 13.54 (C-9 Me).



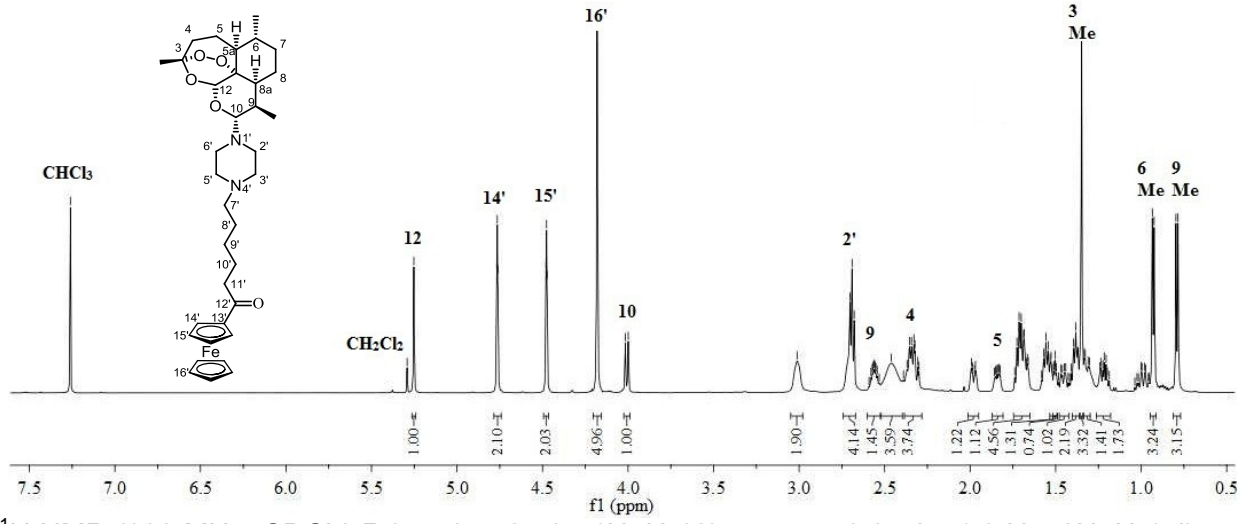
HRMS ESI: m/z $[M+H]^+$ 621.2972 (calculated for $C_{34}H_{49}FeN_2O_5^+$: 621.2991).

10α-[1'-Piperazino-4'-(ferrocene-hexan-1-one)]-10-deoxo-10-dihydroartemisinin ***5***

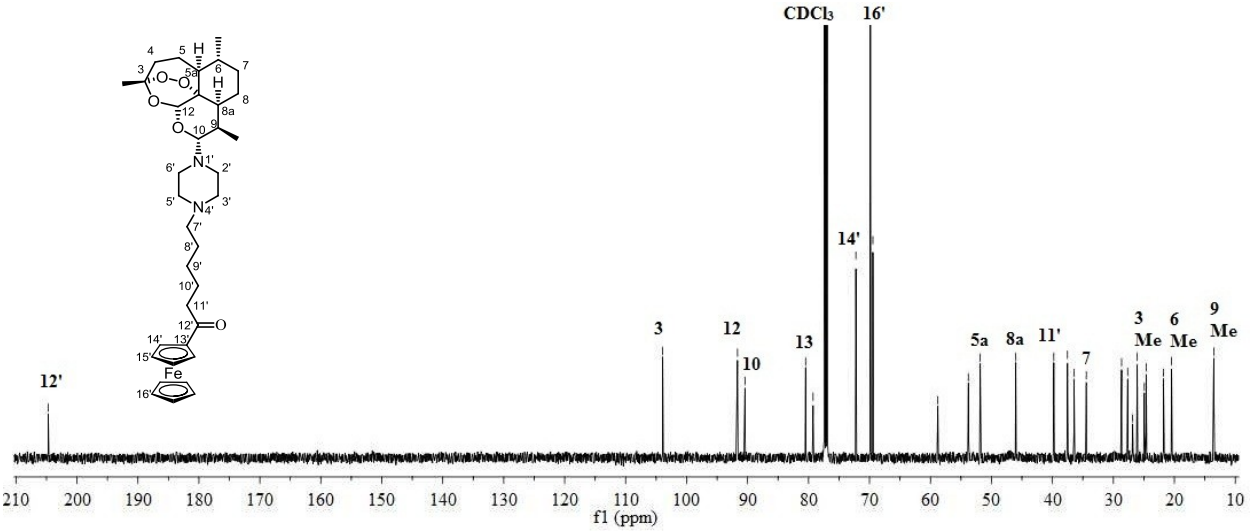
Orange red gum, 0.83 g (47 %), R_f 0.46 (dichloromethane-MeOH 9:1).



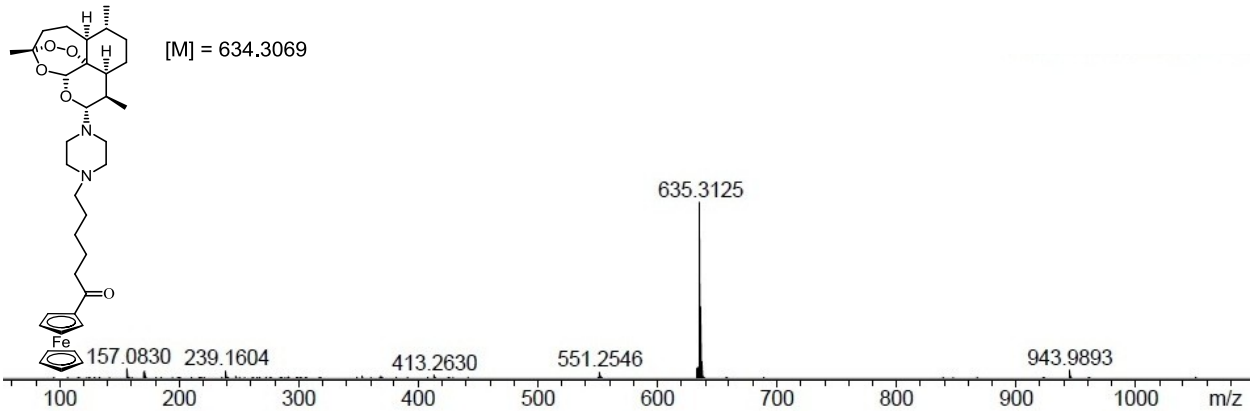
IR (ATR) ν_{max}/cm^{-1}: 3078 (aromatic C-H), 2921, 2859, 2761, 1648 (C=O), 1455, 1444, 1375, 1278, 1188, 1143, 1055, 1040, 1027, 878, 822, 765, 550, 508, 440.



^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.25 (s, 1H, H-12), 4.77-4.76 (t, *J* = 1.8 Hz, 2H, H-14'), 4.48-4.47 (t, *J* = 1.7 Hz, 2H, H-15'), 4.18 (s, 5H, H-16'), 4.02-4.00 (d, *J* = 10.1 Hz, 1H, H-10), 2.70-2.68 (t, *J* = 7.5 Hz, 4H, H-2'), 2.57-2.53 (m, 1H, H-9), 2.37-2.30 (m, 3H, H-4), 1.99-1.97 (dt, *J* = 13.5, 3.2 Hz, 1H, H-4), 1.86-1.82 (m, 1H, H-5), 1.74-1.66 (m, 4H, H-7/H-8/H-10'), 1.52-1.48 (m, 1H, H-8a), 1.47-1.44 (dd, *J* = 12.9, 4.7 Hz, 1H, H-5), 1.35 (s, 3H, H-3 Me), 1.33-1.30 (m, 1H, H-6), 0.93-0.92 (d, *J* = 6.3 Hz, 3H, H-6 Me), 0.80-0.78 (d, *J* = 7.1 Hz, 3H, H-9 Me).



^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 204.70 (C=O), 103.92 (C-3), 91.66 (C-12), 90.43 (C-10), 80.48 (C-13), 79.26 (C-13'), 72.24 (C-14'), 69.86 (C-16'), 69.45 (C-15'), 58.79, 53.78, 51.84 (C-5a), 46.01 (C-8a), 39.77 (C-11'), 37.53 (C-6), 36.46 (C-4), 34.44 (C-7), 28.65 (C-9), 27.65, 26.84, 26.08 (C-3 Me), 24.93 (C-5), 24.61 (C-19), 21.78 (C-8), 20.45 (C-6 Me), 13.53 (C-9 Me).



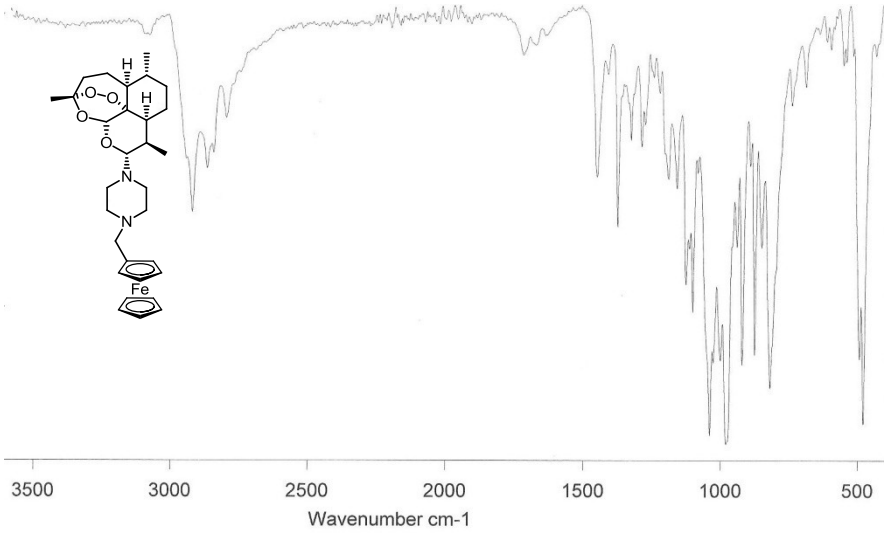
HRMS ESI: m/z $[M+H]^+$ 635.3125 (calculated for $C_{35}H_{51}FeN_2O_5^+$: 635.3147).

5. Synthesis of 10α-[1'-Piperazino-4'-(ferrocenemethyl)]-10-deoxo-10-dihydroartemisinin **6**.

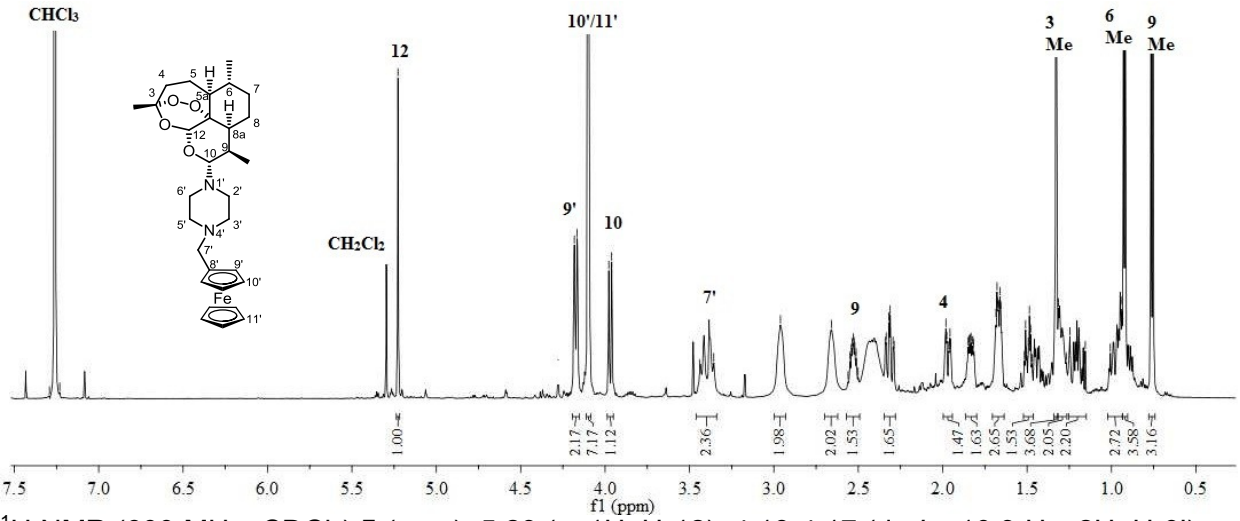
Ferrocenecarboxaldehyde (0.9 mmol, 1 equiv) and **2** (2.7 mmol, 3 equiv) were dissolved in stirred anhydrous THF under N_2 at room temperature. Sodium triacetoxyborohydride (2.3 mmol) was added portion wise to the mixture. The reaction was left to stir overnight at room temperature. The reaction mixture was basified to pH 10 with 1M NaOH. The mixture was extracted with Et_2O (4 x 50 mL) and the combined extracts were dried over magnesium sulfate. The solvent was removed *in vacuo*, and the residue was purified by column chromatography; eluting with dichloromethane-MeOH (9:1) afforded the derivative; analysis by TLC indicated a single compound.

10α-[1'-Piperazino-4'-(ferrocenemethyl)]-10-deoxo-10-dihydroartemisinin ***6***

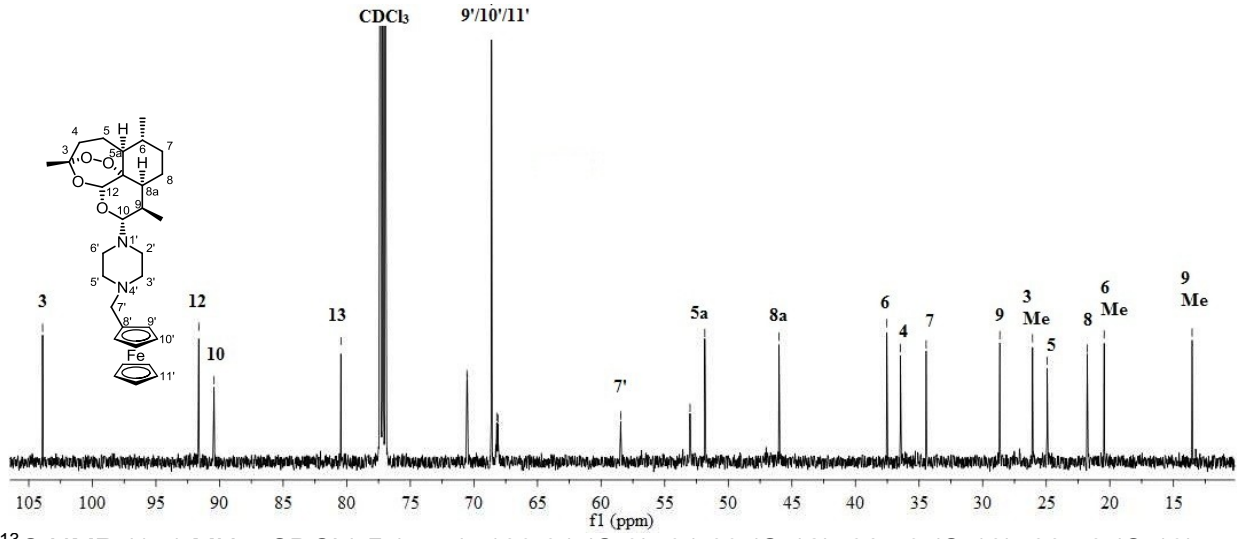
Yellow powder, 0.30 g (61%), R_f 0.53 (dichloromethane-MeOH 9:1), melting point 52.7-56.2 °C.



