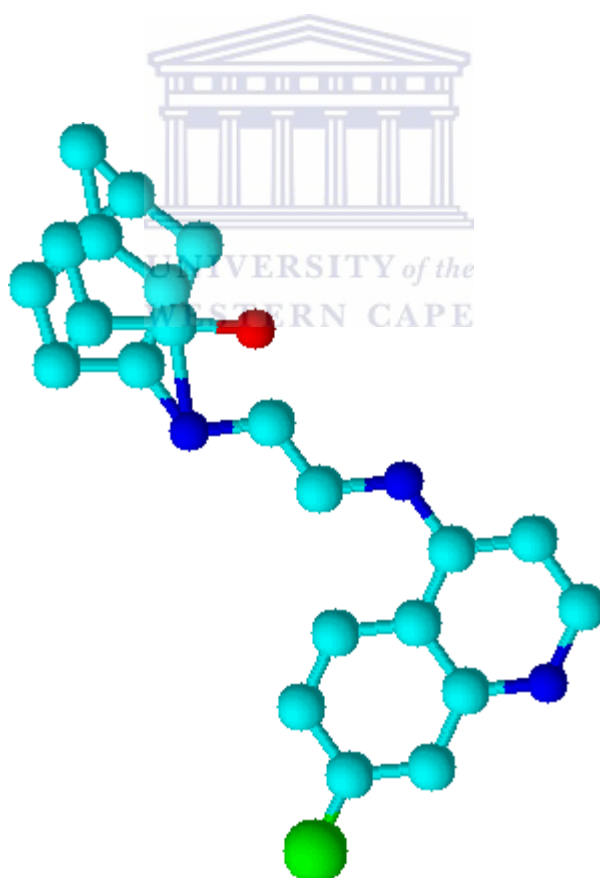


Novel Aminoquinoline-Polycyclic Hybrid Molecules as Potential Antimalarial Agents

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Novel Aminoquinoline-Polycyclic Hybrid Molecules as Potential Antimalarial Agents

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A dissertation submitted in partial fulfilment of the requirements for the degree



Supervisor: Prof. S.F. Malan

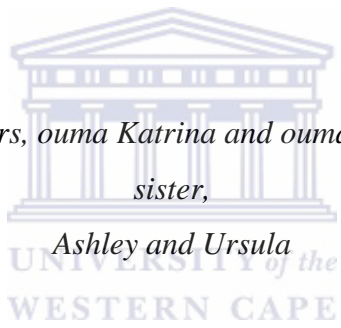
Co-supervisor: Dr. J. Joubert

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PHARMACEUTICAL CHEMISTRY, SCHOOL OF PHARMACY, UNIVERSITY OF THE
WESTERN CAPE, PRIVATE BAG X17, BELLVILLE, 7535

*Dedicated to my late grandmothers, ouma Katrina and ouma Noenie, and my late brother and
sister,*



Ashley and Ursula

UNIVERSITY of the
WESTERN CAPE

Keywords

Malaria

Plasmodium falciparum

4-Aminoquinolines

Chloroquine resistance

p-Glycoprotein (pGP) efflux pump

P. falciparum CQ resistance transporter (PfCRT) protein

Reversed chloroquine compounds

Reversal agent

Polycyclic cage compounds

Pentacycloundecylamines



Declaration

I declare that *Novel aminoquinoline-polycyclic hybrid molecules as potential antimalarial agents* is my own work and that it has not been submitted for any other degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete reference.

Full name of researcher:..... Date:.....



Signed:.....

Abstract

Plasmodium falciparum malaria continues to be a worldwide health problem, especially in developing countries in Africa and is responsible for over a million fatalities per annum. Chloroquine (CQ) is low-cost, safe and was the mainstay aminoquinoline derived chemotherapeutic agent that has been used for many years against blood-stage malaria. However, today the control of malaria has been complicated by increased resistance of the malaria parasite to existing antimalarial agents such as CQ. The primary cause of resistance is mutation in a putative ATP-powered multidrug efflux pump known as the *p*-glycoprotein (pGP) pump, and point mutation in *P. falciparum* CQ resistance transporter (PfCRT) protein. These mutations are responsible for the reduced accumulation of CQ at its primary site of action, the acidic digestive food vacuole of the parasite.

To overcome the challenges of CQ resistance in *P. falciparum*, chemosensitiser offer an attractive approach. Chemosensitisers or reversal agents are structurally diverse molecules that are known to reverse CQ resistance by inhibiting the pGP efflux pump and/or the PfCRT protein associated with CQ export from the digestive vacuole in CQ resistant parasites. Chemosensitisers include the well-studied calcium channel blocker verapamil and antihistaminic agent chlorpheniramine. These drugs have little or no inherent antimalarial activity but have shown to reverse CQ resistance in *P. falciparum* when co-administered with CQ. Because of the channel blocking abilities of pentacycloundecylamines (PCUs) such as NGP1-01, it is postulated that these agents may act as chemosensitisers and circumvent the resistance of the *Plasmodium* parasite against CQ. Therefore as a proof of concept we conducted an experiment using CQ co-administered with different concentrations of NGP1-01 to evaluate the ability of NGP1-01 to act as a chemosensitiser.

Herein, we report the ability of NGP1-01, the prototype pentacycloundecylamine (PCU), to reverse CQ resistance (> 50 %) and act as a chemosensitiser. NGP1-01 alone exhibited very low intrinsic antimalarial activity against both the resistant and sensitive strain (> 2000 nM), with no toxicity to the parasite detected at 10 µM. A statistically significant ($p < 0.05$) dose dependent shift was seen in the CQ IC₅₀ values at both 1 µM and 10 µM concentration of co-administered NGP1-01 against the resistant strain. Based on this finding we set out to synthesise a series of

novel agents comprising of a PCU moiety as the reversal agent (RA) conjugated to a CQ-like aminoquinoline (AM) molecule and evaluate the potential of these PCU-AM derivatives as antimalarial- and/or reversed CQ agents. As recently shown by Peyton *et al.*, (2012), the conjugation of a CQ-like molecule with a RA such as the chemosensitiser imipramine and derivatives thereof is a viable strategy to reverse CQ resistance in multidrug-resistant *P. falciparum*. The novel compounds were obtained by amination and reductive amination reactions. The synthetic procedures involved the conjugation of the Cookson's diketone with different tethered 4-aminoquinoline moieties to yield the respective carbinolamines and the subsequent imines. This was followed by a transannular cyclisation using sodium cyanoborohydride as reducing agent to yield the desired PCU-AM derivatives. The CQ-like AM derivatives were obtained using a novel microwave (MW) irradiation method. Structure elucidation was done by utilising ¹H- and ¹³C NMR spectroscopy as well as IR absorption spectrophotometry and mass spectrometry.

Five PCU-AM reversed CQ derivatives were successfully synthesised and showed significant *in vitro* antimalarial activity against the CQ sensitive strain (NF54). PCU-AM derivatives **1.1** – **1.4** showed antimalarial IC₅₀ values in the ranges of 3.74 – 17.6 ng/mL and 27.6 – 253.5 ng/mL against the CQ-sensitive (NF54) and CQ-resistant strains (Dd2) of *Plasmodium falciparum*, respectively. Compound **1.1** presented with the highest antimalarial activity against both strains and was found to be 5 fold more active against the resistant strain than CQ. The reversed CQ approach resulted in improved resistance reversal and a significantly lower concentration PCU was required compared to NGP1-01 and CQ in combination. This may be attributed to the improved ability of compound **1.1** to actively block the pGP pump and/or the increased permeability thereof because of the lipophilic aza-PCU moiety. Compound **1.1** also showed the lowest RMI value confirming that this compound has the best potential to act as a reversed CQ agent in the series. Cytotoxicity IC₅₀ values observed for compounds **1.1** – **1.4** were in the low micromolar concentrations (2.39 – 9.54 μM) indicating selectivity towards *P. falciparum* (SI = 149 – 2549) and low toxicity compared to the cytotoxic agent emetine (IC₅₀ = 0.061 μM).

These results indicate that PCU channel blockers and PCU-AM derived conjugates can be utilised as lead molecules for further optimisation and development to enhance their therapeutic potential as reversal agents and reversed CQ compounds.

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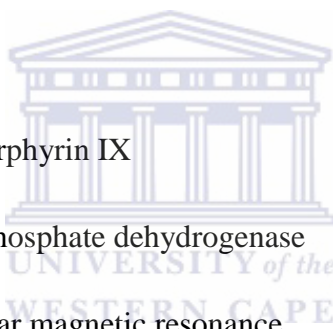
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LIST OF ABBREVIATIONS



ACT	Artemisinin-combined therapy
AM	Aminoquinoline
ATP	Adenosine triphosphate
¹³ C-NMR	Carbon 13 nuclear magnetic resonance
CCB	Calcium channel blocker
CHO	Chinese Hamster Ovarian
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
CH ₃ CN	Acetonitrile
CQ	Chloroquine
CQ ^R	Chloroquine resistant

CQ ^S	Chloroquine sensitive
d	Doublet
dd	Doublet doublet
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DMSO	Dimethyl sulfoxide
DMT	Drug and metabolite transporter
EtOH	Ethanol
FPIX	Ferriprotoporphyrin IX
G6PD	Glucose-6-phosphate dehydrogenase
¹ H-NMR	Proton nuclear magnetic resonance
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
H ₂ O ₂	Hydrogen peroxide
Hrs	Hours
Hz	Hertz
IC ₅₀	Inhibitory concentration 50 %
IR	Infrared radiation
<i>J</i>	Spin-Spin coupling constant (Hz)
m/z	Mass to charge ratio



MDR	Multidrug-resistant
MeOH	Methanol
min	Minutes
MP	Melting point
MW	Microwave
MS	Mass spectrometry
m	Multiplet
NaCNBH ₄	Sodium cyanoborohydride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaHCO ₃	Sodium hydrogen carbonate
Na ₂ SO ₄	Sodium sulfate
PABA	<i>p</i> -aminobenzoic acid
PCU	Pentacycloundecylamines
PCU-AM	Pentacycloundecane-aminoquinoline
PfATP6	Sarcoplasmic/endoplasmic reticulum Ca ²⁺ -ATPase orthologue of <i>Plasmodium falciparum</i>
Pfmdr	<i>Plasmodium falciparum</i> multidrug-resistant gene
PfCRT	<i>Plasmodium falciparum</i> chloroquine transporter protein
PfFKBP	<i>P. falciparum</i> FK506 binding protein
PfHRP	<i>Plasmodium falciparum</i> histidine-rich protein

pGP	<i>p</i> -glycoprotein pump
ppm	Part per million
pLDH	Parasite lactate dehydrogenase
psi	Pounds per square inch
RA	Reversal agents
RCQ	Reverse chloroquine compounds
ROS	Reactive oxygen species
RMI	Response modification index
Ro5	Lipinski's rule of five
rt	Room temperature
RPMI	Roswell Park Memorial Institute (Culture medium)
SAR	Structure-activity relationship
SI	Selectivity index
SERCA	Sarco/endoplasmic reticulum Ca ²⁺ -ATPase
s	Singlet
t	Triplet
THF	Tetrahydrofuran
TMS	Tetramethylsilane
TLC	Thin-layer chromatography
TS	Thymidylate synthase

UV	Ultraviolet
W	Watt
WHO	World Health Organisation
δ	Chemical shift (ppm)
$^{\circ}\text{C}$	Degrees Celsius



CHAPTER 1

INTRODUCTION

1.1 Background

Malaria is one of the world's most devastating parasitic infections and has in recent years become an important focus of research. This infection has an immense effect on economic productivity, livelihood and human settlement patterns (Gallup & Sachs, 2001). The four *Plasmodium* species namely *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* are the major cause of the infection while the vast majority of death in humans, is caused by *falciparum* malaria. The most severe clinical cases are observed amongst children under the age of 5 years, pregnant woman (Rowe *et al.*, 2006) and non-immune individuals travelling to malaria-endemic regions (World Health Organization, 2011). In addition, *P. knowlesi* was recently established as the fifth cause of malaria and its effects are currently observed in Malaysia (Singh *et al.*, 2004; Cox-Singh & Singh, 2008; World Health Organization, 2011).

Chloroquine (CQ) (1) is low-cost, safe and was the mainstay chemotherapeutic agent since its discovery more than 75 years ago for blood-stage malaria treatment. CQ has been used extensively for the latter purpose especially in Africa, an economy that demands inexpensive, efficacious and safe drugs. However, today chloroquine resistant plasmodia, in particular the virulent *P. falciparum*, impede its use (Hyde 2005a). Chloroquine resistant *falciparum* malaria was originally concentrated in Colombia and at the Cambodia-Thailand border during the late 1950's (Lim *et al.*, 2003). Resistance thereafter spread to South America (Cortese *et al.*, 2002), India (Sharma *et al.*, 1996; Mahapatra *et al.*, 2011) and Africa (Wootton *et al.*, 2002; Dondorp *et al.*, 2009). The primary cause of resistance is mutation in a putative ATP-powered multidrug efflux pump known as the *p*-glycoprotein (pGP) pump, and point mutation in the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) protein (Sanchez *et al.*, 2008; Chinappi *et al.*, 2010). These mutations are responsible for the reduced accumulation of CQ at its primary site of action, the acidic digestive food vacuole of the parasite.

The eradication of this infection has become increasingly difficult, especially with the prevailing resistant *falciparum*. Introducing novel chemical entities to the market has exorbitant cost implications especially in impoverished malaria-endemic areas that are in dire need of an immediate cure. Intense and urgent action is thus of paramount importance to fortify current antimalarial drug libraries to circumvent the problem of resistance and to bring a halt to this scourge of an infection.

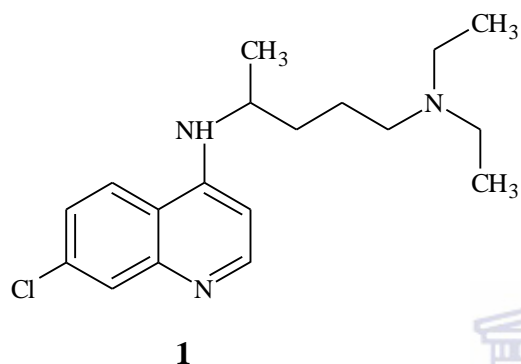


Figure 1.1: Chloroquine (CQ), a blood-stage antimalarial agent



1.2 Rationale

1.2.1 Reversed chloroquine agents

Although many antimalarial agents have been developed with the advances in modern science, there still exists an enormous need for novel and improved antimalarial agents. Reversal agents or chemosensitisers are structurally diverse molecules that are known to reverse CQ resistance, and are well documented to reinstate the antimalarial activity of CQ in *P. falciparum* chloroquine resistant (CQ^R) strains (Krogstad *et al.*, 1987; Millet *et al.*, 2004). In a study conducted by van Schalkwyk *et al.*, (2001), combinations of two or more of these reverse agents at pharmacological concentrations with chloroquine was found to provide clinical relevant reversal activity. The antihistaminic agent, chlorpheniramine (**2**) demonstrated *in vitro* antimalarial activity and reversed chloroquine resistance in *falciparum* malaria in the micromolar range (Nakornchai & Konthiang, 2006). Chlorpheniramine further potentiate the efficacy of CQ in the treatment of acute uncomplicated *falciparum* malaria in children (Sowunmi *et al.*, 1997). Similar CQ reversal activity against *falciparum* CQ^R isolates in an *in vitro* study was observed by the

tricyclic antidepressant, desipramine (**3**) (Basco & Le Bras, 1990). However, during a clinical trial, desipramine in combination with CQ failed to improve the efficacy of CQ against chloroquine-resistant *Plasmodium falciparum in vivo* (Warsame, Wernsdorfer, & Björkman, 1992). Poly-pharmacy approaches thus appeared to be viable therapeutic strategies to restore the antimalarial drug ‘pipeline’. However, it is inadequate and impractical because for these poly-pharmacy combinations to exert meaningful antimalarial and/or reversal activity, unacceptably high concentrations of the reversal agent are generally required. Thus Burgess *et al.* (2006) designed a hybrid molecule comprising of a chloroquine-like aminoquinoline portion and imipramine (**4**), a known PfCRT reversal agent (Miki *et al.*, 1992; Burgess *et al.*, 2010) and termed it a reversed chloroquine (RCQ) molecule. This RCQ molecule successfully inhibited the growth of *falciparum* CQ^R and chloroquine sensitive (CQ^S) parasites *in vitro* and also after oral dosing *in vivo*.

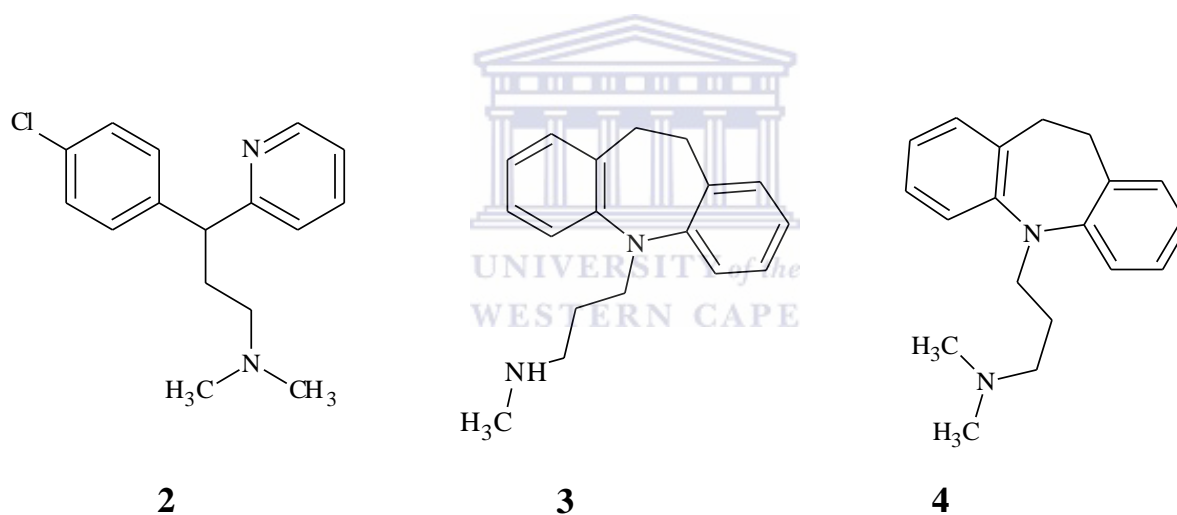


Figure 1.2: Representative reversal agents (**2 – 4**)

In addition, for novel compound to potentially overcome drug resistance in *P. falciparum* CQ^R strains, structural modification of CQ is required (Egan *et al.*, 2000; Madrid *et al.*, 2006; Hocart *et al.*, 2011). This can be achieved by both shortening and lengthening the separation between the two aliphatic amino moieties, and incorporation of molecules with wide variation in size and composition on the terminal amine (Figure 1.3). By incorporating this model Yearick *et al.*, (2008) synthesised a series of 4-amino-7-chloroquinolines derivatives. The tribasic derivatives carrying a short linear side chain with two additional aliphatic tertiary amino functions displayed the best reversal activity against both *P. falciparum* CQ^S and CQ^R strains *in vitro*.

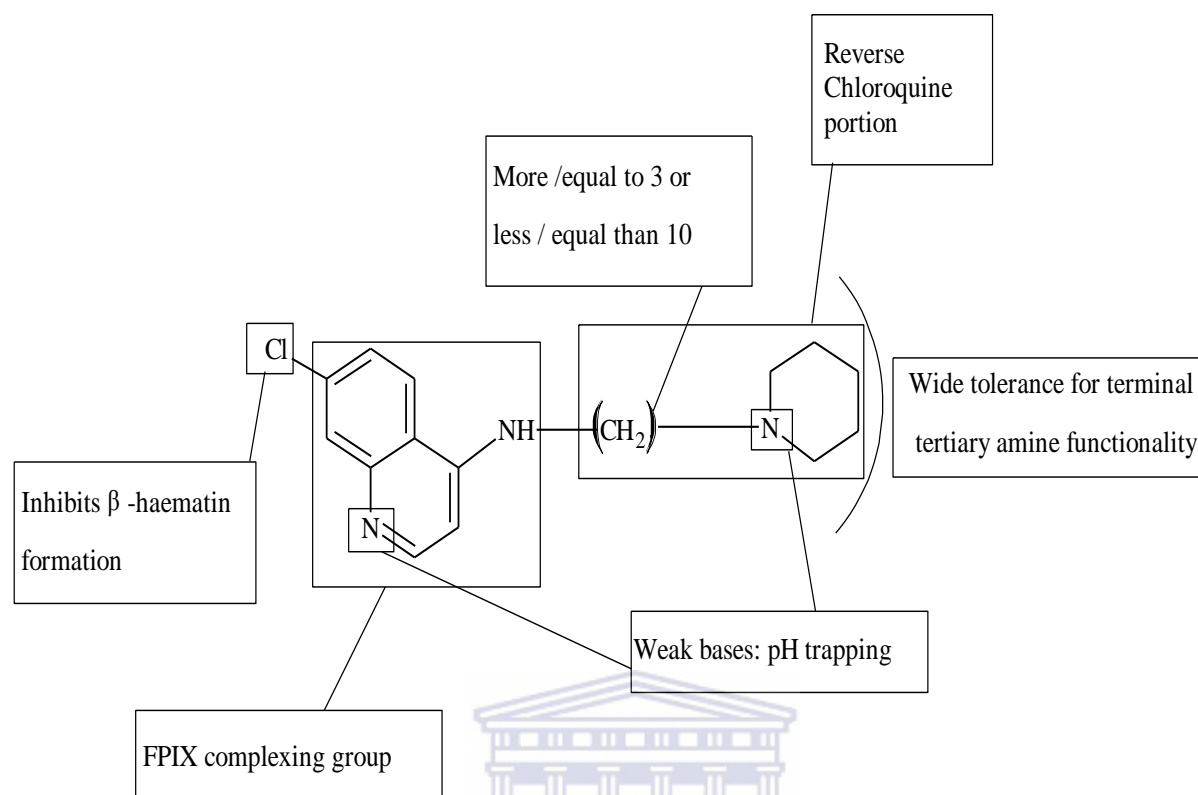


Figure 1.3: Summary of SARs for *P. falciparum* antimalarial- and/or reversed CQ activity (Egan *et al.*, 2000; Madrid *et al.*, 2006; Hocart *et al.*, 2011; Peyton *et al.*, 2012)

1.2.2 Polycyclic cage structures

Polycyclic cage scaffolds have been successfully used in the development of numerous lead compounds demonstrating a variety of important pharmacological activities; examples are antiviral- (Oliver *et al.*, 1991), neuroprotective- (Malan *et al.*, 2003; Kiewert *et al.*, 2006) and anti-tuberculosis (Onajole *et al.*, 2012) agents. These ‘bird-cage’ amines such as amantadine (**5**) and pentacycloundecylamines (**6**) possess significant antiviral activity (Oliver *et al.*, 1991; Stanicova *et al.*, 2001; Smith *et al.*, 2004) and improve and modify the lipid-solubility (Brookes *et al.*, 1992) profile of conjugated parent agents.

Polycyclic amines also have the ability to modulate voltage-gated calcium channels (Van der Schyf *et al.*, 1998; Malan *et al.*, 2000; Joubert *et al.*, 2011), in particular the oxapentacycloundecylamine, benzylamine-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (NGP1-01) (**7**), demonstrated activity comparable to that of nimodipine (Van der Schyf *et al.*, 1986), a

dihydropyridine calcium-channel blocker (CCB). Pentacycloundecylamines are derived from Cookson's diketone (pentacyclo[5.4.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione) obtained from the intramolecular photocyclisation of the Diels-Alder adduct of *p*-benzoquinone and cyclopentadiene (Cookson *et al.*, 1958). One of the ketone groups of the pentacycloundecane dione is allowed to react with an amine to obtain the corresponding carbinolamine. The carbinolamine is then dehydrated under Dean-Stark conditions, yielding the corresponding imine. This imine depending on the reducing agent used, can either be reduced to an oxa- or aza polycyclic cage compound. The inherent calcium channel modulatory activity of polycyclic amines sparked the concept that they may possess relevant drug resistance reversal activity. This resistance reversal property was demonstrated by verapamil (Martin *et al.*, 1987; Adovelande *et al.*, 1998), an *L*-type calcium-channel blocker of the phenylalkylamine class.

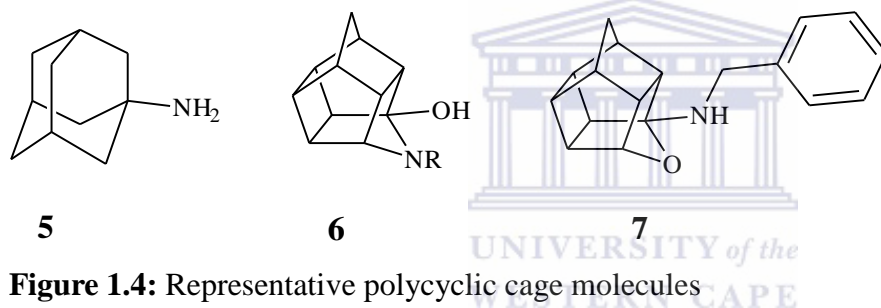


Figure 1.4: Representative polycyclic cage molecules

This non-polycyclic calcium antagonist demonstrated the ability to reverse drug resistance in cancer cell lines (Miller *et al.*, 1991) and also in plasmodia, resistant to amodiaquine and quinine by interfering with the pGP efflux pump (Sidhu *et al.*, 2002). It is thus suggested that polycyclic cage structures, based on their calcium-channel modulating effects, may possess meaningful antimalarial and/or resistance reversal activity (Singh *et al.*, 2004).

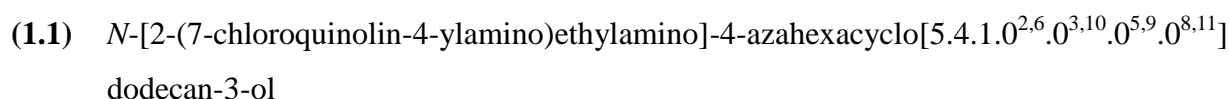
The polycyclic cage may thus be employed as a valuable scaffold to explore the design of potential pharmacological active compounds in the field of malaria and drug resistance.

1.3 Aim of study

The aim of the study was to design and synthesise a novel series of pentacycloundecane-aminoquinoline (PCU-AM) derivatives related to chloroquine and to investigate their resistance reversal potential and antimalarial activity. The antimalarial agents for this study were selected on the basis of their inherent potential RCQ properties and structural similarities to chloroquine as potential antimalarial agents (Figure 1.3; Andrews *et al.*, 2009; Peyton *et al.*, 2012).

The design of the PCU-AM derivatives commenced by selecting an appropriate PCU scaffold. The aza-PCU was considered as the best option since it would enable the design of a terminal tertiary amine portion similar to the structure of CQ. PCU scaffolds also have the potential to increase the permeation of privileged molecules over biological membranes and possibly into the parasite vacuole when covalently bound. Reports have demonstrated the ability of PCU scaffolds to significantly improve the permeability of privileged molecules (Zah *et al.*, 2003; Prins *et al.*, 2009). It is also suggested that the bulky aza-PCU scaffold will protect the terminal tertiary amino group from metabolism through *N*-dealkylation. Previous studies by Stocks *et al.*, (2002) and Madrid *et al.*, (2006) showed that the use of bulkier substituents attached to the terminal amino group of CQ increased the *in vivo* efficacy and also decreased the potential for cross-resistance, most probably by circumventing metabolic *N*-dealkylation (Bray *et al.*, 1996; Kaur *et al.*, 2010). The same effect is expected from the bulky aza-PCU scaffold. A basic centre was also retained in the PCU-AM derivatives, as CQ is postulated to concentrate in the parasite digestive vacuole by virtue of protonation under the acidic conditions found in that compartment (pH of the digestive vacuole is 4.7).

The compounds planned for synthesis include *N*-(7-chloroquinolin-4-yl)ethane-1,2-diamine, *N*-(7-chloroquinolin-4-yl)propane-1,3-diamine, *N*-(7-chloroquinolin-4-yl)butane-1,4-diamine, *N*-(7-chloroquinolin-4-yl)hexane-1,6-diamine and *N*-(7-chloroquinolin-4-yl)octane-1,8-diamine conjugated to pentacyclo[5.4.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione through nucleophilic addition to achieve the corresponding novel reversed CQ- and/or antimalarial agents:



- (1.2) *N*-[3-(7-chloroquinolin-4-ylamino)propylamino]-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecan-3-ol
- (1.3) *N*-[4-(7-chloroquinolin-4-ylamino)butylamino]-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecan-3-ol
- (1.4) *N*-[6-(7-chloroquinolin-4-ylamino)hexylamino]-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecan-3-ol
- (1.5) *N*-[8-(7-chloroquinolin-4-ylamino)octylamino]-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecan-3-ol.

For this study we thus attempted to identify and develop a novel series of PCU-AM derivatives which may potentially be utilised for further *in vitro* and *in vivo* antimalarial assays and to elucidate the molecular mechanism of action of these novel compounds. These compounds may be employed as useful pharmacological tools to investigate the antimalarial activity and/or reversed CQ activity in the quest for more effective antimalarial strategies. As recently shown by Peyton *et al.*, (2012) the conjugation of a CQ-like aminoquinoline (AM) molecule with a reversal agent (RA) such as the chemosensitiser imipramine and derivatives thereof is a viable strategy to reverse CQ resistance in drug-resistant *P. falciparum* (Burgess *et al.*, 2006; Andrews *et al.*, 2010; Burgess *et al.*, 2010).

To achieve the aim, the following will be done:

- Design a model, illustrating the structure-activity relationship (SAR) for the novel compounds to fulfil the requirements as potential reversed CQ agents (Egan *et al.*, 2000; Madrid *et al.*, 2006; Hocart *et al.*, 2011; Peyton *et al.*, 2012);
- Synthesis of selected resistance reversal portions and conjugation thereof to the various chloroquine-like AM moieties;
- To perform structure elucidation of the novel synthesised compounds by means of ¹H-NMR, ¹³C-NMR, MS and IR;
- Synergism evaluation of NGP1-01 for antimalarial activity as single compounds at different concentrations with CQ, as previously done for other calcium channel inhibitors (van Schalkwyk *et al.*, 2001);

- *In vitro* evaluation of the novel PCU-AM hybrid compounds for reversal- and/or antimalarial activity.

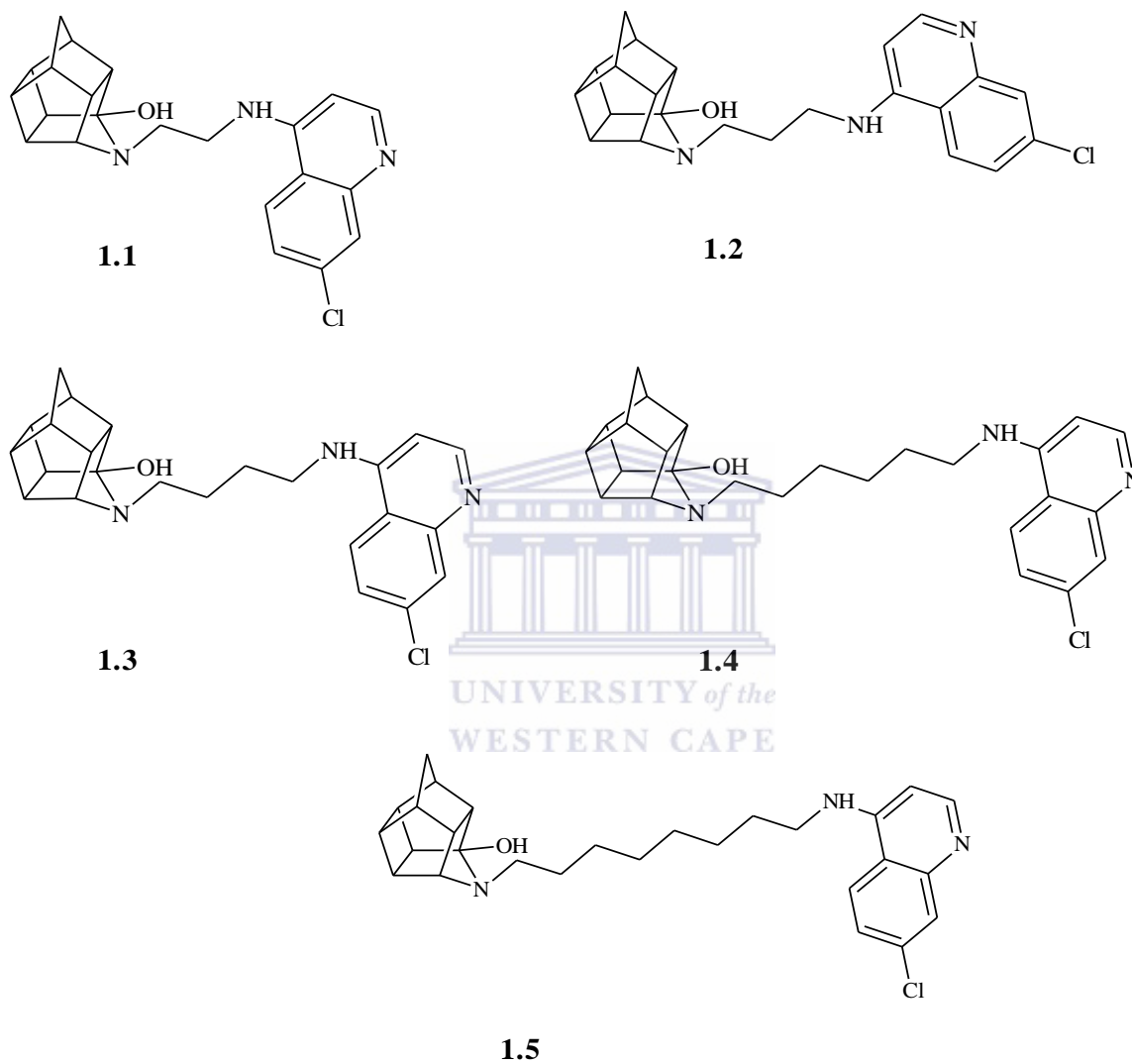


Figure 1.5: Novel pentacycloundecane-aminoquinoline (PCU-AM) derivatives synthesised in this study

1.4 Conclusion

It is expected that these novel PCU-AM derivatives will exhibit good to moderate antimalaria activity and potentially reverse CQ^R in *falciparum* strains as these compounds display structure similarity to the RCQ/RA molecule designed by (Andrews *et al.*, 2009; Peyton *et al.*, 2012). Further, the antimalarial potential of these novel compounds is endorsed by the RCQ SARs model in figure 1.3. Current research in this area seems to support ‘covalent bi-therapy’ i.e. hybrid molecules as the next-generation antimalarial agents. The drug candidates synthesised may potentially delay or circumvent the development of resistance and may be useful in drug design and development for clinical use.



CHAPTER 2

LITERATURE REVIEW

The purpose of this chapter is to briefly describe the malaria parasite's life-cycle and clinical picture. This chapter further attempt to give insight on known- and potential resistance reversal agents and also elaborate on these agents' multi-therapeutic uses as antimalarial agents in the quest for improved and more effective treatment strategies.

2.1. Life-cycle of *Plasmodium* parasite

Malaria is caused by the protozoan genus *Plasmodium* and requires two hosts, the female *Anophele* mosquito (vector) and a human, to complete its complex life-cycle (figure 2.1). The life-cycle starts when an infected female mosquito bites her prey, introducing sporozoite-containing saliva into the blood stream while withdrawing blood. These sporozoites conceal themselves from the host's immune system by travelling *via* the blood stream to the liver. They then invade liver cells, through specific receptor-ligand interaction where they multiply asexually. This is known as the liver-stage. The mature schizonts of the liver-stage cause liver cells to rupture and release thousands of merozoites into the blood stream. The released merozoites initiate the intra-erythrocytic stage which involves invasion of normal erythrocytes, asexual replication and the release of newly formed merozoites. This process takes place repeatedly over 1 – 3 days. Merozoites are responsible for the expanding infective biomass, clinical manifestation and pathology of malaria. In the case of *P. vivax* and *P. ovale*, sporozoites may remain dormant in the liver cells, known as hypnozoites, causing relapses months or years after the initial infection. However, *P. falciparum* and *P. malariae* lacks this liver persistent phase. Alternatively, merozoites of the erythrocytic cycle may differentiate into sexual forms, known as gametocytes. The mechanism for gametocyte formation is unknown. The ingestion of female- and male gametocytes into the mid-gut by an *Anophele* mosquito causes fusion into a zygote, with the eventual development of new sporozoites which invades the mosquito salivary gland epithelium. The *Plasmodium* life-cycle is perpetuated when the mosquito bites a susceptible vertebrate host (Mota & Rodriguez 2004; Ashley *et al.*, 2006).

