SYNTHESIS OF A NOVEL HYBRID MOLECULE FOR POTENTIAL USE AGAINST *Plasmodium Falciparum* PARASITES

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Synthesis of a Novel Hybrid Molecule for the Potential Use against *Plasmodium falciparum* Parasites

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

To the persevering mind and for the love of science.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin Combined Therapy
AL	Artemether Lumefantrine
CC_{50}	Cytotoxicity level
CDI	1,1'-Carbonyldiimidazole
CO_2	Carbon dioxide
COSY	Correlation Spectroscopy
CQ	Chloroquine
CQ^R/CQR	Chloroquine resistant
CQ^S/CQS	Chloroquine sensitive
CTMDR	Centre for Traditional Medicine and Drug Research
DMSO	Dimethyl Sulfoxide
DEPT	Distortionless Enhancement by Polarization Transfer
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
G27	Gauge 27
HMBC	Heteronuclear Multiple Bond Correlation
HOBt	1-hydroxy-benzotriazole
HSQC	Heteronuclear Single Quantum Correlation
IC_{50}	Half maximal inhibitory concentration
ITROMID	Institute of Tropical Medicine and Infectious Diseases
KEMRI	Kenya Medical Research Institute

LM	Lumefantrine
LM^R	Lumefantrine resistant
MDR	Multidrug Resistance
MEM	Minimum Essential Medium
MKU	Mount Kenya University
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)
PBS	Phosphate buffer saline
PfCRT	*Plasmodium falciparum* Chloroquine Resistant Transporter
RA	Reversal agent
RCQ	Reversed Chloroquine
SP	Sulfadoxine Pyrimethamine
WHO	World Health Organization

ABSTRACT

Malaria is ranked seventh among the leading causes of death in sub-Saharan Africa. The major setback to treatment of malaria has been the emergence of parasite resistance to antimalarial drugs especially chloroquine (CQ) which was once held as a gold standard for malaria treatment. Research has shown that compounds referred to as chemosensitizers are capable of reversing resistance to CQ and CQ-like molecules. Thus in keeping with the need to save the limited arsenal of antimalarial drugs and to restore the usefulness of CQ and CQ-like molecules, a rational drug design approach to synthesize a novel antimalarial hybrid drug by covalently linking a quinoline pharmacophore to a chemosensitizer pharmacophore, is presented in this study. A linear synthetic route was used to synthesize the quinoline-chemosensitizer molecule making use of ethylene diamine as the linker. This was followed by *in vitro* evaluation of the molecule for antiplasmodial activity using 3D7 (CQ sensitive) and W2 (multidrug resistant) strains of *P. falciparum* and drug cytotoxicity to Vero cells by the MTT assay. The hybrid molecule was then re-evaluated against lumefantrine resistant (LUM-R) *P. berghei* parasites in mice using the 4-day suppressive test. The successfully synthesised hybrid molecule exhibited activity of 0.66 ± 0.06 µg/ml in 3D7 and 0.62 ± 0.01 µg/ml in W2 strain which was higher than that of the individual precursor molecules (4,7-dichloroquinoline, probenecid) and the combination. There was no cytotoxicity exhibited by the compound on Vero cells. Additionally, treatment with the hybrid compound was not effective in the mice infected with lumefantrine (LM) resistant *P. berghei* when compared to mice treated with LM. The results successfully validate the concept of utilizing a hybrid molecule to combine antiplasmodial activity and resistance reversal activity effective against *P. falciparum* and reflect the likelihood of resistance reversal capability of probenecid *in vitro* as reported previously. However, further structural modification could be considered to improve on the activity of the molecule *in vivo*. An active quinoline pharmacophore such as piperaquine could be used to ensure antiplasmodial activity and an active reversal agent that would allow for resistance reversal activity.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Malaria remains a major public health problem despite it being considered a preventable and treatable infectious disease. It is estimated that 214 million cases and 438,000 deaths, 90% of these occurring in Africa, resulted from malaria in 2015 (WHO, 2015). Malaria is caused by the protozoan blood parasite of the genus *Plasmodium* and five species have been identified as being responsible for malaria namely: *P. vivax, P. malariae, P. ovale, P. knowlesi* and *P. falciparum* with the latter, being the most lethal of the five species.

The fight against malaria dates back to 1898 when Ross and Grassi discovered that transmission of the malaria parasite occurred through the bite of an infected *Anopheles* mosquito. This finding formed the basis of initial vector control measures some of which included; a change in agricultural habits to reduce mosquito breeding sites and application of insecticides such as dichloro-diphenyl-trichloroethane (DDT) (Hay *et al*., 2004). In an effort to step up the fight against malaria, chemotherapy was introduced as a measure to compliment vector control and provide treatment of the disease (Hay *et al*., 2004).