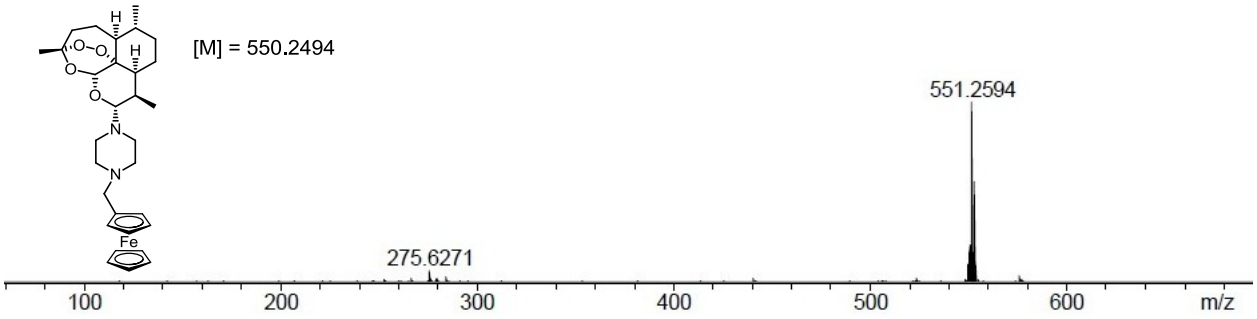
IR (ATR) ν_{max}/cm^{-1}: 3079 (aromatic C-H), 2921, 2868, 2844, 1450, 1375, 1191, 1160, 1103, 1040, 923, 878, 822, 496, 482.



^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.23 (s, 1H, H-12), 4.18-4.17 (d, *J* = 10.3 Hz, 2H, H-9'), 4.10 (s, 7H, H-10'/H-11'), 3.98-3.96 (d, *J* = 10.1 Hz, 1H, H-10), 3.44-3.36 (m, 2H, H-7'), 2.56-2.50 (m, 3H, H-9), 2.96 (s, 2H, H-2'/H-3'), 2.66 (s, 2H, H-2'/H-3'), 2.55-2.51 (m, 1H, H-9), 2.34-2.29 (td, *J* = 13.9, 3.8 Hz, 1H, H-4), 1.98-1.95 (dt, *J* = 13.3, 2.8 Hz, 1H, H-4), 1.85-1.82 (m, 1H, H-5/H-8a), 1.68-1.66 (m, 3H, H-7/H-8), 1.52-1.50 (m, 1H, H-8a), 1.47 (m, 1H, H-5), 1.33 (s, 3H, H-3 Me), 1.31 (s, 3H, H-3Me), 1.31-1.27 (m, 2H, H-6), 1.25-1.16 (m, 2H, H-5a), 1.01-0.94 (m, 2H, H-7), 0.93-0.92 (d, *J* = 6.3 Hz, 3H, H-6 Me), 0.77-0.75 (d, *J* = 7.1 Hz, 3H, H-9 Me).



^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 103.91 (C-3), 91.63 (C-12), 90.43 (C-10), 80.46 (C-13), 70.55, 70.51, 68.62 (C-9'), 68.21 (C-10'), 68.11 (C-11'), 58.46 (C-7'), 53.00, 51.85 (C-5a), 46.01 (C-8a), 37.53 (C-6), 36.46 (C-4), 34.45 (C-7), 28.65 (C-9), 26.08 (C-3 Me), 24.93 (C-5), 21.78 (C-8), 20.45 (C-6 Me), 13.53 (C-9 Me).



HRMS ESI: m/z $[M+H]^+$ 551.2594 (calculated for $C_{30}H_{43}FeN_2O_4^+$: 551.2572).

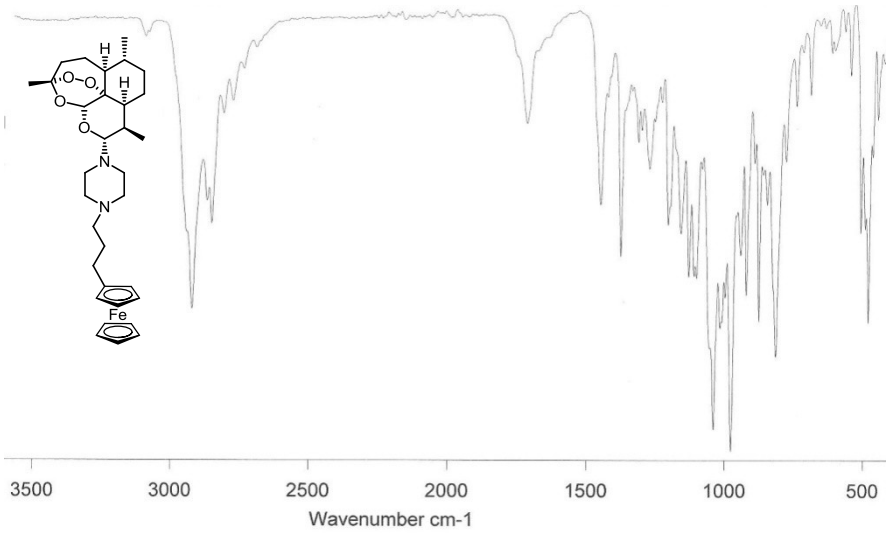
6. General procedure for the synthesis of derivatives **7** – **9**

The artemisinin-piperazine derivative **2** (2.8 mmol 1 equiv) was dissolved in 10 mL of anhydrous acetonitrile with the ferrocenyl alkyl halide (2.8 mmol, 1 equiv) in a reaction flask. To this solution was added DBU (5.6 mmol, 2 equiv) and 0.1 mL DMF. The reaction vessel was placed in a Bruker microwave reactor and was radiated in bursts of 60 watts at 40 °C for 4 minutes. The reaction mixture was then cooled for 30 min and placed in the microwave following the program above. This cycle was repeated until no differences in product formation could be detected by TLC (8 to 10 times). The reaction mixture was then quenched with saturated NH_4Cl (20 mL) and extracted with ethyl acetate (3 x 25 mL). The extracts were combined and washed with brine (25 mL) and dried over magnesium sulfate. The solvent was removed *in vacuo*, and the residue was purified by column

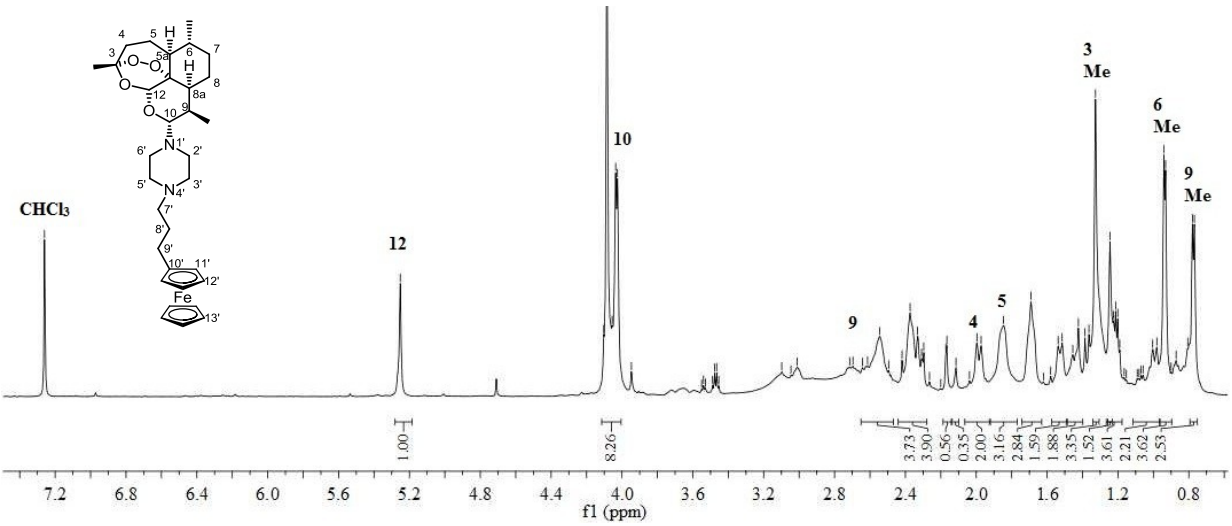
chromatography. Elution with dichloromethane-MeOH (9:1, v/v) afforded the derivatives that as assessed by TLC analyses were single fractions.

10α-[1'-Piperazino-4'-(ferrocenepropyl)]-10-deoxo-10-dihydroartemisinin ***7***

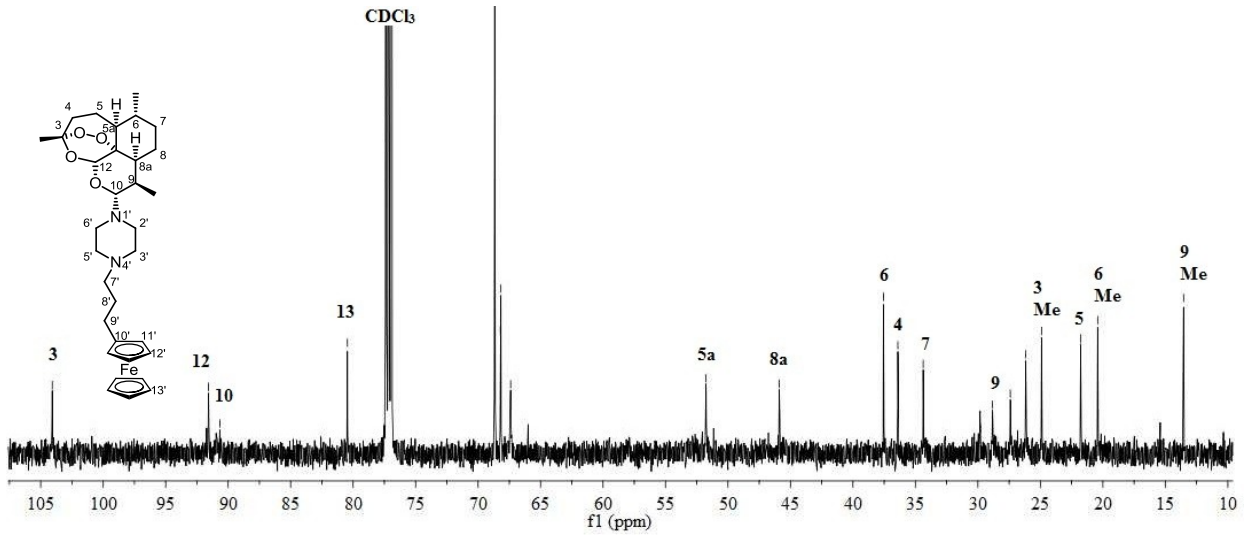
Yellow gum, 0.43 g (27%), R_f 0.55 (dichloromethane-MeOH 9:1).



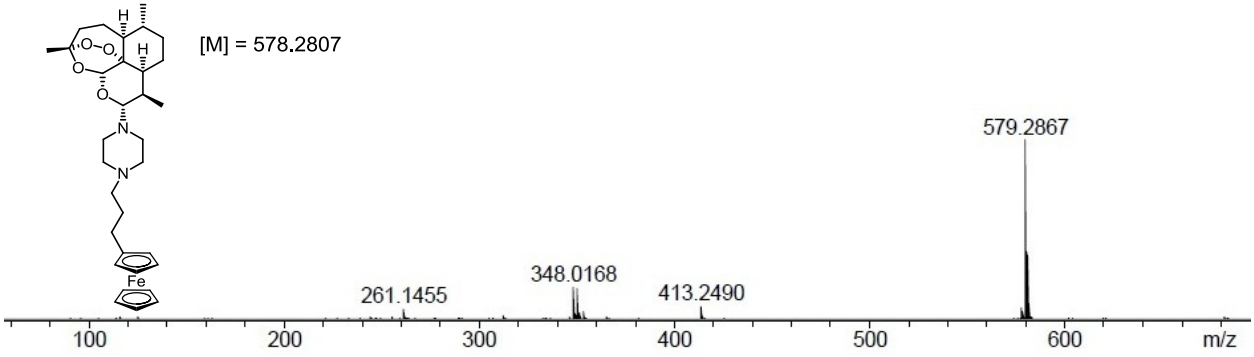
IR (ATR) ν_{max}/cm^{-1}: 3093 (aromatic C-H), 2923, 2852, 1714, 1448, 1375, 1228, 1205, 1159, 1112, 1041, 978, 894, 817, 510, 468.



^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.25 (s, 1H, H-12), 4.10-4.03 (m, 8H, H-10/Fc), 2.64-2.49 (m, 3H, H-9), 2.42-2.26 (m, 3H, H-4), 2.04-1.97 (d, *J* = 14.1 Hz, 2H, H-4), 1.85 (s, 3H, H-5), 1.69 (s, 3H, H-7/H-8), 1.54-1.52 (d, *J* = 13.3 Hz, 1H, H-8a), 1.45-1.36 (m, 2H, H-5), 1.33 (s, 3H, H-3 Me), 1.24-1.15 (m, 3H, H-8/H-6), 1.00-0.98 (m, 2H, H-7), 0.94-0.93 (d, *J* = 5.6 Hz, 3H, H-6 Me), 0.78-0.77 (d, *J* = 6.6 Hz, 3H, H-9 Me).