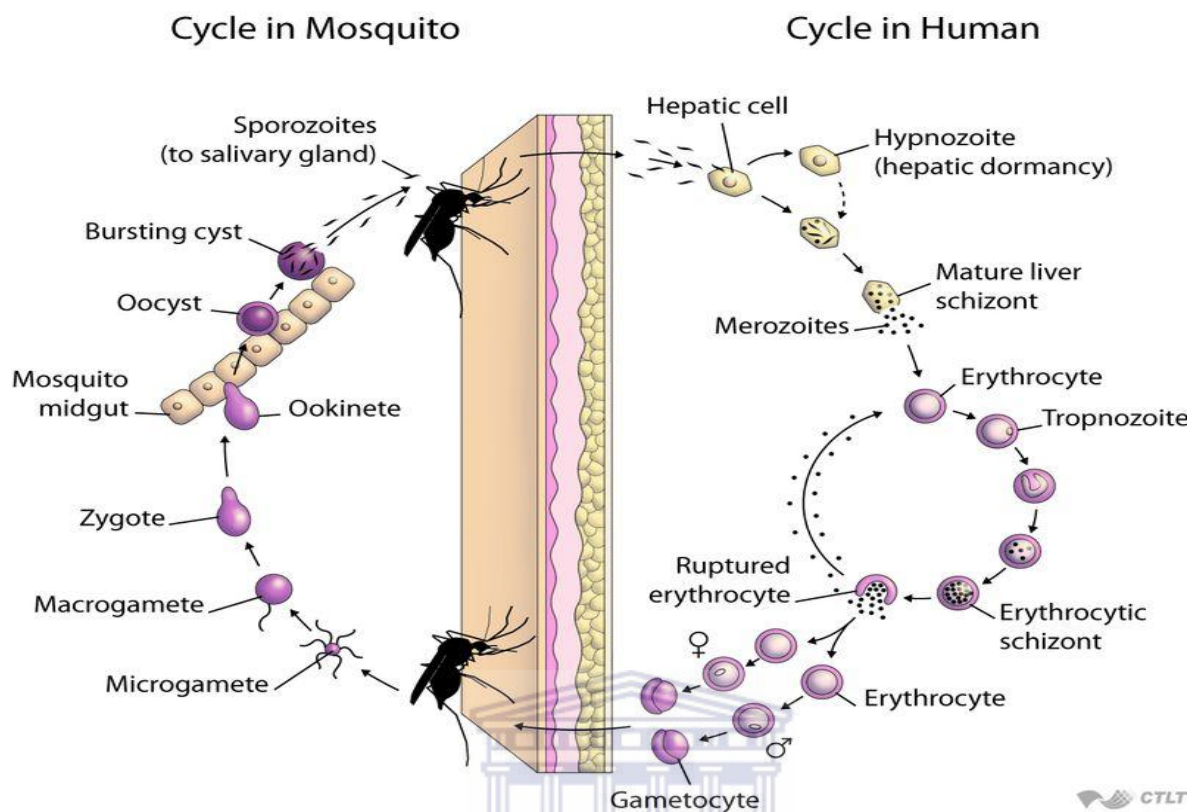


Figure 2.1: Lifecycle of *Plasmodium* parasite (Source: walwest.gr)

2.2 Clinical disease

The severity and clinical disease of the four species of *Plasmodium* varies but the symptoms exhibited, remains the same. In uncomplicated malaria, symptoms are non-specific and difficult to differentiate from febrile illnesses. Uncomplicated malaria is characterised by fever and chills, fatigue and malaise, back and limb pain and nausea (known as malaria paroxysm). These symptoms are consistent with the rupture of schizont-infected erythrocytes in the blood stream of an infected patient (Karunaweera *et al.*, 2007). Failure in prompt treatment of uncomplicated malaria is a major cause of severe malaria. Complicated or severe malaria is associated with malarial anaemia, high fever, hypoglycaemia, renal failure and cerebral malaria. Even if treated, the mortality in patients is 10 – 20 % (Wilairatana *et al.*, 1999). The most severe clinical manifestation and malaria pathology is observed in children, pregnant woman and travellers from non-malarious regions.

Malaria is diagnosed by previously described clinical symptoms and by microscopic examination of the blood smear. Stained thick- and thin blood smears are used to diagnose malaria and to quantify the level of parasitaemia, respectively. Giemsa-stained thin smears are used to differentiate between the species of parasite (Luxemburger *et al.*, 1998).

2.2. Blood-stage antimalarial agents

Malaria has been a scourge of humankind throughout history and has become a global crisis due to emergence of drug resistance to all major classes of antimalarial agents. The devastating effects of malaria have left the global population with only a handful of established and effective antimalarial agents. These include aminoquinoline, antifolates and artemisinin and related derivatives.

2.2.1. Aminoquinolines

Quinine (**8**) and its diastereomer quinidine (**9**) are alkaloids found in the bark of the cinchona tree. The therapeutic potential of quinine as an antimalarial agent was discovered during the 17th century. Dorn *et al.* (1998) demonstrated the blood schizonticidal activity of quinine after the compound inhibited the growth of *falciparum* cultures *in vitro*. It was also shown that quinine possesses good gametocytocidal activity against *falciparum* gametocytes (Chotivanich *et al.*, 2006).

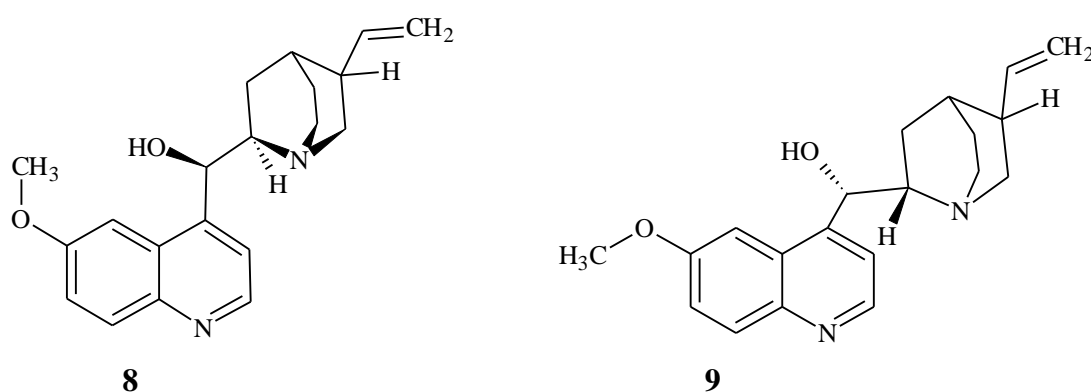


Figure 2.2: *Cinchona* bark derived antimalarial agents, quinine (**8**) and quinidine (**9**)

Methylene blue (**10**) was the first synthetic drug demonstrating intrinsic antiparasitic activity and act as CQ sensitiser by virtue of its selective inhibitory effect on the *falciparum* glutathione reductase enzyme (Schirmer *et al.*, 2003). It also served as lead compound for the development of other synthetic antimalarial agents (figure 2.3). Pamaquine (**11**) is an 8-aminoquinoline derivative developed in 1925 by structural modification to a methylene blue congener (**10a**) with a quinoline heterocycle. Since its discovery, pamaquine, in combination with quinine, demonstrated gametocytocidal activity and also prevented relapse from *vivax* malaria. However pamaquine's high toxicity eroded its therapeutic use (Peters 1999).

Currently, primaquine (**12**) is the only clinical useful tissue schizonticide (Zheng *et al.*, 1992; WHO 2011) capable of radical cure (anti-relapse therapy) against *vivax*- and *ovale* malaria. It also curbs disease transmission from the host to the *Anopholene* mosquito vector. This gametocytocidal activity is observed against all *Plasmodium* forms including CQ^R *falciparum* strains (Rieckmann *et al.*, 1968; Sutanto *et al.*, 2013). Primaquine is obtained by the replacement of the diethylamino group of pamaquine, with an unsubstituted primary amine. Further, the compound also displayed potent synergistic antimalarial activity with CQ (Pukrittayakamee *et al.*, 1994; Bray *et al.*, 2005), comparable to that of known chemosensitiser verapamil, against CQ^R *falciparum* strains *in vitro*. This resistance reversal activity is exerted by inhibition of PfCRT protein at therapeutic concentrations (Bray *et al.*, 2005). Primaquine is a poor blood schizonticide however new 8-aminoquinoline drug candidates under development demonstrated improved activity (Vennerstrom *et al.*, 1999).

Although the mechanism of action of primaquine remains unclear, literature suggests that generation of reactive oxygen species (ROS) such as superoxide- and hydroxyl radicals which interfere with mitochondrial function, are responsible for parasite killing as well as for its toxicology (Fletcher *et al.*, 1988). The latter limits both the prophylactic and therapeutic application of primaquine.

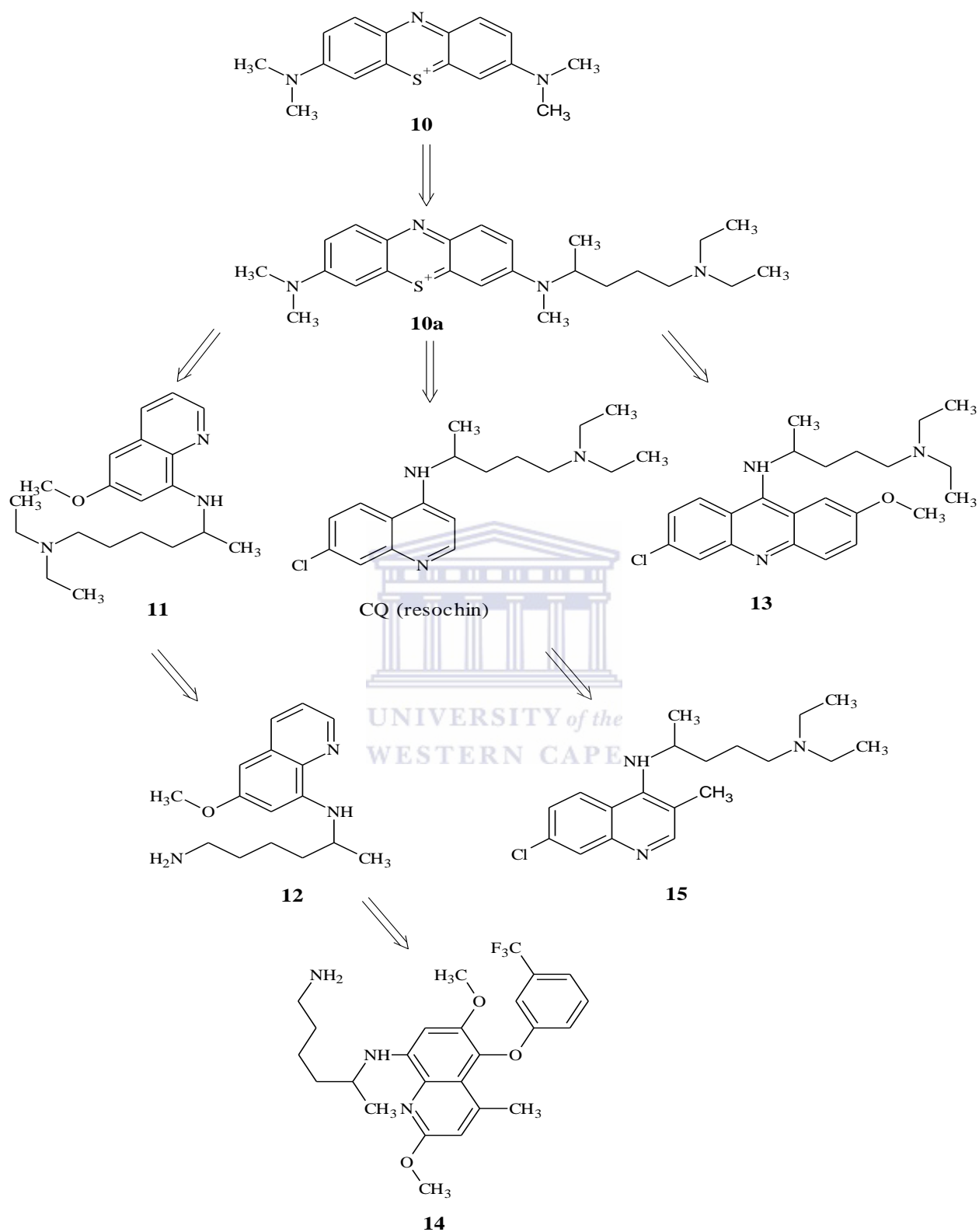


Figure 2.3: Synthetic antimalarial agents derived from methylene blue and methylene blue derivative (10a)

In Elberfeld laboratories in Germany, quinacrine or mepracrine (**13**), a 9-aminoacridine antimalarial agent marketed in 1932 as Atebrin[®] was discovered. Quinacrine was obtained by the conjugation of a diethylaminoisopentylamino side chain to the acridine heterocycle of methylene blue. The compound is active against the blood stages of *P. falciparum* but its toxicity such as discoloration of the skin and eyes (Coatney 1963) limited its use.

It was the structural modification of methyl blue derivative (**10a**) that led to the discovery of the resochin, a 4-aminoquinoline. Resochin displayed potent antiplasmodial activity but its toxicity observed during clinical trials precluded its use and it was thus abandoned for decades. However, resochin and a structurally related analogue, sontoquin or nivaquine (**15**), were re-assessed during World War II. Resochin, later known as chloroquine was found to be relatively well-tolerated and became the most successful single agent for *falciparum* malaria chemotherapy. CQ displayed limited host toxicity, excellent clinical efficacy and is obtained by simple synthesis. Despite all the success of CQ in malaria chemoprophylaxis, resistance in *P. falciparum* and *P. vivax* emerged as a consequence of the compound's heavy use during the 1950's. Chloroquine resistant *falciparum* detected in Africa during the late 1970's, devastated the continent, in terms of the resurged mortality and morbidity amongst children (Trape *et al.*, 1998). Chloroquine resistant *vivax* was only detected in Papua New Guinea in 1989 and subsequently spread to Southeast Asia and South America (Whitby 1997). In the absence of a replacement drug with the low cost, effectiveness and reliability of chloroquine, resistance became a global problem.

Tafenoquine or WR 238605 (**14**) is a 5-phenoxy analogue of chloroquine. This auxophoric substitution is responsible for the observed increase in the lipophilic profile of the drug as well for its enhanced parasite killing activity. The molecule displays improved blood schizonticidal-, anti-relapse- and sporontocidal activity over primaquine, although the gametocytocidal activity is significantly reduced (Peters *et al.*, 1993). Tafenoquine, although safer and better tolerated than primaquine, may still potentially cause haemolysis in glucose-6-phosphate dehydrogenase (G6PD) deficient patients (Brueckner *et al.*, 1998), in particular in African regions. Thus the quest for improved alternatives continued.

Amodiaquine (**16**), a 4-anilinoquinoline, structurally similar to CQ displayed superior antimalaria activity compared to CQ and retained the ability to reverse CQ^R in certain *falciparum* strains. However, further chemoprophylactic use of the compound was aborted due to observed

fatal toxicity such as hepatitis and agranulocytosis. The toxicity was dose-dependent and is believed to be caused by the amodiaquine quinone imine, an electrophilic metabolite which can bind to cellular macromolecules and initiate hypersensitivity reactions (O'Neill *et al.*, 1994).

Further quests for analogues active against *Plasmodium* malaria resulted in the development of improved quinine-related derivatives such as isoquine (**17**), pentaquine (**18**), tebuquine (**19**) and many more. Of all the compounds synthesised, mefloquine (**20**), a 2-aryl substituted chemical structure analogue of quinine showed great promise. Mefloquine is an orally available blood schizonticide and is active against CQ^R *falciparum* (Trenholme *et al.*, 1975) and *vivax* malaria infections (Schmidt *et al.*, 1978). However, in malaria prophylaxis, high doses of the compound exerted serious dose-dependent neuropsychiatric toxicity and together with the advent of resistance reported in 1990 in Thailand, the 4-quinolinemethanol derivative's therapeutic use was limited. Attempts to prevent resistance by adjuvant therapy with antifolates such as pyrimethamine and sulfadoxine were also unsuccessful (Nosten *et al.*, 1991).

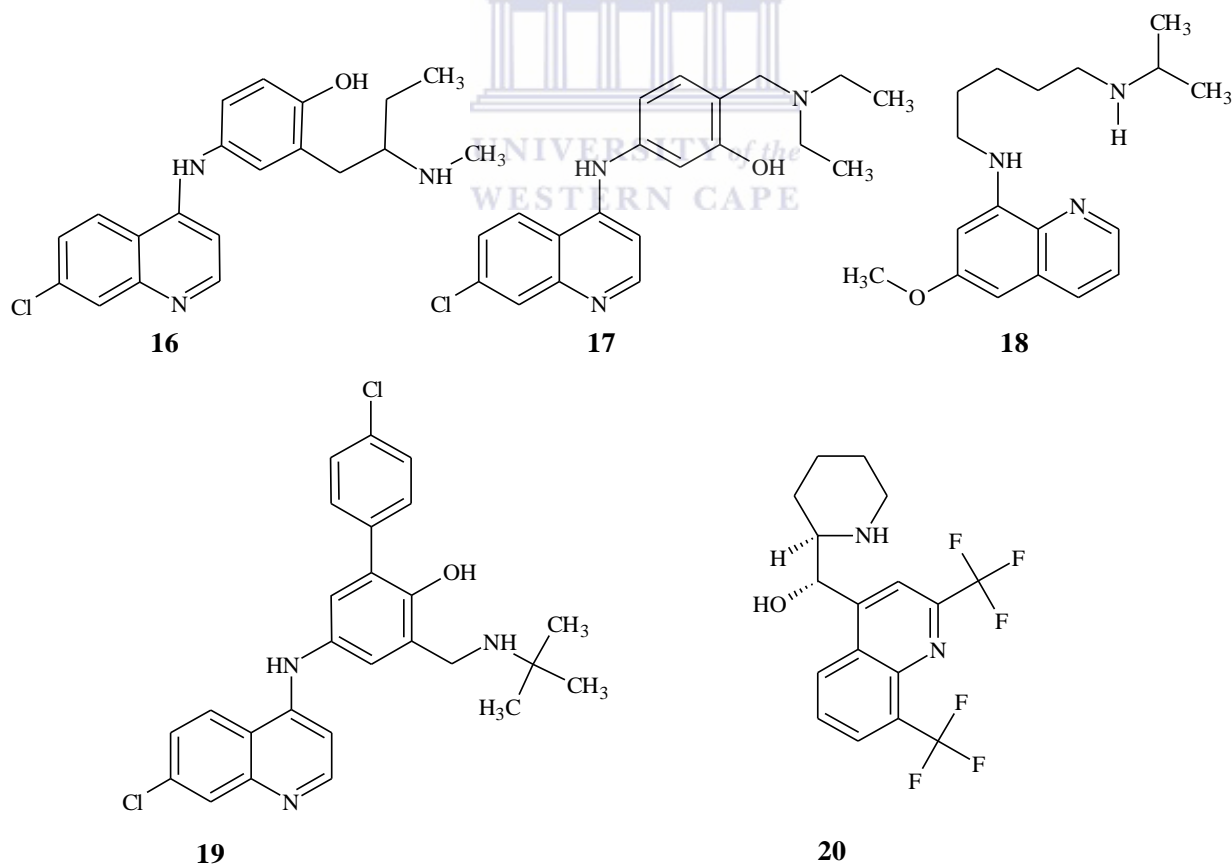


Figure 2.4: Quinoline-based antimalarial agents (**16 – 20**)

Further efforts by Walter Reed Army Institute of Research on the quinoline scaffold led to the discovery of halofantrine (**21**), a substituted phenanthrene analogue. Halofantrine is an orally available blood schizonticide, which is active against CQ^R *falciparum* (Watkins *et al.*, 1988), pyrimethamine-sulfadoxine resistant *falciparum* and *vivax* malaria (Bryson & Goa 1992), but ineffective against mefloquine-resistant *falciparum* malaria (Shanks *et al.*, 1991). The compound also displayed serious dose-dependent cardiotoxicity in patients treated for uncomplicated *falciparum* malaria (Nosten *et al.*, 1993), which as a result, limited its therapeutic use. The amino-alcohol analogue, lumefantrine (**22**), is a 2,4,7,9-substituted flourene which displays lower cardiotoxicity than the parent drug, halofantrine.

Lumefantrine is a schizonticide used in adjuvant therapy with artemether (see figure 2.8), marketed as Coartem[®]. The latter combines the rapid parasite killing of the artemisinin derivative and the slow-acting activity of lumefantrine (van Vugt *et al.*, 2000; Lefevre *et al.*, 2001). Although this promising poly-pharmacy combination has a high cure rate, it is too expensive for use in developing countries in Africa.

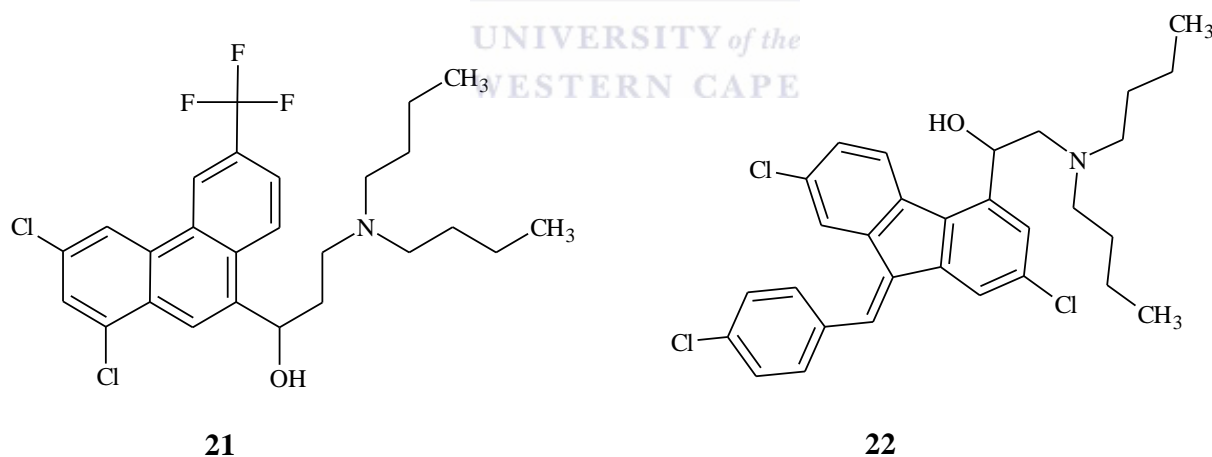


Figure 2.5: Amino-alcohol antimalarial agents (**21** – **22**)

2.2.2. Folate inhibitors

Molecules that act on folate pathways have gained considerable research interest as a result of the widespread CQ resistance. Antifolates play an important role in the asexual blood stages and in disease transmission. They target two major enzymes known as dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR). The former is a promising drug target since its only present in the *Plasmodium* parasite (Nzila *et al.*, 2005), which may allow for the development of selective inhibitors.

Sulfonamides and sulfones are antifolates that mimic *p*-aminobenzoic acid (PABA). They competitively block DHPS-catalysed formation of dihydropteroate in folate pathways. Biguanides, quinazolines, triazines and pyrimethamine are antifolates that inhibit the bifunctional enzyme, DHFR coupled with thymidylate synthase (TS) in the parasite. They block NADPH-mediated reduction of dihydrofolate (DHF) to tetrahydrofolate by DHFR (Wang *et al.*, 1997; Wang *et al.*, 1999). Tetrahydrofolate is an important co-factor for the biosynthesis of purine nucleotides, thymidylate and amino-acids (Ferone 1977, Hyde 2005b).

Sulfachrysoidine or Prontosil[®] (**23**) is an antibacterial agent developed in 1932 that was also found to inhibit parasite DHPS. Reductive cleavage of the azo-component of sulfachrysoidine yielded the bioactive sulfanilamide (**24**). However, the effectiveness of quinoline-based and other synthetic antimalarial agents decreased the usefulness of these sulfa-moieties. Interest in sulfonamides was only re-established in the 1950's when safer and improved analogues were developed such as sulfadoxine (**25**), a PABA analogue (Chulay *et al.*, 1984). Soon after the introduction of the sulfonamides, widespread resistance to this class was noted in Africa (Plowe *et al.*, 1997).

Proguanil (**26**) a biguanide prodrug and cycloguanil (**27**) its active tricyclic triazine metabolite, were good inhibitors of DHFR (Crowther & Levi 1953) and are structurally related to chlorproguanil (**28**) which is used in malaria treatment and prophylaxis. However, point mutations in parasite DHFR decreased the use of proguanil (Reeder *et al.*, 1996).

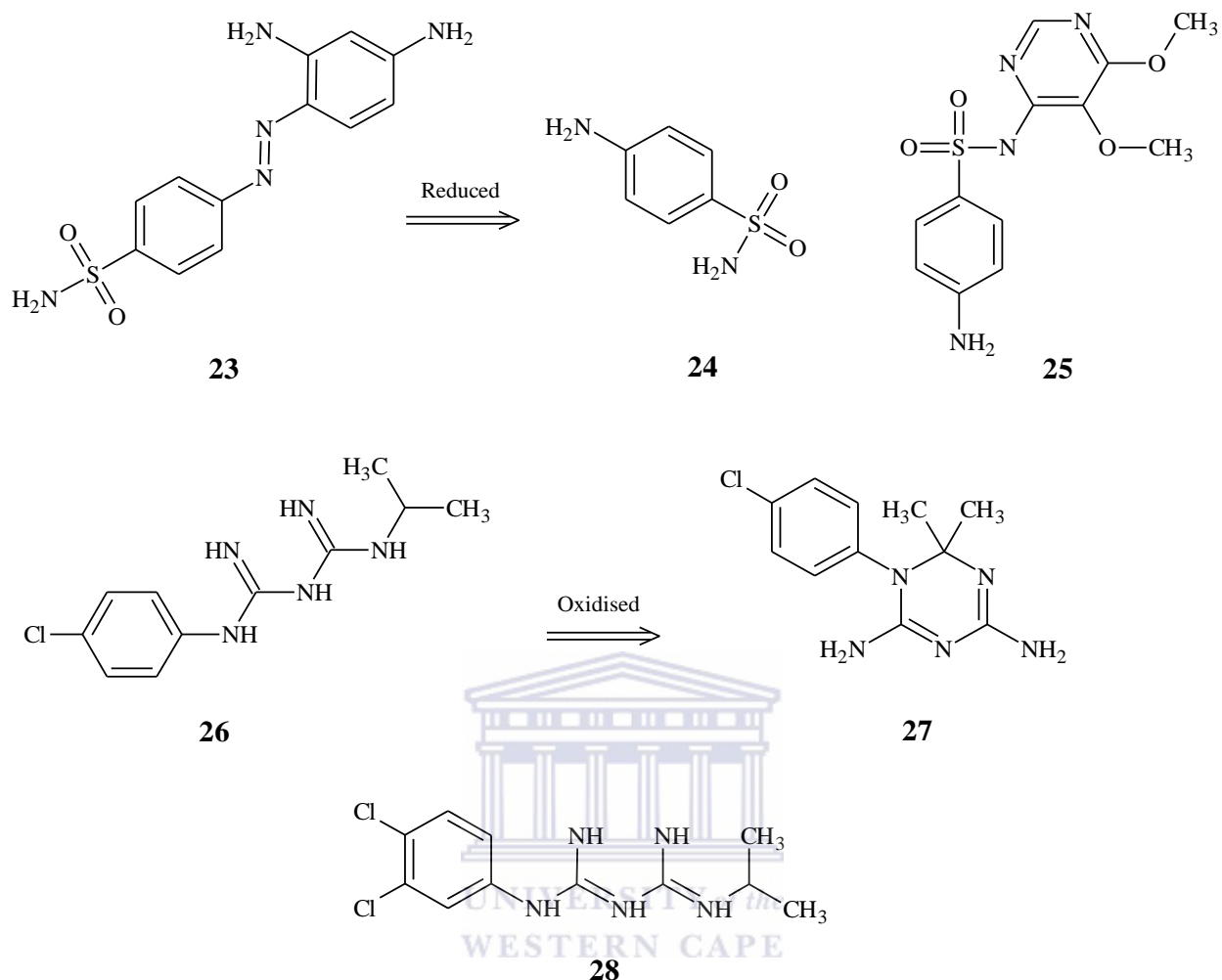


Figure 2.6: Sulfadoxine (25) and other antifolates

Combination therapy was the only available armour to slow widespread resistance in developing African countries. Fansidar[®] which consists of sulfadoxine and pyrimethamine (29), a DHFR inhibitor, demonstrated synergistic antimalarial activity against CQ^R *falciparum* strains *in vitro* (Chulay *et al.*, 1984). However, resistance observed clinically impeded further use of this combination in Africa (Wang *et al.*, 1997). An improved alternative, LapDap[®] is a novel combination comprising of dapsone (30) a leprosy drug and chlorproguanil (active metabolite chlorcycloguanil) which is effective against Fansidar[®] resistant parasites (Nzila-Mounda *et al.*, 1998). Although the combination is safe and effective, recent concerns of emerging resistance reduced its use in Africa. A fixed triple combination of LapDap[®] with artesunate is in clinical development to extend the therapeutic use of this combination (Price & Nosten 2001).

Other useful combinations include Malarone[®], which consists of proguanil and atovaquone (**31**), a hydroxynaphthoquinone. Malarone[®] is effective against blood stages (Looareesuwan *et al.*, 1999) and liver stages of the plasmodia (Berman *et al.*, 2001). Although there have been reports of resistance in Nigeria (Fivelman *et al.*, 2002), widespread use of this adjuvant therapy is restricted due to its high price.

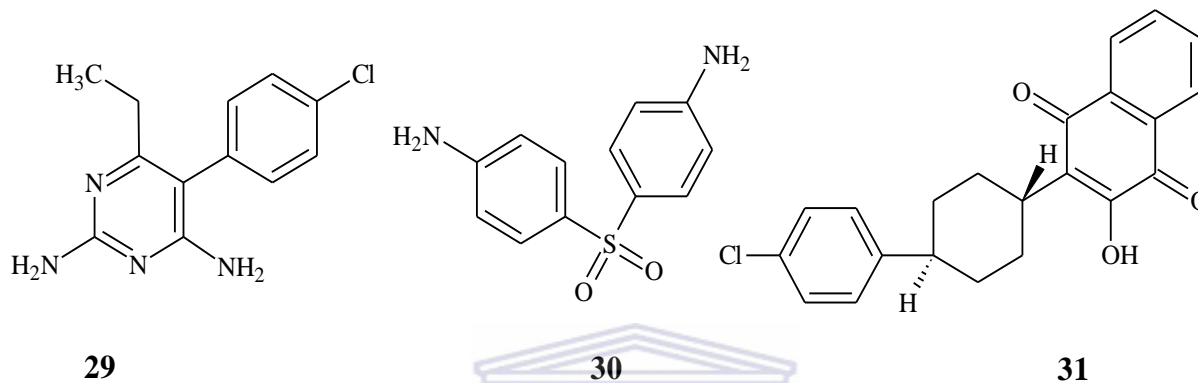


Figure 2.7: Pyrimethamine (**29**) and other synthetic antifolates (**30 – 31**)

2.2.3. Artemisinin and related agents

Artemisinin (**32**) is a sesquiterpene lactone derived from sweet wormwood (*Artemisia annua*) and has been in use since 1971 for malaria chemotherapy in China. It is rapid-acting, effective and possesses substantial antimalarial activity (Haynes & Vonwiller 1994). Its 1,2,4-trioxane system or endoperoxide bridge is responsible for its antimalarial activity although the exact mechanism and drug targets still remains unresolved.

It is suggested that artemisinin and related derivatives act by an Fe(II)-mediated cleavage of endoperoxide leading to the formation of a reactive carbon-centred radical which targets different proteins, including ferriprotoporphyrin IX (FPIX) which is alkylated and destroyed, leading to parasite death (Pandey *et al.*, 1999; Olliaro *et al.*, 2001; Meshnick 2002; Loup *et al.*, 2007). Additionally, Loup and co-workers (2007) have shown that heme-artemisinin adducts, like CQ, are also able to inhibit FPIX detoxification *in vitro* as well as *in vivo* in the presence of a *Plasmodium falciparum* histidine-rich protein (PfHRP2). PfHRP are macromolecules necessary for formation of hemozoin, an inactive biocrystal. This dual mode of action may be useful in preventing development of drug resistance. Furthermore, in a fluorescent study conducted by

Eckstein-Ludwig *et al.* (2003) artemisinin displayed an inhibitory action on the *P. falciparum* orthologue (PfATP6), a sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) calcium transporter protein in *Xenopus* oocytes. This study found that artemisinin and derivatives thereof inhibits PfATP6 outside the food vacuole after activation by Fe²⁺-species to obtain effective parasite killing. This was confirmed in a homology modelling and docking simulation study conducted by Jung *et al.* (2005). They have shown that the primary binding source between artemisinin and related derivatives includes hydrophobic interactions and that the biologically active peroxide bonds were exposed to the outside of the binding pocket.

However, efficacy of artemisinin against multi-drug resistant *falciparum* malaria is limited due to both poor lipid- and water solubility. Thus structural modification of artemisinin by Chinese researchers led to the discovery of an optimised water-soluble hemiacetal derivative dihydroartemisinin (**33**) and further optimisation yielded arteether (**34**) and artemether (**35**), the acetal derivatives. Both the acetal compounds were more potent than the parent drug, artemisinin (Haynes *et al.*, 2004; Haynes *et al.*, 2005), but their therapeutic uses were limited due to shorter plasma half-lives and fatal neurotoxicity observed in animal models (Brewer *et al.*, 1994; Nontprasert *et al.*, 2002).

Despite these derivatives' good lipophilic profile and oral availability, variable plasma levels were observed after intramuscular application of artemether. This stimulated the need for novel analogues with improved physicochemical properties and therapeutic application such as the artesunate (**36**) salt that can be administered intravenously. Artesunate is capable of rapidly reducing parasitaemia and curing cerebral malaria (Lin *et al.*, 1989), however the compound is rapidly biotransformed to dihydroartemisinin, a reduced lactol. This metabolite is associated with a high rate of recrudescence i.e. reoccurrence of asexual parasitaemia and for the artemisinin-derivatives' short half-lives and neurotoxicity (Wesche *et al.*, 1994). This neurotoxicity is more serious in artelinate (**37**), a more potent and potential successor of artesunate. Nonetheless, no further attempt was made to optimise artelinate and it was thus abandoned.

Combining the rapid action of artemisinins with the longer acting non-artemisinins yielded improved and well-tolerated artemisinin-combined therapies (ACTs). However, despite the successes of ACTs such as Coartem[®], concerns of resistance are emerging and the problem is

compounded by combining drugs with unmatched pharmacokinetic profiles (Schlitzer 2007). Other fixed dose combinations include artesunate/mefloquine and artesunate/pyronaridine.