To date there has been no successful vaccine developed for the prevention of malaria though RTS,S/AS01 vaccine has recently shown promising potential (Olotu *et al*., 2016;

RTS,S Clinical Trials Partnership, 2015), thus chemotherapy remains to be seen as the major means that is largely depended upon to combat malaria. Treatment of malaria has since evolved from the use of drugs belonging to three main classes of compounds that is quinoline and its related analogs: quinine, chloroquine, amodiaquine, primaquine, mefloquine and halofantrine; antifolates such as pyrimethamine, proguanil, sulfadoxine and dapsone; and artemisinins such as artemisinin, dihydroartemisinin, artemether, arteether and artesunate (Schlitzer, 2008).

The quinoline class of drugs is undoubtedly one of the most widely used antimalarial drugs dating as far back as the 1930s. The era of quinoline drugs started with quinine which is an extract of the Peruvian *Cinchona* tree bark. Quinine was the first drug used to fight against malaria and though effective for some time it presented issues related to complicated synthesis as well as availability (Butler *et al*., 2010). Quinine eventually served as a template for synthesis of simpler analogs such as chloroquine (CQ). CQ offered the following advantages; cost effectiveness thus making production on large scale feasible, excellent clinical efficacy, limited host toxicity hence was safe for women and children who are considered vulnerable victims of malaria and ease of use and most importantly affordability of the drug.

However, the value of chloroquine along with other quinoline analogs was eroded with the emergence of parasite resistance, though WHO currently recommends CQ for the treatment of *P. vivax* malaria where the drug remains effective (WHO, 2006). Parasite

resistance has been the major setback in the development of antimalarial drugs with most of the quinoline drugs being resistant to *P. falciparum* rendering them useless as first line treatment for malaria (Egan, 2001).

Heme detoxification is widely believed to be the primary target of quinoline antimalarial drugs such as CQ. When feeding in the host red blood cells, the malaria parasite is said to degrade host hemoglobin as a source of amino acids. Degradation of hemoglobin releases heme which is then polymerized by the parasite to inert biocrystals of hemozoin. Therefore accumulation of CQ in the digestive vacuole of the parasite causes it to bind to hematin which is a heme dimer. Consequently, buildup of the toxic heme monomers causes the permeabilizibility of the membrane eventually leading to parasite death (Zhang *et al*., 1999).

Several compounds have since been studied and have demonstrated the promising capability to reverse the antimalarial drug resistance *in vitro* in parasite isolates in animal models and in human malaria (Henry *et al*., 2006). The chemosensitizers belong to different pharmacological classes such as calcium channel blockers, tricyclic antidepressants, antipsychotic calmodulin antagonists, histamine H-1 receptor antagonists, analgesic antipyretic drugs, non-steroidal anti-inflammatory drugs (Pradines *et al*., 2005).

Chemosensitizers have presented the hope of restoring the clinical usefulness of quinoline drugs such as CQ. Noteworthy is that even with the promise of these

chemosensitizers to reverse antimalarial drug resistance, poor *in vivo* pharmacokinetic and pharmacodynamic properties have hindered their adoption into malaria chemotherapy (Burgess *et al*., 2006). Thus it is imperative to take on new research approaches that shall effectively combat the phenomenon of drug resistance and embrace the design of novel drugs with suitable safety profiles and *in vivo* efficacy. These approaches need not necessarily be based on the identification of new drug targets but rather on utilization of validated chemical scaffolds such as quinoline and artemisinin.

The focus of this study was on the rational drug design approach using a single hybrid molecule with a dual functionality. Meunier (2008) defines a hybrid molecule as a "double edged sword" in that it is a single molecule with two pharmacophores having different modes of action that are able to interact with either a single target, two independent targets or two related targets. The two pharmacophores are linked covalently hence the term covalent bitherapy. The two pharmacophores used in the study were quinoline and probenecid.

1.2 Statement of the problem

Poverty has significantly contributed to the morbidity and mortality caused by malaria in sub-Saharan Africa (WHO, 2015). CQ which was for a long time used as a first line treatment for malaria was cost effective, had excellent clinical efficacy, exhibited limited host toxicity and was affordable.

However, the emergence of CQ resistant *P. falciparum* strains necessitated a switch to alternative antimalarial drugs like artemisinin derivatives which are unaffordable for many countries in sub-Saharan Africa (Bosman and Mendis, 2007). The current option for reducing the morbidity and mortality of malaria is chemotherapy. Thus until eradication of malaria becomes a reality, development of effective drugs remains significant in the fight against malaria.