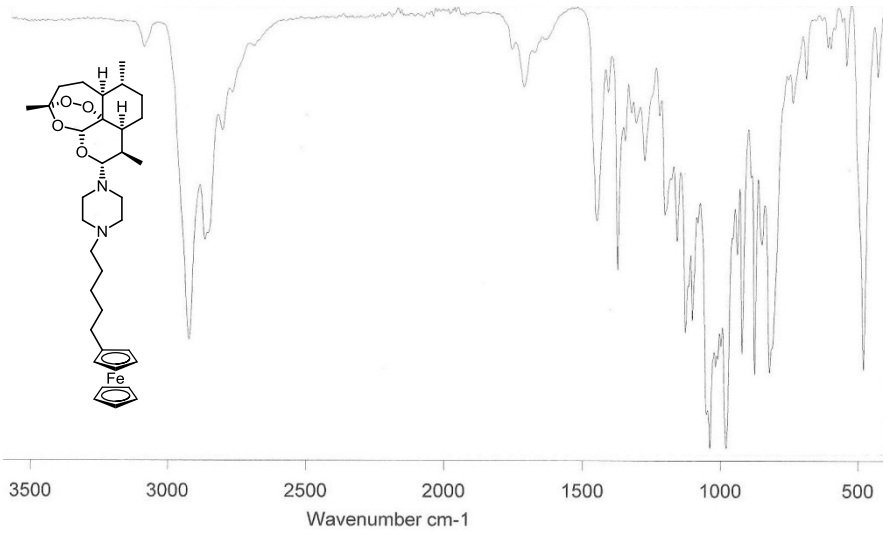
^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 104.07 (C-3), 91.57 (C-12), 90.67 (C-10), 80.46 (C-13), 68.67 (C-11'), 68.22 (C-12'), 68.17 (C-13'), 67.39 (C-9'), 51.76 (C-5a), 45.90 (C-8a), 37.56 (C-6), 36.40 (C-4), 34.38 (C-7), 29.79, 28.83 (C-9), 27.40, 26.18 (C-3 Me), 24.91 (C-5), 21.78 (C-8), 20.42 (C-6 Me), 13.54 (C-9 Me).



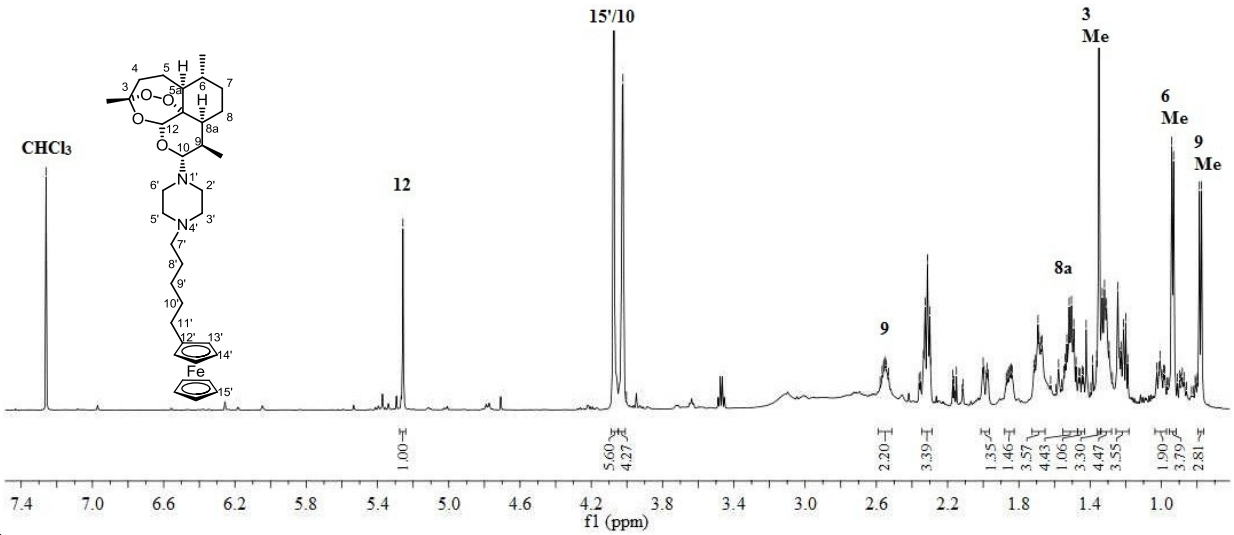
HRMS ESI: m/z $[M+H]^+$ 579.2867 (calculated for $C_{32}H_{47}FeN_2O_4^+$: 579.2885).

10α-[1'-Piperazino-4'-(ferrocenepentyl)]-10-deoxo-10-dihydroartemisinin ***8***

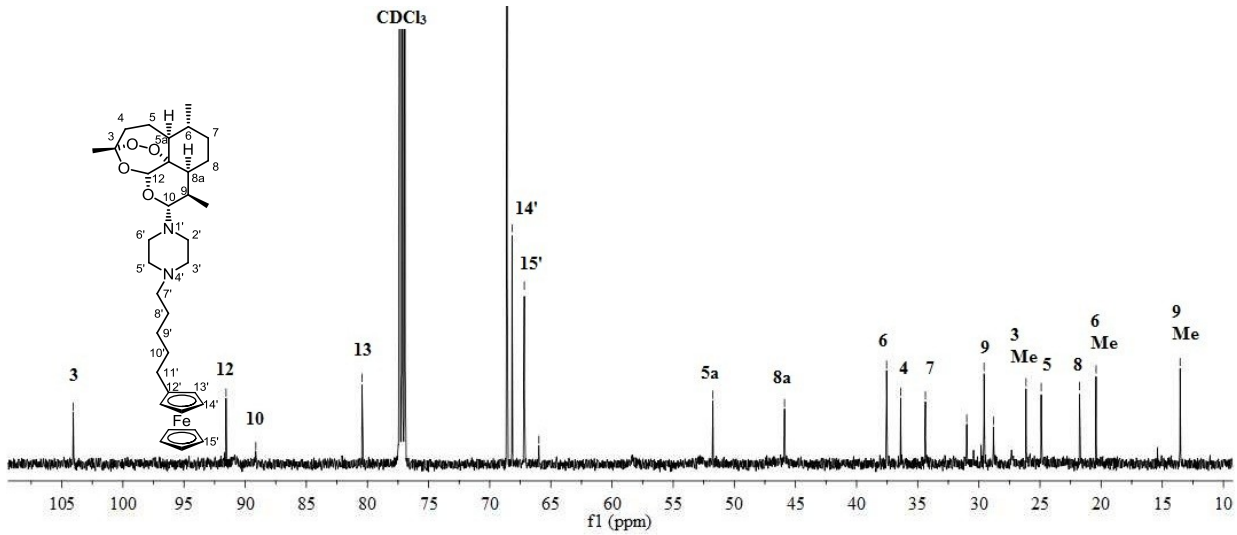
Reddish gum, 0.40 g (24 %), R_f 0.53 (dichloromethane-MeOH 9:1).



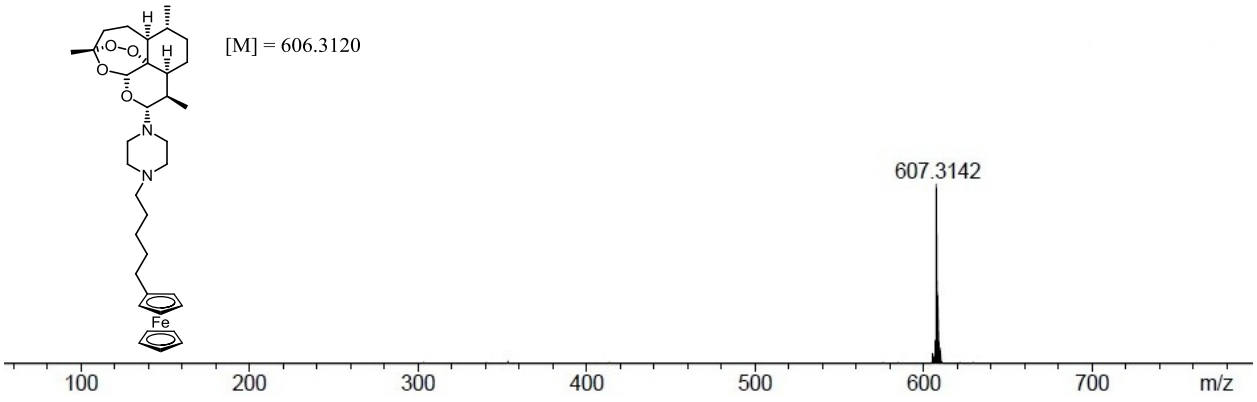
IR (ATR) ν_{max}/cm^{-1}: 3092 (aromatic C-H), 2925, 2869, 1715, 1450, 1375, 1278, 1204, 1160, 1129, 1104, 1040, 981, 924, 879, 824, 484.



^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.26 (s, 1H, H-12), 4.03-4.02 (d, *J* = 1.9 Hz, 4H, H-10/Fc), 2.58-2.53 (m, 2H, H-9), 2.34-2.30 (m, 3H, H-4), 2.00-1.97 (dt, *J* = 13.1, 3.0 Hz, 1H, H-4), 1.87-1.84 (m, 1H, H-4), 1.72-1.67 (m, 3H, H-7/H-8), 1.55-1.48 (m, 4H, H-8a), 1.46-1.44 (m, 1H, H-5), 1.35 (s, 3H, H-3 Me), 1.33-1.29 (m, 4H, H-8), 1.24-1.19 (m, 3H, H-5a), 0.94-0.93 (d, *J* = 6.3 Hz, 3H, H-6 Me), 0.79-0.77 (d, *J* = 7.2 Hz, 3H, H-9 Me).



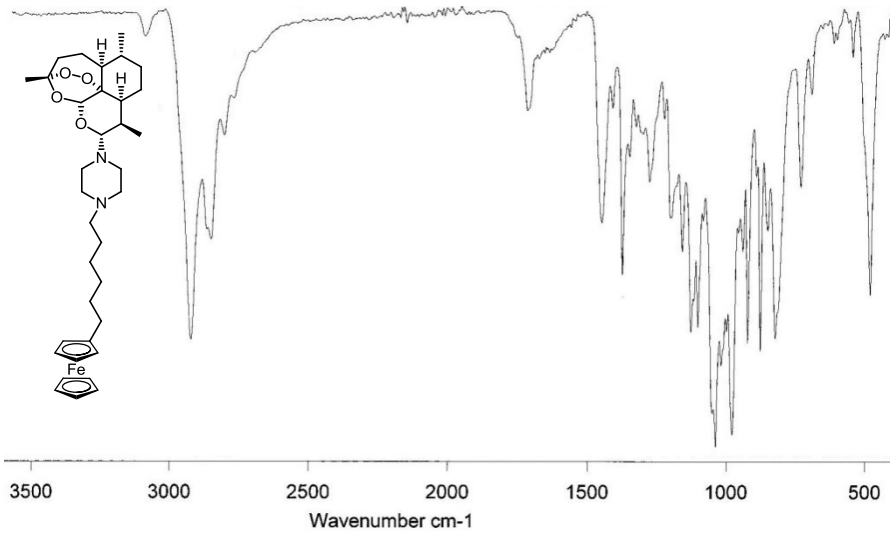
^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 104.06 (C-3), 91.56 (C-12), 89.25 (C-10), 80.46 (C-13), 68.58 (C-13'), 68.17 (C-14'), 67.17 (C-15'), 65.98 (Fc), 51.75 (C-5a), 45.90 (C-8a), 37.55 (C-6), 36.40 (C-4), 34.37 (C-7), 30.99, 29.58, 28.83 (C-9), 26.14 (C-3 Me), 24.90 (C-5), 21.77 (C-8), 20.41 (C-6 Me), 13.54 (C-9 Me).



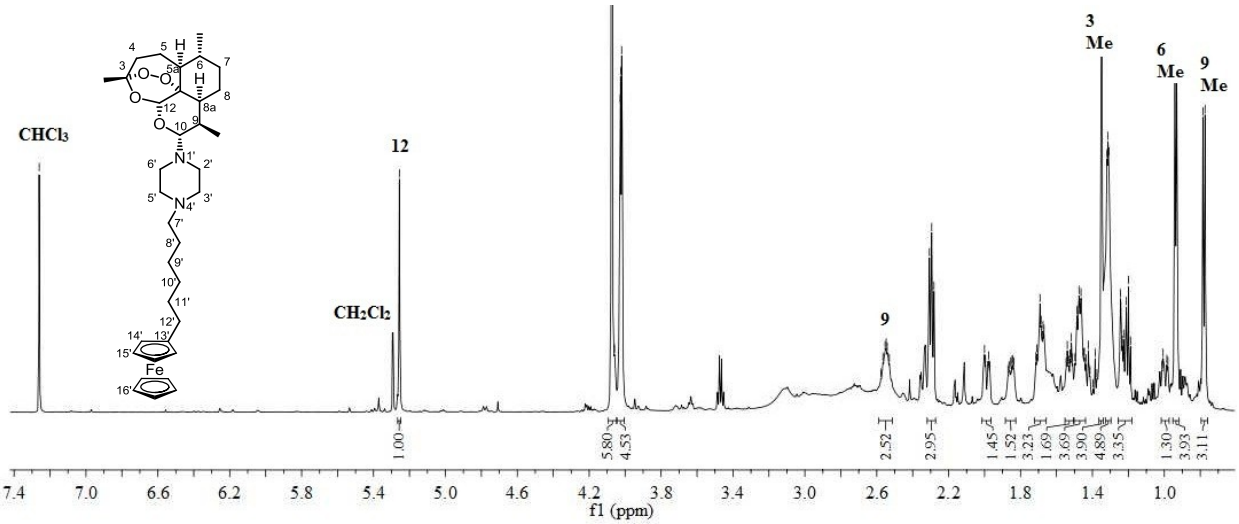
HRMS ESI: m/z $[M+H]^+$ 607.3142 (calculated for $C_{34}H_{51}FeN_2O_4^+$: 607.3198).