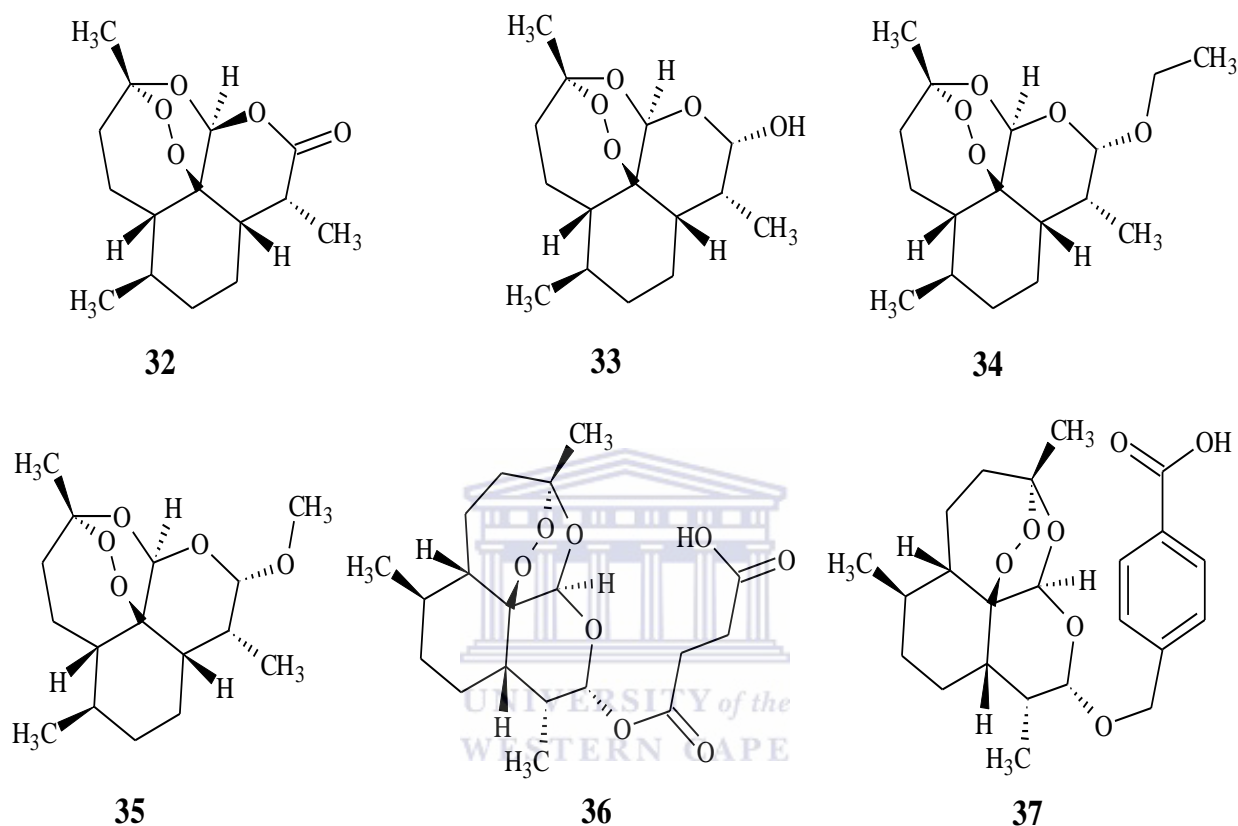


Figure 2.8: Artemisinin (32) and first generation semi-synthetic artemisinins (33 – 37)

Ongoing research on the sesquiterpene lactone scaffold yielded a promising series of second generation semi-synthetic, 10-alkylamino artemisinins. Artemisone (38) is an thiomorpholino-*S,S*-dioxide derivative that due to the polar heterocycle inclusion displayed improved pharmacokinetic properties and lacks neurotoxicity. This promising antimalarial agent demonstrated a 4 fold increase in effectiveness over artesunate and has emerged as a promising molecule for further clinical development (Haynes *et al.*, 2004; Haynes *et al.*, 2005).

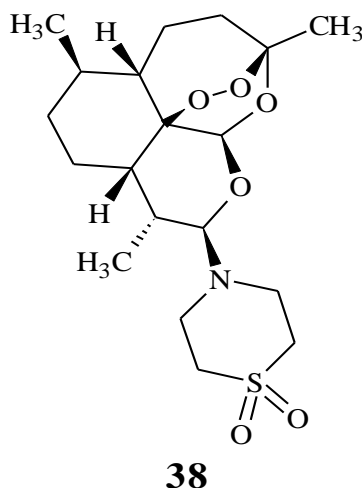


Figure 2.9: Second-generation semi-synthetic artemisinin, artemisone (**38**)

2.3. Chloroquine (CQ): mechanism of action and resistance

CQ was the mainstay chemotherapeutic agent against blood stage malaria which was inexpensive, safe and effective. However, with advent of resistant plasmodia, the therapeutic use of CQ declined in many malaria-endemic regions. In the absence of a suitable replacement agent with the reliability and low-cost of CQ, resistant plasmodia became a widespread problem, in particular in third-world countries. In spite of CQ's successes, its exact mechanism against *Plasmodium* malaria stills remains controversial and its mechanism of resistance is even more elusive.

2.3.1. Potential mechanism of action

a. Alteration of pH in parasite digestive vacuole

During the blood stages, host erythrocytes are invaded and haemoglobin is transported into the acidic food vacuole of the parasite. CQ is a diprotic weak base ($pK_{a1} = 8.1$; $pK_{a2} = 10.2$) that in its unprotonated form diffuses freely through erythrocyte membranes into parasite cytoplasm (pH 7.4). Once in the acidic compartment i.e. food vacuole of parasite (pH 5.2 – 5.4), it becomes diprotonated, membrane impermeable and accumulates.

This vacuolar increase in pH interferes with parasite metabolic processes which eventually result in parasite killing *via* weak-base mechanism (Krogstad *et al.*, 1985; Yayon *et al.*, 1984).

b. Blockage of haematin (FPIX) detoxification

Haemoglobin enclosed in parasite food vacuole is degraded by a cascade of proteolytic enzymes yielding small peptides, free aminoacids (which is absorbed by parasite), and free toxic haem. Oxidation of the central iron yields ferriprotoporphyrin IX (FPIX) radicals which destabilises and lyses parasite membranes. The parasite detoxifies toxic haem by forming FPIX polymers (or dimers) which is then converted into non-toxic hemozoin. CQ acts to block haem detoxification, by complexation with both free toxic haem and FPIX. These complexes interfere with parasite membrane function resulting in reduced haemoglobin degradation and blocks further sequestration of toxic haem, leading to a build-up of toxic haem (Fitch *et al.*, 1982; Goldberg *et al.*, 1990; Sullivan *et al.*, 1996; De *et al.*, 1998 & Egan *et al.*, 2000).

c. Blockage of glutathione-dependent haematin degradation

During oxidation of free haem, a pro-oxidant, from Fe^{2+} to Fe^{3+} , hydrogen peroxide (H_2O_2) and other oxygen radicals are generated leading to oxidative stress in the parasite food vacuole (Postma *et al.*, 1996). Host derived peroxidase and catalase enzymes neutralises H_2O_2 but is also rapidly inactivated by parasite proteolytic enzymes.

Further, glutathione peroxidase and catalase oxidant defence enzymes in eukaryotic cells such as *Plasmodium* parasites also destroy haem in the parasite cytoplasm. CQ and probably CQ-haem- and CQ-FPIX complexes inhibit glutathione peroxidase- and catalase-mediated detoxification of haem thus prolonging H_2O_2 -mediated toxicity leading to irreversible parasite lipid peroxidation and protein damage (Ginsburg *et al.*, 1998; De *et al.*, 1998 & Loria *et al.*, 1999). Although this proposed mechanism was contradicted by Egan *et al.* (2000), it appears that both oxidative and glutathione-mediated haem degradation as well as haem detoxification is inhibited by CQ and certain related aminoquinolines.

Further, despite the widespread chloroquine-resistance in malaria-endemic regions, the quest for novel molecules that act *via* CQ's proposed mechanisms but for which there is no resistance continues.

2.3.2. Mechanism of resistance

CQ^R *Plasmodium* was originally detected in Cambodia-Thailand border, the ‘epicentre’ of resistance and gradually spread to Africa during the 1980’s (Dondorp *et al.*, 2009). Today, CQ is only effective in a handful of countries. Although, the exact mechanism of resistance is poorly understood, some studies demonstrated the involvement of point mutation in the chloroquine transporter protein and an energy dependant efflux pump. Both these key proteins are localised to the membrane of the parasite’s digestive vacuole.

a. *P. falciparum* CQ transporter protein

In CQ^R strains, CQ is removed from its proposed site of action, the digestive food vacuole. Resistance is primarily caused by point mutation in a 10-transmembrane domain transport protein, PfCRT, which is encoded by a *P. falciparum* CQ resistance transporter gene. This transporter protein belongs to the drug and metabolite transporter (DMT) superfamily. In all CQ^R strains, the lysine at position 76 is replaced by a threonine residue in the protein. The K76T mutation induces a neutral charge on the transport membrane allowing access of protonated CQ into cytoplasm, which reduces CQ concentration in the food vacuole. Mutant PfCRT thus acts as a gated channel or pore that causes leakages of dicationic CQ out of parasite digestive vacuole (Lakshmanan *et al.*, 2005; Bray *et al.*, 2006). Furthermore, in CQ^R strains elevated levels of glutathione was observed which further reduces the activity of CQ. However, this limitation can be overcome by the incorporation of glutathione reductase inhibitor (Ginsburg 1998) such as methylene blue (Färber *et al.*, 1998). As a result of these mutations, CQ is unable to block haem polymerisation and glutathione-mediated haem degradation.

b. *p*-Glycoprotein efflux pump

The ATP-powered multidrug efflux pump, *p*-glycoprotein (pGP) of the superfamily, ATP-binding cassette transporters, is encoded by multidrug resistant (MDR) genes. This is the same gene implicated in cancer cell lines. pGP is capable of expelling a variety of structurally and functionally unrelated molecules (Riordan *et al.*, 1985). During blood stages of the *falciparum* parasite, pGP-1, a gene product of MDR (*pfmdr1* gene) is expressed. This *pfmdr1* gene or pGP1 has been implicated in mefloquine-, halofantrine- and quinine resistance (Price *et al.*, 1999). Its involvement in CQ^R however remains unsubstantiated (Sidhu *et al.*, 2006).

Some studies suggest that it may play a compensatory role in both laboratory cell-lines (Price *et al.*, 1999) and CQ^R field isolates under CQ pressure (Mita *et al.*, 2006). Conversely, others studies found that expression of the *pfmdr1* gene in a large number of *falciparum* strains were not responsible for all reported CQ^R cases (Price *et al.*, 1999). The reduced accumulation of CQ in the food vacuole was postulated to be due to loss of an intracellular receptor (Chou *et al.*, 1980), changes in vacuolar pH (Ursos & Roepe 2002), changes in import of CQ (Sanchez *et al.*, 1997) and nuclear receptor-inducible gene regulation (Johnson *et al.*, 2008) instead of a drug transporter (Djimé *et al.*, 2001; Warhurst 2003; Zhang *et al.*, 2004).

2.3.3. Potential *P. falciparum* resistance reversal agents

It is well documented that molecules that lack intrinsic antiplasmodial activity and that are structurally unrelated to known malaria chemotherapeutic agents may be capable of restoring the efficacy of previously useful malaria therapeutics such as CQ and pyrimethamine. Although the exact mechanisms of these agents are controversial and unresolved, their therapeutic uses are necessitated in countries that are in dire need of a cure.

a. Calcium channel blockers (CCB)

Verapamil (**39**) is an *L*-type CCB of the class phenylalkylamines that is used in cardiovascular diseases. This compound is a well-studied chemosensitiser (Martiney *et al.*, 1995) used to reverse multidrug resistance in tumor cell lines (Miller *et al.*, 1991) and in CQ^R parasites but had no effect against CQ^S parasites at the same concentration of 1µM (Martin *et al.*, 1987).

It is suggested that the mutated parasite PfCRT protein affects drug-receptor interaction, drug-haem complex formation and drug accumulation by altering pGP-mediated CQ flux across the food vacuole membrane. Verapamil and related lipophilic derivatives synergistically with CQ reverse resistance in CQ-resistant *falciparum* isolates by interacting with the multidrug resistant glycoprotein pump (Zuguang *et al.*, 1988; Sidhu *et al.*, 2002).

Severe toxicity in humans limits the use of racemic verapamil and closely related analogues. However, this toxicity is restricted only to the S- (–) isomers of the drugs which shows stereospecificity for cardiovascular calcium channels. The R- (+) isomers of the drugs lack this

activity (Zuguang *et al.*, 1988), suggesting promising leads for further development and use. Other CCB that may potentiate CQ antiplasmodial activity in resistant strains include nimodipine (**40**) and nifedipine (**41**).

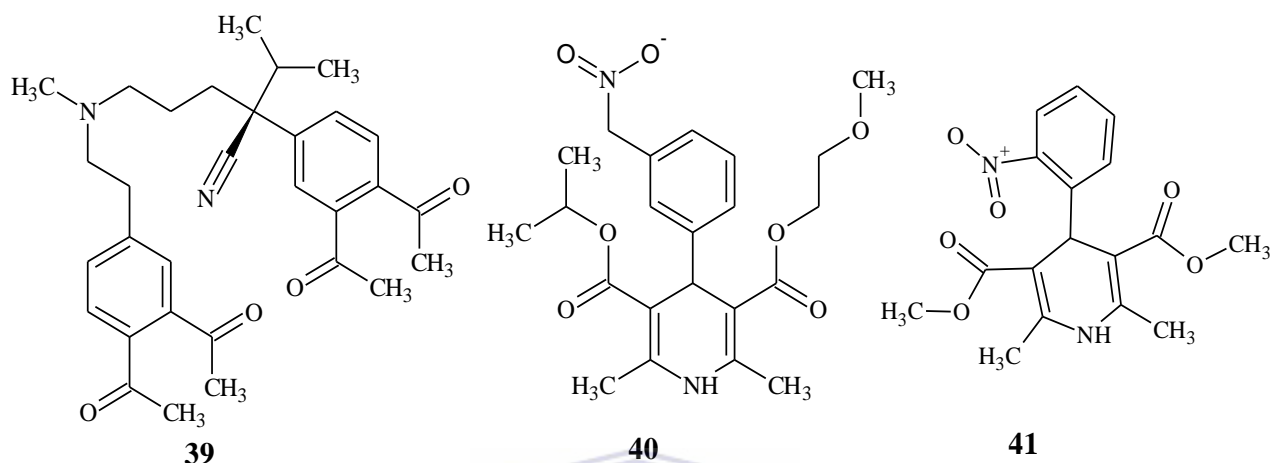


Figure 2.10: Representative CCB capable of reversing CQ-resistance (**39 – 41**)

b. Polycyclic amines

Polycyclic scaffolds have been employed in the design and development of numerous lead compounds ranging from antimalarial- (Vennerstrom *et al.*, 2004; Solaja *et al.*, 2008; Harikishore *et al.*, 2013) to anti-HIV-1 (El-Emam *et al.*, 2004) agents. These polycyclic amines such as adamantylamines and aza/oxa pentacycloundecylamines (PCU) are well-known scaffolds capable of modifying the lipophilic profile of conjugated parent compounds. They possess good calcium channel modulation (Malan *et al.*, 2003) and antiviral activity (Oliver *et al.*, 1991).

The prototype oxa-PCU, NGP1-01 (**7**), displayed similar CCB activity to nimodipine, a known CCB (Van der Schyf *et al.*, 1986). Nimodipine is also a known chemosensitizer used to reverse multidrug resistance in both *Plasmodium*- and cancer cell lines. It is believed that all CCB possess this MDR reversal property although the exact mechanism remains elusive. NGP1-01 and amantadine display remarkable structural similarity (Oliver *et al.*, 1999) and both compounds may thus be exploited as potential antimalarial and/or resistance reversal agents.

Further, by employing a tritiated hypoxanthine uptake method, amantadine (**5**) demonstrated activity against CQ^R FCR-3 *P. falciparum* strains ($IC_{50} = 5.35 \pm 1.15 \mu M$) *in vitro*. It was also

observed that amantadine in combination with CQ exerted slight synergistic activity in CQ^S and CQ^R isolates (Evan & Havlik 1993). The compound also displayed the ability to potentiate the activity of both quinine and CQ in resistant strains (Evan & Havlik 1994). Amantadine acts primarily by modulating membrane properties (Evan & Havlik 1996) altering lipid-protein interaction leading to ion-leakage from the digestive food vacuole and resulting in parasite killing (Miller *et al.*, 1983).

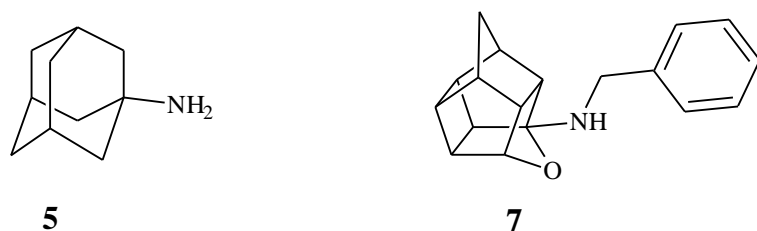
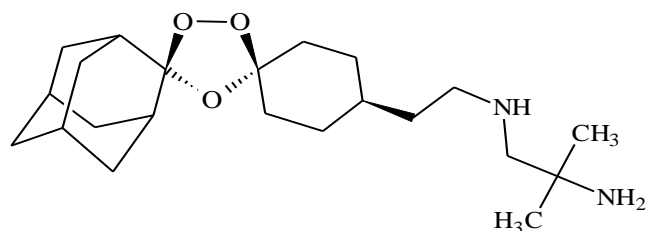


Figure 2.11: Representative potential reversal agents, amantadine (5) and NGP1-01 (7)

Recently, spiroadamantane 1,2,4-trioxolanes were obtained by an acid-catalysed condensation of adamantanone with β -hydroxyperoxides, incorporating a spiroadamantane peroxide (Griesbeck *et al.*, 2005). Optimisation of these compounds led to the discovery of the ozonide, arterolane (OZ277) (42), which demonstrated comparable activity to artemether (35) when evaluated against *P. falciparum in vitro*. Ozonides are highly reactive intermediates of the ozonolysis reaction (Griesbaum *et al.*, 1997) utilising *O*-methyl-2-adamantanone oxime as precursor with a suitable ketone (Griesbeck *et al.*, 2005). When arterolane was evaluated against a CQ-resistant isolate ($IC_{50} = 0.47$ nM) from Gabon, the compound displayed superior activity compared to other known endoperoxide antimalaria agents (Kreidenweis *et al.*, 2006). The compound is currently in phase II clinical trials.



OZ277 (arterolane) 42

Figure 2.12: Spiroadamantane 1,2,4-trioxolane antimalarial agent, arterolane (42).

In another study, Harikishore *et al.* (2013) synthesised a novel series of adamantylamide compounds that inhibited the activity of *P. falciparum* FK506 binding protein (PfFKBP) in the nanomolar ranges *in vitro*. PfFKBP is a member of the FKBP family that mediates protein-protein interactions regulating various physiological processes such as neurotrophic activity, receptor signalling, protein stability, calcium homeostasis and malaria. The series of compounds comprised of supradamal (**43**) and related derivatives (**44** – **45**) that also inhibited *P. falciparum* 3D7 strain and parasite trophozoite stages. However, PfFKBP is highly homologous to the human FKBP family members such as FKBP12 and FKBP 51 and further studies are required to obtain selective inhibitors of *Plasmodium* FKBP's.

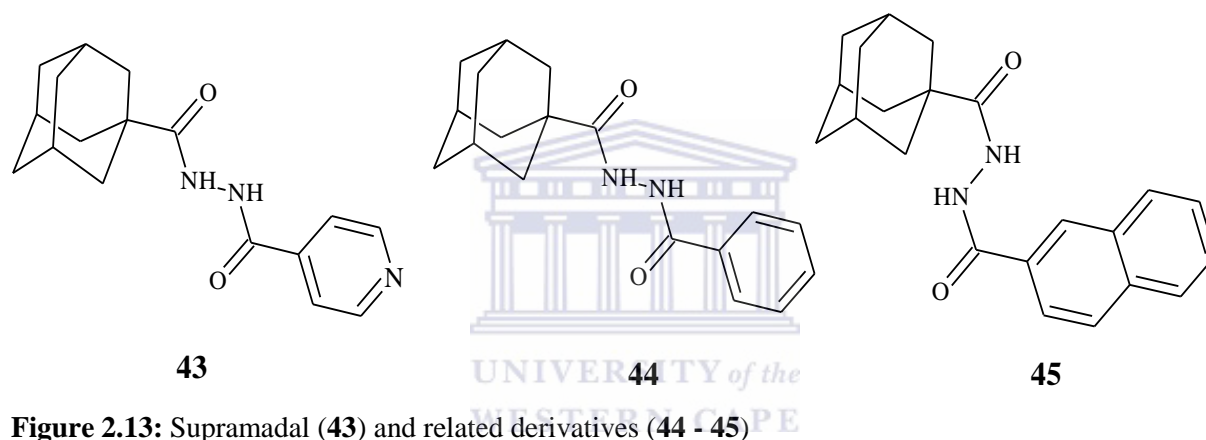


Figure 2.13: Supramadal (**43**) and related derivatives (**44** - **45**)

c. Antihistaminic agents

Chlorpheniramine is a histamine H₁ receptor antagonist that has been extensively studied in combination with known malaria chemotherapeutic agents such as CQ.

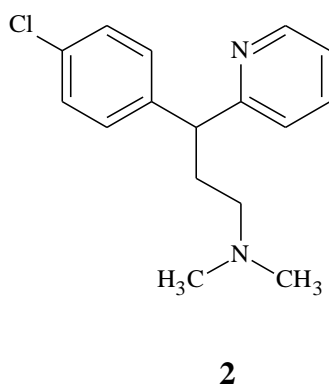


Figure 2.14: Chlorpheniramine (**2**), an antihistaminic agent

This first-generation alkylamine antihistamine has been shown to increase the efficacy of CQ in uncomplicated *P. falciparum* malaria treatment amongst children in Nigeria. This compound also displayed similar CQ-resistance reversal activity to that of verapamil against parasite isolates (Sowunmi & Oduola *et al.*, 1997; Sowunmi *et al.*, 1997). Patients treated with pyrimethamine-sulfadoxine in combination with CQ and chlorpheniramine displayed reduced gametocytaemia compared to pyrimethamine-sulfadoxine used alone (Sowunmi *et al.*, 1998).

In a kinetic study conducted by Okonkwo *et al.* (1999), treatment with a chlorpheniramine-CQ combination increased parasite clearance and increased cure rate compared to CQ monotherapy. Combinations of this molecule were well-tolerated however its therapeutic use still remains unclear.

Approaches such as systematic high-throughput screening led to the identification of a non-sedating antihistamine, astemizole (**46**), as antimalarial lead compound (Chong *et al.*, 2006). The compound displayed *in vitro* activity against three *falciparum* parasite strains in the micromolar ranges but weaker than that of CQ. After oral dosing, astemizole is converted to a more active metabolite *o*-desmethyastemizole which has a 95% oral bioavailability. Desmethyastemizole (**47**) in an *in vivo* antimalarial test, displayed a higher reduction in parasitaemia compared to astemizole, in mice infected with CQ^S *P. vinckei* strains. However, when both these agents were evaluated against CQ^R *P. yoelii* infected mice, their activity was equipotent and recrudescence was observed after treatment was stopped.

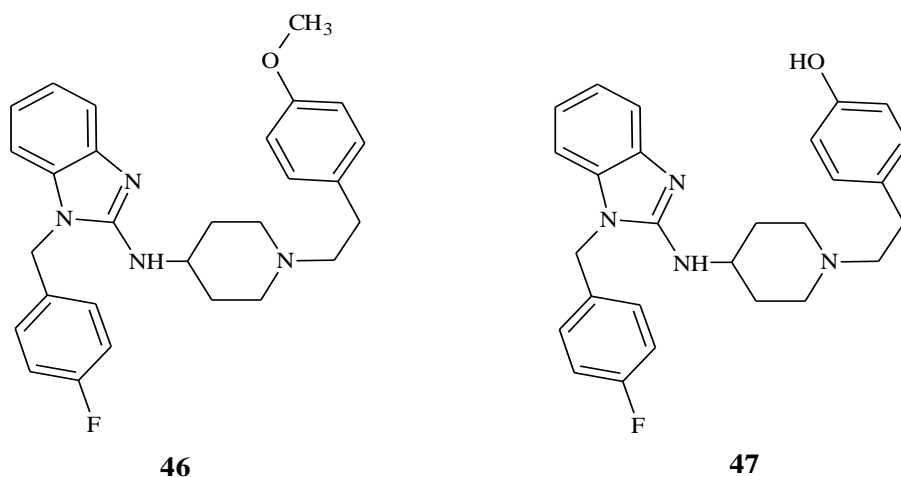


Figure 2.15: Antihistamine, astemizole (**46**) and its *o*-desmethyl derivative (**47**)

2.4. Quinoline-based antimalarial agents

Recent studies in rational antimalarial drug design appear to endorse hybrid molecules as the next-generation antimalarial agents. One such approach involves the hybridisation of two diverse pharmacophoric molecules into a single hybrid agent which can be further optimised for clinical use.

A novel series of trioxaquine (figure 2.16) antimalarial agents were developed by Singh, Malik & Puri (2004) which consists of a 1,2,4-trioxane linked *via* an intermediate chain to an aminoquinoline moiety. These hybrids combine the haem alkylation of the trioxane moiety, and haem stacking and inhibition of haem polymerisation of the aminoquinoline portion. After oral dosing, the trioxaquine hybrid molecules were more active than the parent trioxane and 4-aminoquinoline, when evaluated against MDR *P. yoelii* in a mice model. The antimalarial activity of trioxaquine molecules may potentially be additive or synergistic. However, the trioxaquine hybrid molecules displayed stability problems and poor water- and lipid solubility. Other derivatives of trioxaquinines also displayed excellent antimalarial activity against CQ^S and CQ^R *falciparum* strains (IC₅₀ = 5 – 19 nM) *in vitro* and cleared parasitaemia in *P. vinckei* infected mice after intraperitoneal administration of 20 mg/kg/day (Dechy-Cabaret *et al.*, 2004).

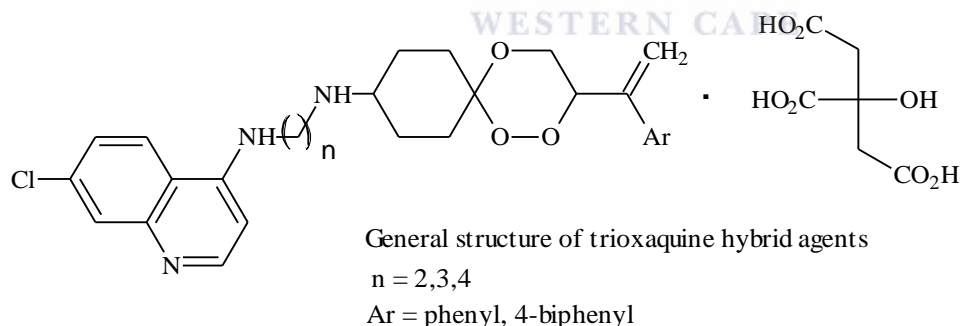


Figure 2.16: Representative structure of trioxaquine hybrid agents

In other multifunctional drug design approaches the endoperoxide core was covalently linked to quinoline entities *via* esterification. The reduced lactol dihydroartemisinin was hybridised with a modified quinine portion to obtain an ester ‘mutual prodrug’ hybrid (**48**) (figure 2.17). This hybrid molecule displayed a 3 fold increase in activity compared to the 1:1 mixture of quinine and artemisinin, indicating preserved parasite killing. The compound also inhibited growth of *falciparum* 3D7 cultures at a lower concentration compared to the parent compounds alone

(Walsh *et al.*, 2007). Recently, Araújo *et al.* (2009) synthesised a series of 1,2,4-trioxolaquine hybrid antimalarial agents that are related to trioxaquinines but more potent. A novel hybrid (**49**) (figure 2.18) demonstrated improved *in vitro* antiparasitic activity compared to both CQ and artemisinin against *falciparum* isolates.

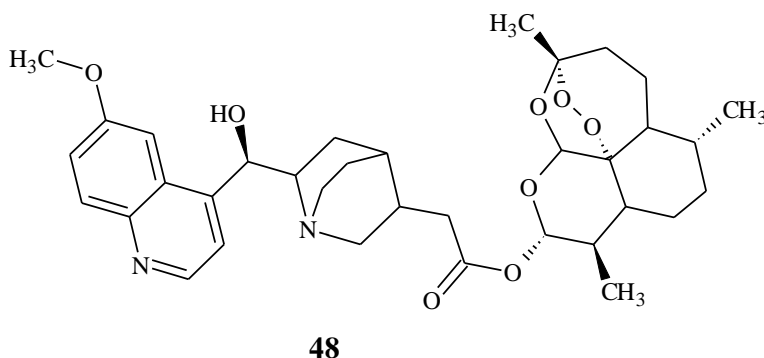


Figure 2.17: Artemisinin-quinine ‘mutual prodrug’ hybrid prototype

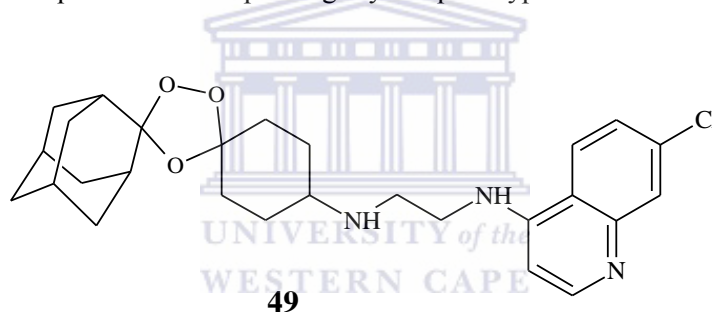


Figure 2.18: Potent trioxolaquine hybrid antimalarial agent

Burgess *et al.* (2006) worked on a similar multi-functional approach, known as RCQ molecules. The prototype consisted of a CQ-like aminoquinoline and imipramine a known antidepressant and well-studied chemosensitiser. This aminoquinoline-imipramine hybrid (**50**) (figure 2.19) molecule is effective against both CQ^S (IC₅₀ = 3 nM) and CQ^R (IC₅₀ = 5 nM) *falciparum* strains *in vitro*, and in an animal model cured *P. chabaudi* infected mice, after oral dosing (99 % suppression) with no toxicity. This quinoline-chemosensitiser dual inhibitor exerted its effects by inhibition of PfCRT protein and pGP pump which are associated with increased CQ export. Despite the novel derivative’s potent activity and efficacy, the molecule was too lipophilic (CLogP = 8.9) and may exhibit potential bio-availability problems.

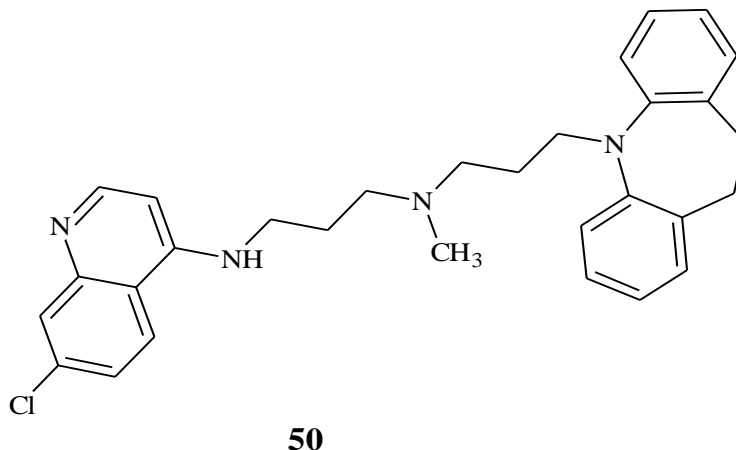
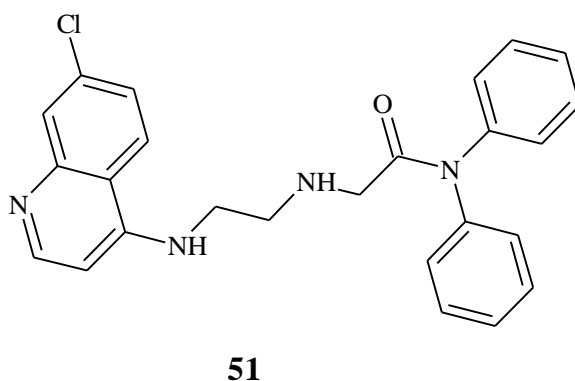


Figure 2.19: Aminoquinoline-imipramine hybrid antimalarial agent

Andrews *et al.* (2010), attempted to optimise the RCQ molecule's features by systematic structural modification. All 12 compounds synthesised in their study displayed activity ($IC_{50} \leq 125$ nM) against D6 (CQ^S) and Dd2 (CQ^R) *falciparum* strains. Only a few of these compounds displayed an improved lipid-solubility profile while retaining their activity (figure 2.20).

The lipophilicity indicator or ClogP should not be more than 5 in order to facilitate good biological membrane penetration for a drug to be orally bio-available (Lipinski 2002). The reduced ClogP of these dibenzylamide antimalarial agents, in particular compound **51**, may thus serve as lead in the quest for novel orally available antimalarial agents.



ClogP 5.2, CQ^S ($IC_{50} = 5$ nM), CQ^R ($IC_{50} = 13$ nM)

Figure 2.20: Optimised dibenzylamide antimalarial (**51**) agent

The quest for improved antiplasmodial agents led to investigation of adamantyl moieties. The adamantylated 4-amino-7-chloroquinoline hybrid (**52**) (figure 2.21) was evaluated against multidrug resistant *falciparum* TM91C23 strain ($IC_{50} = 7.39$ nM) *in vitro* and displayed a 17-fold increase in activity compared to CQ ($IC_{50} = 124.24$ nM; Solaja *et al.*, 2008).

The lysosomotropic hypothesis of antimalarial drug action postulates that amantadine, a weak base and amphiphilic agent, is trapped by a similar mechanism to CQ in the parasite food vacuole. In this lysosome-like environment, the weak base is protonated and hence membrane impermeable. This leads to an increase in vacuolar pH which blocks haemoglobin metabolism by the parasite that result in parasite killing in erythrocytes (Miller *et al.*, 1983; Evan & Havlik 1993). Amantadine presumably also directly interacts with a lipophilic pocket (Evan & Havlik 1996) within PfCRT as another possible mode of action. However, due to its kinetic profile amantadine requires unacceptably high concentrations to exert useful antiplasmodial activity as a single agent and this may limit its use.

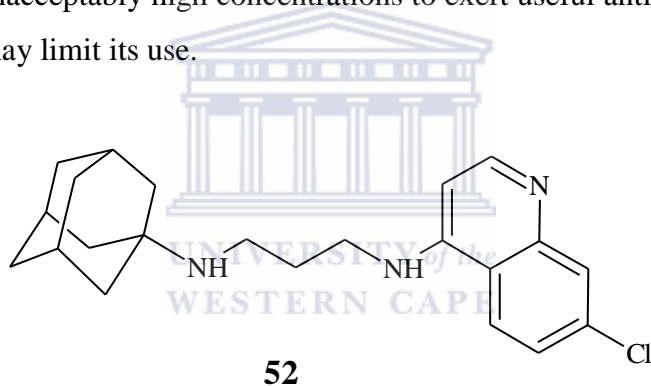


Figure 2.21: Representative adamantyl-aminoquinoline hybrid (**52**)

Isatin functionalised with electrophilic groups are known *falciparum* cysteine proteases- or falcipain-2 inhibitors that prevent haemoglobin degradation by parasites. Chiyanzu *et al.* (2005) incorporated this scaffolds' unique antiplasmodial activity and designed a series of aminoquinoline-based isatin derivatives. Electrophilic groups such as the thiosemicarbazone moiety located in these aminoquinoline-based isatin hybrids provided reactive sites (imine and thiol carbonyl) for alkylation/arylation of the enzyme cysteine thiolate.

All hybrids in this series displayed good antiplasmodial activity while two derivatives displayed activity superior to that of CQ. These two quinoline-ethylene isatin (**53** – **54**) derivatives were active against both CQ^R- and CQ^S strains of *falciparum*, suggesting involvement of other mechanisms and/or mode of parasite killing apart from falcipain-2 inhibition.

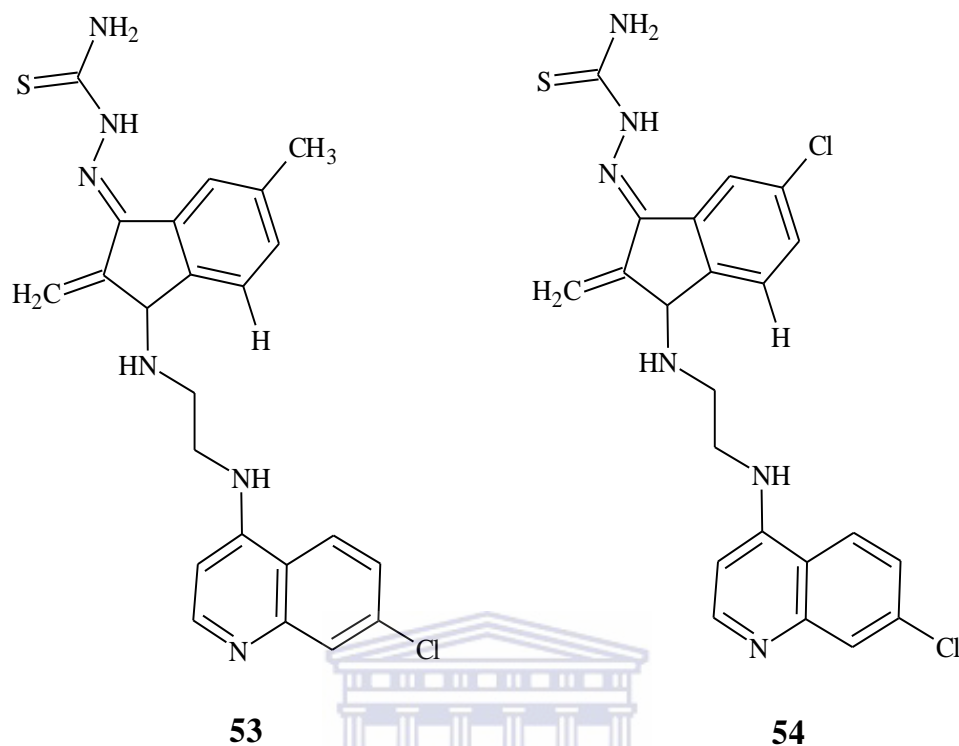


Figure 2.22: Representative quinoline-ethylene isatin hybrid molecules (**53** – **54**)

In continuing with theme of hybrid molecules as multi-therapeutic strategies for malaria chemotherapy, the squaric (**55**) scaffold was evaluated. Ribeiro and co-workers expanded on a series of squaric-quinoline conjugates and screened them against erythrocytic parasites *in vitro*. A few of these derivatives (**56** – **57**) displayed antiplasmodial activity superior to that of CQ, against CQ^R *falciparum* W2 isolates with no toxicity. However, a mixture of CQ:squaric acid in a ratio of 1:1 displayed activity weaker than some hybrids but equipotent to CQ. The 2:1 mixture displayed activity comparable to that of the potent hybrids suggesting that the aminoquinoline moiety plays a significant role and that the squaric scaffold act by some other mechanism, probably as chemosensitiser (Ribeiro *et al.*, 2013).