In Kenya, the Ministry of Health (MoH) has had to change first line recommendations for the treatment of malaria twice; in 1998 when sulfadoxine pyrimethamine (SP) replaced CQ due to the development of resistance and in mid-June 2001 when SP was reported to have a record treatment failure in Kenya above 25% prompting the second change of policy from SP to ACT (Amin *et al*., 2007). The major concerns raised before the implementation of ACT were that Artemether-Lumefantrine (AL) sold under the brand name Coartem® was singly sourced that is from Novartis. Secondly the global supply of artemisinin was in jeopardy and the cost of AL was significantly more than the previous first line choices for treatment of malaria despite subsidized arrangements between WHO and Novartis (Shretta *et al*., 2000). However to increase competition and value for money 75% of Kenya's annual AL order is sourced through an international open tender, a move that has seen the entry of companies such as Ajanta that have AL retailing at half the cost of those directly sourced from Norvatis (Kangwana *et al.*, 2009).

Evidently, there is a growing need for antimalarial drug development. However, the greatest challenge of this process is drug development cost. Firstly, antimalarial drugs should be affordable such that they are routinely available to populations in need in developing countries. Secondly, emphasis on antimalarial drug use is placed in Africa where endemic populations exist. Implying that the malaria market is primarily in poor countries which due to limited resources have limited investment in drug discovery and development so alternatives that may preclude excess costs ought to be considered. Apart from finances, the other challenges are related to compliance issues and development of resistance. As such antimalarial drugs that are dosed orally and amenable to a single-dose administration, with short curative regimens ideally 1-3 days in length are desired. Further to this desired antimalarial drugs should be effective, safe and well tolerated especially in the vulnerable groups like women and children. So side effects if any should be no worse than the best tolerated drugs (Nwaka and Hudson, 2006).

Various approaches have been proposed for drug design and among them is the rational design of hybrid drugs that incorporate two pharmacophores with independent activity (Na-Bangchang and Karbwang, 2009; Muregi *et al*., 2011). Considering that the characteristics of CQ are difficult to abandon, a proposal to make use of the quinoline pharmacophore that gave rise to CQ and covalently link it to a compound capable of reversing CQ resistance that is a chemosensitizer. The mechanism of chloroquine resistance although controversial has often been compared to multidrug resistance in

mammalian cells mediated by intrinsic membrane protein drug transporters such as *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) (Henry *et al*., 2008). Studies on various compounds termed as chemosensitizers such as verapamil and imipramine have been able to demonstrate their ability to reverse CQ resistance in parasite isolates *in vitro,* in animal isolates and in human models.

1.3 Justification of the study

Africa bears the largest burden of malaria, and therefore presence of alternative drugs such as CQ that are affordable and accessible is desirable. Resistance to CQ has been attributed to the gene encoding the digestive vacuole membrane that is *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) which results in decreased drug concentration at the target without altering the target itself (Fidock *et al*., 2000). Therefore in this case as opposed to where drug resistance occurs as a result of protein target mutations as observed in antifolates, the target can be restored. The restoration of sensitivity to quinoline resistant drugs has been proven possible with the use of chemosensitizers (Henry *et al*., 2006).

The use of different pharmacophores such as those of quinoline and trioxanes has been incorporated in rational drug design using single hybrid molecules with dual functionality. Due to its advanced mode of action and high selectivity the hybrid molecule based chemotherapy has emerged as a beneficial tool in the contemporary trend of antimalarial drug discovery. Burgess and his colleagues were able to

demonstrate the feasibility of this concept when they designed a hybrid molecule that linked a quinoline moiety to a reversal agent (RA) - imipramine and they termed it a reversed chloroquine molecule (RCQ) (Burgess *et al*., 2006).

Despite these efforts a clear choice of potential resistance reversal agents or preclinical studies leading to the development of novel resistance reversing hybrid drugs is yet to be seen, even after the discovery of antimalarial chemosensitizers. In light of this, the development of a quinoline - chemosensitizer hybrid drug taking advantage of the restoration of previously used drugs is proposed herein.

1.4 Research hypothesis

The quinoline-chemosensitizer hybrid will be able to reverse CQ/multidrug resistance.

1.5 Objectives

1.5.1 The overall objective

To determine the antiplasmodial activity and safety of a quinoline hybrid molecule.