10α-[1'-Piperazino-4'-(ferrocenehexyl)]-10-deoxo-10-dihydroartemisinin ***9***

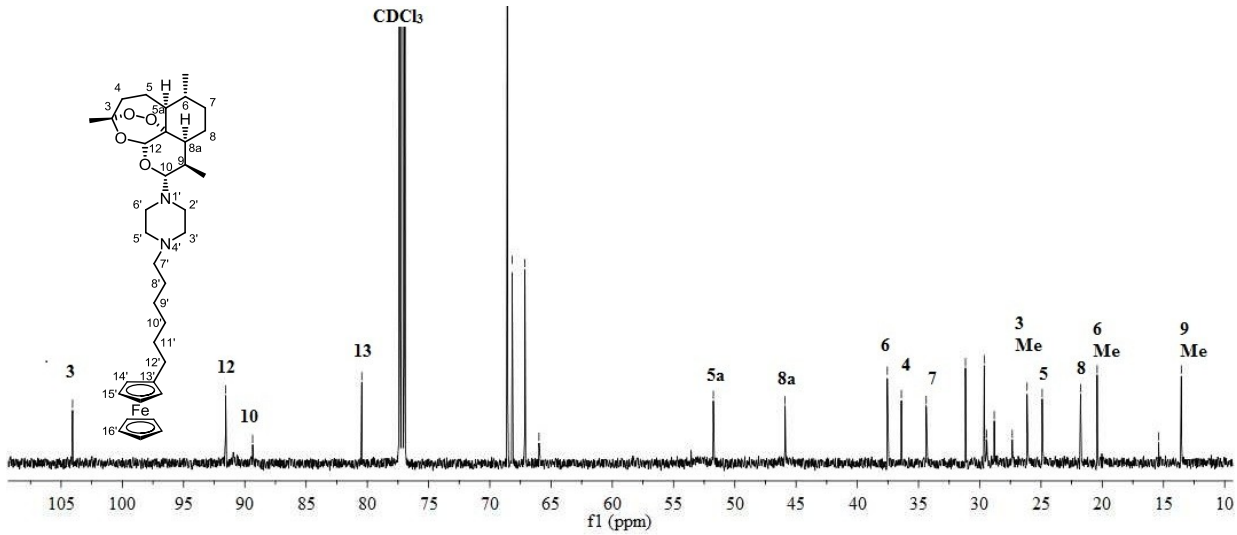
Red gum, 0.39 g (23 %), R_f 0.55 (dichloromethane-MeOH 9:1).



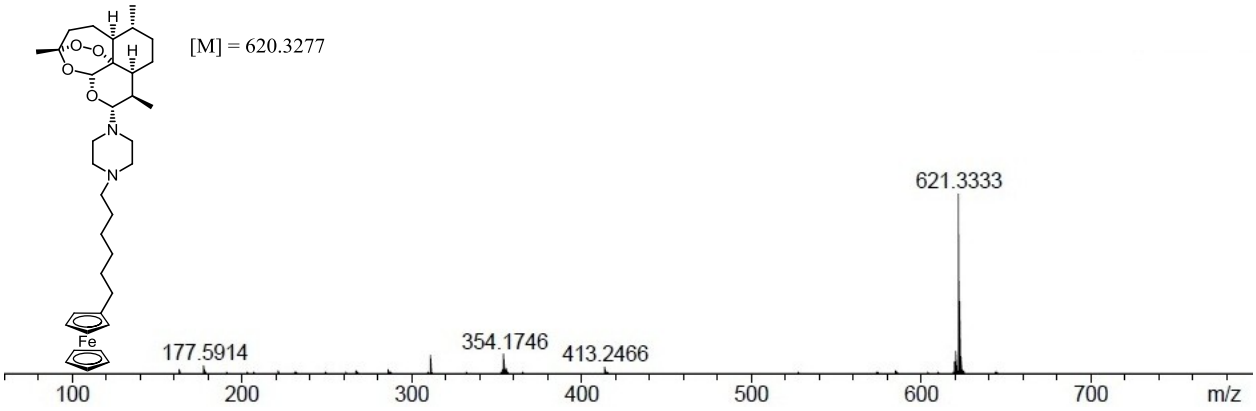
IR (ATR) ν_{max}/cm^{-1}: 3091 (aromatic C-H), 2924, 2852, 2805, 1716, 1480, 1375, 1278, 1204, 1160, 1128, 1104, 1040, 980, 925, 879, 825, 484.



^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.26 (s, 1H, H-12), 4.07-4.06 (s, 6H, Fc), 4.03-4.02 (dd, *J* = 5.2, 1.2 Hz, 4H, Fc/H-10), 2.58-2.53 (m, 2H, H-9), 2.31-2.28 (m, 3H, H-4), 2.00-1.97 (dt, *J* = 13.7, 3.1 Hz, 1H, H-4), 1.87-1.84 (m, 1H, H-5), 1.71-1.67 (m, 3H, H-7/H-8), 1.55-1.51 (m, 3H, H-8a), 1.50-1.49 (m, 3H, H-5), 1.35 (s, 3H, H-3 Me), 1.32-1.31 (m, 5H, H-8), 1.24-1.19 (m, 3H, H-5a), 1.01-0.98 (m, 1H, H-5a), 0.94-0.93 (d, *J* = 6.2 Hz, 3H, H-6 Me), 0.79-0.77 (d, *J* = 7.1 Hz, 3H, H-9 Me).



^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 104.06 (C-3), 91.56 (C-12), 89.36 (C-10), 80.46 (C-13), 68.57 (C-14'), 68.18 (C-15'), 67.14 (C-16'), 65.99 (Fc), 51.75 (C-5a), 45.89 (C-8a), 37.56 (C-6), 36.40 (C-4), 34.37 (C-7), 31.17, 29.64, 29.43, 28.82, 27.35, 26.14 (C-3 Me), 24.90 (C-5), 21.77 (C-8), 20.42 (C-6 Me), 13.55 (C-9 Me).



HRMS ESI: m/z $[M+H]^+$ 621.3333 (calculated for $C_{35}H_{53}FeN_2O_4^+$: 621.3355).

5 References:

1. Wentzel JF, Lombard MJ, Du Plessis LH, Zandberg L. Evaluation of the cytotoxic properties, gene expression profiles and secondary signalling responses of cultured cells exposed to fumonisin B1, deoxynivalenol and zearalenone mycotoxins. *Arch Toxicol.* 2017; 91(5): 2265-2282.
2. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983; 65(1-2): 55-63.

3. Verlinden BK, Niemand J, Snyman J, et al. Discovery of novel alkylated (bis) urea and (bis) thiourea polyamine analogues with potent antimalarial activities. *J Med Chem.* 2011; 54(19): 6624-6633.
4. Lau CK, Tardif S, Dufresne C, Scheigetz J. Reductive deoxygenation of aryl aldehydes and ketones by tert-butylamine-borane and aluminum chloride. *J Org Chem.* 1989; 54(2): 491-494.

Adddendum B:
Supporting data for Chapter 5

Synthesis, in vitro antimalarial activities and cytotoxicities of amino-artemisinin-1, 2-disubstituted ferrocene derivatives

Christo de Lange[a], Dina Coertzen[b], Frans J. Smit[a], Johannes F. Wenzel[a], Ho Ning Wong[a], Lyn-Marie Birkholtz[b], Richard K. Haynes[a*] and David D. N'Da[a*]

[a]Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom 2520, South Africa.
bDepartment of Biochemistry, Genetics and Microbiology, Institute for Sustainable Malaria Control, University of Pretoria, Pretoria 0002, South Africa.

Table of contents

1. General procedures

Dihydroartemisinin (DHA) (a mixture of 10-α and 10-β epimers) was purchased from Changzhou Kaixuan Chemical Co (Chunjiang,China). Piperazine, oxalyl chloride, piperidine, morpholine, thiomorpholine, ferrocenecarboxaldehyde, potassium *tert*-butoxide, triethylamine and sodium triacetoxyborohydride were purchased from Sigma-Aldrich (Johannesburg, South Africa). Methanol, magnesium sulfate, diethyl ether, dichloromethane and toluene were purchased from ACE chemicals (Johannesburg, South Africa). Sodium hydroxide was purchased from Saarchem (Krugersdorp, South Africa). All the chemicals and reagents were of analytical grade. Diethyl ether and tetrahydrofuran was dried and distilled from a sodium-benzophenone distil, dichloromethane and toluene were dried and distilled from calcium hydride. Chemicals were used without further purification.

The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Advance™ III 600 spectrometer at a frequency of 600 MHz and 150 MHz, respectively, in $CDCl_3$. Chemical shifts are reported on the δ scale (ppm) downfield from tetramethylsilane (δ=0.0 ppm) using the residual solvent signal at δ=7.26 ppm (^{1}H) or δ=77.00 ppm (^{13}C) as internal standard.

High resolution mass spectrometry (HRMS) was recorded on a Bruker MicroTOF Q II mass spectrometer, equipped with an atmospheric pressure chemical ionisation (APCI) or an electrospray ionisation (ESI) source, set at 200 °C or 180 °C, respectively, using Bruker Compass DataAnalysis 4.0 software. A full scan from 50 to 1500 *m*/*z* was performed at a capillary voltage of 4500 V, an end plate offset voltage of −500 V, with the nebulizer set at 1.6 Bar and 0.4 Bar, respectively, and a collision cell RF voltage of 100 Vpp.

Mass spectra (MS) were recorded in positive mode on a Thermo Electron LXQ™ ion trap mass spectrometer, equipped with Xcalibur 2.2 data acquisition and analysis software. The MS had an APCI source set at 300 °C, and was direct infusion with a Harvard syringe pump utilized at a flow rate of 10 µL/min. A full scan from 100 to 1200 amu was achieved in 1 s, with a capillary voltage of 7 V, while the corona discharge was 10 µA.

Infrared (IR) spectra were recorded on a Bruker Alpha-P FTIR instrument using the Attenuated Total Reflectance (ATR) technique. Thin layer chromatography (TLC) was performed, using silica gel plates ($60F_{254}$), obtained from Merck (Johannesburg, South Africa). Column chromatography was performed, using silica gel 60, 70-230 mesh ASTM, supplied by Macherey-Nagel (Germany).

2. Biological evaluation

2.1 *In vitro* efficacy studies on asexual *P. falciparum* parasites

The *P. falciparum* parasites were maintained at 37 °C in human erythrocytes (O+) suspended in complete culture medium [RPMI 1640 medium (Sigma-Aldrich) supplemented with 25 mM HEPES (Sigma-Aldrich), 20 mM D-glucose (Sigma-Aldrich), 200 µM hypoxanthine (Sigma-Aldrich), 0.2% sodium bicarbonate, 24 µg/mL Gentamycin (Sigma-Aldrich) and 0.5% AlbuMAX II] under an atmosphere of 90% N_2, 5% O_2, and 5% CO_2.[1] The *in vitro* ring-stage intra-erythrocytic *P. falciparum* NF54 parasite cultures (genotyped drug sensitive) (200 µL at 1% haematocrit, 1% parasitaemia) were treated with compounds at 5 and 1 µM. The controls for this assay included chloroquine disulfate (1 µM as positive control) and complete RPMI media (as negative control) and grown for 96 h at 37 °C under the 90% N_2, 5% O_2, and 5% CO_2 gas mixture in 96-well plates. At the conclusion of the 96 h growth period, equal volumes (100 µL each) of the *P. falciparum* parasite cultures were combined with SYBR Green I lysis buffer (0.2 µL/mL 10 000xSYBR Green I, Invitrogen; 20 mM Tris, pH 7.5; 5 mM EDTA; 0.008% (w/v) saponin; 0.08% (v/v) Triton X-100). The samples were incubated at 37 °C for 1 h after which the fluorescence was measured using a Fluoroskan Ascent FL microplate fluorometer (Thermo Scientific, excitation at 485 nm and emission at 538 nm). The 'background' fluorescence (i.e. that measured in the samples derived from chloroquine-treated iRBC samples in which parasite proliferation was completely inhibited) was subtracted from the total fluorescence measured for each sample to provide a measure of parasite proliferation. Data obtained were analyzed in Excel and sigmoidal dose-response curves were plotted using GraphPad 5.0. Experiments are always performed in technical triplicates for at least three independent biological replicates (n=3).

2.2 Determination of gametocytocidal activities [2]

The luciferase reporter assay was established to enable accurate, reliable and quantifiable investigations of the stage-specific action of gametocytocidal compounds for both the early and late gametocytes using the NF54-PfS16-GFP-Luc marker cell line. Drug assays were set up on day 5 and 10 (representing >90% of either early stage II/III or mature stage IV/V gametocytes, respectively). In each instance, assays were set up using a 2 – 3% gametocytaemia, 1.5% haematocrit culture and 48 h drug pressure in a gas chamber (90% N_2, 5% O_2, and 5% CO_2) at 37 ºC. Luciferase activity was determined in 20 µL parasite lysates by adding 50 µL luciferin substrate (Promega Luciferase Assay System) at room temperature and detection of resultant bioluminescence at an integration constant of 10 s with the GloMax®-Multi+ Detection System with Instinct® Software. Methylene blue (5 µM)

is routinely included as a control. Dual point screens are routinely performed as technical triplicates for a single biological assay.

2.3 *In vitro* anticancer and cytotoxicity screening[3]

A375 (ATCC® CRL-1619™ Human malignant melanoma) and Hek293 cells (ATCC® CRL-1573™ Human embryonic kidney cells) were cultured in Dulbecco's modified essential medium (DMEM; Hyclone, GE healthcare, South Logan, UT, USA) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% 200 mM L-Glutamine and 1% non-essential amino acids (Lonza, Basel, Switzerland). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. For compound treatment, cells were seeded in a 96 well plate and cultured until 80-90% confluent. Stock solutions for compounds were prepared in DMSO preheated to 40 °C. All subsequent dilutions were prepared in serum free DMEM and vehicle controls were included in all experiments.

The 3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to determine *in vitro* cell viability.[4] A375 and Hek293 cells were seeded in a 96-well plate and incubated until cells were ~ 90% confluent. After 24 hours exposure to the compounds (12 - 1800 µM), growth medium was removed, cells rinsed twice with 1 x phosphate buffered saline (PBS) and 100 µL fresh serum free medium containing 5 mg/ml MTT solution was added. Cells were then incubated for 4 hours at 37 °C, after which the MTT was carefully removed and replaced with 100 µL dimethyl sulfoxide (DMSO). After 1 hour of incubation at 37 °C, cell viability was determined using a microplate reader (SpextraMac Paradigm) at an absorbance wavelength of 550 nm and background wavelength of 630 nm with DMSO measured as a blank. Cell viability is expressed as a percentage relative to the untreated control, which is assumed to be 100 % viable. As a positive control, cells were treated with 0.01% Triton-X 100 (Sigma-Aldrich, St Louis, MO, USA) for 4 hours. Using the MTT assay data, IC_{50} values was calculated using GraphPad Prism 5. In brief, data was normalized to the negative controls (presumed to be 100% viable), followed by the log-transformation of the concentration values. The curve was fitted using the log (inhibitor) vs. response function and the IC_{50} values calculated. Experiments were done at least in triplicate.