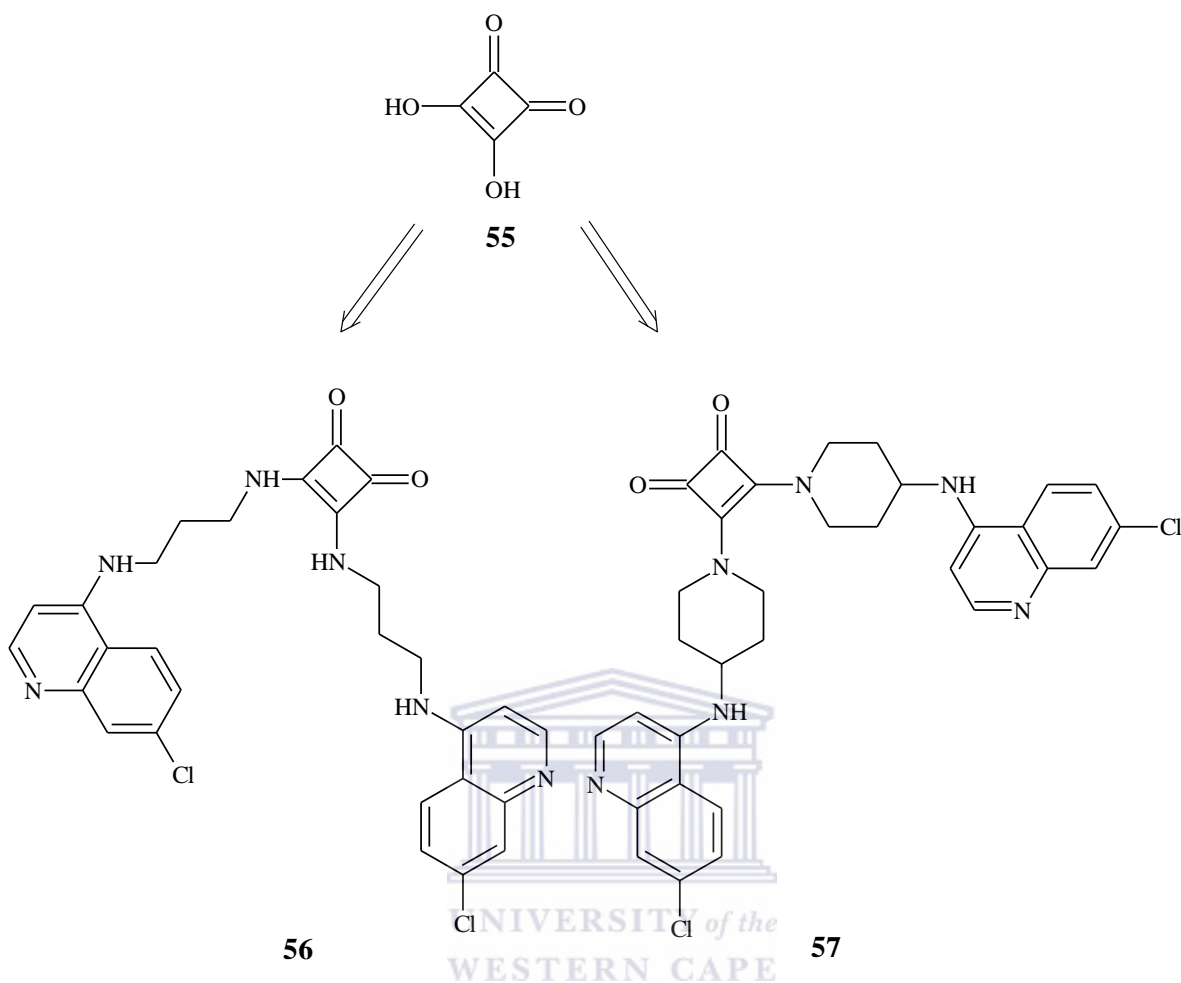


Figure 2.23: Squaric acid (**55**) and squaric-aminoquinoline hybrid molecules (**56 – 57**)

2.5. Conclusion

Malaria has been a scourge of social and economic burden and is currently emerging as a global problem. The alarming escalation of parasites resistant to commercially available malaria chemotherapeutic agents has left the global population with a handful of effective drugs. The challenge of resistance raised further concerns due to recent reports of resistance to artemisinin-combined therapies (ACTs), which is currently the mainstay antimalarial treatment option in endemic regions (Noedl *et al.*, 2008; Dondorp *et al.*, 2009). The identification of viable drug targets against the *Plasmodium* has thus gained considerable research interest.

Viable options for rational drug design includes the mutant PfcRT (Howard *et al.*, 2002 & Sanchez *et al.*, 2005), a transmembrane protein that is related to another target, the MDR

glycoprotein pump (pGP) (Zuguang *et al.*, 1988; Sidhu *et al.*, 2002), and cysteine proteases/falcipain (Rosenthal *et al.*, 1996; Na *et al.*, 2004), a critical parasite haemoglobolytic enzyme. Other potential drug targets include *Plasmodium* binding pockets such as PfATP6, PfHRP2 and PfFKBP (Jung *et al.*, 2003; Eckstein-Ludwig *et al.*, 2003; Loup *et al.*, 2007 & Harikishore *et al.*, 2013) and oxidant defence inhibitors (Ginsburg 1998; Färber *et al.*, 1998).

Research focussed on these pertinent *Plasmodium* drug targets led to the discovery of a wide range of scaffolds that may be employed as potential antiparasitic agents. Polycyclic amines such as adamantylamines and the structurally related pentacycloundecylamines (PCUs) (Oliver *et al.*, 1991) like NGP1-01 are examples of useful leads with chemosensitising- i.e. RCQ activity, probably due to their intrinsic CCB activity. These multifunctional molecules may potentially restore or potentiate CQ's activity in CQ^R- as well CQ^S parasites (Evans & Havlik 1993). This potentiation was first observed with verapamil (Krogstad *et al.*, 1987), a better-studied non-polycyclic CCB. Adamantyl-based molecules have also shown great promise as antiparasitic agents as described (Kreidenweis *et al.*, 2006; Araújo *et al.*, 2009). It is thus evident that similar antiparasitic activity may be expected from the structurally related oxa-PCUs such as NGP1-01 when incorporated as part of a multi-functional drug strategy.

These multi-therapeutic- and/or hybridisation approaches have resulted in the successful development of numerous novel antimalarial agents. However, there still remains a growing need for optimised and improved molecules capable of radical cure against all malaria pathogens. We are thus optimistic that the incorporation of polycyclic scaffolds will yield useful resistance reversal agents which may be further explored in the quest for improved antimalarial and/or reversal agents.

CHAPTER 3

SYNTHETIC PROCEDURES

3.1. Standard experimental procedures

3.1.1. Instrumentation

Nuclear magnetic resonance spectroscopy (NMR): ^1H and ^{13}C NMR spectra were determined using a Varian Gemini 200 spectrometer at a frequency of 200 MHz and 50 MHz, respectively. Tetramethylsilane (TMS) was used as an internal standard. All chemical shifts are reported in parts per million (ppm) relative to the signal from TMS ($\delta = 0$) added to an appropriate deuterated solvent. The following abbreviations are used to describe the multiplicity of the respective signals: s - singlet, bs - broad singlet, d - doublet, dd - doublet of doublets, t - triplet, q - quartet and m - multiplet. Spectra of selected compounds are included in annexure A.

Infrared spectroscopy (IR): The IR spectra were recorded on a Perkin Elmer Spectrum 400 spectrometer, fitted with a diamond attenuated total reflectance (ATR) attachment. Relevant spectra are included in annexure A.

Mass spectroscopy (MS): The MS spectra were recorded on a Perkin Elmer Flexar SQ 300 mass spectrometer by means of direct injection with a syringe pump. Relevant spectra are included in annexure A.

Melting point determination (MP): Melting points were determined using a Stuart SMP-10 melting point apparatus and capillary tubes. The melting points are uncorrected.

Microwave reactor: Microwave synthetic procedures were performed utilising a CEM DiscoverTM focused closed vessel reactor.

3.1.2. Chromatographic techniques

Thin layer chromatography (TLC): Analytical TLC was performed on a 0.20 mm thick aluminium silica gel sheets (Alugram® SIL G/UV254, Kieselgel 60, Macherey-Nagel, Düren,

Germany). Visualisation was achieved by using an UV light (254 nm and 366 nm), ethanol solution of ninhydrin or iodine vapours, with mobile phases prepared on a volume-to-volume basis.

3.1.3. Materials

Unless otherwise specified, all materials were obtained from commercial suppliers and used without purification. Solvents were dried using standard methods.

3.2. Synthesis of selected molecules

The well-described Cookson's diketone, pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione, was synthesised according to the published method (Cookson *et al.*, 1958, 1964). The reaction involved the formation of the Diels-Alder adduct and subsequent photocyclisation to yield the polycyclic cage structure. Figure 3.1 gives a schematic representation of the synthetic route that was followed. This structure served as primary basis in all further synthetic preparations.

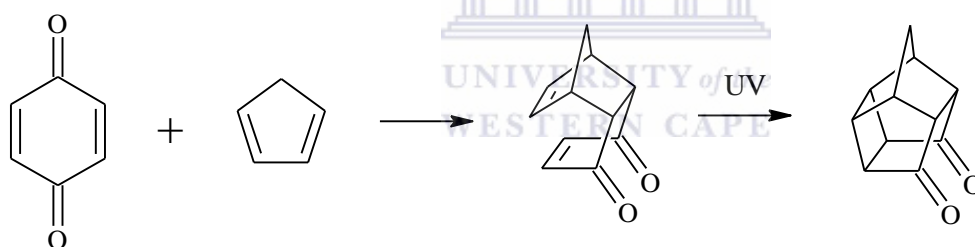
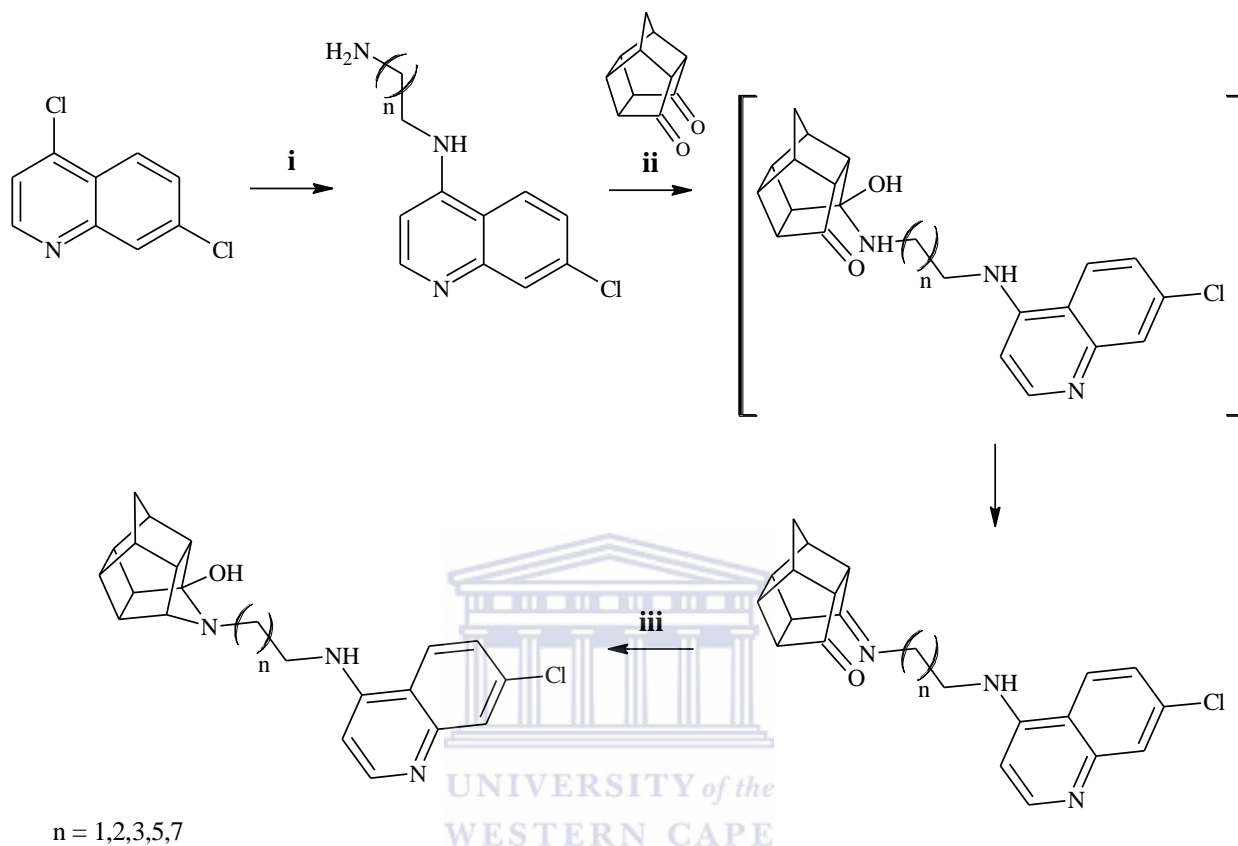


Figure 3.1: Synthesis of Cookson's diketone (Cookson *et al.*, 1958, 1964)

3.2.1. General approach – Amination and amidation

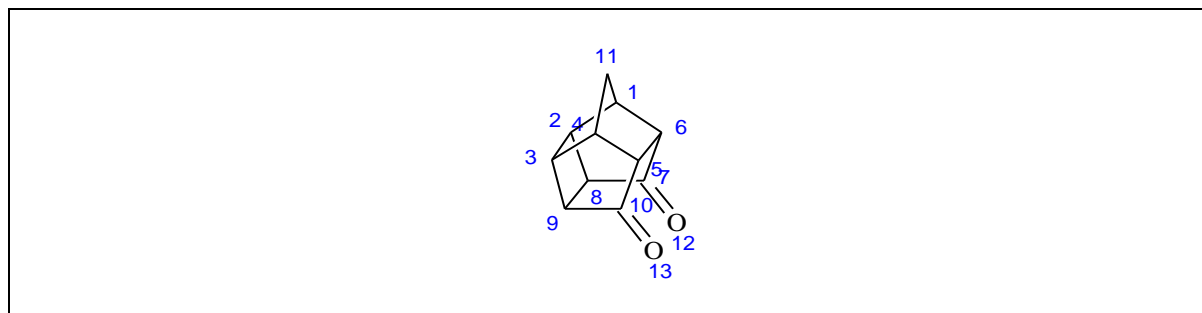
The designed pentacycloundecane-aminoquinoline (PCU-AM) reversed CQ agents were synthesised by conjugating the Cookson's diketone with different tethered 4-aminoquinoline moieties (scheme 1, **i**) to yield the respective carbinolamines (scheme 1, **ii**) and the subsequent corresponding imine. This was followed by a transannular cyclisation using sodium cyanoborohydride as a reducing agent to yield the desired PCU-AM derivatives (scheme 1, **iii**).

The 4-aminoquinolines were obtained by employing a novel microwave (MW) irradiation method.



Scheme 1: Reagents and conditions: (i) Alkyl diamine, CH₃CN, MW, 150 °C, 150 W, 150 psi, 30 min; (ii) Cookson's diketone, anhydrous THF, 5 °C, 60 min; (iii) MeOH, NaCNBH₄, rt, 4-6 hours

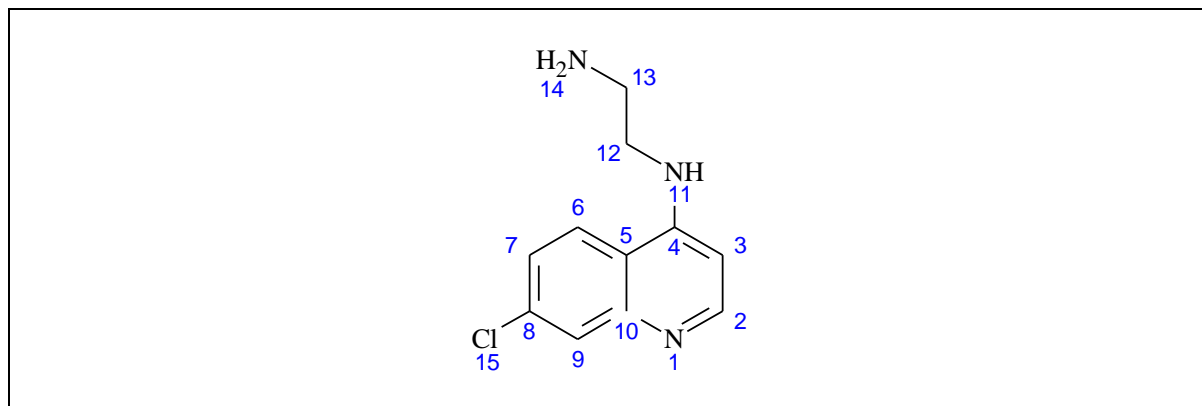
3.2.2. Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione



Synthesis: *p*-Benzoquinone (10.00 g, 0.0925 mol) was dissolved in 100 ml dried benzene on an external ice bath (0 - 5 °C). Freshly monomerised cyclopentadiene (12.45 g, 0.0925 mol) was slowly and stoichiometrically added whilst protecting the reaction mixture from light by means of foil. The reaction mixture was monitored by means of TLC and presumed complete as soon as the *p*-benzoquinone spot was no longer visible of the TLC plate. The photosensitive reaction mixture was stirred for 60 minutes on an external ice bath (0 – 5 °C). Activated charcoal was added and the mixture was stirred at room temperature for 30 minutes. This was done in order for fine impurities to be absorbed from the reaction mixture. The mixture was vacuum-filtered through Celite® to produce a clear yellow solution followed by *in vacuo* evaporation of benzene, resulting in the formation of intensely coloured amber oil. Excess solvent was allowed to fully evaporate in a dark fume hood to afford the yellow Diels-Alder adduct crystals. The crystals were dissolved in ethyl acetate (4 g per 100 ml) and irradiated with UV light for 6 hours, using a photochemical reactor. Decolouration of the solution indicated that cyclisation of the adduct was complete. Evaporation of the solution afforded a light yellow residue, which was purified by Soxhlett extraction in cyclohexane to produce the cage compound as fine white crystals (Yield: 7.5828 g, 0.0435 mol, 47 %). The physical characteristics of these crystals correlated with that in Cookson *et al.* (1958, 1964).

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3.2.3. *N*-(7-chloroquinolin-4-yl)ethane-1,2-diamine

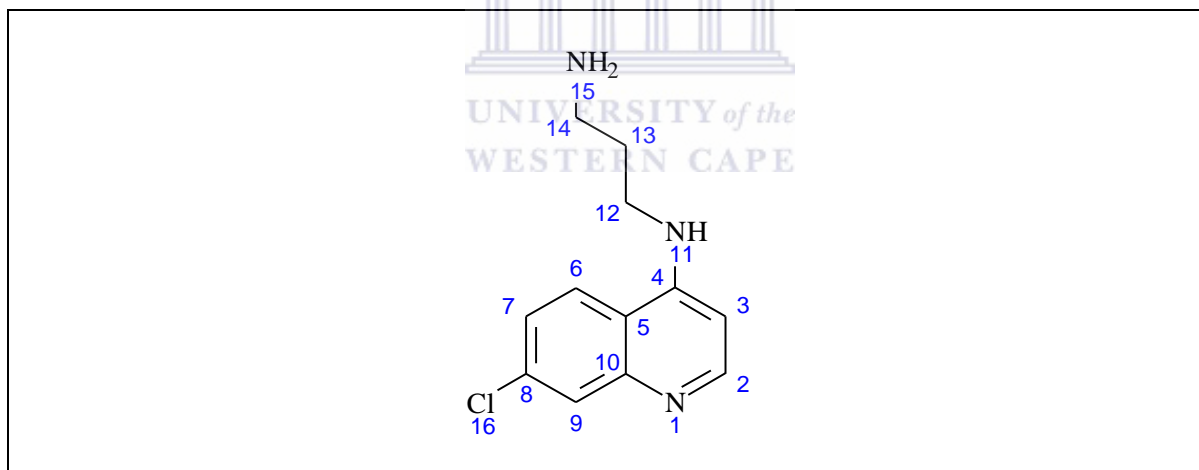


Synthesis: Dichloroquinoline (1 g, 5.05 mmol) and 1,2-diaminoethane (1.7 ml, 25.4 mmol) in 4 ml acetonitrile as solvent were reacted in a closed vessel microwave reactor at 150 °C, 150 W, 150 psi for 30 minutes. The cooled reaction mixture was basified with 30 ml 5 % aqueous

NaHCO₃ then taken-up in 60 ml dichloromethane (20 ml x 3). The combined organic layer was successfully washed in 20 ml water followed by 15 ml brine and finally dried over anhydrous Na₂SO₄. The solvent was removed *in vacuo*. *N*-(7-chloro-4-quinolyyl)-1,2-diaminoethane (Yield: 0.66 g, 2.97 mmol, 58.9 %) was obtained as pale yellow crystals and used without further purification or the residue can be precipitated by the addition of hexane:chloroform (80:20). The physical characteristics were the same as previously described (Yearick *et al.*, 2008; Sunduru *et al.*, 2009).

Physical data: C₁₁H₁₂ClN₃; **MP:** 134 – 137 °C; **R_f** (MeOH:NH₃OH) 0.59, **¹H-NMR** (200 MHz, DMSO-d) δ_H (Spectrum 1): 8.38 (d, 1H, *J* 5.4, H-2), 8.29 (d, 1H, *J* 8.8, H-6), 7.78 (s, 1H, *J* 2.2, H-9), 7.44 (dd, *J* 2.2, H-6, H-7), 6.49 (d, 1H, *J* 5.4, H-3), 3.25 (t, *J* 5.4, H-13), 2.81 (t, *J* 6.2, H-12).

3.2.4. *N*-(7-chloroquinolin-4-yl)propane-1,3-diamine

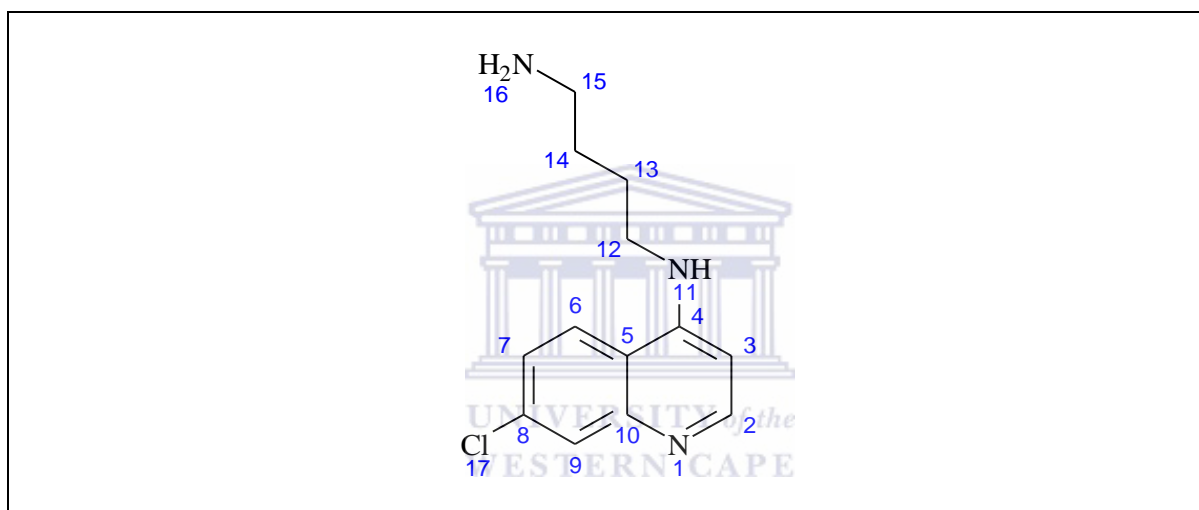


Synthesis: Dichloroquinoline (1 g, 5.05 mmol) and 1,3-diaminopropane (2.1 ml, 25.2 mmol) in 4 ml acetonitrile as solvent were reacted in a closed vessel microwave reactor at 150 °C, 150 W, 150 psi for 30 minutes. The cooled reaction mixture was basified with 30 ml 5% aqueous NaHCO₃ then taken-up in 60 ml dichloromethane (20 ml x 3). The combined organic layer was successfully washed in 20 ml water followed by 15 ml brine and finally dried over anhydrous Na₂SO₄. The solvent was removed *in vacuo*. *N*-(7-chloro-4-quinolyyl)-1,3-diaminopropane (Yield: 0.9491 g, 4.03 mmol, 79.7%) was obtained as pale yellow crystals and used without

further purification or the residue can be precipitated by the addition of hexane:chloroform (80:20). The physical characteristics were the same as previously described (Yearick *et al.*, 2008; Sunduru *et al.*, 2009).

Physical data: C₁₂H₁₄ClN₃, **MP:**128 – 135 °C; **R_f** (MeOH:NH₃OH) 0.37, **¹H-NMR** (200 MHz, CDCl₃) δ_H (Spectrum 2): 8.30 (d, 1H, H-2), 8.00 (d, 1H, H-6), 7.75 (s, 1H, H-9), 7.40 (dd, 1H, H-6, H-7), 6.47 (d, 1H, H-3), 3.40 (t, 2H, H-12), 2.80 (t, *J* 7, 2H, H-14), 1.90 (m, 2H, H-13).

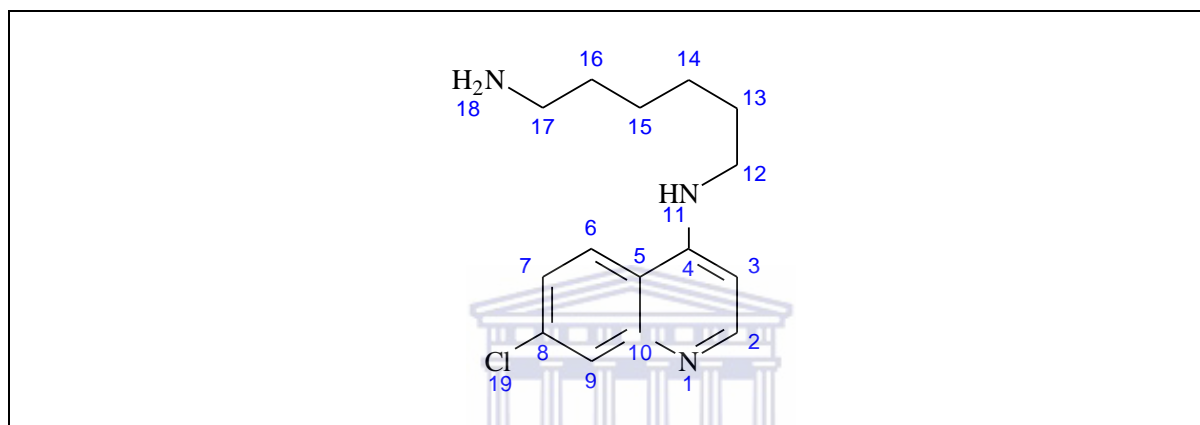
3.2.5. *N*-(7-chloroquinolin-4-yl)butane-1,4-diamine



Synthesis: Dichloroquinoline (1 g, 5.05 mmol) and 1,4-diaminobutane (2.5 ml, 25.4 mmol) in 3 ml acetonitrile as solvent were reacted in a closed vessel microwave reactor at 150 °C, 150 W, 150 psi for 30 minutes. The cooled reaction mixture was basified with 30 ml 5% aqueous NaHCO₃ then taken-up in 60 ml dichloromethane (20 ml x 3). The combined organic layer was successfully washed in 20 ml water followed by 15 ml brine and finally dried over anhydrous Na₂SO₄. The solvent was removed *in vacuo*. *N*-(7-chloro-4-quinoly)-1,4-diaminobutane (Yield: 0.66 g, 2.64 mmol, 52.3 %) was obtained as pale yellow solids and used without further purification or the residue can be precipitated by the addition of hexane:chloroform (80:20). The physical characteristics were the same as previously described (Yearick *et al.*, 2008; Sunduru *et al.*, 2009).

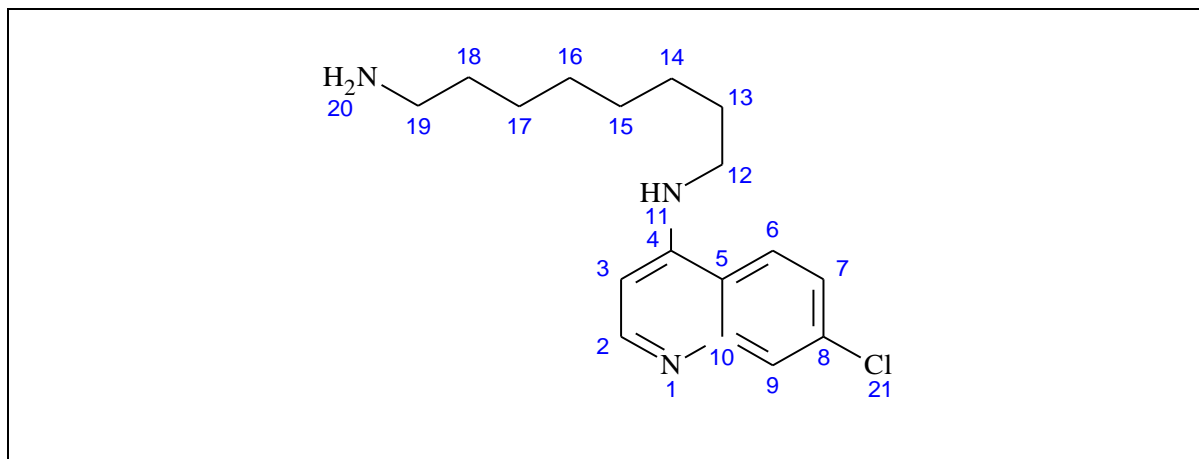
Physical data: C₁₃H₁₆ClN₃, **MP:** 46 – 49 °C; **R_f** (MeOH:NH₃OH) 0.32, **¹H-NMR** (200 MHz, DMSO-d) δ_H (Spectrum 3): 8.37 (d, *J* 5.6, 1H, H-2), 8.27 (d, *J* 9.2, 1H, H-6), 7.77 (d, *J* 2.2, 1H, H-9), 7.42 (dd, *J* 1.8 and 2.2, 1H, H-7), 6.44 (d, *J* 5.4, 1H, H-3), 3.24 (t, *J* 6.8, 2H, H-12), 2.59 (t, *J* 6.6, 2H, H-15), 1.70 (m, 2H, H-13), 1.48 (m, 2H, H-14).

3.2.6. *N*-(7-chloroquinolin-4-yl)hexane-1,6-diamine



Synthesis: Dichloroquinoline (1 g, 5.05 mmol) and 1,6-diaminohexane (3.3 ml, 25.4 mmol) in 2 ml acetonitrile as solvent were reacted in a closed vessel microwave reactor at 150 °C, 150 W, 150 psi for 30 minutes. The cooled reaction mixture was basified with 25 ml 5% aqueous NaHCO₃ then taken-up in 60 ml dichloromethane (20 ml x 3). The combined organic layer was successfully washed in 20 ml water followed by 15 ml brine and finally dried over anhydrous Na₂SO₄. The solvent was removed *in vacuo*. *N*-(7-chloro-4-quinolyl)-1,6-diaminohexane (Yield: 1.25 g, 4.50 mmol, 89.1 %) was obtained as yellow waxy solids and used without further purification or the residue can be precipitated by the addition of hexane:chloroform (80:20). The physical characteristics were the same as previously described (Yearick *et al.*, 2008; Sunduru *et al.*, 2009).

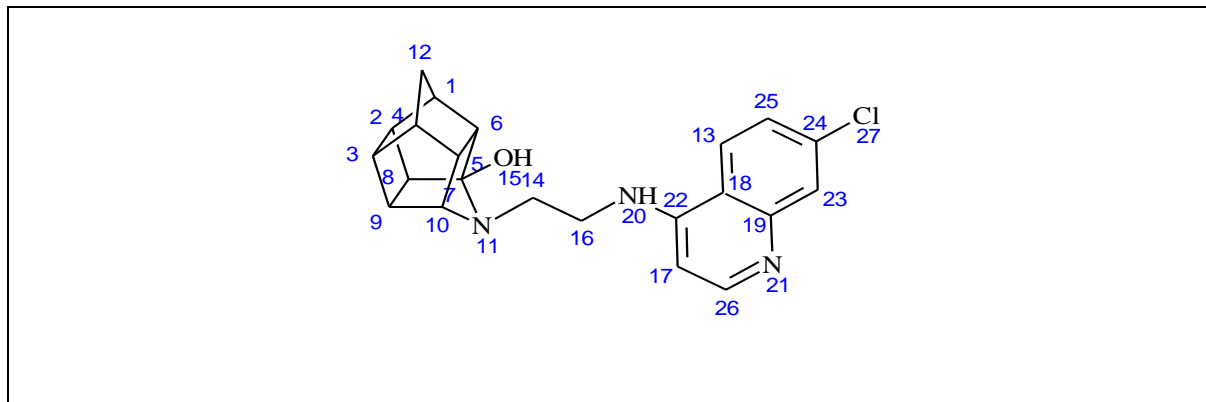
Physical data: C₁₅H₂₀ClN₃, **MP:** 134 – 139 °C; **R_f** (MeOH:NH₃OH) 0.35, **¹H-NMR** (200 MHz, DMSO-d) δ_H (Spectrum 4): 8.05 (d, *J* 5.4, 1H, H-2), 7.70 (d, *J* 8.8, 1H, H-6), 7.51 (d, *J* 2.0, 1H, H-9), 7.07 (dd, *J* 2.2 and 1.8, 1H, H-7), 6.12 (d, *J* 5.6, 1H, H-3), 3.05 (t, *J* 2.2, 2H, H-12), 2.90 - 2.79 (m, 4H), 2.448 – 2.379 (m, 6H, CH₂'s).

3.2.7. *N*-(7-chloroquinolin-4-yl)octane-1,8-diamine

Synthesis: Dichloroquinoline (1 g, 5.05 mmol) and 1,8-diaminooctane (3.6 ml, 25.4 mmol) in 2 ml acetonitrile as solvent were reacted in a closed vessel microwave reactor at 150 °C, 150 W, 150 psi for 30 minutes. The cooled reaction mixture was basified with 30 ml 5% aqueous NaHCO₃ then taken-up in 60 ml dichloromethane (20 ml x 3). The combined organic layer was successfully washed in 20 ml water followed by 15 ml brine and finally dried over anhydrous Na₂SO₄. The solvent was removed *in vacuo*. *N*-(7-chloro-4-quinoly)l-1,8-diaminooctane (Yield: 1.15 g, 3.75 mmol, 74 %) was obtained as a yellow oil and used without further purification or the residue can be precipitated by the addition of hexane:chloroform (80:20). The physical characteristics were the same as previously described (Yearick *et al.*, 2008; Sunduru *et al.*, 2009).

Physical data: C₁₇H₂₄ClN₃; **MP:** 127 – 131 °C; **R_f** (MeOH:NH₃OH) 0.40; **¹H-NMR** (200 MHz, CDCl₃/MeOD) δ_H (Spectrum 5): 8.28 (d, *J* 5.6, 1H, H-2), 7.75 (d, *J* 2.2, 1H, H-6), 7.71 (d, *J* 9.2, 1H, H-9), 7.27 (d, *J* 2.2, 1H, H-7), 6.29 (d, *J* 5.4, 1H, H-3), 3.06 (t, *J* 7.0, 1H, H-12), 2.54 (d, *J* 6.6, 1H, H-19), 1.34 – 1.33 (m, 10H, CH₂'s).