1.5.2 Specific objectives

i. To determine properties of a synthesized quinoline hybrid molecule.

ii. To determine susceptibility of the hybrid molecule against *P. falciparum* CQ sensitive and CQ resistant strains *in vitro.*

iii. To determine growth inhibition of the hybrid molecule against *P. berghei* LM^R parasites *in vivo.*

iv. To determine cytotoxicity and acute toxicity of the hybrid molecule *in vitro* and *in vivo*.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria

Malaria is a preventable infectious disease caused by the protozoan blood parasite of the genus *Plasmodium*. The known species that cause human malaria are *P. ovale*, *P. malariae*, *P. vivax* and *P. falciparum*. Arguably the major contributor to malaria morbidity and mortality is increasing resistance of malaria parasite to available drugs with resistance being primarily seen in *Plasmodium falciparum* the most virulent parasite.

Therefore, there is growing need for development of new antimalarial drugs that are capable of circumventing increasing resistance (Ridley, 2002). This review covers some of the previously used antimalarial drugs and approaches that are being taken on to address the development of new antimalarial drugs.

2.2 Malaria life cycle

The human malaria life cycle is known to be digenetic, implying that it is completed in two hosts, that is the asexual cycle in humans and the sexual cycle in the female *Anopheles* mosquito. The cycle begins when the female *Anopheles* mosquito injects sporozoites into the subcutaneous tissue, which then travel to the liver. A period of incubation then follows in the liver within which the sporozoites invade the hepatocytes resulting in their growth and multiplication. As they near the end of incubation, tissue

schizonts rupture releasing merozoites that enter the blood stream and infect the erythrocytes. In the erythrocytes the parasites grow from 'ring' to mature trophozoite, then schizonts and finally to merozoites. The rupture of erythrocytes causes the release of merozoites into the blood stream. It is at this point that manifestations of clinical symptoms such as anaemia, shivering and fever are observed. The released merozoites then go on to infect healthy erythrocytes whereas some of the merozoites are changed into gametocytes. Gametocytes are ingested by the female *Anopheles* mosquito during a blood meal on an infected person. The gametocytes then undergo sexual reproduction to produce sporozoites. These sporozoites eventually migrate to the salivary glands of the female *Anopheles* mosquito where they are injected into a healthy person during a blood meal. This way the infective cycle of the malaria parasite continues (Kumar *et al.*, 2003).

2.3 Classification of antimalarial drugs

Antimalarial drugs are classified by the stages of the malaria life cycle that are targeted by the drug (WHO, 2015) namely:

i. **Blood schizonticides:** These are said to act on the asexual intraerythrocytic stages of the parasite; consequently terminating clinical attacks of malaria. Drugs of this class include: quinine, CQ, mefloquine, halofantrine, pyrimethamine, sulfadoxine, sulfones and tetracycline derivatives.

ii. **Tissue schizonticides:** These act on the pre-erythrocytic forms of the malaria parasite in essence acting as causal prophylactic drugs. However given that it is

difficult to definitively point out the infection before clinical symptoms manifest, this mode of drug action is considered theoretical rather than practical. Primaquine and to a lesser extent pyrimethamine are the drugs that belong to this class.

iii. **Hypnozoiticides:** These act by killing persistent intra-hepatic stages of *P. vivax* and *P. ovale* which are the two strains that exhibit dormant liver stages. The drug mainly found in this class is primaquine.

iv. **Gametocytocides:** This class of drugs targets the sexual forms of all species of malaria parasite, hence preventing transmission from human to mosquito. Quinine, CQ and amodiaquine (AQ) are active on gametocytes of *P. vivax* and *P. malariae* but not *P. falciparum,* however primaquine has gametocytocidal activity against all human malaria parasites.

2.4 Drugs used against malaria

2.4.1 Arylaminoalcohols and Quinolines

2.4.1.1 Quinine

Quinine (**1**) is one of the most ancient antimalarial drugs. It is the active alkaloid that was isolated in 1820 from the bark of the Cinchona tree; the other alkaloids include quinidine (**2**) which is a diastereomer of quinine, cinchonine and cinchonidine (Butler *et al*., 2010). Quinine is proposed to act principally on the mature trophozoite stage of parasite development. Thus like other structurally similar antimalarials, quinine kills the

sexual stages of *P. vivax*, *P. malariae* and *P. ovale* but not mature gametocytes of *P. falciparum*. The mechanism of action of quinine though not fully understood is thought to be similar to that of CQ (**7**) thus it accumulates in the parasites digestive vacuole ultimately inhibiting the detoxification of heme (Fitch, 2004). Studies have shown that resistance to quinine has been associated with three genes namely: PfCRT (*P. falciparum* chloroquine resistance transporter), PfMDR1 (*P. falciparum* multidrug resistance transporter 1), and PfNHE1(*P. falciparum* sodium/proton exchanger 1) which all encode for transporter proteins (Cooper *et al*., 2002; Sidhu *et al*., 2005; Cooper *et al.*, 2007; Nkuruma *et al.,* 2009). Despite this quinine still remains an important drug for the treatment of severe malaria as preparations for intravenous applications are available (Pasvol, 2005) and as a second line treatment in combination with antibiotics such as tetracycline, doxycycline or clindamycine (WHO, 2006).