3. General experimental procedures

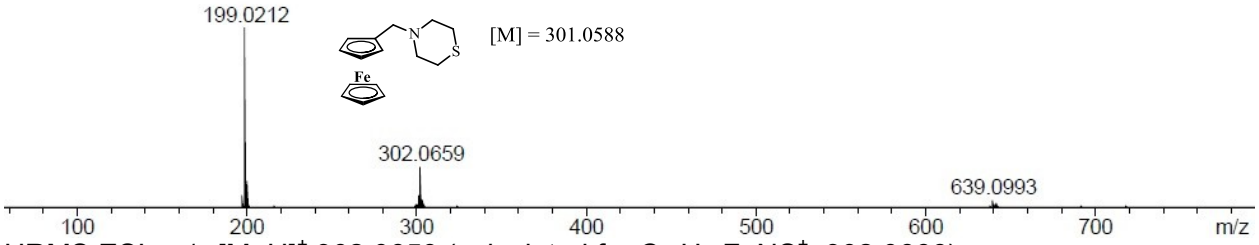
3.1 Reductive amination of ferrocenecarboxaldehyde

A stirred solution of ferrocenecarboxaldehyde (11 mmol, 1 equiv) in anhydrous dichloromethane (50 mL) under nitrogen was treated with the secondary amine (12.2 mmol,

1.1 equiv.). The solution was treated portionwise with sodium triacetoxyborohydride (25 mmol, 2.2 equiv) after which it was left to stir for 4 h at room temperature under N_2. The reaction mixture was poured onto ice, and basified to pH 10 with aqueous NaOH (1M, 5-10 mL), and extracted with diethyl ether until the extract was colourless. The combined extracts were dried ($MgSO_4$), and filtered. The filtrate was evaporated under reduced pressure, and the residue was submitted to column chromatography over silica gel. Eluting with dichloromethane-MeOH (9:1) afforded the derivatives.

(Thiomorpholinomethyl)ferrocene

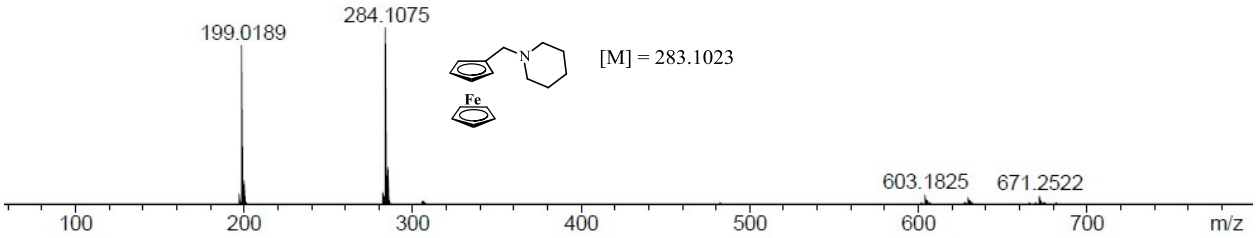
Yellow powder, 2.38 g (72%), melting point 101.4-105.3 °C.



HRMS ESI: m/z $[M+H]^+$ 302.0659 (calculated for $C_{15}H_{20}FeNS^+$: 302.0666)

(Piperidinomethyl)ferrocene

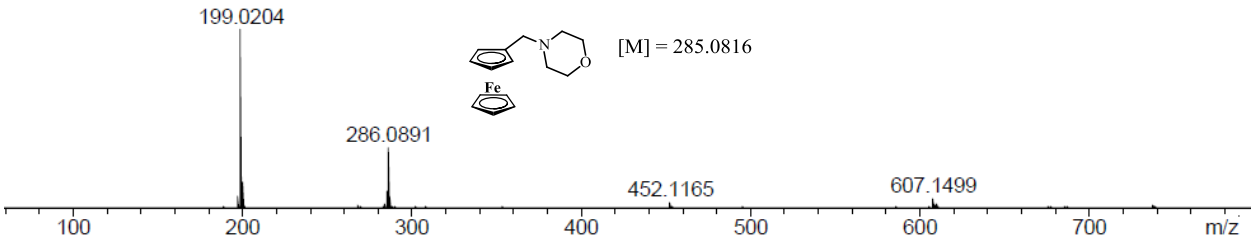
Yellow powder, 2.42 g (78%), melting point 105.7-108.2 °C.



HRMS ESI: m/z $[M+H]^+$ 284.1075 (calculated for $C_{16}H_{22}FeN^+$: 284.1102)

(Morpholinomethyl)ferrocene

Yellow powder, 2.28 g (80%), melting point 108.1-110.8 °C.



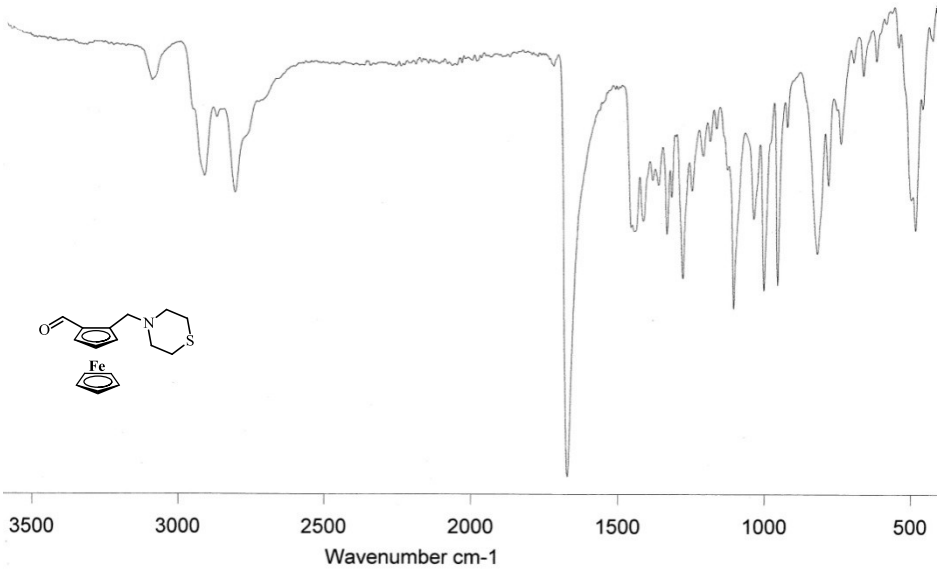
HRMS (ESI): *m/z* $[M+H]^+$ 286.0891 (calculated for $C_{15}H_{20}FeNO^+$: 286.0894)

3.2 Synthesis of aminoferrocene aldehydes

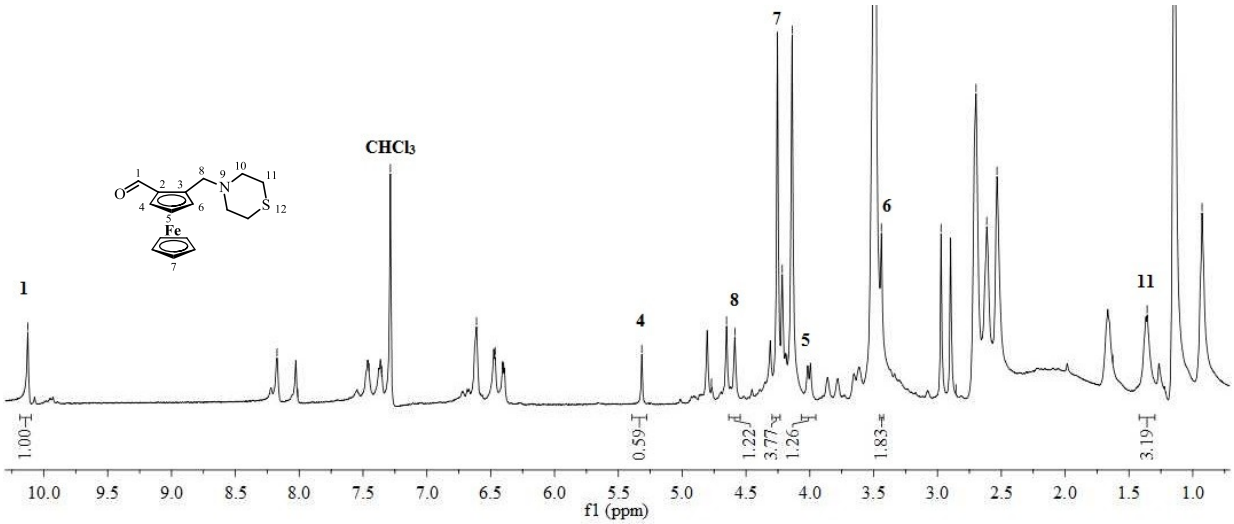
The aminoferrocene (33.2 mmol, 1 equiv.) together with 0.3 mmol potassium *tert*-butoxide (0.1 equiv.) was dissolved in anhydrous diethyl ether (20 mL) under argon at room temperature, and with stirring, *n*-butyllithium (36.5 mmol, 1.1 equiv) was slowly added at room temperature. The reaction mixture was left under argon to stir for 16 h. DMF (99.6 mmol, 3 equiv) was slowly added to the reaction mixture that was left to stir for an additional 4 hr. The reaction mixture was quenched with of ice water (15 mL), and extracted with diethyl ether until the extract remained clear. The combined extracts were dried ($MgSO_4$) and then filtered. The filtrate was evaporated under reduced pressure to dryness, with the flask enclosed in foil to protect the contents from light. The residue was purified by column chromatography over silica gel, with the column also enclosed in foil; eluting with diethyl ether-hexane-triethylamine (7:2:1) afforded the derivatives. Whilst most derivatives were stable during storage, the thiomorpholine-formyl derivative described below, because of instability, was used immediately in the ensuing reductive amination reaction below.

(Thiomorpholinomethyl)-2-formylferrocene

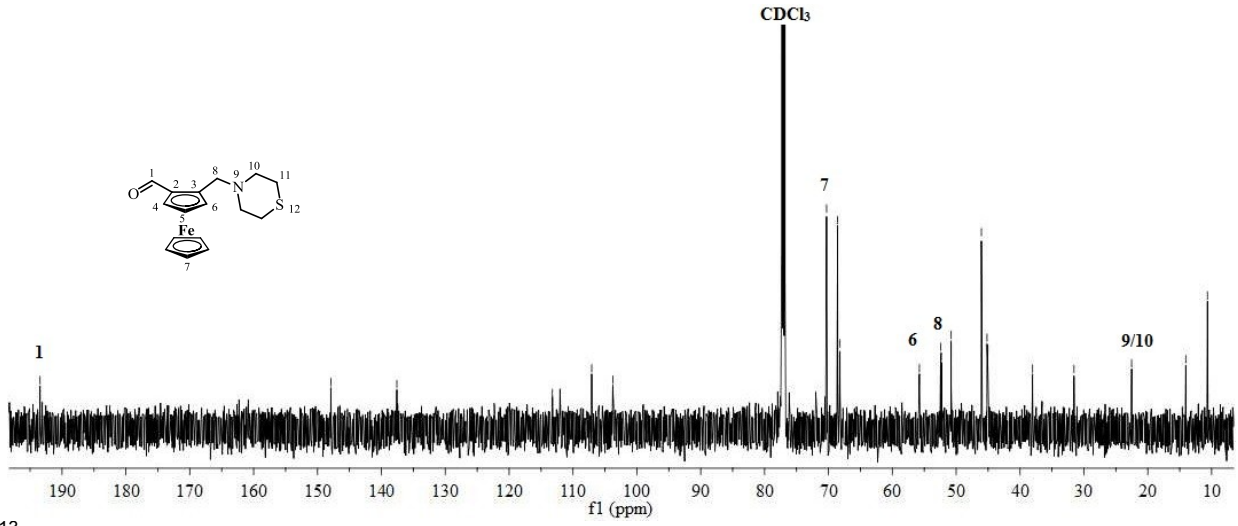
Red oil, 2.51 g (23%).



IR (ATR) $\tilde{\nu}_{max}/cm^{-1}$: 3090, 2909, 2804 (H–C=O), 1670, 1412, 1332, 1278, 1104, 1036, 1001, 954, 820, 783, 742, 485.



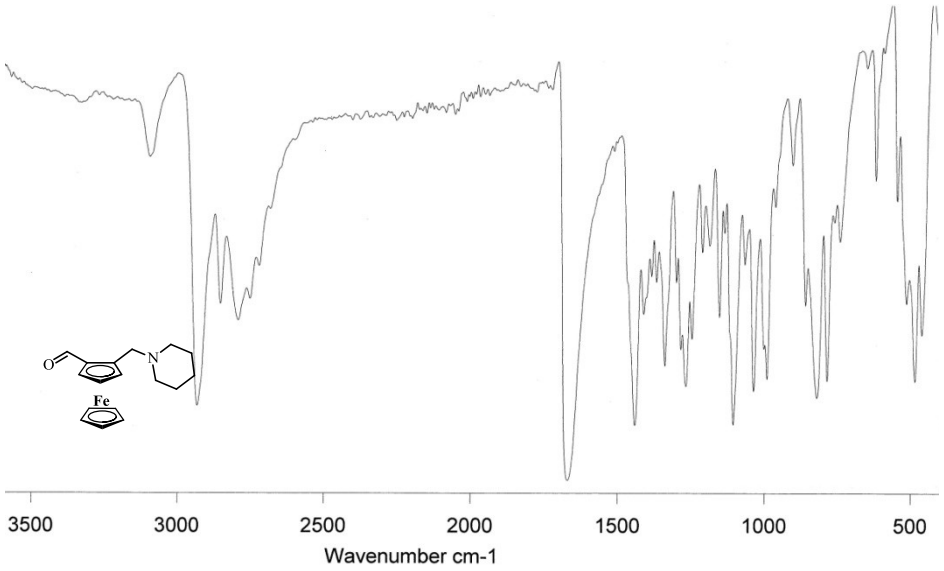
^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 10.13 (s, 1H, H-1), 5.32 (s, 1H, H-4), 4.59 (H-8), 4.22 (H-7), 4.01 (H-5), 3.44 (H-6), 1.36 (H-11).