3.2.8. *N*-[2-(7-chloroquinolin-4-ylamino)ethylamino]-4-azaheptacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecan-3-ol (1.1)

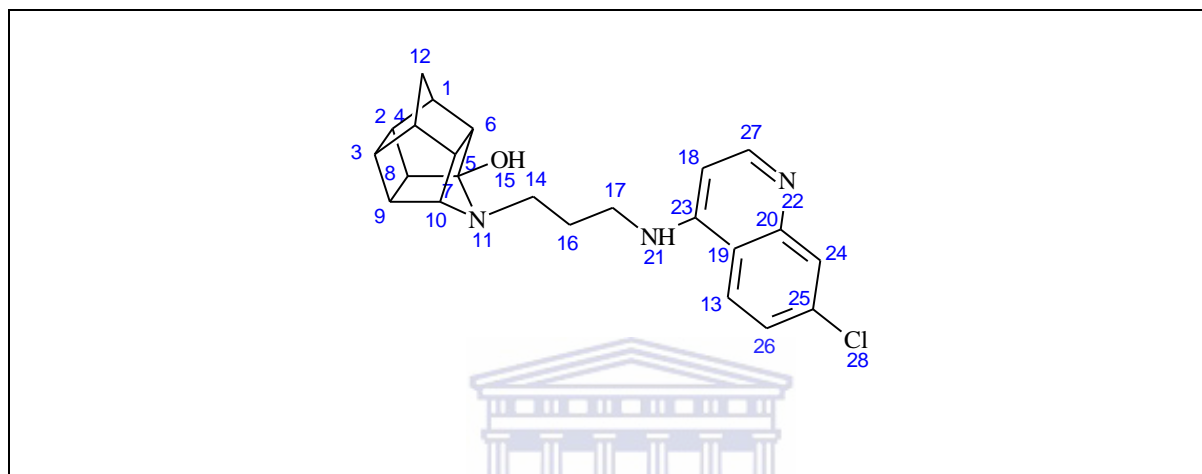


Synthesis: Pentacyclo[5.4.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (0.50 g, 2.87 mmol) was dissolved in 10 ml of dry tetrahydrofuran (THF) and cooled down to 5 °C while stirring in an external ice bath. *N*-(7-chloroquinolin-4-yl)ethane-1,2-diamine (0.64 g, 2.87 mmol) was added slowly with continued stirring of the reaction mixture at 5 °C. The carbinolamine started precipitating after approximately 15 minutes, but the reaction was allowed to reach completion for an additional 45 minutes. The reaction mixture was removed from the ice bath and added to a solution of 3 ml glacial acetic acid in 50 ml methanol. Sodium cyanoborohydride (0.20 g, 3.157 mmol) was added in small portions to the reaction mixture, as reducing agent, while stirring continued at room temperature for 4 – 6 hours. The reaction mixture was concentrated *in vacuo* and 20 ml water was added to remaining residue. Sodium bicarbonate (NaHCO₃; 2 – 4 g) was added until CO₂ gas evolution ceased. The reaction mixture was washed successively with dichloromethane (DCM; 2 x 30 ml), followed by brine (2 x 20 ml) and finally dried over anhydrous Na₂SO₄ and filtered. DCM was removed *in vacuo* and the residue was recrystallised from hexane:ethyl acetate (60:40) yielding the product as light yellow crystals (Yield: 0.20 g, 0.53 mmol, 18.3 %).

Physical data: C₂₂H₂₂ClN₃O; **MP:** 148 – 154 °C; **R_f** (MeOH:DCM) 0.17; **¹H-NMR** (200 MHz, DMSO) δH (Spectrum 6): 8.26 (d, 1H, *J* = 5.32 Hz), 7.83 (d, 1H, *J* = 2.02), 7.36 (s, 1H), 7.10 (dd, 1H, *J* = 1.86, 8.89 Hz), 6.17 (d, 1H, *J* = 5.37 Hz), 3.4 – 2.97 (m, 6H), 2.53-2.04 (m, 8H), 1.79:1.41 (AB-q, 2H, *J* = 10.4 Hz); **¹³C-NMR** (50 MHz, CDCl₃) δC (Spectrum 7): 151.71, 148.95, 148.77, 133.46, 127.28, 124.16, 124.01, 117.35, 98.67, 66.03, 54.41, 50.22, 44.99, 44.56,

44.19, 42.76, 41.34, 41.12, 40.68; **MS** (ESI-MS) m/z (Spectrum 8): 380.17 $[(M+H)^+]$, 100 %], 382.18 $[(M+H+2)^+]$, 30 %]; **IR** (ATR, cm^{-1}) ν_{max} (Spectrum 9): 3100, 2955, 730, 682 cm^{-1} .

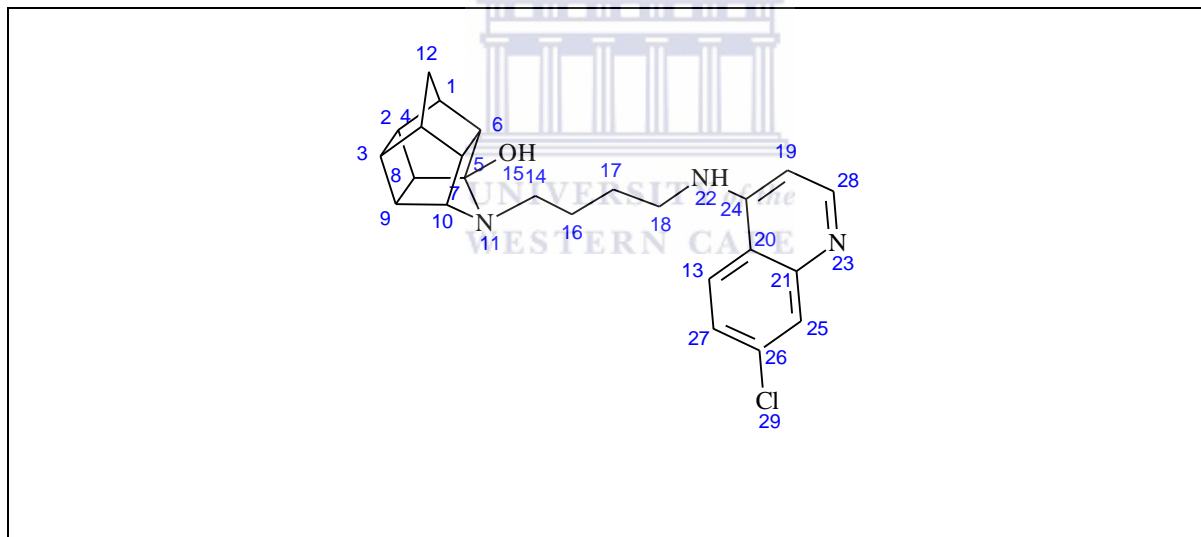
3.2.9. *N*-[3-(7-chloroquinolin-4-ylamino)propylamino]-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecan-3-ol (1.2)



Synthesis: Pentacyclo[5.4.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (0.50 g, 2.87 mmol) was dissolved in 10 ml of dry tetrahydrofuran and cooled down to 5 °C while stirring in an external ice bath. *N*-(7-chloroquinolin-4-yl)propane-1,3-diamine (0.68 g, 2.87 mmol) was added slowly with continued stirring of the reaction mixture at 5 °C. The carbinolamine started precipitating after approximately 15 minutes, but the reaction was allowed to reach completion for an additional 45 min. The reaction mixture was removed from the ice bath and added a solution of 3 ml glacial acetic acid in 50 ml methanol. NaCNBH_4 (0.20 g, 3.157 mmol) was added in small portions to the reaction mixture, as reducing agent, while stirring continued at room temperature for 4 – 6 hours. The reaction mixture was concentrated *in vacuo* and 20 ml water was added to remaining residue. NaHCO_3 (2 – 4 g) was added until CO_2 gas evolution ceased. The reaction mixture was washed successively with a 1:1 mixture of methanol:dichloromethane (MeOH/DCM; 2 x 30 ml), followed by brine (2 x 20 ml) and finally dried over anhydrous Na_2SO_4 . MeOH/DCM was removed *in vacuo* and the residue was recrystallised from hexane:ethyl acetate (60:40) yielding the product as light yellow crystals (Yield: 0.19 g, 0.48 mmol, 17 %)

Physical data: C₂₃H₂₄ClN₃O; **MP/decomposition:** 210 – 213 °C; **R_f** (MeOH:DCM) 0.08; **¹H-NMR** (200 MHz, DMSO-d) δ_H (Spectrum 10): 8.09 (d, 1H, *J* 5.4, H-27), 7.88 (d, 1H, *J* 3.6, H-13), 7.47 (s, 1H, *J* 2.2, H-24), 7.15 (d, 1H, *J* 9.0, H-26), 6.23 (d, 1H, *J* 4.8, H-18), 4.23 – 3.85 (m, 2H, H-10, H-17), 3.19 – 3.04 (m, 4H, H-14, H-16), 2.49 – 1.90 (m, 7H, H-1,2,3,4,6,8,9), 1.57 (AB-q, 2H, *J* 7, 9.6, H-5a, 5b); **¹³C-NMR** (50 MHz, DMSO-d) δ_C (Spectrum 11): 152.19, 151.10, 149.10, 134.83, 127.29, 124.94, 124.36, 117.99, 99.12, 81.08, 71.43, 66.14, 56.31, 55.18, 54.05, 50.83, 50.04, 45.95, 45.38, 44.92; **MS** (ESI-MS) *m/z* (Spectrum 12): 394.17 [(M+H)⁺, 100 %], 395.18 [(M+H+2)⁺, 30 %]; **IR** (ATR, cm⁻¹) ν_{max} (Spectrum 13): 3183, 2957, 730, 701 cm⁻¹.

3.2.10. *N*-[4-(7-chloroquinolin-4-ylamino)butylamino]-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecan-3-ol (1.3)

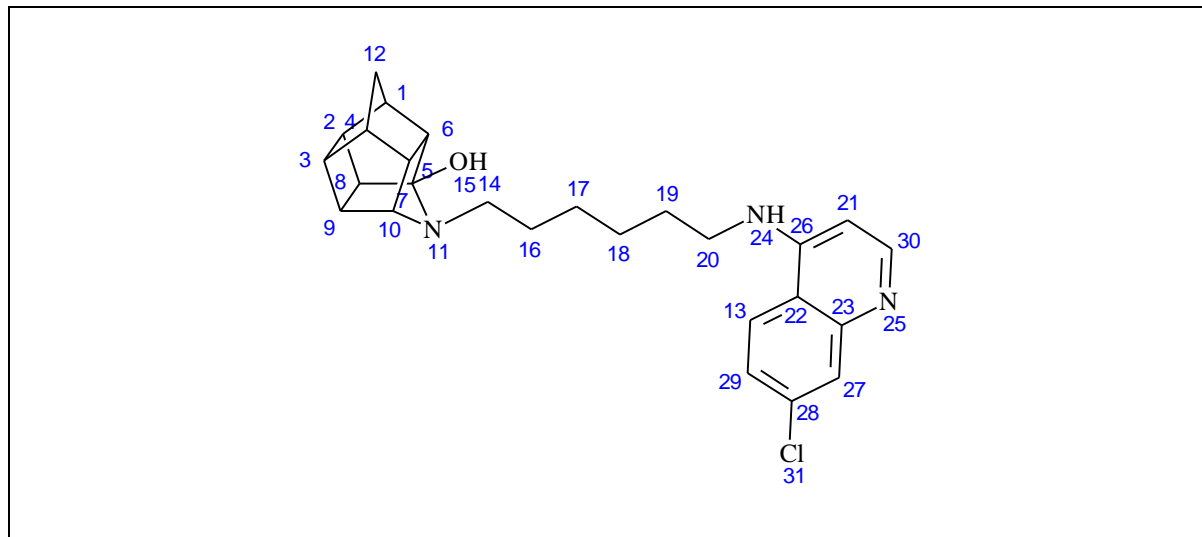


Synthesis: Pentacyclo[5.4.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (0.50 g, 2.87 mmol) was dissolved in 10 ml of dry THF and cooled down to 5 °C while stirring in an external ice bath. *N*-(7-chloroquinolin-4-yl)butane-1,4-diamine (0.72 g, 2.87 mmol) was added slowly with continued stirring of the reaction mixture at 5 °C. The carbinolamine started precipitating after approximately 15 minutes, but the reaction was allowed to reach completion for an additional 45 min. The reaction mixture was removed from ice bath and added a solution of 3 ml glacial acetic acid in 50 ml methanol. NaCNBH₄ (0.20 g, 3.157 mmol) was added in small portions to the

reaction mixture, as reducing agent, while stirring continued at room temperature for 4-6 hours. The reaction mixture was concentrated *in vacuo* and 20 ml water was added to remaining residue. NaHCO₃ powder (2 – 4 g) was added until CO₂ gas evolution ceased. The reaction mixture was washed successively with a mixture of 1:1 MeOH/DCM (2 x 30 ml), followed by brine (2 x 20 ml) and finally dried over anhydrous Na₂SO₄. MeOH/DCM was removed *in vacuo* and yielded the product as a brown oil (Yield: 0.68 g, 1.67 mmol, 58 %).

Physical data: C₂₄H₂₆ClN₃O; **MP:** oil; **R_f** (MeOH:DCM) 0.06; **¹H-NMR** (200 MHz, DMSO:CD₃OD) δH (Spectrum 14): 8.13-8.09 (d, 1H, *J* = 5.39 Hz), 7.91 (d, 1H, *J* = 1.98 Hz) 7.56 (s, 1H), 7.17 (dd, 1H, *J* = 1.94, 8.82 Hz), 6.30 (d, 1H, *J* = 5.37 Hz), 3.57 (t, 2 H), 3.14 – 3.51 (m, 4H), 2.76 – 2.28 (m, 8H) 1.56 – 1.28 (m, 6H); **¹³C-NMR** (50 MHz, CDCl₃) δC (Spectrum 15): 152.21, 150.31, 149.00, 135.80, 127.29, 125.50, 124.37, 99.40, 66.34, 55.10, 51.02, 45.85, 45.01, 44.39, 43.06, 42.52, 41.99, 26.68, 25.32; **MS** (ESI-MS) *m/z* (Spectrum 16): 410.19 [(M+H)⁺, 100 %], 412.20 [(M+H+2)⁺, 30 %]; **IR** (ATR, cm⁻¹) *v*_{max} (Spectrum 17): 3200, 2956, 748, 707 cm⁻¹.

3.2.11. N-[6-(7-chloroquinolin-4-ylamino)hexylamino]-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecan-3-ol (1.4)

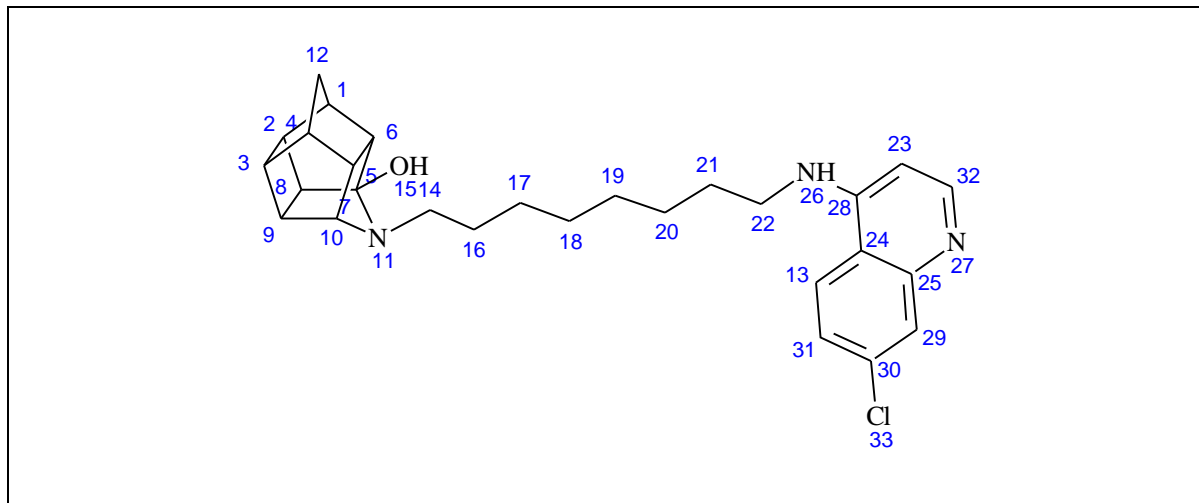


Synthesis: Pentacyclo[5.4.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (0.50 g, 2.87 mmol) was dissolved in 10 ml of dry THF and cooled down to 5 °C while stirring in an external ice bath. *N*-(7-

chloroquinolin-4-yl)hexane-1,6-diamine (0.8551 g, 2.87 mmol) was added slowly with continued stirring of the reaction mixture at 5 °C. The carbinolamine started precipitating after approximately 15 minutes, but the reaction was allowed to reach completion for an additional 45 min. The reaction mixture was removed from ice bath and added a solution of 3 ml glacial acetic acid in 50 ml methanol. NaCNBH₄ (0.20 g, 3.157 mmol) was added in small portions to the reaction mixture, as reducing agent, while continued stirring at room temperature for 4 – 6 hours. The reaction mixture was concentrated *in vacuo* and 20 ml water was added to remaining residue. NaHCO₃ (2 – 4 g) was added until CO₂ gas evolution ceased. The reaction mixture was washed successively with a 1:1 mixture of MeOH/DCM (2 x 30 ml), followed by brine (2 x 20 ml) and finally dried over anhydrous Na₂SO₄. MeOH/DCM was removed *in vacuo* and yielded the product as a light brown oil (Yield: 0.18 g, 0.41 mmol, 14.39 %).

Physical data: C₂₆H₃₀ClN₃O; **MP:** oil; **R_f** (MeOH:DCM) 0.11; **¹H-NMR** (200 MHz, DMSO:CD₃OD) δH (Spectrum 18): 8.12 (d, 1H, *J* = 5.37 Hz), 7.92 (d, 1H, *J* = 1.99 Hz) 7.56 (s, 1H), 7.17 (dd, 1H, *J* = 1.93, 8.83 Hz), 6.27 (d, 1H, *J* = 5.37 Hz), 3.53 (t, 2 H), 3.14 – 3.08 (m, 4H), 2.50 – 2.05 (m, 10H), 1.50 – 1.18 (m, 8H); **¹³C-NMR** (50 MHz, CDCl₃) δC (Spectrum 19): 152.21, 152.06, 149.25, 135.56, 127.28, 125.46, 124.42, 118.30, 99.34, 66.20, 54.64, 50.73, 45.90, 45.50, 44.72, 44.58, 43.70, 43.40 42.38, 41.77, 28.60, 27.43, 26.94; **MS** (ESI-MS) *m/z* (Spectrum 20): 436.22 [(M+H)⁺, 100 %], 438.22 [(M+H+2)⁺, 30 %]; **IR** (ATR, cm⁻¹) *v*_{max} (Spectrum 21): 3167, 2956, 748, 707 cm⁻¹.

3.2.12. *N*-[8-(7-chloroquinolin-4-ylamino)octylamino]-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecan-3-ol (1.5)



Synthesis: Pentacyclo[5.4.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (0.50 g, 2.87 mmol) was dissolved in 10 ml of dry THF and cooled down to 5 °C while stirring in an external ice bath. *N*-(7-chloroquinolin-4-yl)octane-1,8-diamine (0.88 g, 2.87 mmol) was added slowly with continued stirring of the reaction mixture at 5 °C. The carbinolamine started precipitating after approximately 15 minutes, but the reaction was allowed to reach completion for an additional 45 minutes. The reaction mixture was removed from ice bath and added a solution of 3 ml glacial acetic acid in 50 ml methanol. NaCNBH₄ (0.20 g, 3.157 mmol) was added in small portions to reaction mixture, as reducing agent, while stirring continued at room temperature for 4 – 6 hours. The reaction mixture was concentrated *in vacuo* and 20 ml water was added to remaining residue. NaHCO₃ (2 – 4 g) was added until CO₂ gas evolution ceased. The reaction mixture was washed successively with dichloromethane (2 x 30 ml), followed by brine (2 x 20 ml) and finally dried over anhydrous Na₂SO₄. Removed dichloromethane *in vacuo* and yielded the product as a light brown oil (Yield: 0.171 g, 0.369 mmol, 12.8 %).

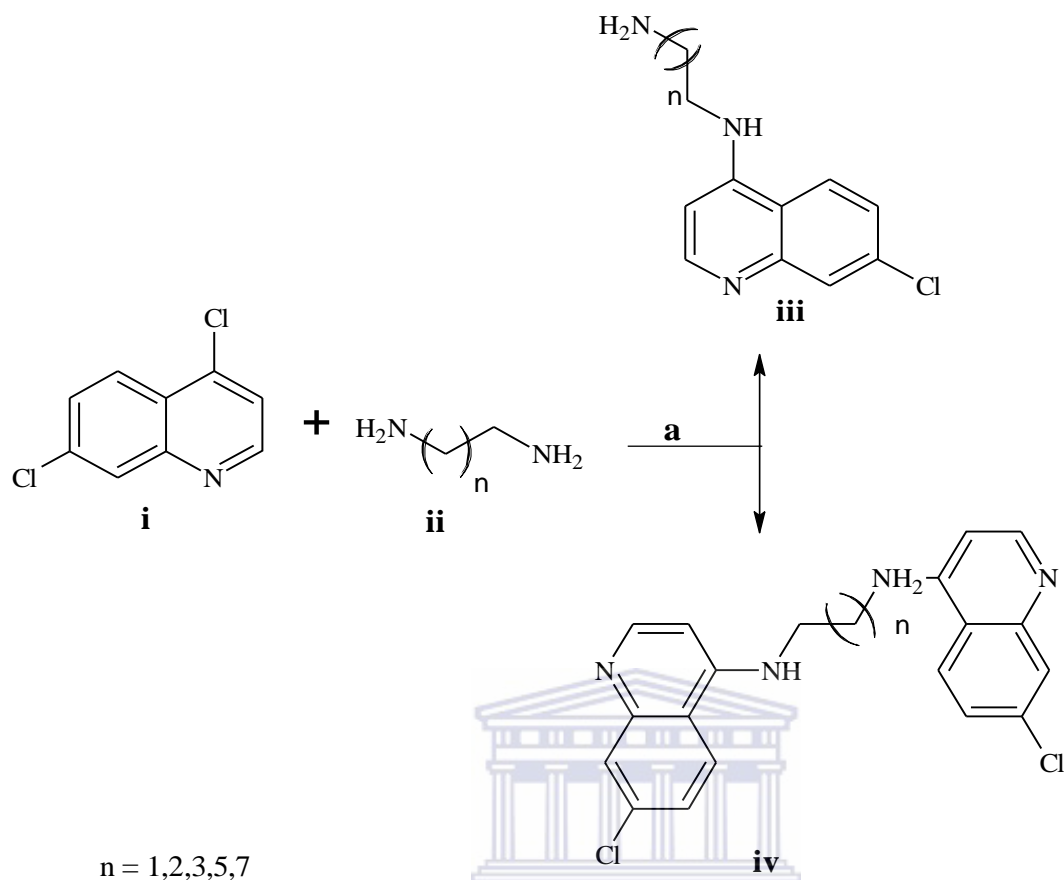
Physical data: C₂₈H₃₄ClN₃O; **MP:** oil; **R_f** (MeOH:DCM) 0.14; **¹H-NMR** (200 MHz, DMSO-d) δ_H (Spectrum 22): 7.62 (d, 1H, *J* 5.8, H-32), 7.48 (d, 1H, *J* 8.8, H-13), 7.01 (s, 1H, H-29), 6.68 (d, 1H, *J* 9.0, H-31), 5.76 (d, 1H, *J* 5.8, H-23), 3.04 (t, 1H, H-10), 2.60 – 2.45 (m, 4H, H-14,22), 2.32 – 1.76 (m, 11H, H-1,2,3,4,5,6,8,9,16), 1.16 – 0.58 (m, 10H, H-17,18,19,20,21); **¹³C-NMR** (50 MHz, DMSO-d) δ_C (Spectrum 23): 152.14, 151.43, 149.15, 134.83, 127.22, 124.91, 124.41, 117.98, 109.71, 99.05, 65.78, 54.88, 54.70, 54.60, 50.48, 46.60, 46.21, 44.54, 44.45, 43.96,

43.03, 42.11, 41.96, 41.62, 41.50; **MS** (ESI-MS) m/z (Spectrum 24): 464.25 [(M+H)⁺, 100 %], 465.25 [(M+H+2)⁺, 30 %]; **IR** (ATR, cm⁻¹) ν_{\max} (Spectrum 25): 3266, 2931, 769, 701 cm⁻¹.

3.3 Challenges during synthetic procedure

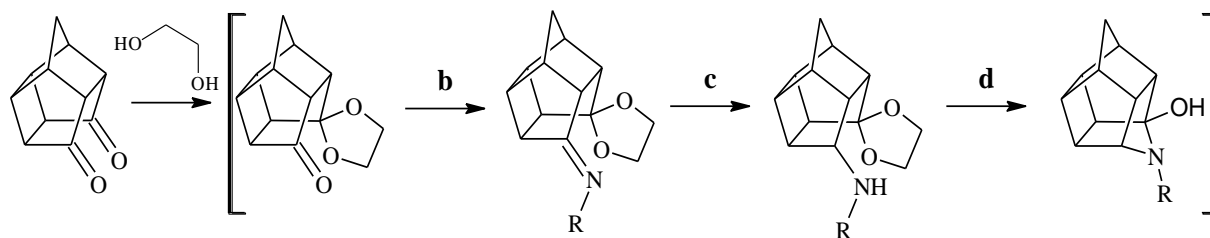
The majority of the reactions employed to obtain the target pentacycloundecane-aminoquinoline (PCU-AM) derivatives were amination and reductive amination reactions. The synthetic work commenced with the synthesis of the different tethered aminoquinoline intermediates. Various reagent and reaction conditions were attempted to generate these intermediates but only the major synthetic problems encountered will be elaborated on and discussed.

Using normal thermal conditions (scheme 2), dichloroquinoline (scheme 2, **i**) was reacted with stoichiometric quantities of the various alkyldiamines (scheme 2, **ii**). Thin-layer chromatography (TLC) plates used to monitor the reactions indicated incomplete or limited formation of the desired aminoquinoline intermediates (scheme 2, **iii**). The alkyldiamines were fixed at the base of the chromatogram (despite the various eluents systems used) and by-products were also visible when visualised with ninhydrin, iodine vapour and exposure to UV light. Considering the poor leaving tendency of the 4-chloro group of dichloroquinoline, longer reaction run-time and incorporation of aprotic solvents such acetonitrile were used but the reactions proved unsuccessful. In addition, incorporation of catalysts such as sodium iodide and bicarbonate employed to accelerate the nucleophilic substitution may have inadvertently resulted in formation of bis aminoquinoline (scheme 2, **iv**) compounds which may explain the observed lack of reactivity. Furthermore, as the aliphatic chain length of the alkyldiamines increased, the more by-products was formed and the more difficult identification of the intermediates became. Normal thermal synthetic routes were thus abandoned.



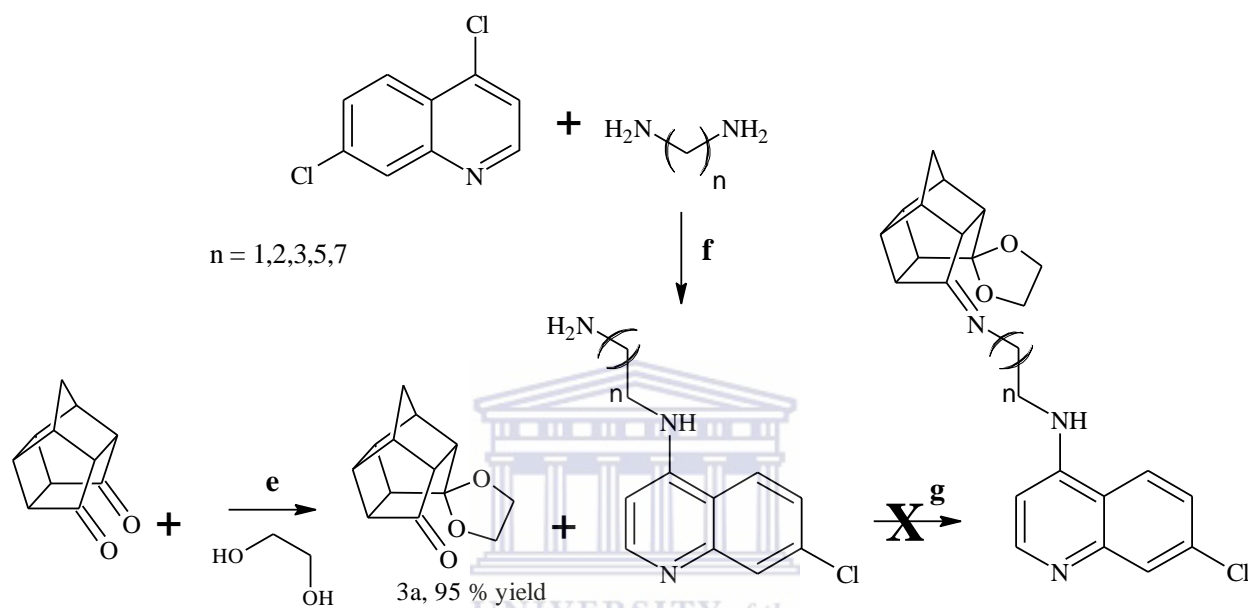
Scheme 2: Reagents and conditions: (a) Reflux, 80 – 150 °C, acetonitrile, 6 – 10 hrs.

When our focus moved to microwave irradiated synthetic methods (chapter 3, scheme 1) to synthesise desired AM molecule, a significant change was observed in terms of reduced reaction time, increased yield and low solvent consumption. The five AM intermediates were successfully obtained with purification of compounds achieved by recrystallisation. The targeted PCU-AM derivatives were initially planned for synthesis by utilising a modified method described by Banister *et al.* (scheme 3; 2011).



Scheme 3: Reagents and conditions: (a) R–NH₂, EtOH, 100 °C, 18 h; (b) NaBH₄, EtOH, rt, 8 hrs; (c) aq 4 M HCl, acetone, rt, 12 hrs, basic work-up (Banister *et al.* 2011).

The Cookson's diketone was successfully converted to the monoketal cage (scheme 3, 3a) with good yield ($\pm 95\%$) by using a novel open-vessel microwave irradiation method (scheme 3, d) instead of normal thermal conditions. The microwave irradiation method here had the advantage of increasing yields, significantly reducing reaction run-time and offers better control over reaction temperature.



Scheme 4: Reagents and conditions: (e) MW, 150 °C, 150 W, 15 min, Benzene, -H₂O; (f) Alkyldiamine, CH₃CN, MW, 150 °C, 150 W, 150 psi, 30 min; (g) MW, EtOH, 100 °C, 150 W, 2–4 hrs.

However, conjugation of the diketone monoethylene acetal (scheme 4, 3a) with different 4-aminoquinoline moieties under pressure as described by Banister *et al.* (2013) was incomplete as indicated by thin-layer chromatography (TLC). In order to improve the yield, the reaction time was prolonged but no change was observed on the TLC and there was no indication of the corresponding imine being formed (scheme 3, f). When the temperature was increased above 100 °C in a closed-vessel reactor, the acetal moiety degraded. Prolongation of reaction run-time also failed to aid in achieving the corresponding imine. Steric hindrance appears to be a possible reason for the reaction not reaching completion (scheme 4, g). SN₂ reactions typically require high temperatures (especially where poor leaving groups are involved) which is not practical especially where thermolabile polycyclic hydrocarbon such as the monoketal cage are concerned.

In an interesting turn of events we were able to successfully synthesise our final five aza PCU-AM derivatives. Successful coupling of the differently tethered AM moieties was achieved through direction conjugation with the Cookson's diketone at low temperature with THF as the solvent on an external ice bath, yielding the corresponding carbinolamine in quantitative yields. This can be explained by strong nucleophilic attack of the amine on the deshielded ketone of the Cookson's diketone (scheme 1, **ii**) as opposed to the highly shielded and steric hindrance imparted by the Cookson's monoethylene acetal (scheme 4, **g**). Subsequently through transannular cyclisation, using NaCNBH₄ as reducing agent, the final aza PCU-AM hybrid molecule was produced.

3.4. Conclusion

The compounds were successfully synthesised, resulting in 5 novel PCU-AM derivatives. The compounds all contain a bulky aza-PCU scaffold which will protect the terminal tertiary amino group thereby 1) circumventing metabolic *N*-dealkylation, 2) increase the *in vivo* efficacy and also decrease the potential for cross-resistance. The yields of the synthetic procedures ranged from 12 % to 58 %. The lower yields were attributed to the formation of various possible by-products during the synthetic procedure as well as inadequate purification of the product mixture during recrystallisation. Yields could be improved through optimisation of synthetic procedures and purification techniques as well as further purification of remaining fractions of the product mixture by employing column chromatography.

The structures were characterised using the analytical instrumentation and techniques described at the beginning of this chapter. Characteristic signals observed for each specific compound, gave confirmation of their structure. In order to meet the objective of this study, the synthesised aza PCU-AM derivatives were subjected to antimalarial assays and cytotoxicity studies. The results of these studies are discussed in chapter 4.

CHAPTER 4

BIOLOGICAL EVALUATION AND RESULTS

The medicinal chemistry of polycyclic scaffolds has been extensively explored and has led to the development of a variety of multipotent and potentially useful therapeutics. Reports have shown that the lipophilic antiviral (Oliver *et al.*, 1991) and antiparkinsonian (Joubert *et al.*, 2008) polycyclic derivative amantadine, demonstrated useful antimalarial activity *in vitro* (Koff *et al.*, 1980; Miller *et al.*, 1983; Evan & Havlik 1993). We thus postulated that the structurally similar analogue NGP1-01 (Oliver *et al.*, 1991) may also possess antimalarial activity and/or resistance reversal activity.

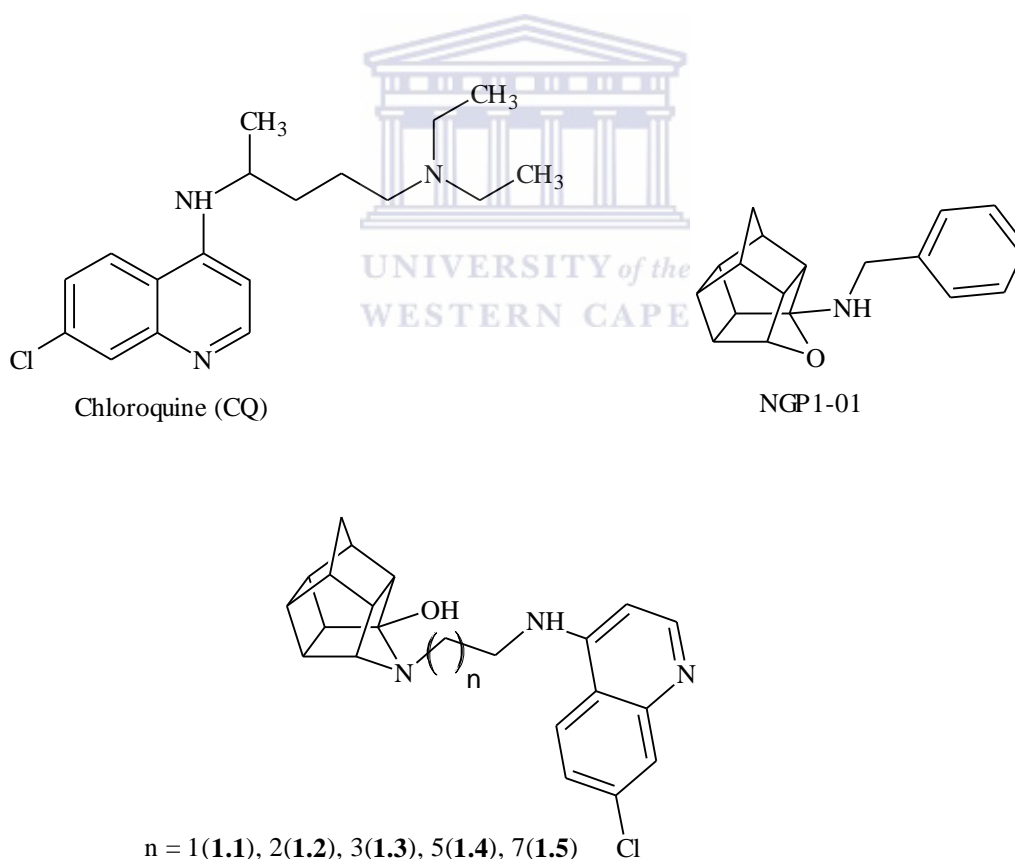


Figure 4.1: Structure of chloroquine (CQ), NGP1-01 and the synthesised pentacycloundecane-aminoquinoline (PCU-AM) derivatives (1.1 – 1.5)

The aim of this study was to design and synthesise a series of novel aza pentacycloundecane-aminoquinoline (PCU-AM) derivatives and to evaluate these compounds for antimalarial activity.