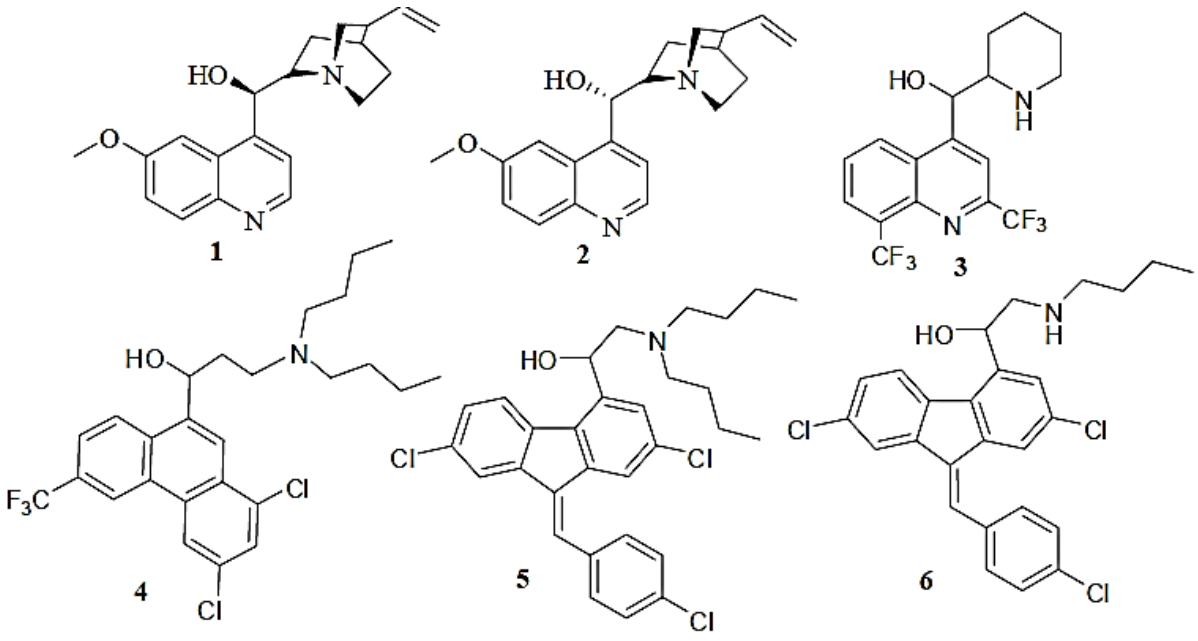


Figure 0.1: Aryl aminoalcohol antimalarial drugs: 1-Quinine, 2-Quinidine, 3-Mefloquine, 4-Halofantrine, 5-Lumefantrine and 6-Desbutyllumefantrine

2.4.1.2 Mefloquine

Mefloquine (**3**) which is a 4-methanolquinoline is an analog of quinine that has a longer half-life that is 14-18 days as opposed to 10 hrs of quinine (Stepniewska and White, 2008). It is the only registered drug effective in a single dose though resistance as in other arylaminoalcohols has been problematic. It is proposed that the resistance to mefloquine is as a result of amplification of PfMDR1 that leads to the over expression of this digestive vacuole membrane transporter (Cowman *et al*., 1994).

2.4.1.3 Lumefantrine

Lumefantrine (**5**) also known as bentflumetol is an arylamino alcohol antimalarial that has a half-life of about 3-5 days (WHO, 2010). Since its absorption is said to vary

between individuals, fatty meals are advised to enhance oral absorption (Ashley *et al*., 2007). Lumefantrine has shown *in vitro* synergism with artemether (Alin *et al*., 1999), as such is a widely used ACT globally for the treatment of *Plasmodium falciparum* malaria (WHO, 2010). The other classes of quinoline drugs that have been part of malaria chemotherapy history are the 4-aminoquinolines with the major drugs being chloroquine and amodiaquine.