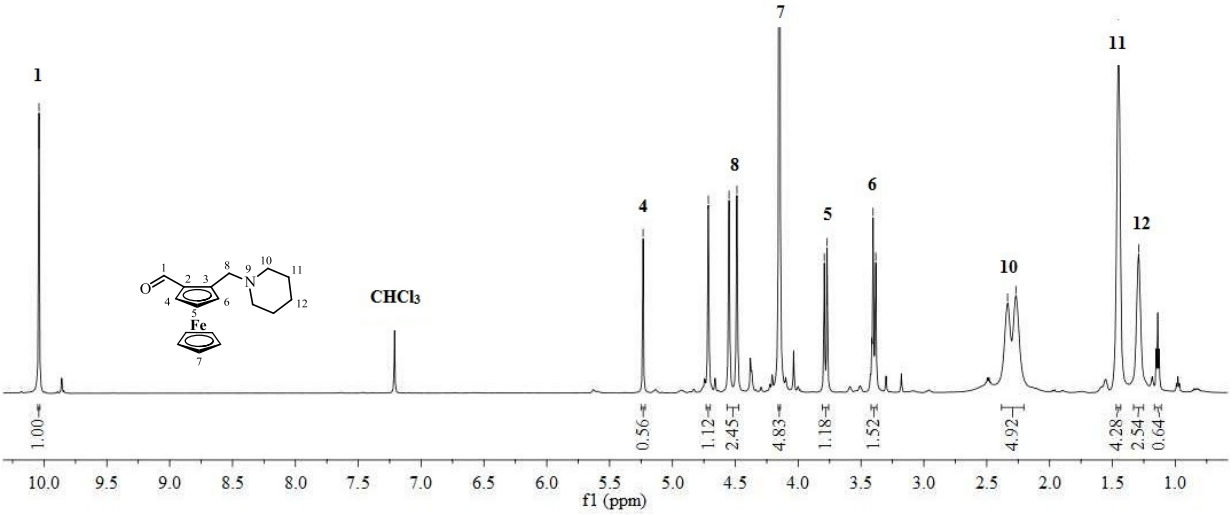
^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 193.45 (C-1), 70.29 (C-7), 52.43 (C-6), 50.78 (C-8), 22.53 (C-9/C-10).

(Piperidinomethyl)-2-formylferrocene

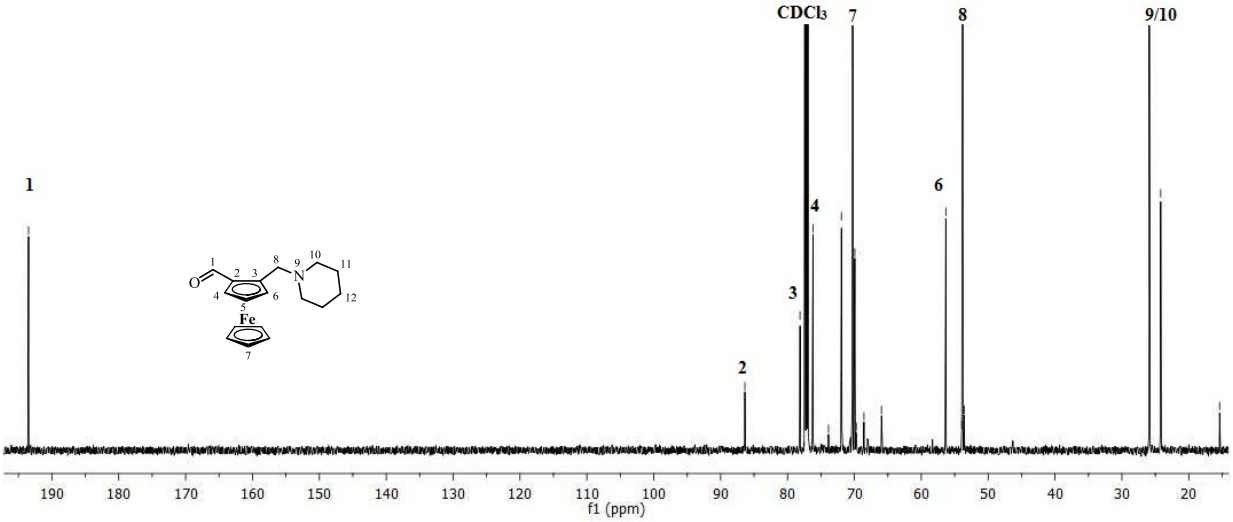
Red oil, 2.68 g (26%).



IR (ATR) $\tilde{\nu}_{max}$/cm^{-1}: 3093, 2930, 2850, 2790 (H–C=O), 2750, 1669, 1439, 1409, 1337, 1265, 1246, 1151, 1035, 989, 859, 819, 785, 485, 462.



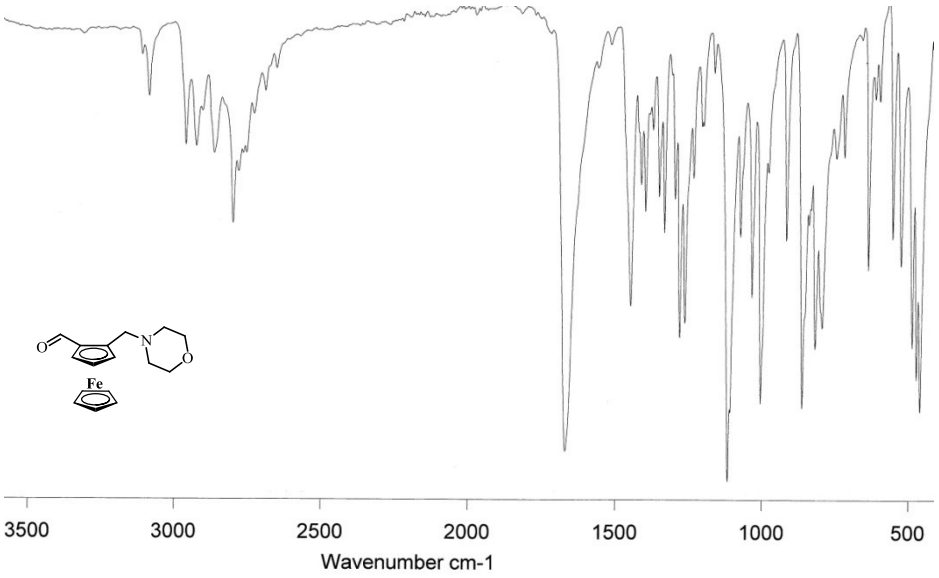
^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): .10.08 (s, 1H, H-1), 5.29 (s, 1H,), 4.77 (s, 1H,), 4.61-4.55 (d, *J* = 4.5, 2H,), 4.22 (s, 5H,), 3.94-3.92 (d, *J* = 3.9, 1H,), 3.61 (s, 4H,), 3.43 (s, 1H,), 2.48-2.41 (d, *J* = 2.4, 4H,), 1.20 (m,).



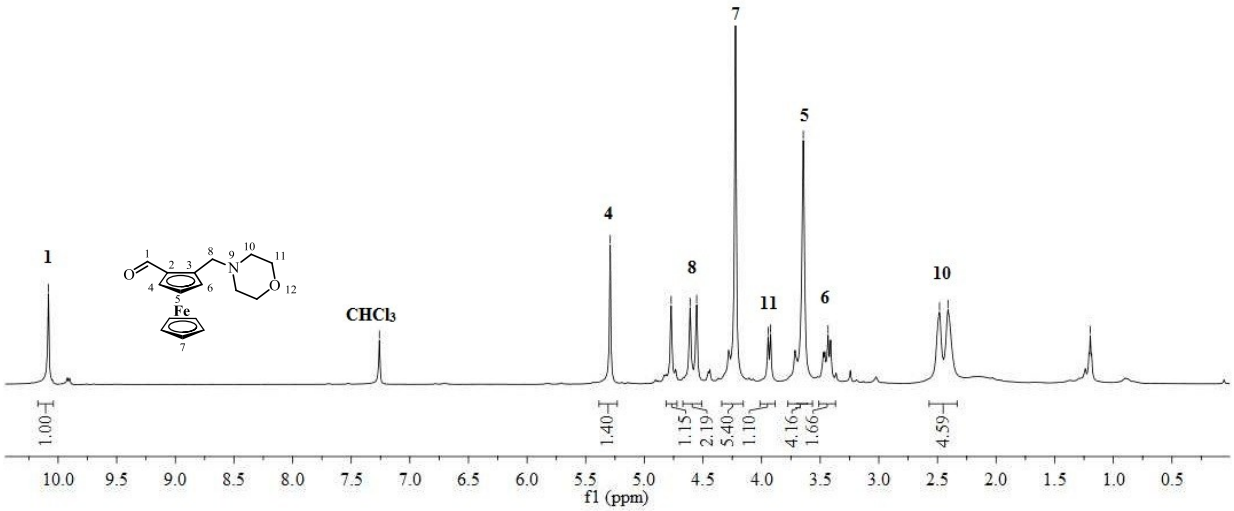
^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm):193.49 (C-1), 86.38 (C-2), 78.14 (C-3), 76.19 (C-4), 71.94 (C-6), 70.29 (C-7), 69.95, 65.98, 56.33 (C-8), 53.81 (C-10), 25.91 (C-11), 24.19 (C-12), 15.38.

(Morpholinomethyl)-2-formylferrocene

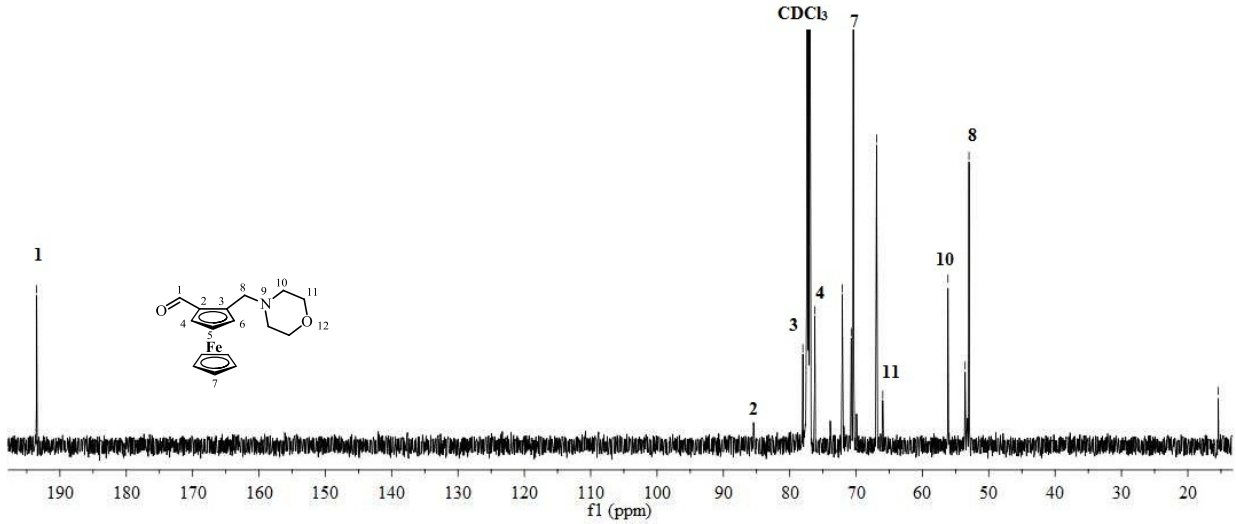
Red oil, 3.32 g (32%).



IR (ATR) $\tilde{\nu}_{max}/cm^{-1}$: 3083, 2957, 2921, 2795 (H–C=O), 1665, 1442, 1393, 1346, 1328, 1276, 1258, 1112, 1068, 1000, 973, 913, 859, 815, 744, 634, 551, 523, 484, 458.



^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): .10.08 (s, 1H, H-1), 5.29 (s, 1H,H-4), 4.77 (s, 1H,), 4.61-4.55 (d, *J* = 4.5, 2H, H-8), 4.22 (s, 5H, H-7), 3.94-3.92 (d, *J* = 3.9, 1H, H-11), 3.61 (s, 4H, H-5), 3.43 (s, 1H, H-6), 2.48-2.41 (d, *J* = 2.4, 4H, H-10), 1.20 (m,).



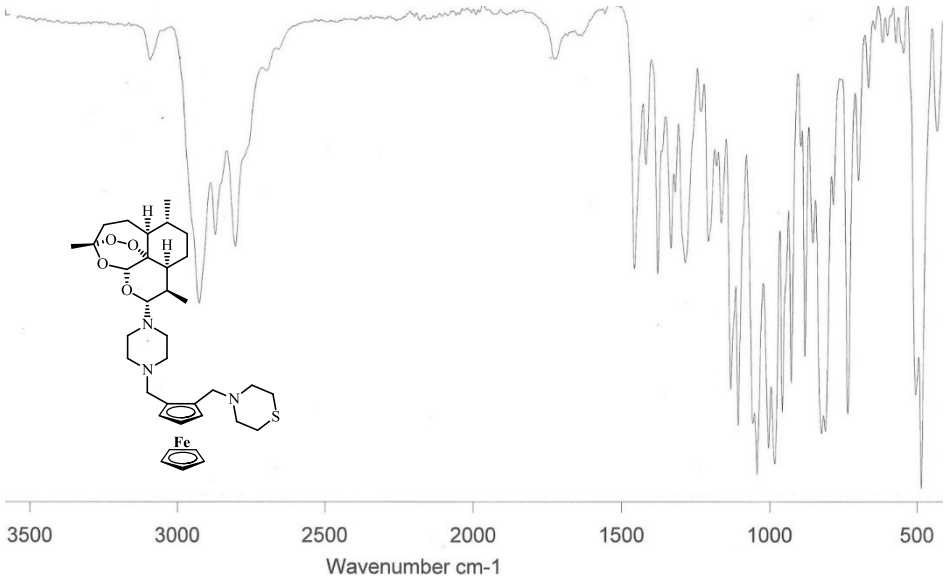
^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm):193.50 (C-1), 86.38 (C-2), 78.02 (C-3), 76.22 (C-4), 72.08 (C-6), 70.73 (C-5), 70.39 (C-7), 66.91 (C-11), 65.98, 56.15 (C-10), 53.57, 52.98 (C-8), 15.40.