In this study, the prototype pentacycloundecylamine NGP1-01 was evaluated for intrinsic antiplasmodial- and reversed chloroquine activity. In the synergism study, NGP1-01 was evaluated for synergistic antiplasmodial activity with CQ in a cell-based assay (isolated Dd2 cultures). The polycyclic structure was also hybridised with various aminoquinoline (AM) cores resulting in a single molecule with a dual mode of action that targets different sites within the *Plasmodium* parasite, which is a desired property for malaria chemotherapy. It has become common practice to use a combination of drugs to treat infectious diseases in order to prevent emergence of resistance to the individual drugs and improve efficacy due to synergistic effects of the drugs. This approach additionally prevents recognition of CQ by modification of its structure which may allow CQ uptake into the digestive vacuole of the *Plasmodium* parasite and thus concentrate and exerts its inhibitory effect (Madrid *et al.* 2006; Muregi & Ishih 2010).

Hybridisation thus minimise the possibility of cross-resistance, optimise antimalarial potential and provides an attractive drug design approach for the development of therapeutics. It is also expected that the novel PCU-AM derivatives will display good antiplasmodial and/or reversal activity and enhanced pharmacodynamic and kinetic profiles.

4.1. Antimalarial Activity Determination

4.1.1. Introduction

A modified method of the parasite lactate dehydrogenase (pLDH) assay described by Makler (1993) was employed for the quantitative determination of the antimalarial activity of the novel PCU-AM derivatives against both chloroquine sensitive (NF54) and resistant (Dd2) strains of *P. falciparum*. From the inhibition data, the 50 % inhibitory concentration (IC₅₀) values were calculated and compared. The antiplasmodial assay employed in this study not only allows for continuous *in vitro* growth in human erythrocytes of *P. falciparum* but may also be used to screen for new drugs, to isolate and characterize strains and clones, and to identify immunogenic

antigens for ultimate use in a vaccine (Trager 1987 & Trager *et al.*, 1987). Toxicity studies were conducted against a Chinese Hamster Ovarian (CHO) cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)-assay to assess cell viability. The assay is dependent on the ability of viable cells to metabolise a water-soluble tetrazolium salt into a water-insoluble formazan product (Twentyman & Luscombe 1987).

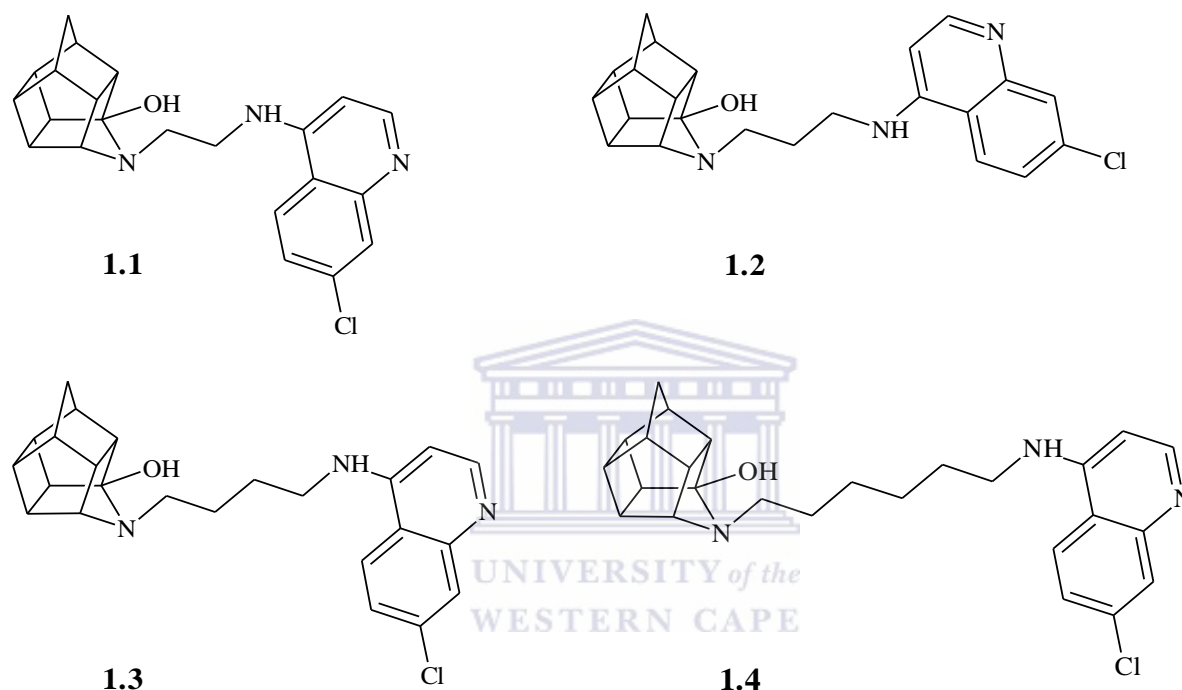


Figure 4.2: PCU-AM hybrid molecules evaluated for antimalarial activity

4.2. Materials and methods

4.2.1. Cells and *P. falciparum* parasite: All stock parasite cultures were maintained using the method of Trager and Jensen (1976). The chloroquine sensitive (CQ^S) NF54 and chloroquine resistant (CQ^R) strain Dd2 of *P. falciparum* and normal type A human red blood cells (2 % hematocrit) suspended in complete tissue culture medium (RPMI 1640 containing 25 mM HEPES buffer, 20 µg/ml of gentamicin, 27 mM bicarbonate and 10 % normal type A human serum) were used for the assay.

4.2.2. Synergism study: To determine intrinsic antimalarial activity, triplicate dose-response experiments were performed using decreasing concentrations of the test compound starting from 2000 ng/mL as described below. For resistance reversal, the test samples were tested in triplicate at 1 μ M and 10 μ M, by addition of a fixed amount of the compound to a dose-response experiment with the reference compound, chloroquine (CQ). All experiments were carried out against the chloroquine-resistant *P. falciparum* isolate Dd2. Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of Trager and Jensen (1976). Quantitative assessment of antimalarial activity *in vitro* was determined via the pLDH assay using a modified method described by Makler (1993).

The test samples were prepared to a 20 mg/ml stock solution in 100 % DMSO and sonicated to enhance solubility. Stock solutions were stored at -20 °C. Further dilutions were prepared on the day of the experiment. CQ was used as the reference drug for the resistance reversal experiment and artesunate, a potent artemisinin-derived antimalarial agent, was used as a control for the antimalarial activity. A full CQ dose-response was performed with the test compound added at the two different concentrations to determine the shift in the CQ concentration inhibiting 50 % of parasite growth (IC_{50} value). CQ and artesunate were tested at a starting concentration of 1000 ng/ml, which was then serially diluted 2-fold in complete medium to give 10 concentrations; with the lowest concentration approximating 2 ng/ml. Immediately thereafter, a fixed amount of either 10 μ M or 1 μ M of the test compound was added to each well containing CQ, and compared to a control dose-response experiment of CQ without the test compound. The highest concentration of solvent to which the parasites were exposed to had no measurable effect on the parasite viability (data not shown). The IC_{50} values were obtained using a non-linear dose-response curve fitting analysis with Graph Pad Prism v.4.0 software.

4.2.3. pLDH assay: The sensitivity assay was initiated by adjusting the initial parasitemia to 1-2 % with the normal type A human red blood cell suspension. The test samples were prepared to a 20 mg/ml stock solution in 100 % DMSO and sonicated to enhance solubility. Samples were tested as a suspension if not completely dissolved. Stock solutions were stored at -20 °C. Further dilutions were prepared on the day of the experiment. Chloroquine (CQ) and artesunate were used as the reference drug in all experiments. The diluted parasite suspension was dispensed in triplicate at 0.2 ml/well into a 96-well, flat-bottomed microtiter plates. At a starting concentration

of 1000 ng/ml, test samples were serially diluted 2-fold in complete medium to give 10 concentrations; with the lowest concentration being 2 ng/ml. The same dilution technique was used for all samples. The highest concentration of solvent to which the parasites were exposed to had no measurable effect on the parasite viability (data not shown).

The cultures were incubated at 37 °C for 48 hours in 3 % O₂, 6 % CO₂ and 91 % N₂. At the conclusion of the incubation period the cultures were carefully re-suspended and aliquots were removed for spectrophotometrical analysis of pLDH activity (Makler *et al.*, 1993). The IC₅₀-values were obtained using a non-linear dose-response curve fitting analysis with Graph Pad Prism v.4.0 software.

4.2.4. Cytotoxicity (MTT) assay: Test samples were screened for *in vitro* cytotoxicity against CHO cell line using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) MTT-assay. The MTT-assay is used as a colorimetric assay for cellular growth and survival, and compares well with other available assays (Mosman 1983; Rubinstein *et al.*, 1990). The tetrazolium salt MTT was used to measure all growth and chemosensitivity. The test samples were tested in triplicate on one occasion.

The same stock solutions prepared for antimalarial testing were used for cytotoxicity testing. Test compounds were stored at -20 °C until use. Dilutions were prepared on the day of the experiment. Emetine was used as the reference drug in all experiments. The initial concentration of emetine was 100 µg/ml, which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 0.001 µg/ml. The same dilution technique was applied to the all test samples. The highest concentration of solvent to which the cells were exposed to had no measurable effect on the cell viability (data not shown). The IC₅₀ values were obtained from full dose-response curves, using a non-linear dose-response curve fitting analysis with GraphPad Prism v.4 software.

4.3. Results and discussion

4.3.1. Synergism study

PCUs such as NGP1-01 are well-studied multichannel blocking agents (Geldenhuys *et al.*, 2004 & Geldenhuys *et al.*, 2007), we thus postulated that these agents may act as chemosensitisers and

circumvent the resistance of the plasmodia parasite against CQ by inhibiting the *p*-glycoprotein efflux pump (Singh *et al.*, 2004) to enable the accumulation of CQ inside the parasite digestive vacuole. Therefore as a proof of concept we conducted an experiment using CQ co-administered with different concentrations of NGP1-01 to evaluate the ability of NGP1-01 to act as a chemosensitiser. NGP1-01 alone exhibited very low intrinsic antimalarial activity against the resistant strain (> 2000 nM), with no toxicity to the parasite detected at 10 μ M (Mosmann 1983; Rubinstein *et al.*, 1990; Geldenhuys *et al.*, 2007). A statistically significant ($p < 0.05$) dose dependent shift was seen in the CQ IC₅₀-values at both a 1 μ M and 10 μ M concentration of co-administered NGP1-01 against the resistant strain as shown in Table 4.1. Reported shifts with other known chemosensitisers such as desipramine have been significantly higher however, with IC₅₀-values of CQ being reduced by up to 95% *in vitro* (equivalent to an RMI value of 0.05; Bitonti *et al.*, 1993, 1994). For comparison, the antimalarial reference artesunate displayed potent activity with an IC₅₀-value of 3.7 ng/mL. In a recent report, Solaja *et al.*, (2008) have shown that adamantylated 4-amino-7-chloroquinoline hybrid molecules display potent *in vitro* antimalarial activity. Structural similarity between NGP1-01 and amantadine (Oliver *et al.*, 1999) thus suggest that NGP1-01 may also exert its resistance reversal and/or antimalarial activity by modulating local charge density of the parasite membrane and lysosomotropic effect (Evan & Havlik 1993). It was also interesting to note that the unconjugated (non-hybrid) NGP1-01 was able to display some reversal activity against the CQ-resistant strain Dd2 of *P. falciparum*.

Table 4.1: *In vitro* antimalarial activity of NGP1-01 alone & in combination with CQ

Sample	Strain	Mean IC ₅₀ (ng/ml)	RMI*
NGP1-01	Dd2	>2000	-
CQ+1 μ M NGP1-01	Dd2	60.7 \pm 4.1	0.60
CQ+10 μ M NGP1-01	Dd2	48.7 \pm 5.3	0.48
CQ	Dd2	101.8 \pm 4.9	1.00
Artesunate	Dd2	3.7 \pm 1.1	-

*Response Modification Index; the ratio of the altered IC₅₀ using (CQ + test compound) to that of CQ alone. Lower values indicate better reversal.

This is the first time, to the best of our knowledge, that a PCU compound has been evaluated for and exhibited potential as a chemosensitiser. A number of PCU compounds previously designed by our group have shown significantly more potent channel blocking activity than NGP1-01 and may potentially present with better chemosensitising ability (Geldenhuys *et al.*, 2004; Geldenhuys *et al.*, 2007). We are currently exploring the potential of these compounds as chemosensitising agents. This synergism study indicates NGP1-01 as a weak chemosensitising agent however, on-going attempts to improve and optimise the molecule led to the concept of ‘therapeutic’ hybridisation. The approach involves the covalent linking of the polycyclic structure with various CQ-like derivatives forming a single ‘dual-acting’ hybrid agent which may potentially reverse CQ resistance thus ushering a novel class of antimalarial agents to the malaria armamentarium.

4.3.2. Parasite lactate dehydrogenase (pLDH) and Cytotoxicity

Based on the chemosensitising ability of NGP1-01, our next objective was to attempt to design potential reversed CQ agents comprising of a PCU moiety as the reversal agent (RA) conjugated to a CQ-like aminoquinoline (AM) molecule and to evaluate the potential of these PCU-AM derivatives as reversed CQ agents (Figure 4.2).

The series of novel synthesised PCU-AM derivatives (**1.1**, **1.2**, **1.3** and **1.4**) displayed antimalarial activity in the same range as both CQ and artesunate, against the CQ sensitive NF54 strain (Table 4.2). Compound **1.5** was not evaluated because of insufficient purity for biological evaluation. Compound **1.1**, the most active derivative in the series, displayed significant antiplasmodial activity when evaluated against the CQ-sensitive (NF54:IC₅₀ = 3.74 ng/mL) and CQ resistant (Dd2:IC₅₀ = 27.6 ng/mL) strain, demonstrating a better activity profile than CQ against both strains of the *Plasmodium* parasites and exhibited an activity profile comparable to that of antimalarial reference artesunate (Table 4.2). Compound **1.2** displayed good activity against the CQ-sensitive (NF54:IC₅₀ = 15.6 ng/mL) strain however its activity profile was significantly reduced in the CQ-resistant strain (Dd2:IC₅₀ = 121 ng/mL). Compound **1.3** and **1.4** both demonstrated good activity against the CQ-sensitive NF54 strain (IC₅₀ = 8.45 and 17.6 ng/mL) respectively however, their activity profile was significantly reduced against the CQ-resistant Dd2 strain of *P. falciparum* (187 and 253 ng/mL, respectively). When compared to the

lead compound NGP1-01, all PCU-AMs evaluated in this study, displayed better antimalarial activity profile against the CQ-resistant Dd2 strain of *falciparum*. Compound **1.1** also showed the lowest RMI value which further supports that this PCU-AM derivative has the best potential to act as a reversed CQ agent in the series. Cytotoxicity IC_{50} values observed for compounds **1.1** – **1.4** were in the low micro molar concentrations (2.39 – 9.54 μ M) indicating selectivity towards *P. falciparum* (SI = 149 – 2549, Table 4.2) and low toxicity compared to the cytotoxic agent emetine (IC_{50} = 0.061 μ M).

Table 4.2: *In vitro* IC_{50} -values of PCU-AM derivatives and reference compounds with standard deviations of antiplasmodial activity and cytotoxicity

Compound	NF54 IC_{50} (ng/ml)	Dd2 IC_{50} (ng/ml)	CHO IC_{50} (μ g/ml)	RI	SI	LogP
1.1	3.74 \pm 0.13	27.6 \pm 5.1	9.54 \pm 0.23	7	2549	2.11
1.2	15.6 \pm 1.82	121 \pm 0.03	4.01 \pm 1.45	8	256	2.34
1.3	8.45 \pm 0.27	187 \pm 35.4	2.39 \pm 0.56	22	283	2.72
1.4	17.6 \pm 1.02	253 \pm 4.0	2.63 \pm 0.12	14	149	3.59
CQ	5.49 \pm 0.72	123.5 \pm 6.98	ND	23	ND	4.69
Artesunate	<2	2.18	ND	ND	ND	ND
Emetine	ND	ND	0.061 \pm 0.01	ND	ND	ND

Selectivity index (SI) = IC_{50} CHO/ IC_{50} NF54; Resistance index (RI) = IC_{50} Dd2/ IC_{50} NF54. LogP values using ACD/ChemSketch. ND = not determined. CHO = Chinese Hamster Ovarian.

The calculated pKa values for compounds **1.1** – **1.4** were all above 7.0 (calculated using Molecular Operating Environment) and would result in > 99% protonation at pH 4.7. The PCU-AM derivatives also abided to the Rule of Five (Ro5; Lipinski 2004) and in all cases lipophilicity was in the same range as CQ (LogP = 2 – 4, Table 4.2). This will aid in the further development of these compounds with desired drug-like properties. As for preliminary structure-activity relationship (SARs), a chain length of two carbon atoms, as in compound **1.1**, was found to be optimum for antiplasmodial activity. An increase in the carbon-chain length between the CQ-like

AM portion and the aza-PCU reduced the activity. This decrease in activity is greater in the resistant strain as seen by the decrease in activity of compounds **1.3** and **1.4**. The reduced antiplasmodial activity may be as result of: 1) an increased aliphatic chain length that modified the lipophilic profile of the compounds may have in advertently trapped the aza PCU-AM derivatives in the membrane of parasite; 2) alternatively the compounds may have been subjected to a pGP-mediated efflux (Riordan *et al.*, 1985; Zhang *et al.*, 2004). However, when the activity of compound **1.1** is compared with the results observed in the combination study (Table 4.1) it is clear that this reversed CQ agent yielded significantly improved antiplasmodial activity.

The 'therapeutic' hybridisation approach employed in this study resulted in more effective and improved antimalarial agents compared to lead compounds, NGP1-01 and chloroquine. The approach allows for incorporation of multiple bio-active components into a single hybrid molecule with viable antimalarial and/or resistance reversal potential.

4.4. Conclusion

PCU-AM derivatives designed in this study potentiated the antimalarial activity of CQ with varying IC₅₀-values. Compound **1.1**, the most potent derivative in the series, displayed moderate antimalarial activity against both strains of *P. falciparum* (NF54:IC₅₀ = 3.74 ng/mL and Dd2:IC₅₀ = 27.6 ng/mL) and was found to be 5 fold more active against the resistant strain (Dd2) than CQ. In a separate experiment, the polycyclic amine NGP1-01 was shown to reverse CQ resistance and act as a chemosensitiser. The reversed CQ approach however resulted in improved resistance reversal and a significantly lower concentration PCU was required compared to the NGP1-01 and CQ in combination. This may be attributed to the improved ability of compound **1.1** to actively block the *p*-glycoprotein pump and/or to the increased permeability thereof because of the lipophilic aza-PCU moiety.

CHAPTER 5

SUMMARY AND CONCLUSION

5.1. Introduction

Malaria is one of the world's most devastating parasitic infections and has in recent years become an important focus of research. This infection has an immense effect on economic productivity, livelihood and human settlement patterns (Gallup & Sachs 2001) and is responsible for over a million fatalities per annum. Chloroquine (CQ) is a low-cost, safe and the mainstay aminoquinoline derived chemotherapeutic agent that has been used for many years against blood-stage malaria. However, today CQ resistant plasmodia, in particular the virulent *P. falciparum* impede its use. The primary cause of resistance is mutation in a putative ATP-powered multidrug efflux pump known as the *p*-glycoprotein pump, and point mutation in *P. falciparum* CQ resistance transporter (PfCRT) protein. These mutations resulted in significant reduced accumulation of CQ at its primary site of action.

In attempt to circumvent the challenges of prevailing CQ resistance in *P. falciparum*, chemosensitisers offer an attractive approach. Chemosensitisers are structurally diverse molecules that are known to reinstate the efficacy of CQ in resistant *Plasmodium* species by inhibiting the pGP efflux pump and/or the PfCRT protein associated with CQ export from the digestive vacuole in CQ resistant *Plasmodium* parasites. Chemosensitisers include the antihistaminic agent chlorpheniramine and calcium channel blockers such as verapamil. These drugs have little or no inherent antimalarial activity but have been shown to reverse CQ resistance in *P. falciparum* when co-administered with CQ.

Understanding this process and finding effective antimalarial- and/or reversed CQ agents for it formed the rational of this study. To reach the study objective a series of novel PCU-AM compounds were synthesised and evaluated *in vitro* for antimalarial and/or reversed CQ activity. These structures revealed promising *in vitro* activity and this study and further investigations of the novel reversal agents will contribute to the better understanding of the mechanisms involved in malaria pathogenesis and contribute to potential therapeutic compounds in this field.

5.2. Synthesis

Synthesis of the pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione resulted in a yield of 47 % and this diketone was used as the basis for further substitutions. The polycyclic structure was applied to various tethered CQ-like aminoquinoline derivatives to enable the design of a terminal tertiary amine portion similar to the structure of CQ which may potentially improve the permeability of privileged molecules. It is also suggested that the bulky aza-PCU scaffold will protect the terminal tertiary amino group from metabolism through *N*-dealkylation. The aminoquinoline moieties were conjugated to the polycyclic structure by amination and reductive amination reaction (nucleophilic addition). Five novel PCU-AM compounds were synthesised with percentage yields ranging between 12 % and 58 %. Recrystallisation was mostly used in the purification of the compounds. Purification of some of the structures proved to be a challenge due to the formation of various unidentified impurities. This complicated the extraction, purification and recrystallisation processes and contributed to the low yields of some of the compounds. NMR and IR spectra showed the characteristic signals and MS confirmed the molecular masses of the compounds.

5.3. Biological Evaluation

In an *in vitro* antiplasmodial assay the prototype pentacycloundecyl-derived NGP1-01 was evaluated for intrinsic antimalarial activity against CQ resistant Dd2 of *P. falciparum*. Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of Trager and Jensen (1976). During this evaluation the polycyclic amine was co-administered at various concentrations with CQ to evaluate the ability of NGP1-01 to act as reversal agent and/or chemosensitiser and from this inhibition data the IC₅₀ values were determined and compared. It was observed that NGP1-01 when co-administered with CQ displayed meaningful reversed CQ activity, in the micromolar range, against the resistant strain Dd2. As a further objective, the polycyclic structure was conjugated to different tethered CQ-like aminoquinolines and these novel PCU-AM derivatives were evaluated for antimalarial activity and/or reversed CQ activity. Quantitative determination of the activity profile of the novel derivatives were evaluated by employing an *in vitro* antimalarial pLDH assay (Makler 1993) against both CQ^R Dd2 and CQ^S NF54 strains of *P. falciparum* while toxicity studies were

conducted using MTT-assay (Mosman *et al.*, 1983; Rubinstein *et al.*, 1990). From this inhibition data the IC₅₀ values were calculated and compared.

The newly synthesised compounds, structures **1.1**, **1.2**, **1.3**, and **1.4** demonstrated improved reversal activity and/or antimalarial activity, and significantly lower concentrations of the PCU was required compared to NGP1-01 co-administered with CQ. Compound **1.1**, the short-chain derivative was the most active in the series with an activity profile 5 fold more potent than CQ against the resistant strain Dd2. It is postulated that this may be attributed to the improved ability of compound **1.1** to actively block the *p*-glycoprotein pump and/or the increased permeability thereof because of the lipophilic aza-PCU moiety. Compound **1.1** also showed the lowest RMI value confirming that this compound has the best potential to act as a reversed CQ agent in the series. Cytotoxicity IC₅₀ values observed for compounds **1.1** – **1.4** were in the low micro molar concentrations (2.39 – 9.54 µM) indicating selectivity towards *P. falciparum*.

5.4. Conclusion

In malaria chemotherapy, widespread chloroquine-resistant *falciparum* malaria has left the global population with limited useful antimalarial agents. The quest for novel and improved chemosensitiser and/or reversal agents thus continues.

NGP1-01 alone displayed poor intrinsic antiplasmodial activity against CQ^R Dd2 strain of *falciparum* however, a significant dose-response was observed in the IC₅₀ value of CQ when NGP1-01 was co-administered with CQ. Based on these findings the pentacycloundecyl substructure (PCU) was covalently conjugated with various tethered aminoquinoline (AM) moieties to achieve 5 novel reversal agents which mimicked the structure of CQ. These compounds could thus have potential as useful pharmacological tools to investigate antimalarial- and/or reversed CQ activity in the quest for effective antimalarial strategies. The 5 novel reversal agents synthesised in this study displayed antiplasmodial activity profiles superior to that of NGP1-01, with a lower concentration of this reversal moiety required. In this novel series, the short-chain aza derivative compound **1.1** displayed the best activity against both CQ resistant (Dd2) and sensitive (NF54) strains of *falciparum*. Compound **1.1** also displayed activity comparable to that of the highly active artemisinin-derived artesunate and was 5 fold more active

than CQ against CQ^S NF54 strain thus making compound **1.1** and related derivatives (compounds **1.2** – **1.4**) promising lead compounds in malaria drug discovery.

These results indicate that the PCU channel blockers and PCU-AM derived conjugates can be utilized as lead molecules for further optimization and development to enhance their therapeutic potential as chemosensitisers and reversed CQ agents. In addition, to produce a more detailed analysis of the SARs of the novel PCU-AM and their therapeutic potential, future studies essentially need to include: 1) structural optimisation studies of compound **1.1**; 2) investigation or elucidation of the mechanistic or molecular mode of action of compound **1.1** and related derivatives for better understanding of the molecules' antiparasitic effect against *P. falciparum*; 3) molecular modelling studies which may add valuable insights into the rational design of other PCU-AM molecules; and 4) cysteine proteases/falcipain- (Rosenthal *et al.*, 1996) and oxidant defence assays (Ginsburg 1998; Färber *et al.*, 1998).

This additional work on this novel class of antimalarial agents will lend impetus to malaria chemotherapeutic development, in particular in African regions, which are in an immediate need of a cure. The polycyclic structure that was applied as carrier molecules, thus not only serve as pharmacokinetic enhancer but also to improve pharmacodynamics. With the described antiplasmodial and/or reversed CQ activity of the polycyclic structures and taking the above aspects into account, these novel compounds may find application as multipotent drugs in malaria chemotherapy. The polycyclic cage thus appears to be a useful scaffold to explore in order to design potential pharmacological active compounds in the field of malaria.

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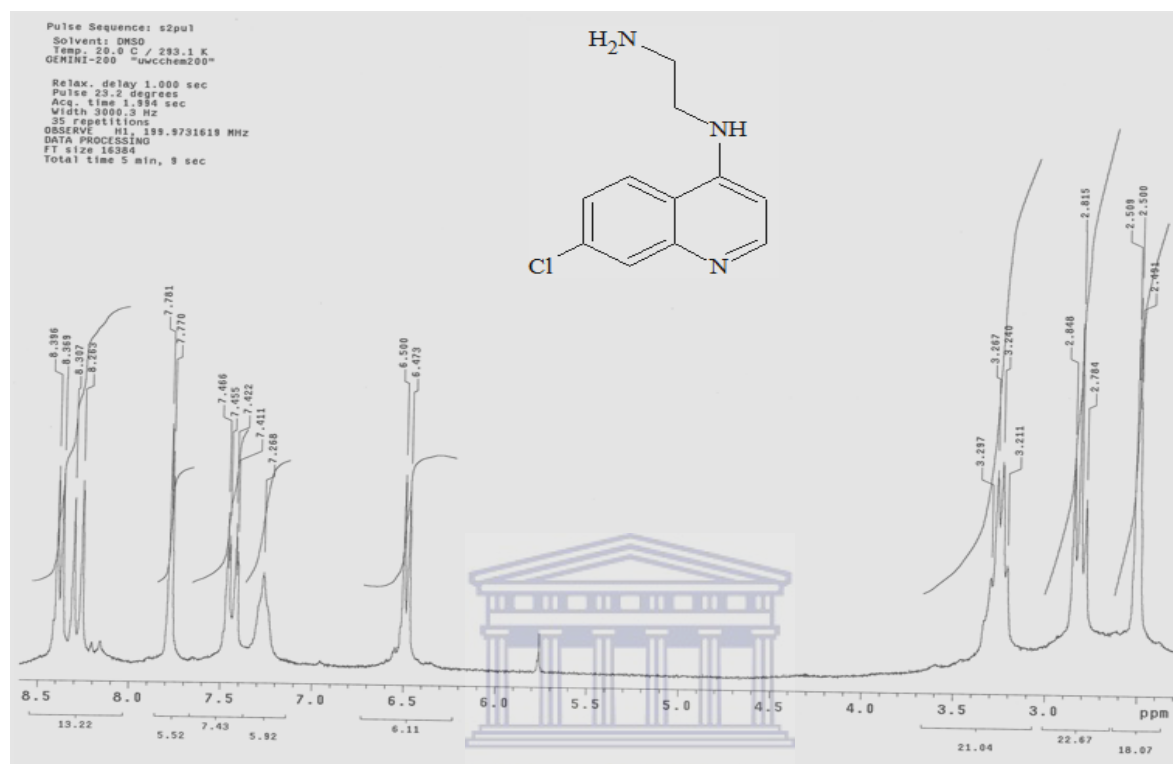
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ANNEXURE A
SPECTRAL DATA ^1H -NMR, ^{13}C -NMR, MS and IR



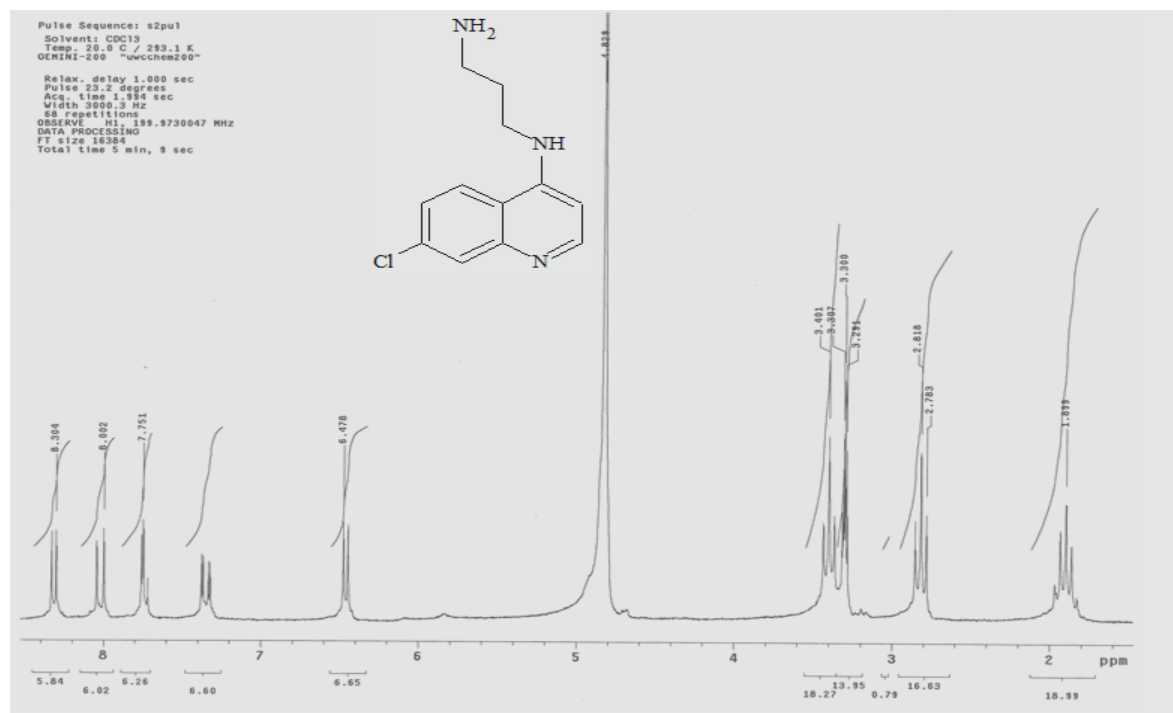
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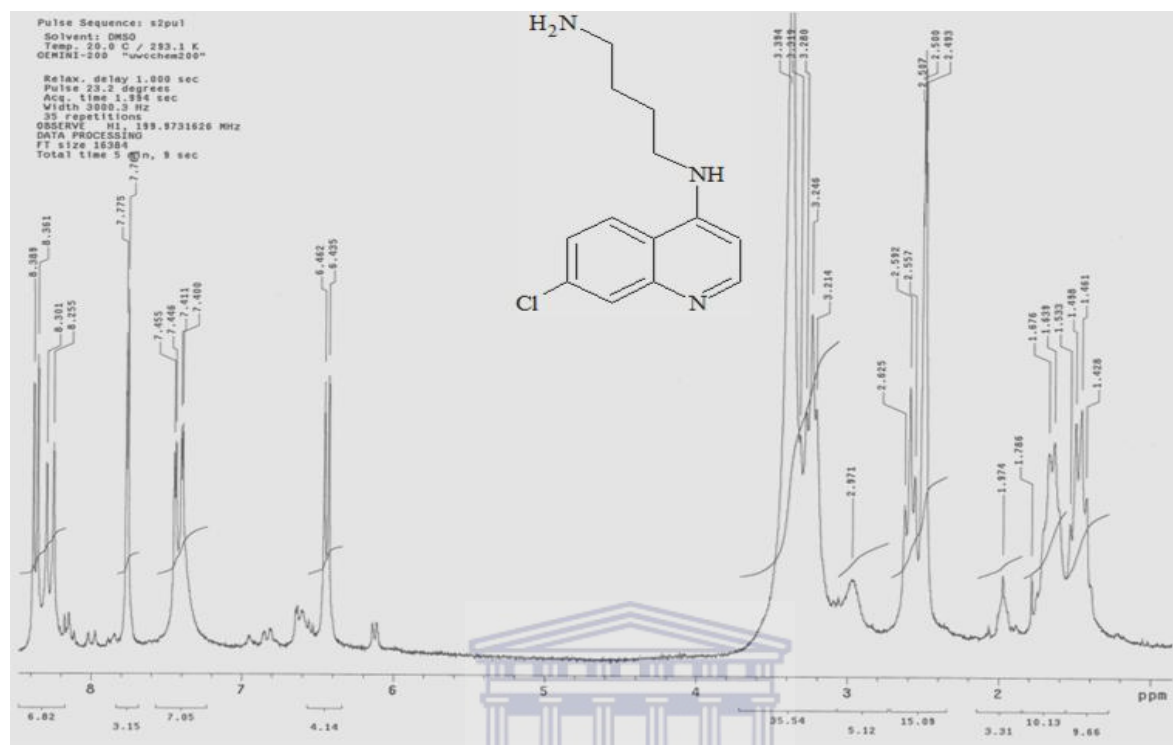