2.4.1.4 Chloroquine

Chloroquine (**7**) is one of the most extensively used drugs for the treatment and prevention of malaria. Its extensive use as a successful single drug for the treatment and prophylaxis of malaria is attributed to its efficacy, affordability and safety especially for pregnant women and children, who are considered vulnerable to malaria thus making it a gold standard for the treatment of malaria for the past century (AlKadi, 2007). The mechanism of action of CQ has been an area of great interest and a number of modes of action have been proposed. However in the widely accepted mode of action it is proposed that CQ which is a weak basic compound (pKa 8.1 and 10.2) is diprotonated upon entry into the acidic digestive vacuole (pH 5-5.4). This in turn leads to build up of CQ in the digestive vacuole due to it being unable to traverse across the membrane once in the digestive vacuole (Martin *et al*., 2009). Continued accumulation of CQ in the digestive vacuole causes it to bind to heme. Heme which is toxic to the parasite is produced following the digestion of erythrocytic hemoglobin and in order to combat the toxic effects of heme, the parasite polymerizes it to an insoluble polymer called

hemozoin (Egan, 2008). Therefore, binding of CQ to heme forms a complex with ferriprotoporphyrin IX which prevents the detoxification of the latter by the parasite leading to build up of heme and eventual parasite death (Zhang *et al*., 1999).

The massive use and to some extent misuse of CQ has led to the emergence of CQ resistant parasites rendering it useless as a first line treatment for malaria. CQ resistance is believed to be as a result of a mutation in the membrane transport protein PfCRT where a threonine residue replaces lysine at position 76 of the membrane (Sidhu *et al*., 2002). CQ has also been found to have the longest half-life of all the antimalarials that is approximately 60 days, a factor that has contributed to selection for drug resistant parasites, because the parasite remains exposed to CQ that has fallen below therapeutic concentration for a long time.

2.4.1.5 Amodiaquine

Amodiaquine (AMQ) (**8**) is a mannich base 4-aminoquinoline and its mode of action is thought to be similar to that of CQ thus not surprising are the reports of cross resistance between CQ and amodiaquine brought about by mutations in PfCRT and PfMDR1 that are associated with diminished susceptibility of both drugs (Sá *et al*., 2009). It is also believed that antimalarial activity of amodiaquine could be as a result of the primary metabolite, monodesethylamodiaquine that has a half-life of 9-18 days as opposed to the precursor drug amodiaquine that has a half-life of 3 hours (Stepniewska and White, 2008).

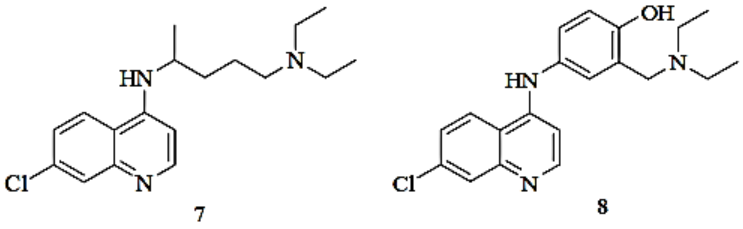


Figure 0.2: 4-aminoquinoline class of drugs: 7-Chloroquine, 8-Amodiaquine

2.4.1.6 Primaquine

Primaquine (**9**) belongs to the 8-aminoquinoline class of quinoline antimalarials. Although its activity against asexual blood stages of *P. falciparum* is deemed too low to be therapeutically significant, it is the only approved therapy for anti-relapse therapy of *P. vivax* hypnozoite liver stages (Wells *et al*., 2010). Nonetheless primaquine is contraindicated in glucose-6-phosphate dehydrogenase deficient group of people, due to potentially life threatening heamolysis that is thought to be a side effect in these people (Taylor and White, 2004).

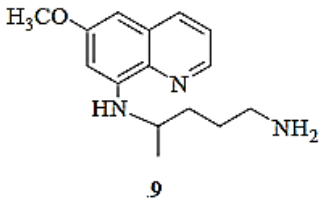


Figure 0.3: Preferred drug for the elimination of hypnozoites, 9 - Primaquine.

2.4.2 Antifolates

These are drugs which mainly inhibit two key enzymes of the folate biosynthetic pathway that is dihydroprotease synthase (DHPS) and dihydrofolate reductase (DHFR).

In malaria chemotherapy, the drugs that have been used are sulfadoxine and dapsone that inhibit *Pf*DHPS and pyrimethamine and proguanil which inhibit *Pf*DHFR activity (Nzila *et al*., 2005). Next to CQ the combination of sulfadoxine with pyrimethamine (SP) under the brand name Fansidar™ served as an equally important antimalarial. Like CQ, SP was a highly effective, cheap, well tolerated drug. In addition, it had good compliance as it was administered in a single dose (Uhlemann and Krishna, 2005). The combination of DHPS and DHFR inhibitors are suggested to exert their toxic effect in the late erythrocytic schizont stage the point at which inhibition of parasite DNA synthesis is suggested to occur (Gregson and Plowe, 2005). Resistance to antifolates is attributed primarily to point mutations in both target enzymes (Uhlemann and Krishna, 2005).