3.3 Synthesis of amino-artemisinin-1,2-disubstituted ferrocene derivatives

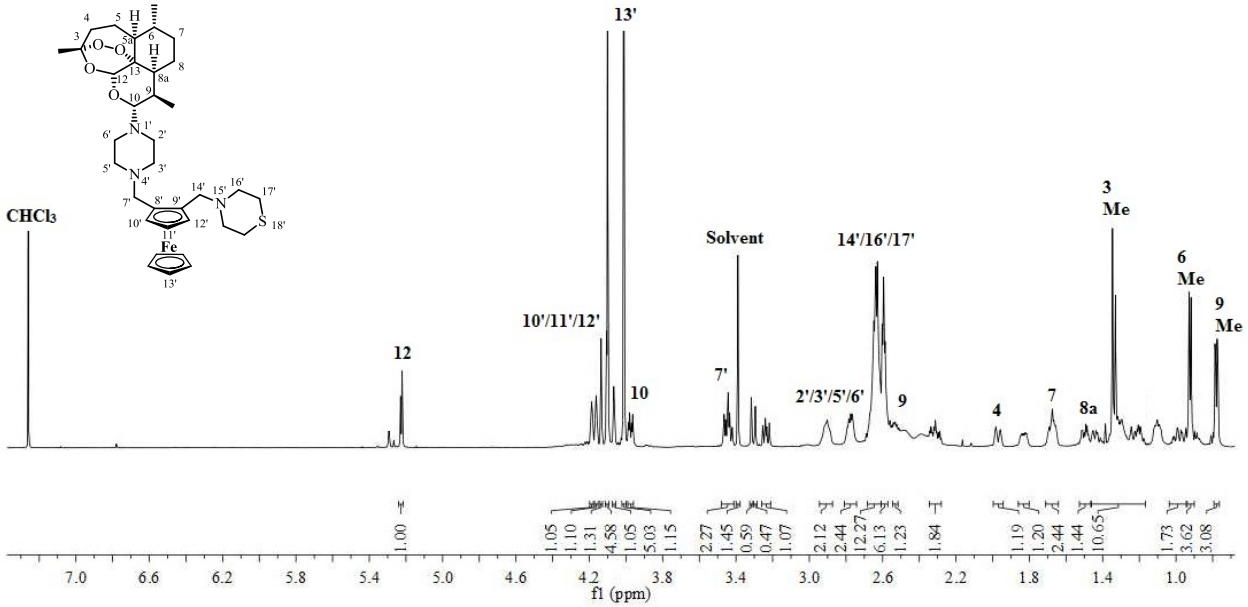
10α-(1'-Piperazino)-10-deoxo-10-dihydroartemisinin **2** (2 mmol, 3 equiv.) and the corresponding aminoferrocenealdehyde (0.6 mmol, 1 equiv.) were dissolved in anhydrous THF (22 mL) under nitrogen at room temperature. The resulting solution was stirred and treated portionwise with sodium triacetoxyborohydride (2 mmol, 3 equiv.). The reaction mixture was left to stir overnight at room temperature under nitrogen. The reaction mixture was basified to pH 10 by treatment with aqueous NaOH (1M, 5-10 mL), and extracted with diethyl ether (4 x 50 mL). The extracts were combined and dried ($MgSO_4$), and then filtered. The filtrate was evaporated under reduced pressure and the residue was purified by column chromatography over silica gel with dichloromethane-MeOH-triethylamine (9:1:1) to give the derivatives.

10α-[1'-Piperazino-4'-(8'-thiomorpholinomethyl ferrocenemethyl)]-10-deoxo-10-dihydro-artemisinin **3**

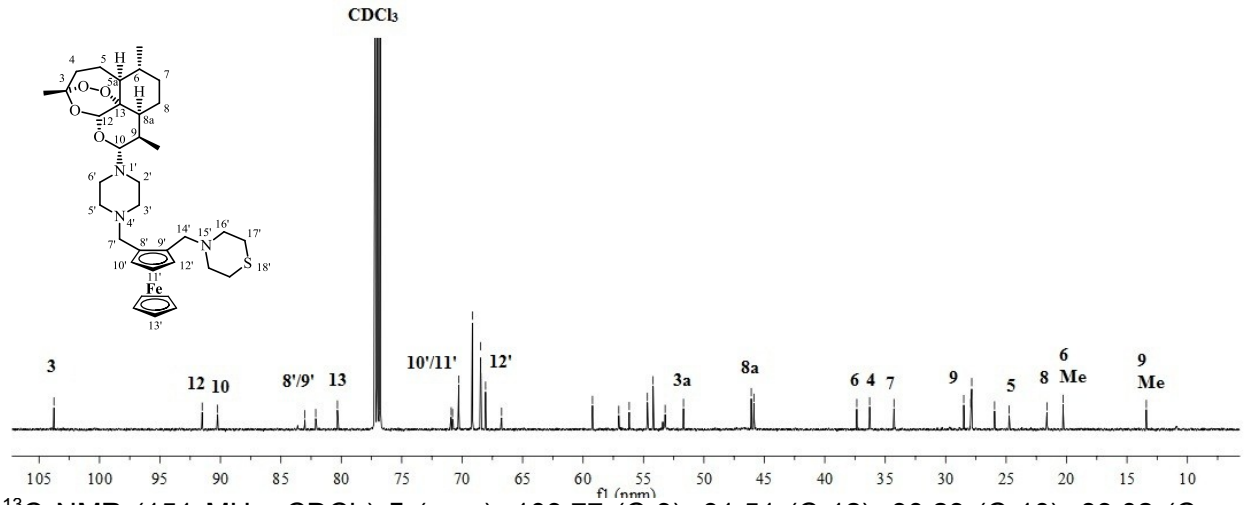
Red gum, 0.07 g (18%), R_f 0.47 (dichloromethane-MeOH 9:1).



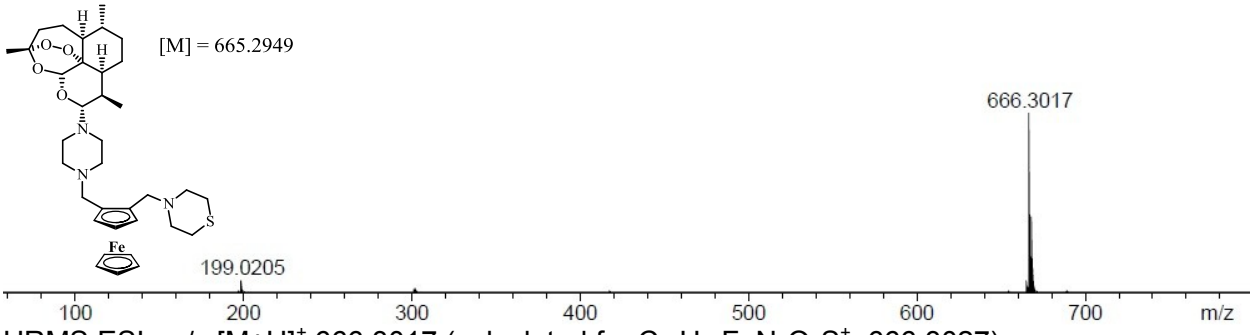
IR (ATR) ν_{max}/cm^{-1}: 3091, 2923, 2869, 2801, 1723, 1453, 1374, 1161, 1053, 979, 784, 733, 503, 483.



^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.23 (d, *J* = 4.8 Hz, 1H, H-12), 4.19 (m, 1H, H-10'/H-11'/H-12'), 4.16 (m, 1H, H-10'/H-11'/H-12'), 4.10 (s, 5H), 4.07-4.06 (m, 1H, H-10'/H-11'/H-12'), 4.01 (s, 5H, H-13'), 3.98-3.97 (dd, *J* = 10.1, 3.7 Hz, 1H, H-10), 3.47-3.43 (m, 2H, H-7'), 2.91-2.90 (m, 2H, H-2'/H-3'/H-5'/H-6'), 2.79-2.77 (m, 2H, H-2'/H-3'/H-5'/H-6'), 2.65-2.62 (m, 12H, H-14'/H-16'/H-17'), 2.55-2.51 (m, 1H, H-9), 2.34-2.28 (td, *J* = 9.8, 4.4 Hz, 1H, H-4), 1.98-1.96 (d, *J* = 14.5 Hz, 1H, H-4), 1.85-1.81 (m, 1H, H-5), 1.70-1.66 (m, 2H, H-7), 1.52-1.47 (m, 1H, H-8a), 1.46-1.43 (m, 1H, H-5), 1.35 (d, *J* = 9.5 Hz, 3H, H-3Me), 1.32-1.30 (m, 1H, H-6), 0.93-0.92 (d, *J* = 6.3 Hz, 3H, H-6Me), 0.79-0.77 (dd, *J* = 7.1, 2.6 Hz, 3H, H-9Me).



^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 103.77 (C-3), 91.51 (C-12), 90.23 (C-10), 83.02 (C-6'/C-7'), 82.11 (C-6'/C-7'), 80.32 (C-13), 70.91 (C-8'/C-9'), 70.80 (C-8'/C-9'), 70.28, 69.15 (C-10'), 68.47, 68.06 (C-10'), 66.74 (C-8'/C-9'), 59.22, 57.04 (C-12'/C-16'/C-17'), 56.18 (C-12'/C-16'/C-17'), 54.67 (C-2'/C-3'), 54.21 (C-2'/C-3'), 53.21 (C-5'/C-11'), 51.69 (C-5a), 46.08 (C-8a), 45.85 (C-6), 37.35, 36.29 (C-4), 34.28 (C-7), 28.50 (C-9), 27.84, 25.95 (C-3Me), 24.75 (C-5), 21.62 (C-8), 20.28 (C-6Me), 13.40 (C-9Me).

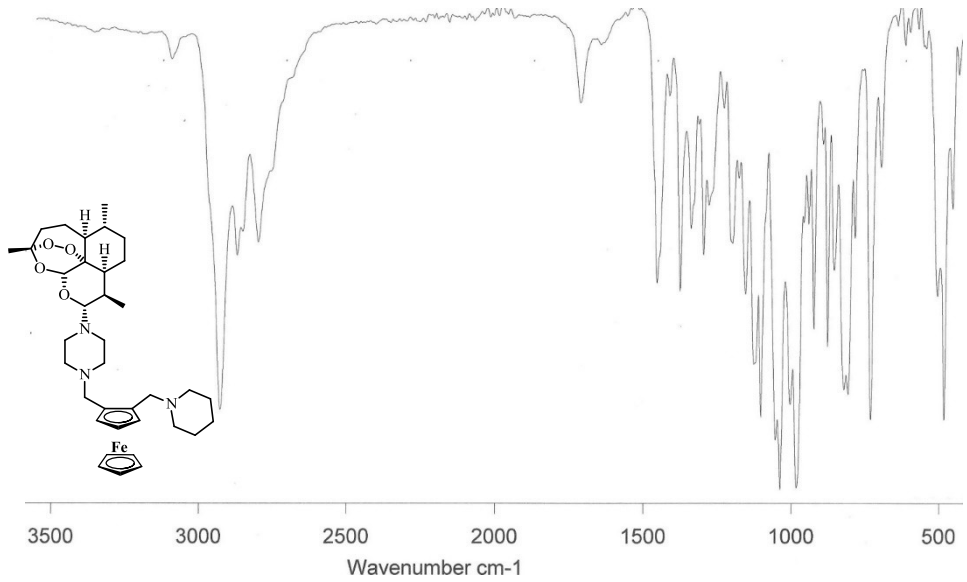


HRMS ESI: m/z $[M+H]^+$ 666.3017 (calculated for $C_{35}H_{52}FeN_3O_4S^+$: 666.3027)

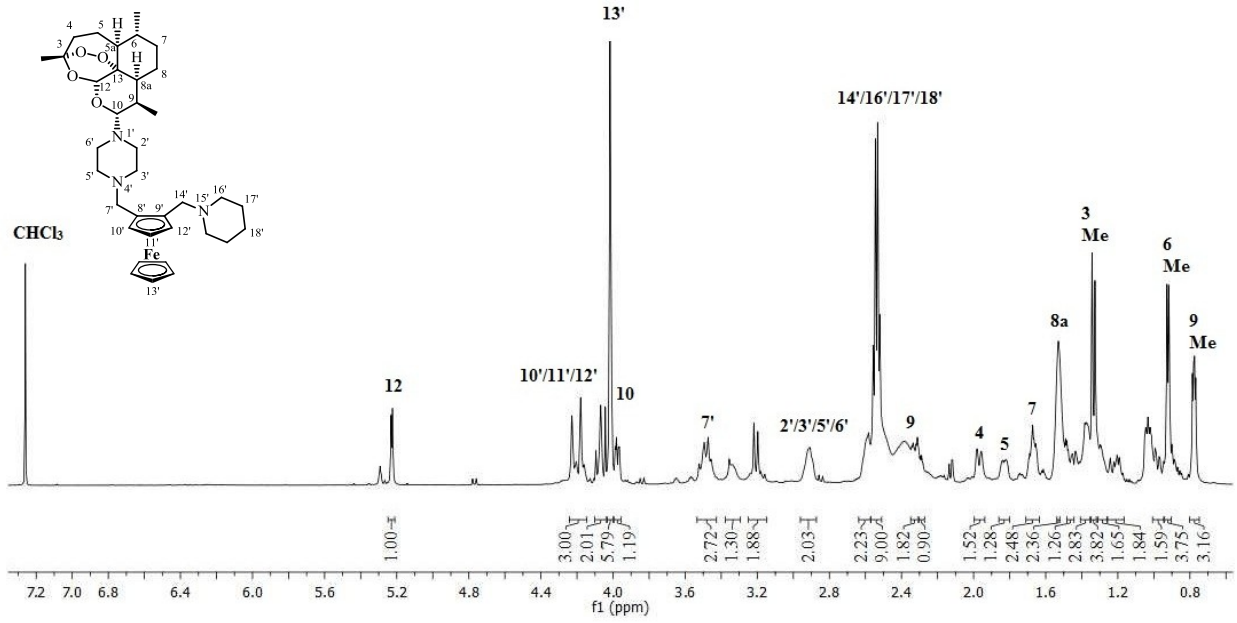
10α-[1'-Piperazino-4'-(8'-piperidinomethyl ferrocenemethyl)]-10-deoxo-10-dihydroartemisinin

4

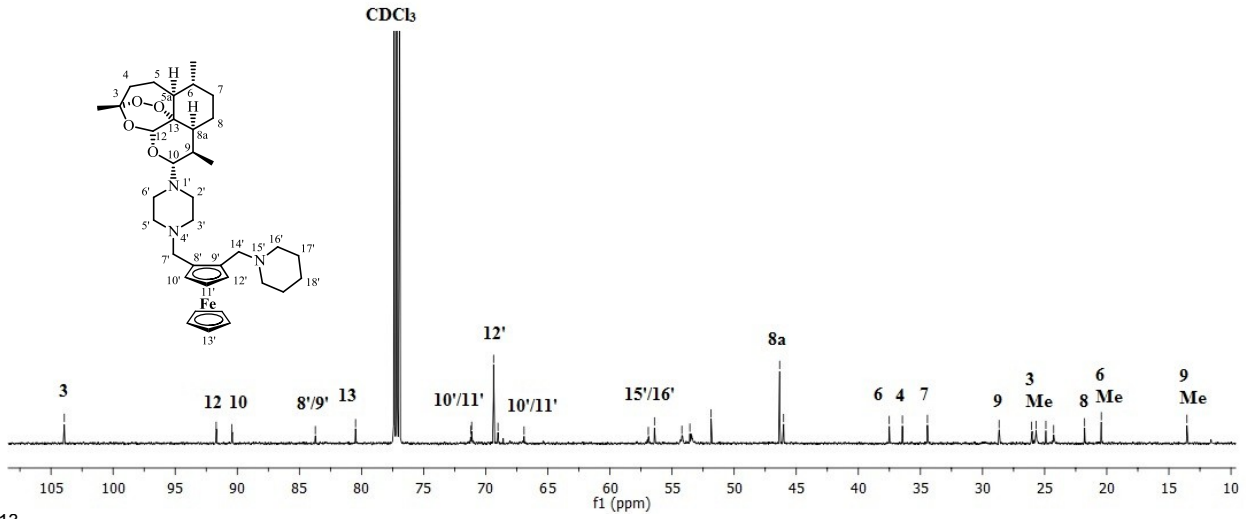
Red gum, 0.08 g (23%), R_f 0.53 (dichloromethane-MeOH 9:1).