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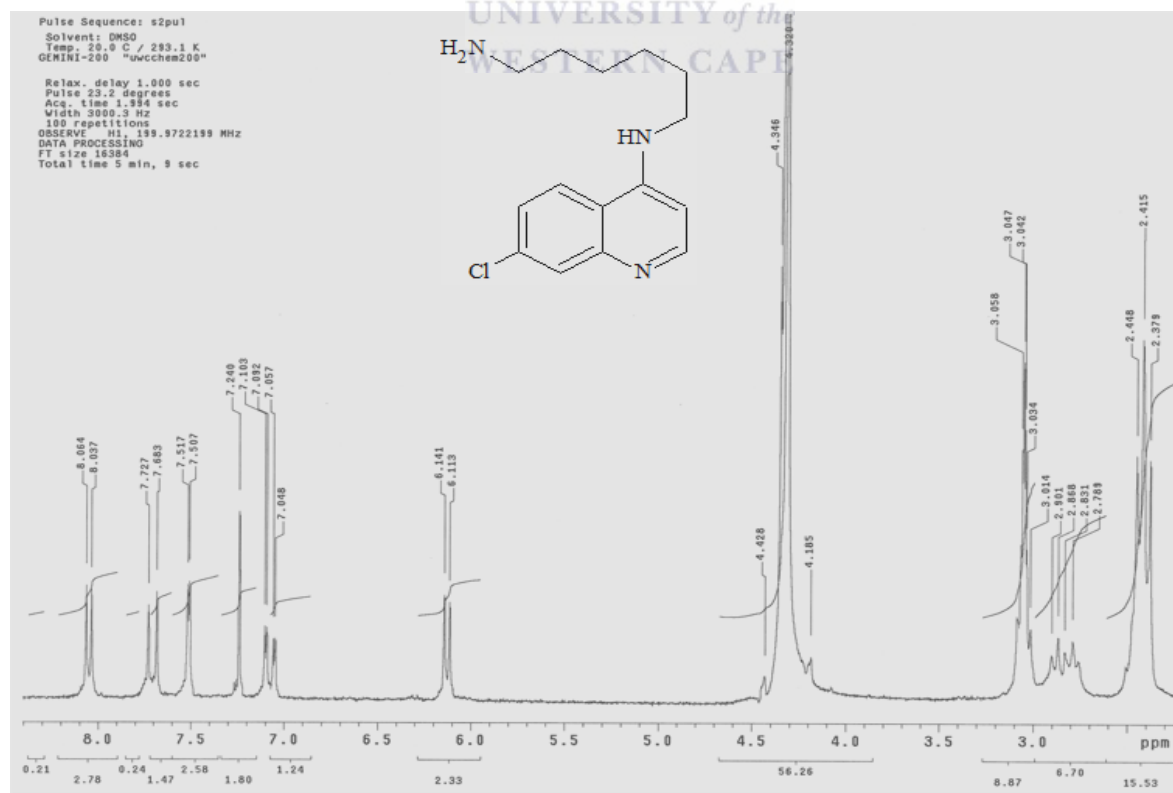
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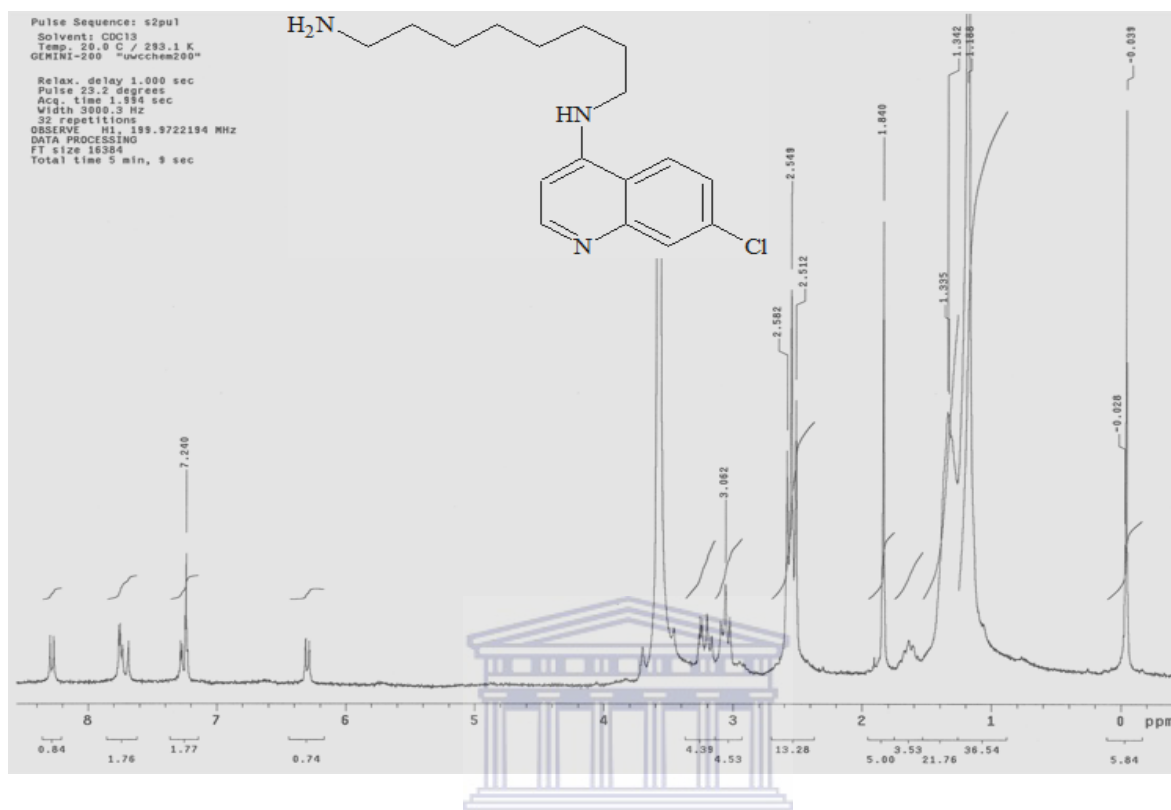
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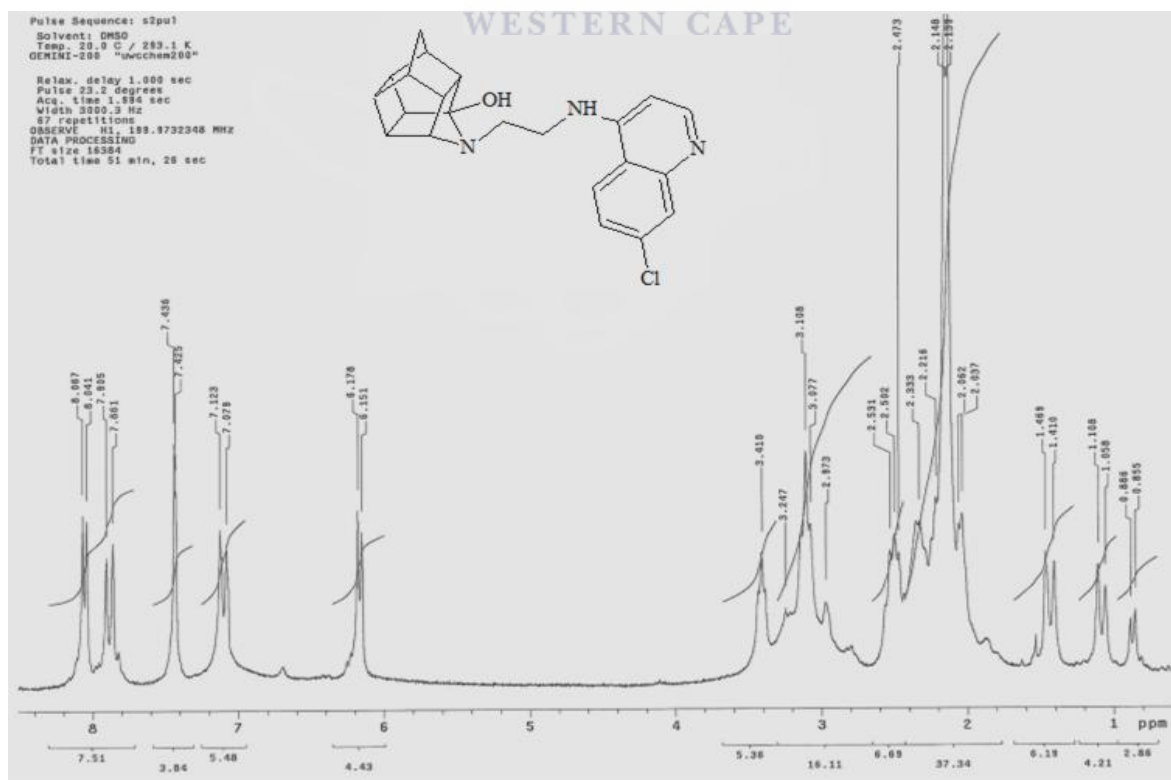
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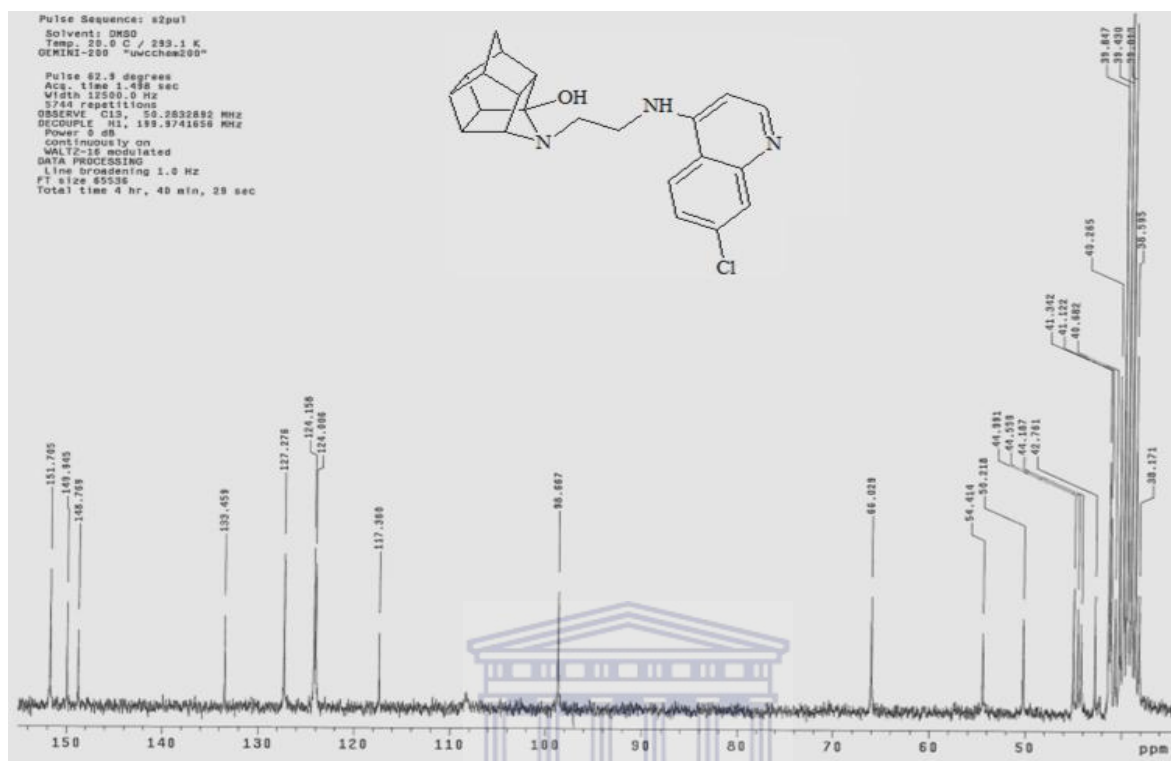
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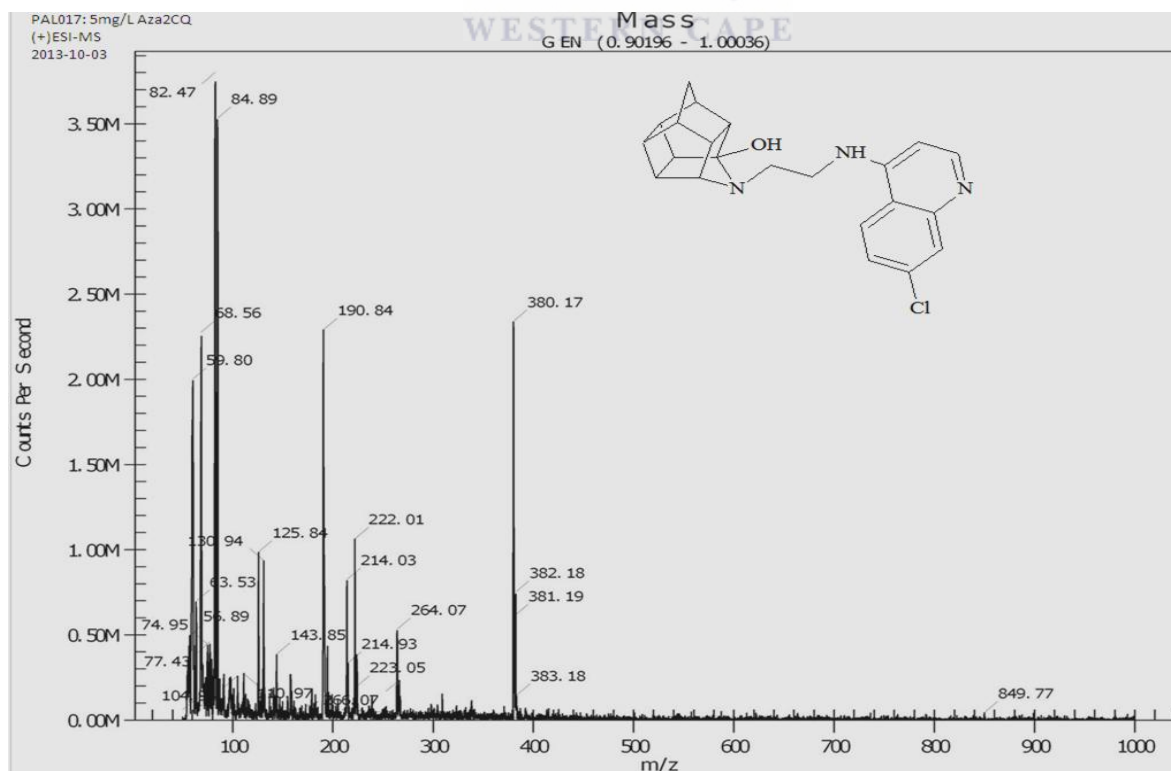
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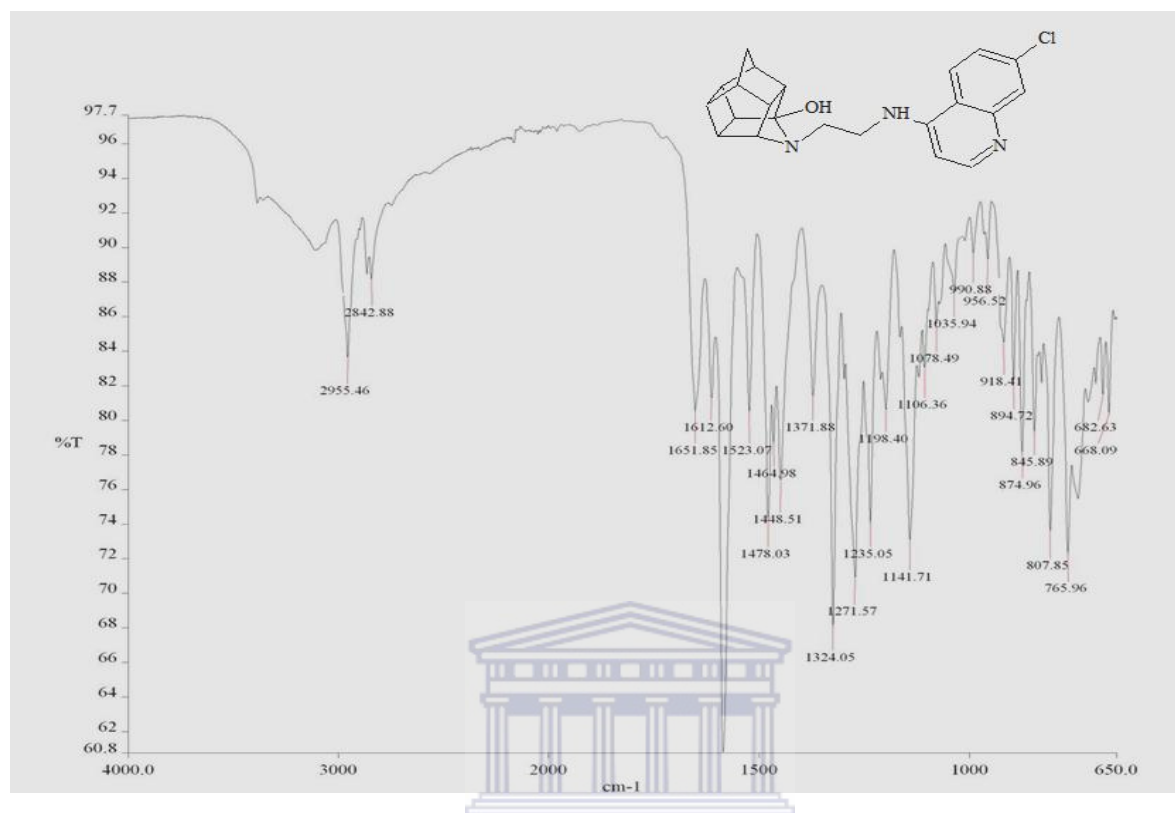
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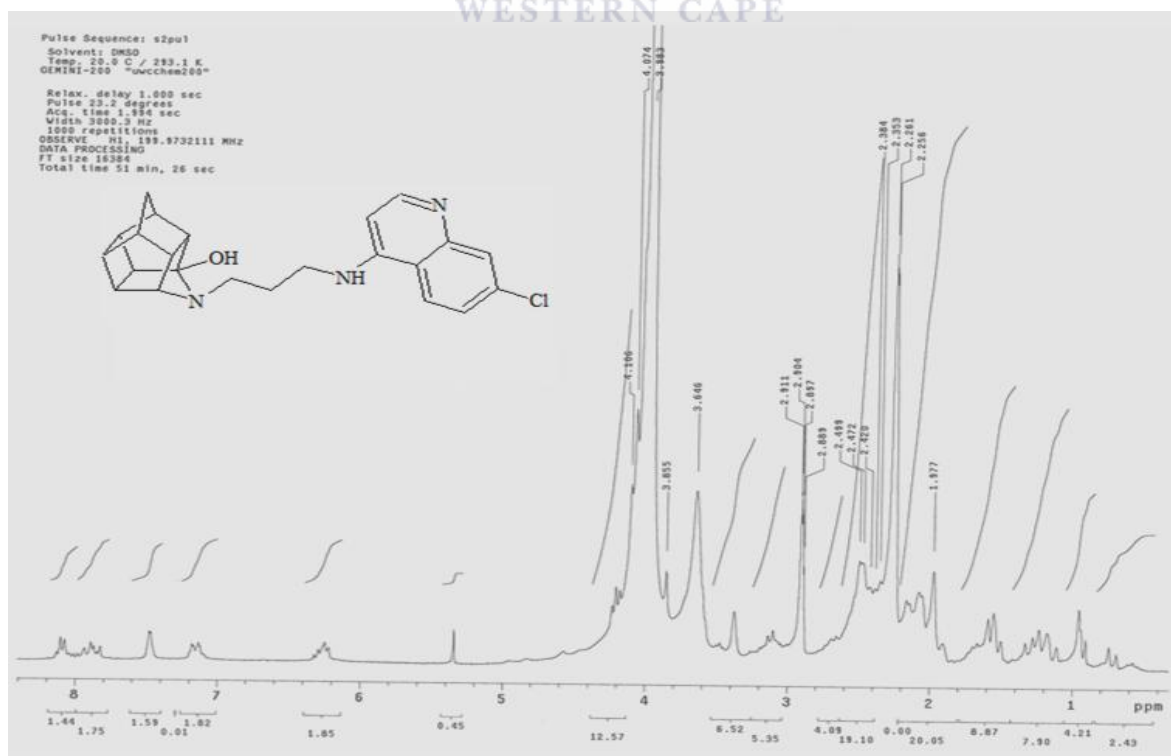
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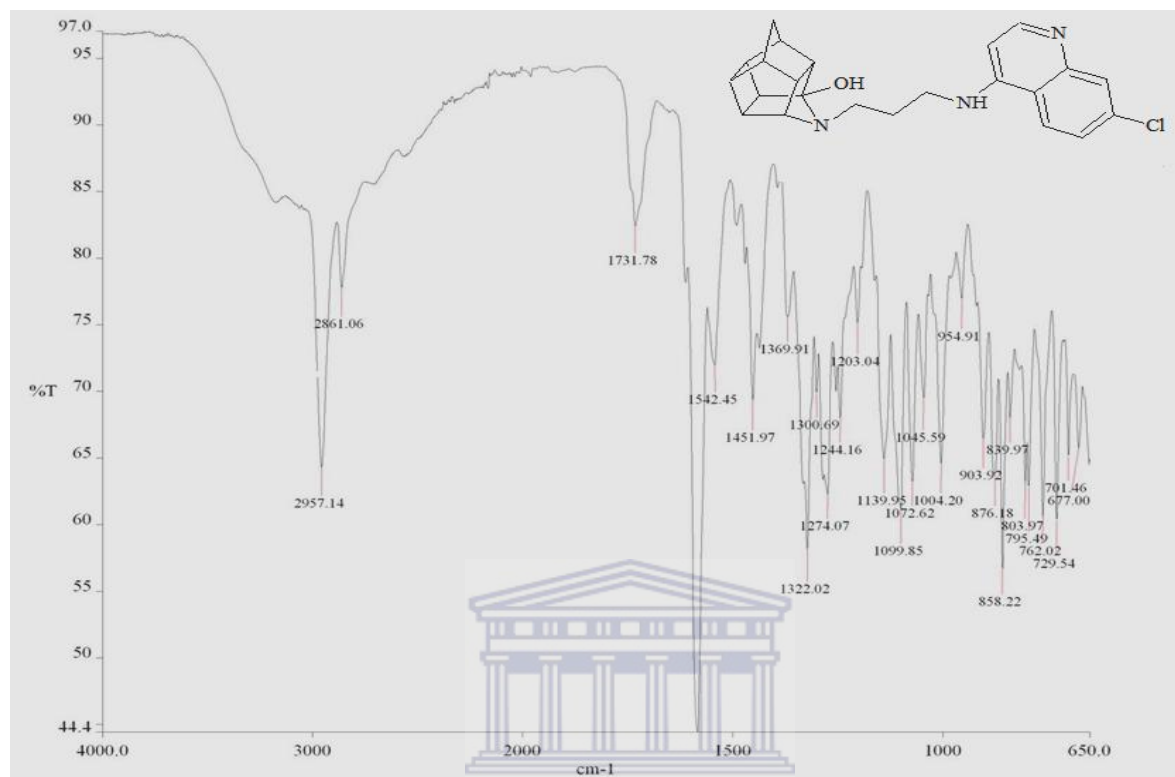
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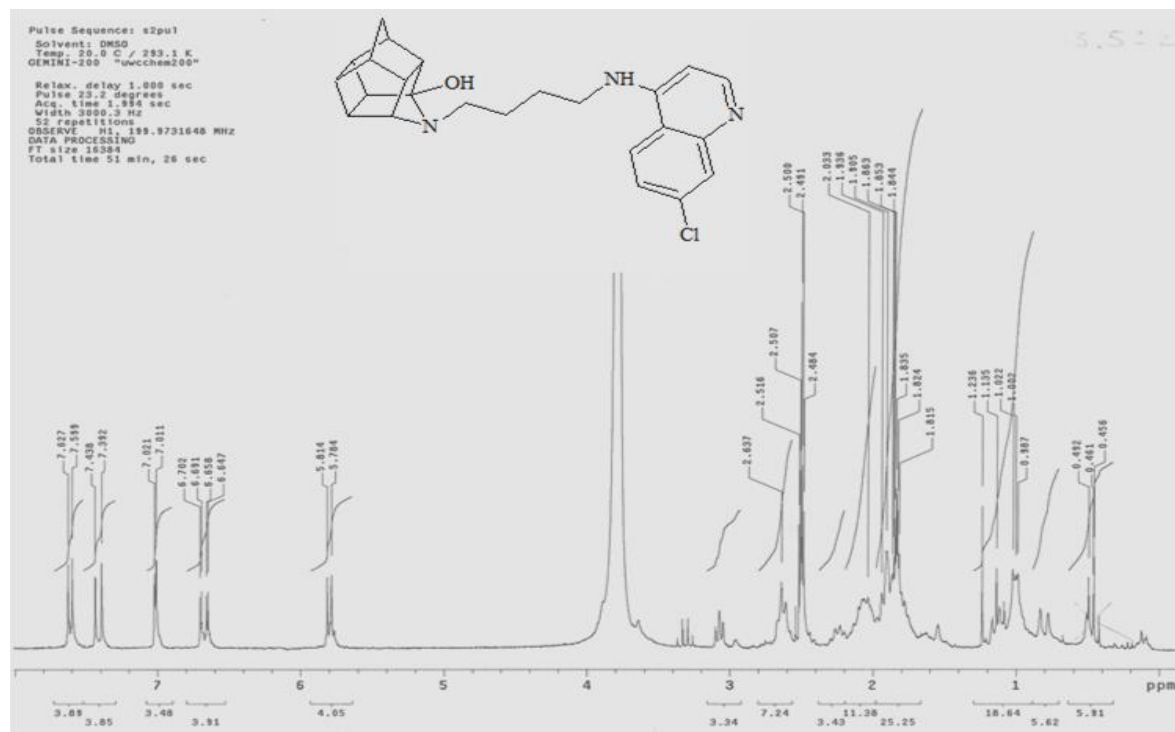
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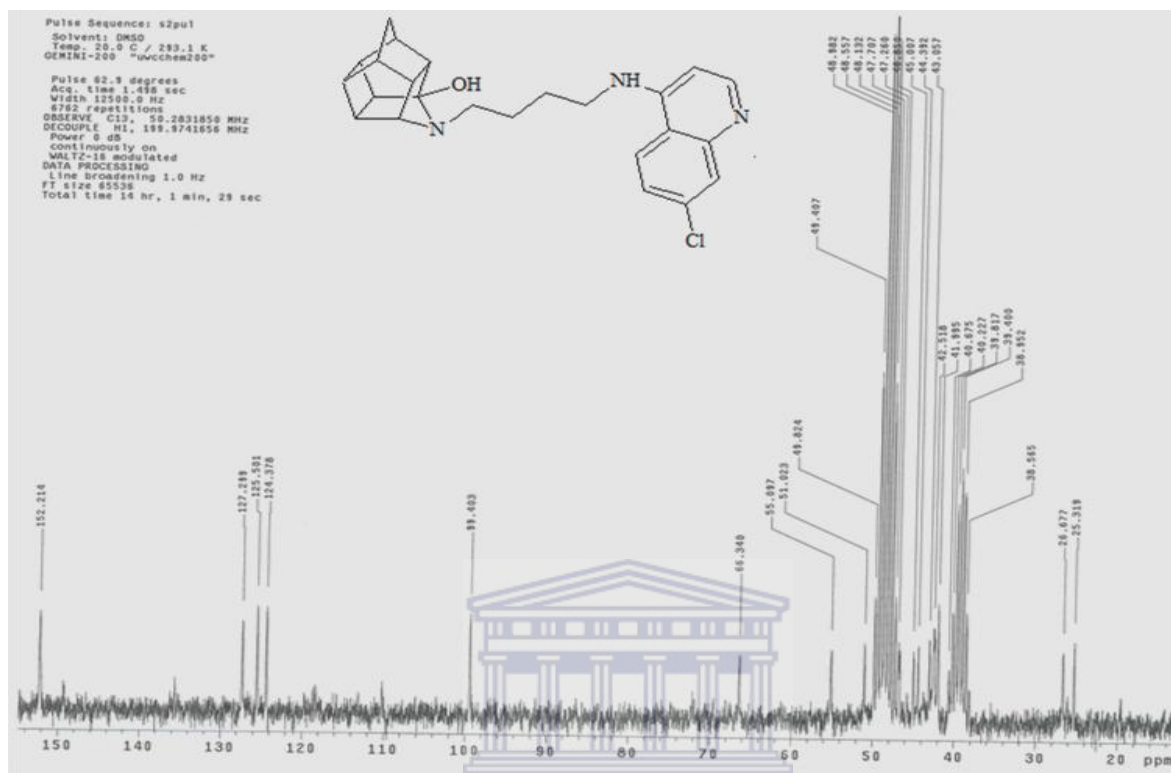
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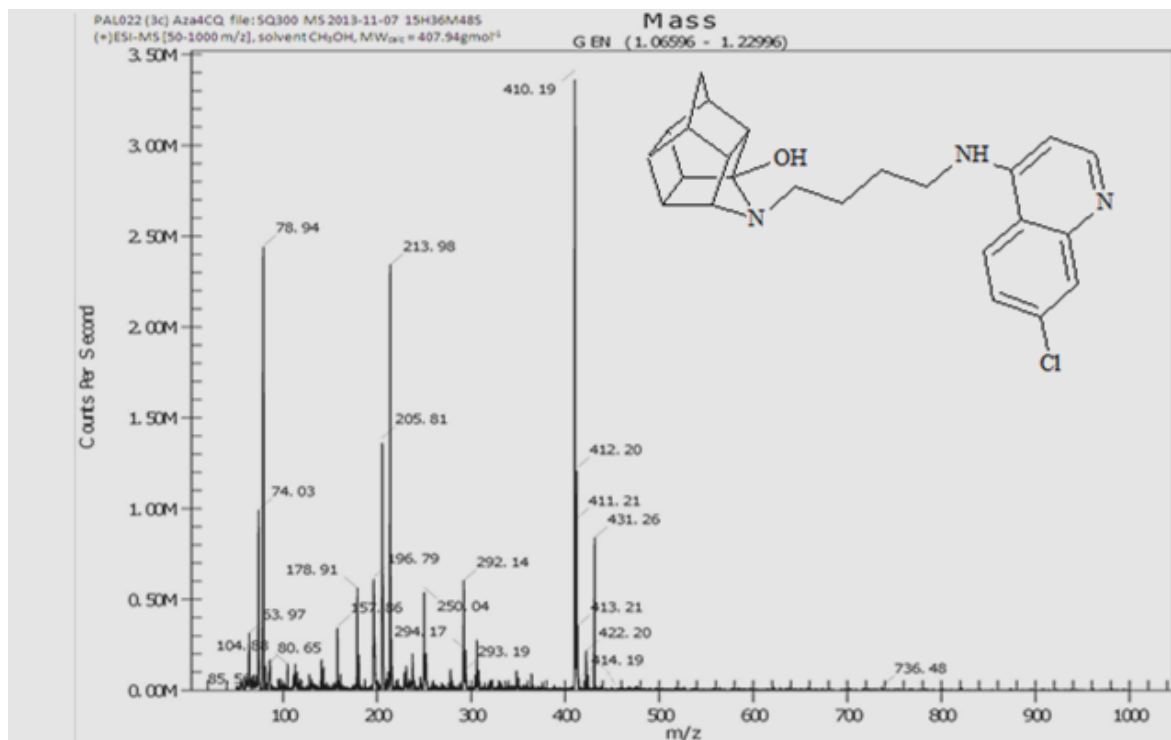
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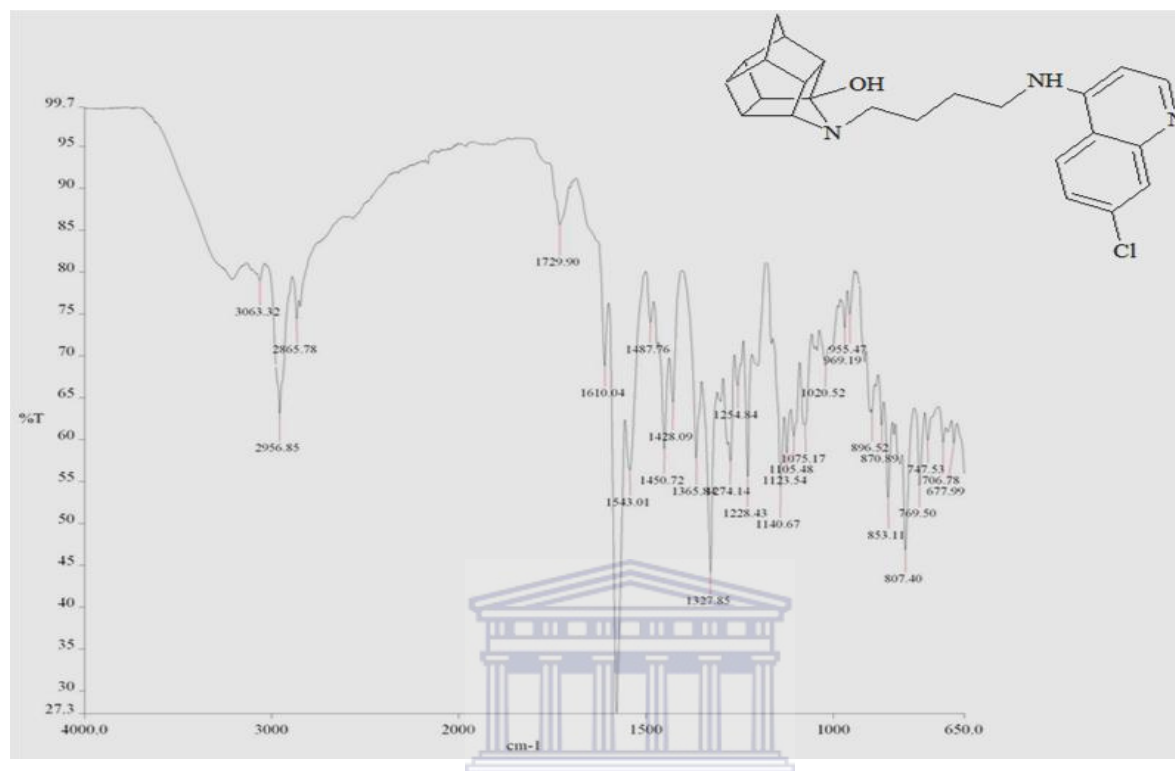
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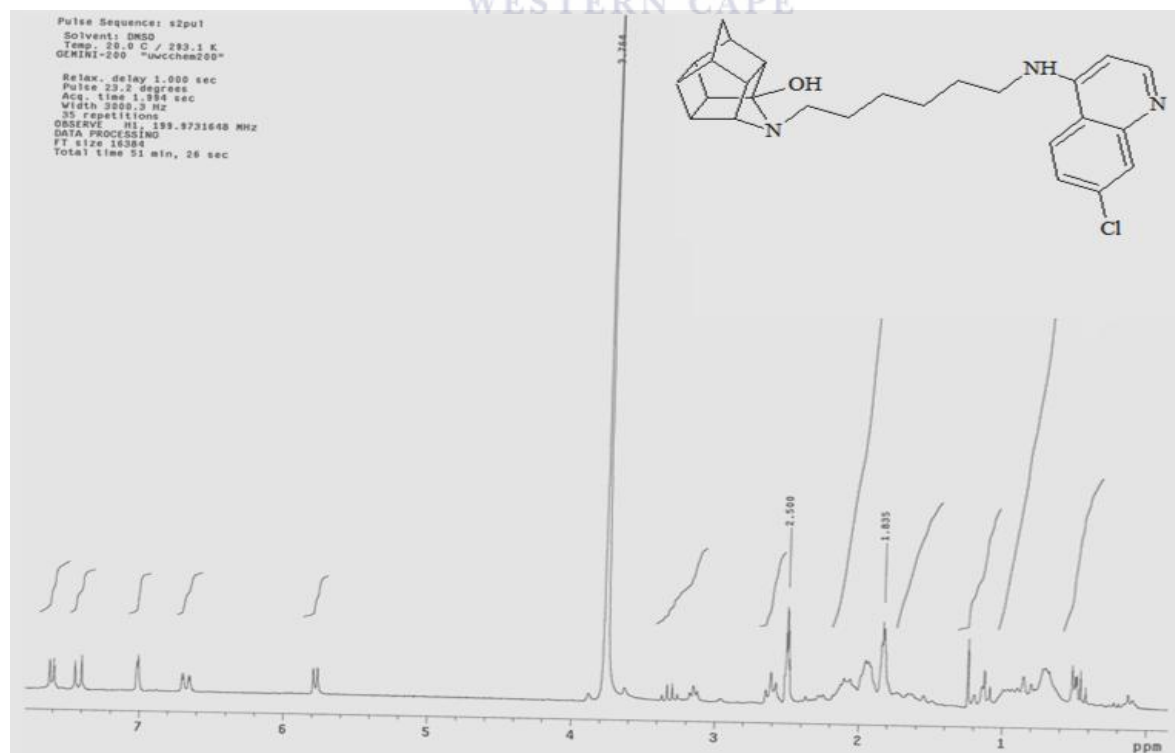
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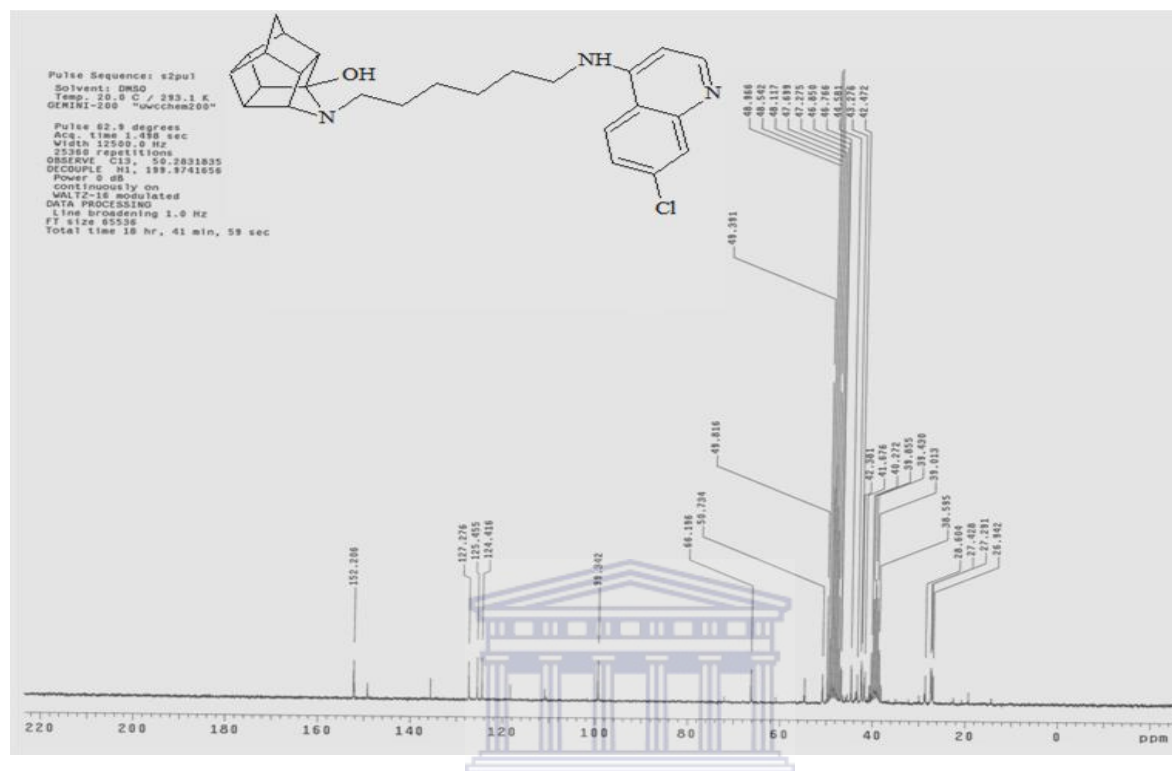
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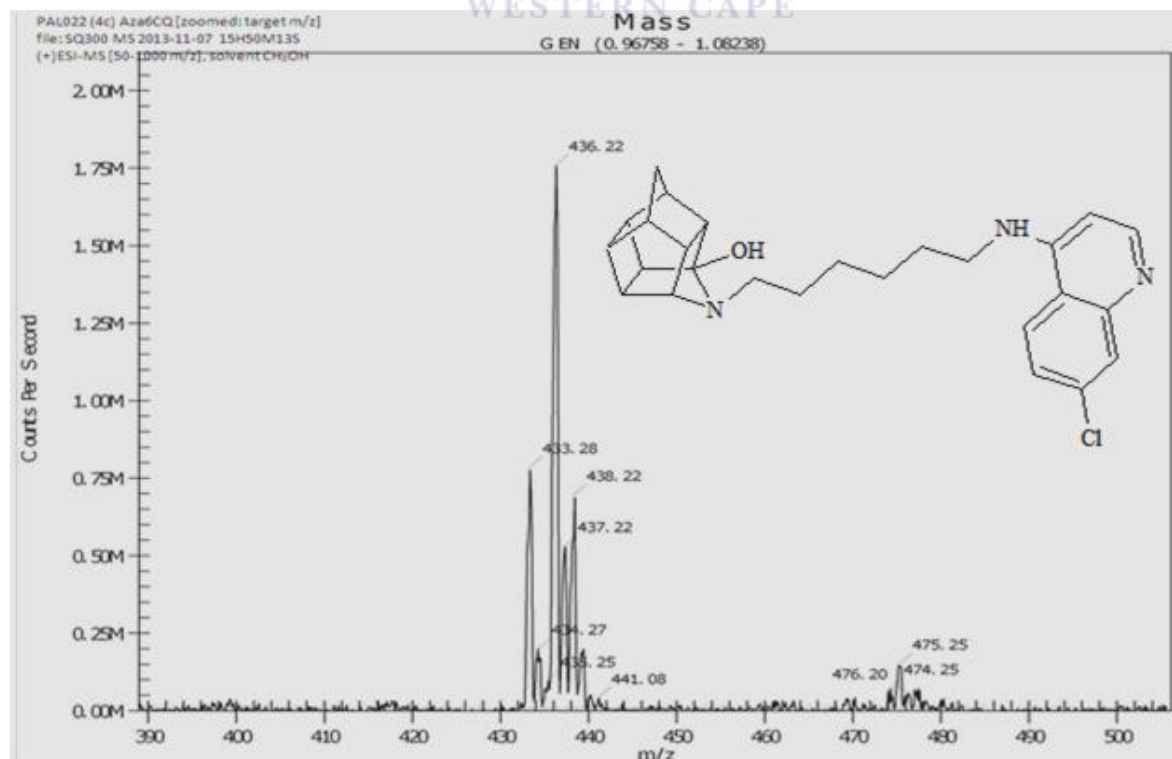
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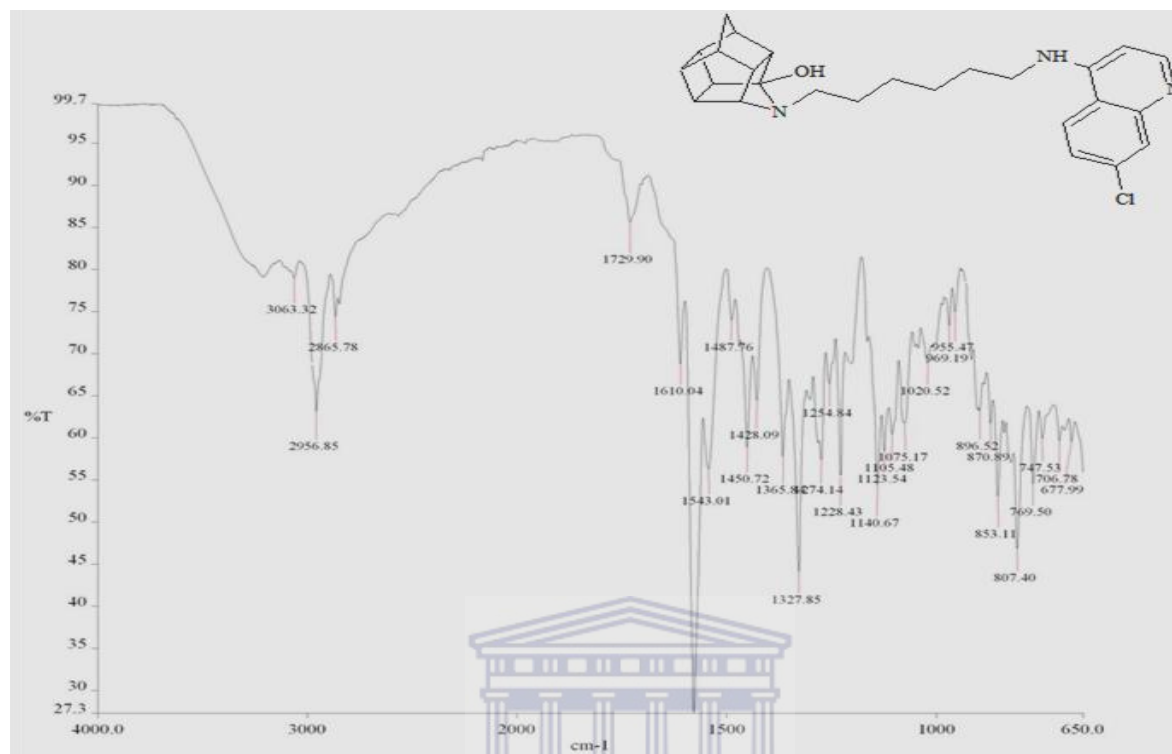
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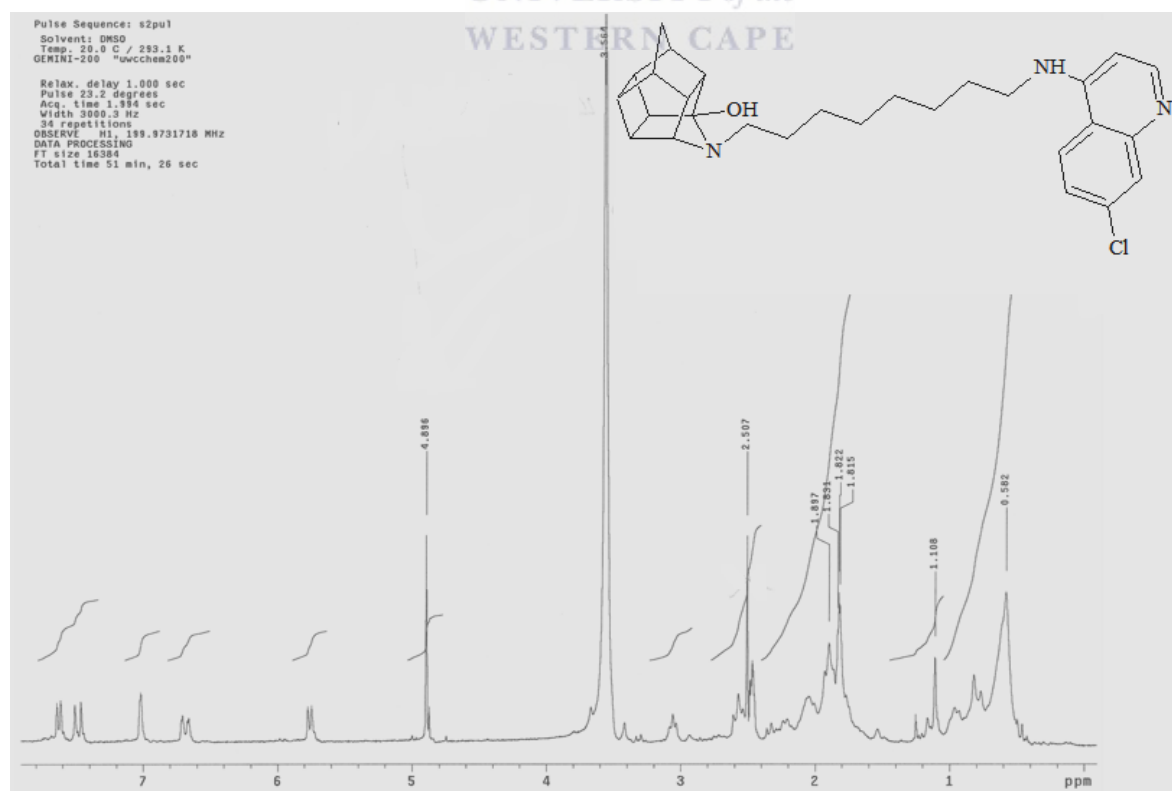
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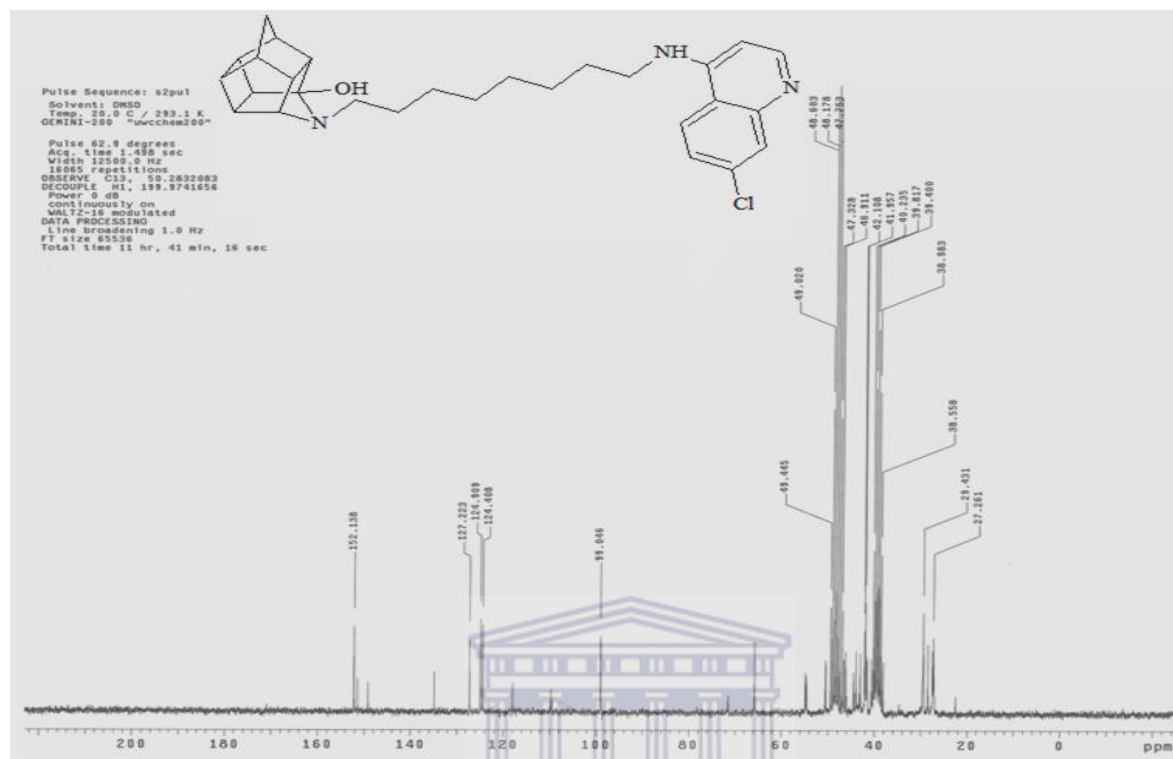
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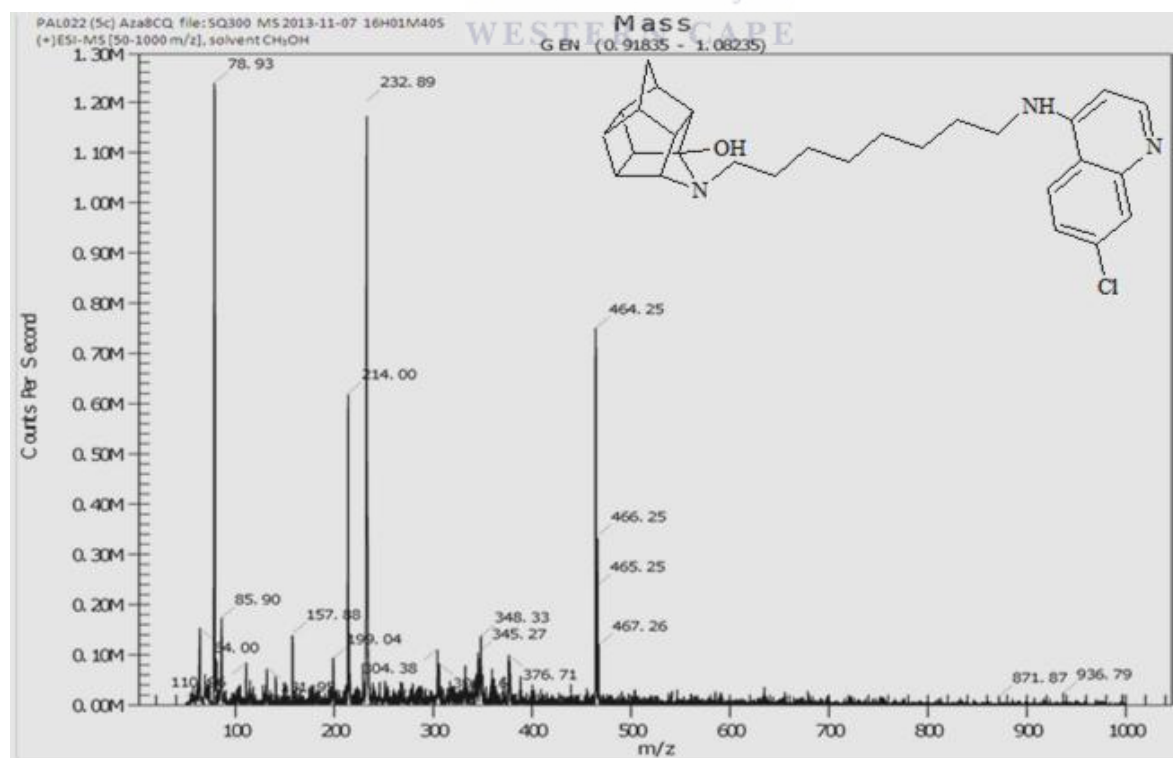
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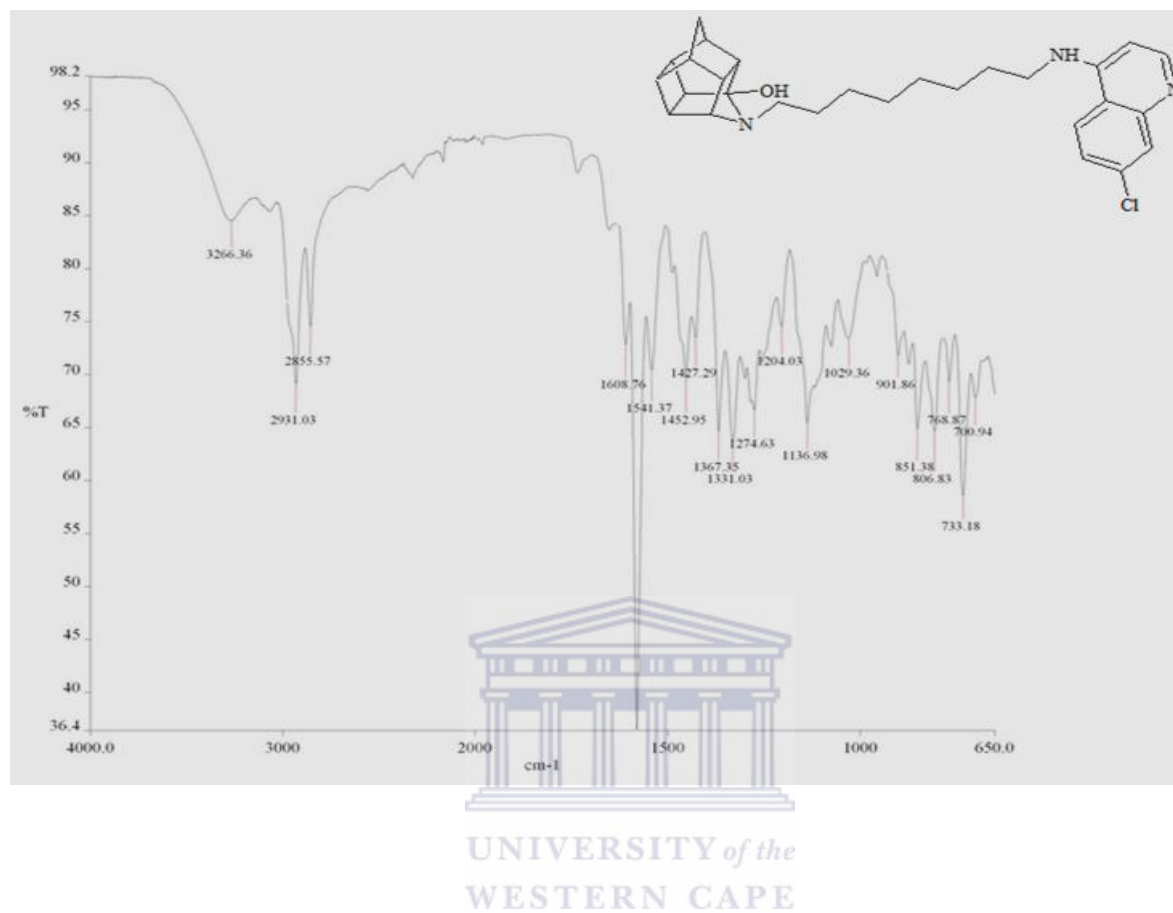
Spectrum 23:



Spectrum 24:



Spectrum 25



ANNEXURE B

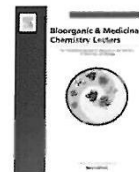
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Pentacycloundecylamines and conjugates thereof as chemosensitizers and reversed chloroquine agents

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ABSTRACT

The control of malaria has been complicated by increased resistance of the malaria parasite to existing antimalarials such as chloroquine (CQ). Herein, we report the ability of NGP1-01, the prototype pentacycloundecylamine (PCU), to reverse CQ resistance (>50%) and act as a chemosensitizer. Based on this finding we set out to synthesize a small series of novel agents comprising of a PCU moiety as the reversal agent conjugated to a CQ-like aminoquinoline (AM) molecule and evaluate the potential of these PCU-AM derivatives as reversed CQ agents. PCU-AM derivatives **1–3** showed anti-plasmodial IC₅₀ values in the ranges of 3.74–17.6 nM and 27.6–253.5 nM against CQ-sensitive (D10) and CQ-resistant strains (Dd2) of *Plasmodium falciparum*, respectively. Compound **1** presented with the best antiplasmodial activity at low nM concentrations against both strains and was found to be 5 fold more active against the resistant strain than CQ. Compound **1** can be considered as a lead compound to develop reversed CQ agents with improved pharmacodynamic and pharmacokinetic properties.

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Plasmodium falciparum malaria continues to be a worldwide health problem, especially in developing countries in Africa and is responsible for over a million fatalities per annum.¹ Chloroquine (CQ) is a low-cost, safe and mainstay aminoquinoline derived chemotherapeutic agent that has been used for many years against blood-stage malaria.^{2,3} However, today CQ resistant plasmodia, in particular the virulent *P. falciparum* impede its use.³ The primary cause of resistance is mutation in a putative ATP-powered multi-drug efflux pump known as the *p*-glycoprotein pump, and point mutation in *P. falciparum* CQ resistance transporter (*PfCQRT*) protein. These mutations are responsible for the reduced accumulation of CQ at its primary site of action, the acidic digestive food vacuole of the parasite.^{4–6}

To overcome the challenges of CQ resistance in *P. falciparum*, chemosensitizers offer an attractive approach. Chemosensitizers are structurally diverse molecules that are known to reverse CQ resistance by inhibiting the *p*-glycoprotein efflux pump and/or the *PfCQRT* protein associated with CQ export from the digestive vacuole in CQ resistant parasites.^{7–9} Chemosensitizers include the antihistaminic agent chlorpheniramine and calcium channel blockers such as imipramine and verapamil. These drugs have little or no inherent antimalarial activity but have shown to reverse CQ resistance in *P. falciparum* when co-administered with CQ.¹⁰

Because of the channel blocking abilities of PCUs, such as NGP1-01 (Fig. 1),^{11,12} it is postulated that these agents may act as chemosensitizers and circumvent the resistance of the plasmodia parasite against CQ by inhibiting the *p*-glycoprotein efflux pump and enable the accumulation of CQ inside the parasite digestive vacuole. Therefore as a proof of concept we conducted an experiment using CQ co-administered with different concentrations NGP1-01 to evaluate the ability of NGP1-01 to act as a chemosensitizer. In vitro antiplasmodial activity was determined against the CQ sensitive D10 strain and CQ resistant Dd2 strain of *Plasmodium falciparum* using a well established method.^{13–15} NGP1-01 alone exhibited very low intrinsic antimalarial activity against both the resistant and sensitive strain (>2000 nM), with no toxicity to the parasite detected at 10 μM.^{16–18} A statistically significant (*p* < 0.05) dose dependent shift was seen in the CQ IC₅₀ values at both a 1 μM and 10 μM concentration of co-administered NGP1-01 against the resistant strain as shown in Table 1. This is the first time, to the best of our knowledge, that a PCU compound has been evaluated for and exhibited potential as a chemosensitizer. A number of PCU compounds previously designed by our group have shown significantly more potent channel blocking activity than NGP1-01 and may potentially present with better chemosensitizing ability.^{11,12,19} We are currently exploring the potential of these compounds as chemosensitizing agents.

Based on the chemosensitizing ability of NGP1-01, our next objective was to attempt to design potential reversed CQ agents

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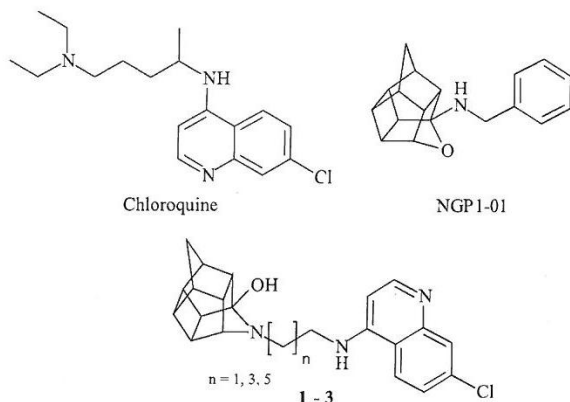


Figure 1. Structure of chloroquine, NGP1-01 and the synthesised PCU-AM derivatives (1–3).

Table 1

In vitro antiplasmodial experiment using NGP1-01 and CQ separately and in combination

Compound	Strain	IC ₅₀ (nM)	SD	RMI
NGP1-01	D10	>2000	–	–
NGP1-01	Dd2	>2000	–	–
CQ ²⁰	Dd2	101.8	4.9	1
CQ + 1 μM NGP1-01	Dd2	60.7	4.1	0.60
CQ + 10 μM NGP1-01	Dd2	48.8	5.3	0.48

Response modification index, the ratio of the altered IC₅₀ using CQ and NGP1-01 to that of CQ alone. Lower values indicate better reversal.

comprising of a PCU moiety as the reversal agent (RA) conjugated to a CQ-like aminoquinoline (AM) molecule and to evaluate the potential of these PCU-AM derivatives as reversed CQ agents. As recently shown by Peyton and co-workers, the conjugation of a CQ-like molecule with a RA such as the chemosensitizer imipramine and derivatives thereof is a viable strategy to reverse CQ resistance in drug-resistant *P. falciparum*.^{21–23} The design of the PCU-AM derivatives (1–3) commenced by selecting an appropriate PCU scaffold. The aza-PCU was considered as the best option because it would enable the design of a terminal tertiary amine portion similar to the structure of CQ. PCU scaffolds also have the potential to increase the permeation of privileged molecules over biological membranes and possibly into the parasite vacuole when covalently bound. Studies by Zah et al., 2003 and Prins et al., 2009 illustrated the ability of PCU scaffolds to significantly improve the permeability of privileged molecules.^{24,25} It is also suggested that the bulky aza-PCU scaffold will protect the terminal tertiary amino group from metabolism via N-dealkylation. N-dealkylation of CQ reduces its lipid solubility and may lead to an increase in the potential for cross-resistance with CQ.^{26,27} Previous studies^{28,29}

showed that the use of bulkier substituents attached to the terminal amino group of CQ increased the in vivo efficacy and also decreased the potential for cross-resistance, most probably by circumventing metabolic N-dealkylation. The same effect is expected with the bulky aza-PCU scaffold. A basic center was also retained in the PCU-AM derivatives, as CQ is postulated to concentrate in the parasite digestive vacuole by virtue of protonation under the acidic conditions found in that compartment (pH of the digestive vacuole is 4.7). The calculated pK_a values for compounds 1–3 were all above 7.0 (calculated using Molecular Operating Environment) and would result in >99% protonation at pH 4.7. The PCU-AM derivatives also abided to the Ro5,³⁰ and in all cases lipophilicity was in the same range as CQ (LogP = 2–4, Table 2). This will aid in the further development of these compounds with desired drug-like properties.

The designed PCU-AM derivatives were synthesised by conjugating the Cookson's diketone with different tethered 4-aminoquinoline moieties to yield the respective carbinolamines (Scheme 1, ii) and the subsequent imines. This was followed by a transannular cyclization using sodium cyanoborohydride as reducing agent to yield the desired PCU-aminoquinoline molecules (1–3, Scheme 1, iii).³¹ The 4-aminoquinolines were obtained using a novel microwave (MW) irradiation method (Scheme 1, i).³²

In the series of novel synthesised PCU-AM derivatives, compound 1 had significant antimalarial activity when tested against the CQ sensitive (IC₅₀ = 3.74 nM) and CQ resistant strain (IC₅₀ = 27.6 nM), showing greater activity than CQ against both strains of the parasite (Table 2).^{13–15} Compounds 2 and 3 displayed good activity against the CQ-sensitive strain (IC₅₀ = 8.45 and 17.6 nM, respectively) however their activity profile was significantly reduced in the CQ-resistant strain (IC₅₀ = 187 and 253 nM, respectively). Compound 1 also showed the lowest RMI value confirming that this compound has the best potential to act as a reversed CQ agent in the series. Cytotoxicity IC₅₀ values observed for compounds 1–3 were in the low micro molar concentrations (2.39–9.54 μM) indicating selectivity towards *P. falciparum* (SI = 149–2549, Table 2) and low toxicity compared to the cytotoxic agent emetine (IC₅₀ = 0.061 μM).¹⁶ A chain length of two carbon atoms, as in compound 1, was found to be optimum for antiplasmodial activity. An increase in the carbon-chain length between the aminoquinoline-like portion and the aza-PCU reduced the activity. This decrease in activity is greater in the resistant strain as seen by the decrease in activity of compounds 2 and 3. When the activity of compound 1 is compared with the results observed in the combination study (Table 1) it is clear that this reversed CQ agent yielded significantly improved antiplasmodial activity.

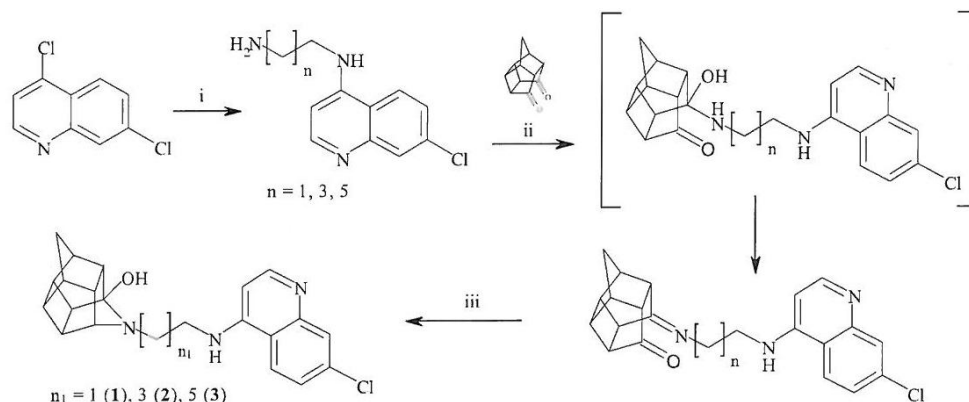
In conclusion, PCU-AM reversed CQ derivatives were synthesised and showed significant in vitro antimalarial activity against the CQ sensitive strain (D10). Compound 1 showed the highest antiplasmodial activity against both strains tested and was found to be 5 fold more active against the resistant strain (Dd2) than CQ. In a separate experiment, the polycyclic amine, NGP1-01, was shown to reverse CQ resistance and act as a chemosensitizer.

Table 2

In vitro IC₅₀ values of the PCU-AM derivatives and reference compounds with standard deviations (SD) of antiplasmodial activity and cytotoxicity

Compound	D10: IC ₅₀ (nM)	SD	Dd2: IC ₅₀ (nM)	SD	RI	CHO: IC ₅₀ (nM)	SD	SI	LogP
1	3.74	0.13	27.6	5.1	7	9540	0.23	2549	2.11
2	8.45	0.27	187	35.4	22	2390	0.56	283	2.72
3	17.6	1.02	253	4.0	14	2630	0.12	149	3.59
CQ ²⁰	5.49	0.72	123.5	6.98	23	ND	ND	ND	4.69
Emetine	ND	ND	ND	ND	ND	61	0.01	ND	ND

Resistance index (RI) = IC₅₀ Dd2/IC₅₀ D10. Selectivity index (SI) = IC₅₀ CHO/IC₅₀ D10. ND = not determined. CHO = Chinese Hamster Ovarian. LogP values calculated using ACD/ChemSketch.



Scheme 1. Reagents and conditions: (i) alkyl diamine, CH₃CN, MW, 150 °C, 150 W, 150 psi, 30 min; (ii) Cooksen's diketone, anhydrous THF, 5 °C, 1 h; (iii) MeOH, NaCNBH₃, rt, 4–6 h, 1–3.

The reversed CQ approach however resulted in improved resistance reversal and a significantly lower concentration PCU was required compared to the NGP1-01 and CQ in combination. This may be attributed to the improved ability of compound **1** to actively block the *p*-glycoprotein pump and/or the increased permeability thereof because of the lipophilic aza-PCU moiety. These results indicate that PCU channel blockers and PCU-AM conjugates can be utilized as lead molecules for further optimization and development to enhance their therapeutic potential as chemosensitizers and reversed CQ agents.

Acknowledgments

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- General procedure for the synthesis of 1–3:* Pentacyclo[5.4.0^{2,6}.0^{3,10}.0^{5,9}.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecan-3-ol **1**. ¹H NMR (200 MHz, DMSO) δ_H: 8.26 (d, 1H, J = 5.32 Hz), 7.83 (d, 1H, J = 2.02), 7.36 (s, 1H), 7.10 (dd, 1H, J = 1.86, 8.89 Hz), 6.17 (d, 1H, J = 5.37 Hz), 3.41–2.97 (m, 6H), 2.53–2.04 (m, 8H) 1.79:1.41 (AB-q, 2H, J = 10.4 Hz); ¹³C NMR (50 MHz, CDCl₃) δ_C: 151.71, 148.95, 148.77, 133.46, 127.28, 124.16, 124.01, 117.35, 98.67, 66.03, 54.41, 50.22, 44.99, 44.56, 44.19, 42.76, 41.34, 41.12; MS (ESI-MS) m/z: 380.17 [(M+H)⁺, 100%], 382.18 [(M+H+2)⁺, 30%]. *N*-[4-(7-chloroquinolin-4-ylamino)butylamino]-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecan-3-ol **2**. ¹H NMR (200 MHz, DMSO-CD₃O) δ_H: 8.13–8.09 (d, 1H, J = 5.39 Hz), 7.91 (d, 1H, J = 1.98 Hz) 7.56 (s, 1H), 7.17 (dd, 1H, J = 1.94, 8.82 Hz), 6.30 (d, 1H, J = 5.37 Hz), 3.57 (t, 2 H), 3.14–3.51 (m, 4H), 2.76–2.28 (m, 8H) 1.56–1.28 (m, 6H); ¹³C NMR (50 MHz, CDCl₃) δ_C: 152.21, 150.31, 149.00, 135.80, 127.29, 125.50, 124.37, 99.40, 66.34, 55.10, 51.02, 45.85, 45.01, 44.39, 43.06, 42.52, 41.99, 26.68, 25.32; MS (ESI-MS) m/z: 410.19 [(M+H)⁺, 100%], 412.20 [(M+H+2)⁺, 30%]. *N*-[6-(7-chloroquinolin-4-ylamino)hexylamino]-4-azahexacyclo[5.4.1.0^{2,6}.0^{3,10}.0^{5,9}.0^{8,11}]dodecan-3-ol **3**. ¹H NMR (200 MHz, DMSO/CD₃O) δ_H: 8.12 (d, 1H, J = 5.37 Hz), 7.92 (d, 1H, J = 1.99 Hz) 7.56 (s, 1H), 7.17 (dd, 1H, J = 1.93, 8.83 Hz), 6.27 (d, 1H, J = 5.37 Hz), 3.53 (t, 2 H), 3.14–3.08 (m, 4H), 2.50–2.05 (m, 10H) 1.50–1.18 (m, 8H); ¹³C NMR (50 MHz, CDCl₃) δ_C: 152.21, 152.06, 149.25, 135.56, 127.28,

- 125.46, 124.42, 118.30, 99.34, 66.20, 54.64, 50.73, 45.90, 45.50, 44.72, 44.58, 43.70, 43.40, 42.38, 41.77, 28.60, 27.43, 26.94; MS (ESI-MS) *m/z*: 436.22 [(M+H)⁺, 100%], 438.22 [(M+H+2)⁺, 30%].
32. *General procedure for the synthesis of N¹-(7-chloroquinolin-4yl)diamines:* 4,7-Dichloroquinoline (5.05 mmol) and the appropriate diamine (25.4 mmol) in 4 ml acetonitrile was placed in a closed vessel microwave reactor at 150 °C, 150 W, 150 psi and irradiated for 30 min. After irradiation the reaction mixture was basified with 30 ml 5% aqueous NaHCO₃ and extracted with DCM (3 × 20 ml). The combined organic layer was successfully washed with 20 ml water and by 15 ml brine, dried over anhydrous Na₂SO₄ and filtered. The solvent was removed in vacuo and the residue precipitated by addition of hexane/chloroform (80:20) yielding the desired N¹-(7-chloroquinolin-4yl)diamines. The physical characteristics were exactly the same as previously described.^{3,34}
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