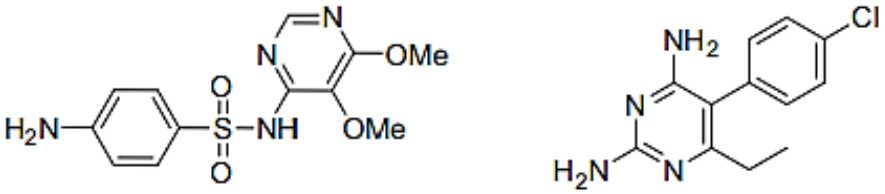


Figure 0.4: Antifolate drugs 10 - Sulfadoxine, 11 - Pyrimethamine previously used as first line therapies

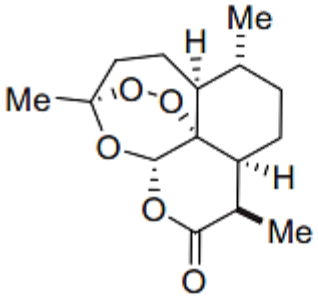
2.4.3 Artemisinins

The discovery of artemisinins has done well to allay some of the fears brought about by malaria parasite resistance. Artemisinin (**12**), which is an extract of *Artemisia annua*

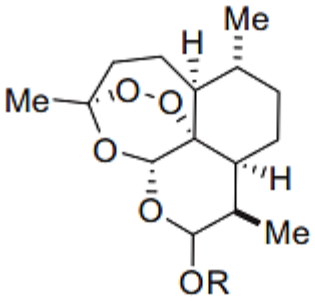
plant, is a sesquisterpene trioxane lactone whose endoperoxide is widely believed to be essential for antimalarial activity and it has been found that the absence of the endoperoxide bridge makes the drug devoid of antimalarial activity. The antimalarial action of artemisinin is thought to involve the cleavage of the peroxide bond by Fe (II) found in the heme proteins thus generating toxic oxygen radicals (O'Neill and Posner, 2004). Artemisinin and its derivatives: dihydroartemisinin (**13**) (DHA), artemether (**14**) and artesunate (**15**), have proved to be one of the most effective and potent antimalarials to date, in essence superseding the previously recommended CQ which in turn was replaced by SP. Results of a study by Dondorp and colleagues, have suggested the use of parenteral artesunate as the drug of choice for treatment of severe malaria to replace quinine (Dondorp *et al.*, 2010).

Despite this, artemisinin drugs are limited by their short half-life that is between 0.5 hrs and 1.5hrs which has attributed to recrudescence that occurs when administered as a monotherapy (Butler and Wu, 1992). As such, the WHO made the recommendation of artemisinin based combined therapy (ACT) as a policy standard for the first line treatment of malaria. Malaria combined therapy entails co-formulation of two or more agents into a single tablet as opposed to the traditional cocktail therapy. In the case of ACT, it is a combination of artemisinin based drugs which have been shown to kill parasites quickly albeit their rapid excretion from the body with an antimalarial drug that has a longer half-life, thus enabling full eradication of the parasite and preventing recrudescence (Eastman and Fidock, 2009). Some of the commonly used ACTs include:

artemether-lumefantrine, artesunate-mefloquine, artesunate-amodiaquine, artesunate-sulfaoxine-pyrimethamine, dihydroartemisinin-piperaquine and artesunate-pyronadine (WHO, 2010).



12: Artemisinin



13: Dihydroartemisinin: R = H

14: Artemether: R = Me

15: Artesunate: R = -CO-CH_2-CH_2-CO_2^-

Figure 0.5: Artemisinin and its derivatives

2.5 Current approaches to antimalarial chemotherapy

There is growing need for the discovery of new antimalarial drugs and with cost being a major concern, various approaches that utilize shortcuts to generate new compounds for study at a relatively low cost should be taken on. These approaches need not necessarily be based on the identification of new drug targets but rather on utilization of validated chemical scaffolds such as quinoline and artemisinin.

2.5.1 Optimization of therapy with existing agents

Drug combinations that have independent modes of action have been devised as a way to delay development and spread of resistant malaria parasites. Ideally, a combination

regimen that prevents resistance development should include at least two agents against which parasite resistance has not yet developed and those with similar pharmacokinetics such that low blood levels of a single agent will not be present at any one time (Martinelli *et al*., 2008). Given that this is difficult to achieve, combinations make use of old and newer agents to optimize the activity of co-formulated drugs. Some of the WHO recommended ACT WHO-recommended ACTs for uncomplicated *P. falciparum* malaria include: artemether-lumefantrine, artesunate-AMQ, artesunate-mefloquine, DHA-piperaquine and artesunate-sulfadoxine pyrimethamine (WHO, 2006).