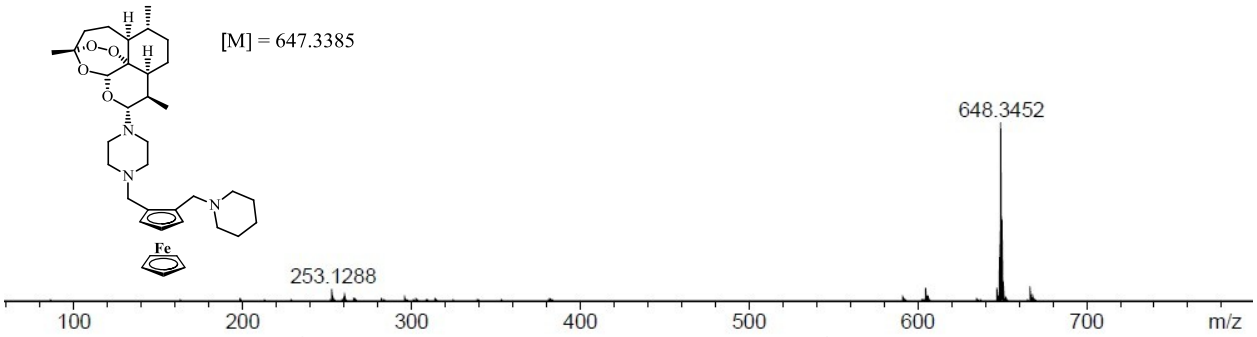
IR (ATR) $\tilde{\nu}_{max}/cm^{-1}$: 3092, 2928, 2870, 2798, 1712, 1453, 1375, 1296, 1103, 1039, 984, 734, 485.



^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.23 (d, *J* = 4.4 Hz, 1H, H-12), 4.23-4.16 (m, 3H, H-10'/H-11'/H-12'), 4.07 (m, 2H, H-10'/H-11'/H-12'), 4.02 (s, 5H, H-13'), 3.98-3.97 (m, 1H, H-10), 3.52-3.46 (s, 2H, H-7'), 2.91 (s, 2H, H-2'/H-3'/H-5'/H-6'), 2.60-2.58 (m, 12H, H-14'/H-16'/H-17'/H-18'), 2.56-2.51 (m, 1H, H-9), 2.34-2.29 (m, 1H, H-4), 1.98-1.96 (d, 1H, H-4), 1.85-1.81 (m, 1H, H-5), 1.69-1.65 (m, 2H, H-7), 1.53 (s, 2H, H-8a), 1.48-1.45 (m, 1H, H-5), 1.34-1.33 (d, *J* = 9.6 Hz, 3H, H-3Me), 1.30-1.26 (m, 1H, H-6), 1.24-1.19 (m, 1H, H-5a), 0.99-0.97 (m, 1H, H-7), 0.93-0.92 (d, *J* = 6.2 Hz, 3H, H-6Me), 0.79-0.77 (dd, *J* = 10.8 Hz, 3H, H-9Me).



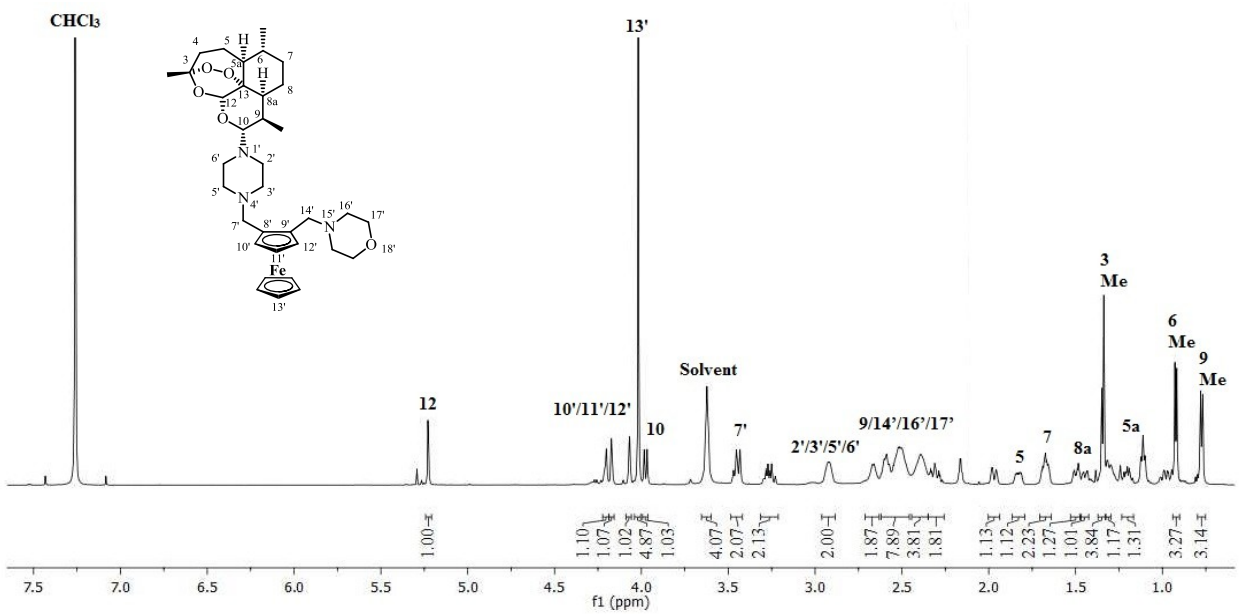
^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 103.92 (C-3), 91.70 (C-12), 90.44 (C-10), 83.72 (C-6'/C-7'), 80.48 (C-13), 71.21 (C-8'/C-9'), 69.37 (C-10'), 69.01, 66.91 (C-8'/C9'), 56.91 (C-16'/C-17'), 56,41 (C-16'/C-17'), 53.56, 53.44, 51.85 (C-5a), 46.02 (C-8a), 37.51 (C-6), 36.45 (C-4), 34.44 (C-7), 28.67 (C-9), 26.05 (C-3Me), 25.69, 24.90 (C-5), 24.27, 21.78 (C-8), 20.44 (C-6Me), 13.54 (C-9Me).



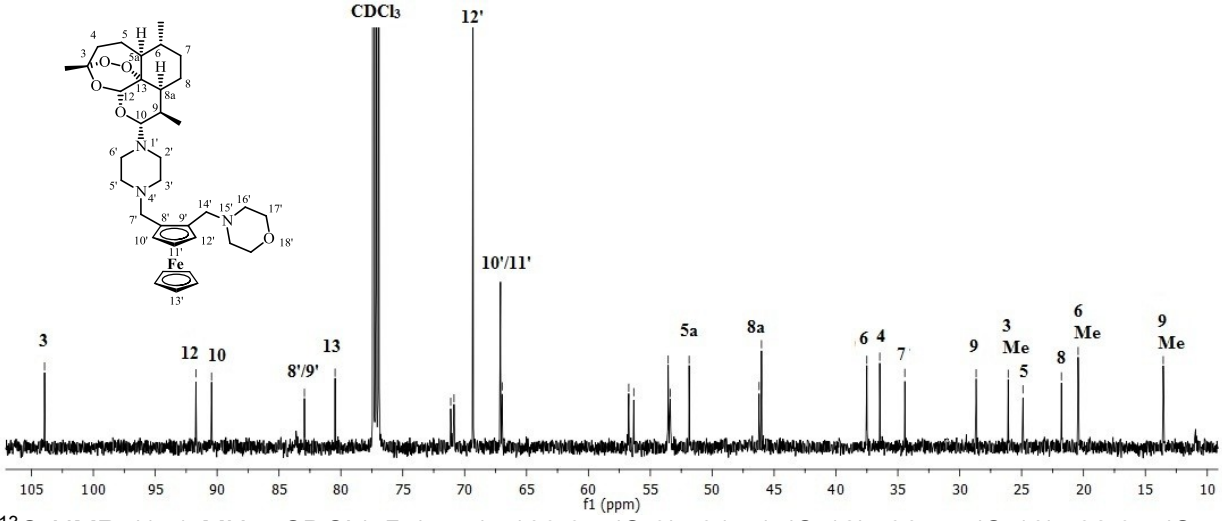
HRMS ESI: m/z $[M+H]^+$ 648.3452 (calculated for $C_{36}H_{54}FeN_3O_4^+$: 648.3460).

10α-[1'-Piperazino-4'-(8'-morpholinomethyl ferrocenemethyl)]-10-deoxo-10-dihydroartemisinin, **5**

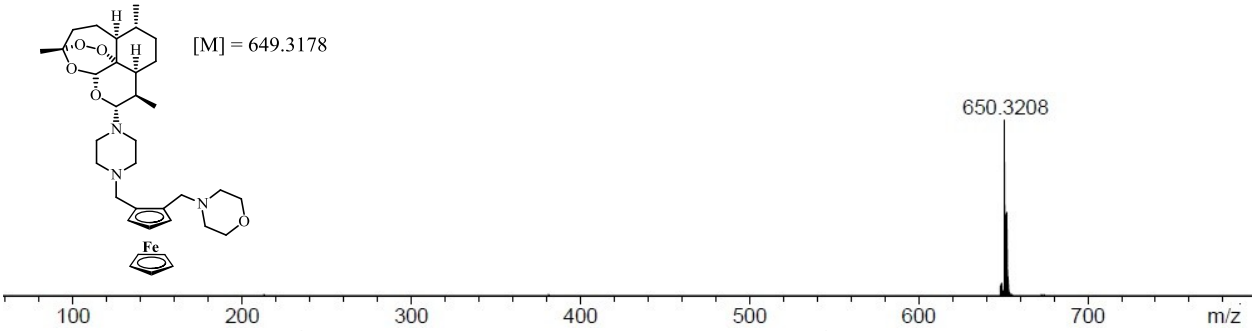
Red gum, 0.10 g (26%), R_f 0.43 (dichloromethane-MeOH 9:1).



^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.23-5.22 (d, *J* = 2.8 Hz, 1H, H-12), 4.20 (s, 1H, H-10'/H-11'/H-12'), 4.17 (s, 1H, H-10'/H-11'/H-12'), 4.07 (s, 1H, H-10'/H-11'/H-12'), 4.02 (s, 5H, H-13'), 3.98-3.97 (d, *J* = 10.1 Hz, 1H, H-10), 3.47-3.43 (m, 2H, H-7'), 2.92 (s, 2H, H-2'/H-3'/H-5'/H-6'), 2.71-2.66 (m, 2H, H-2'/H-3'/H-5'/H-6'), 2.60-2.50 (m, 8H, H-9/H-14'/H-16'/H-17'), 2.33-2.28 (m, 2H, H-4), 1.98-1.96 (d, *J* = 14.4 Hz, 1H, H-4), 1.85-1.81 (m, 1H, H-5), 1.69-1.65 (m, 2H, H-7), 1.51-1.48 (m, 1H, H-8a),1.46-1.41 (m, 1H, H-5), 1.35-1.34 (d, *J* = 6.9 Hz, 3H, H-3Me), 1.32-1.31 (m, 1H, H-8), 1.31-1.30 (m, 1H, H-6), 1.22-1.17 (m, 1H, H-5a), 0.93-0.92 (d, *J* = 6.3 Hz, 3H, H-6Me), 0.78-0.77 (dd, *J* = 7.1, 1.9 Hz, 3H, H-9Me).



^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 103.94 (C-3), 91.71 (C-12), 90.45 (C-10), 82.95 (C-6'/C-7'), 80.47 (C-13), 71.21 (C-8'/C-9'), 70.86 (C-8'/C-9'), 69.33 (C-10'), 66.97 (C-8'/C9'), 56.74, 56,34, 53.55, 53.39, 51.86 (C-5a), 46.02 (C-8a), 37.51 (C-6), 36.46 (C-4), 34.44 (C-7), 28.67 (C-9), 26.09 (C-3Me), 24.89 (C-5), 21.79 (C-8), 20.44 (C-6Me), 13.56 (C-9Me).



HRMS ESI: m/z $[M+H]^+$ 650.3208 (calculated for $C_{35}H_{52}FeN_3O_5^+$: 650.3256).

4. References:

1 Verlinden BK, Niemand J, Snyman J, et al. Discovery of novel alkylated (bis) urea and (bis) thiourea polyamine analogues with potent antimalarial activities. *J Med Chem.* 2011; 54(19): 6624-6633.

2 Reader J, Botha M, Theron A, et al. Nowhere to hide: interrogating different metabolic parameters of Plasmodium falciparum gametocytes in a transmission blocking drug discovery pipeline towards malaria elimination. *Malaria Journal.* 2015;14(1): 213.

3 Wentzel JF, Lombard MJ, Du Plessis LH, Zandberg L. Evaluation of the cytotoxic properties, gene expression profiles and secondary signalling responses of cultured cells exposed to fumonisin B1, deoxynivalenol and zearalenone mycotoxins. *Arch Toxicol.* 2017; 91(5): 2265-2282.

4 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983; 65(1-2): 55-63.