Amodiaquine which is a 4-aminoquinoline structurally related to chloroquine has been shown to effectively kill chloroquine resistant parasites, though as mentioned earlier AMQ efficacy has also been limited by parasite resistance. As such various studies have been able to demonstrate the synergistic activity of combining AMQ with SP. Even though both drugs show reduced efficacy as single entities, this combination provides for readily available inexpensive drugs with similar pharmacokinetics thus maintaining the efficacy of the drugs as a combination therapy (Staedke *et al*., 2001; Schellenberg *et al*., 2002).

The other combinations that have shown great promise are those of artemisinin analogs and longer acting antimalarial drugs. Although artemisinin is considered a highly effective antimalarial it is limited by its short half-life which is responsible for recrudescence. Artemether a derivative of artemisinin has been combined with

lumefantrine which is related to halofantrine and this has turned out to be the most widely used ACT (Lefevre *et al*., 2001). Artesunate has also been studied in combination with sulfadoxine/pyrimethamine (von Seidlein *et al*., 2000) and amodiaquine (Adjuik *et al*., 2002) and they have both shown good efficacy though the underlying resistance of the artesunate partner drugs could lead to late recrudescence as reported by Dorsey and co-workers (Dorsey *et al*., 2002). Recently the three drug combination of chlorproguanil hydrochloride-dapsone-artesunate (CDA) an ideal combination was withdrawn at phase III clinical trials due to toxicity associated with dapsone this just going to highlight some of the challenges of developing drug combinations. Moreover, due to being expensive and having different pharmacokinetics combinations of artemisinin derivatives are not ideal for widespread use in many areas especially in Africa.

2.5.2 Development of analogs of existing agents

This approach entails chemically modifying existing antimalarials in a bid to enhance their activity. The 4-aminoquinoline, chloroquine became the most widely used drug by the 1940s after its first synthesis in 1934 though their value was diminished by the emergence of widespread resistance of malaria parasite (Loeb *et al*., 1946). Thus the search for CQ analogs that work against CQR parasites was seen as a logical plan. Maintaining this line of thought, De *et al*. (1996) tested the activity of 4-aminiquinoline analogs out of which they found that AQ-13 which is a close structural analog of chloroquine containing a diaminoalkyl side chain with 3 carbons displayed low

nanomolar potency against CQR parasites. AQ-13 has undergone phase I clinical trials with results indicating minimal difference in toxicity compared with CQ (Mzayek *et al*., 2007). Another notable 4-aminoquinoline that has undergone structural modification is amodiaquine. Though structurally related to CQ it has displayed its effectiveness in killing CQR parasites however upon oxidation by cytochrome P450 enzymes, it forms a quinine imine which is believed to be responsible for hepatoxicity and its ability to cause agranuclocytosis (O'Neill *et al*., 2003). A chemical modification of amodiaquine that entailed swapping the hydroxyl aniline ring led to the formation of isoquine which was further optimized to N-*t*-butyl-isoquine. Minimal accumulation of isoquine in rat liver compared to amodiaquine following studies with radioactive compounds led to the conclusion that isoquine did not form covalent adducts with the liver (O'Neill *et al*., 2003).

The second generation ozonide OZ439 which is a synthetic peroxide antimalarial has successfully completed Phase I clinical trials where it has been shown to be safe at doses upto1600 mg. The modification from an 8'-alkyl of the first generation ozonide OZ277 to an 8'-aryl group has contributed to a 50-fold stability of the O-O bond towards Fe(II) that translated to an increased half-life ($t_{1/2}$) of 20 hrs in mice and 25-30hrs in humans. In essence, making OZ439 able to deliver single curative doses as opposed to artemisinin that has to be combined with a second longer acting antimalarial. Phase II trials of this drug are underway (Charman *et al*., 2011). Considering that the precursor

molecule artemisinin is limited by cost, the design of fully synthetic endoperoxides that are less expensive is seen as a noble effort.

2.5.3 Natural products

Compounds derived from plants have for decades provided malaria chemotherapy. Some of the most widely used drugs for treatment of severe malaria are derivatives of plants that is quinine and artemisinin. The practice of using medicinal plants in native malarious regions has grown over many generations thus it is important to gain a better understanding of medicinal plants. The evidently growing interest in natural products and their use as potential antimalarials has been tested in various studies (Gathirwa *et al*., 2007; Orwa *et al*., 2013) that have suggested the presence of compounds in plant materials capable of killing malaria parasites. Therefore as with quinolines and artemisinins, it is likely that antimalarial natural products could provide lead compounds for the semi-synthetic or fully synthetic production of new drugs.

2.5.4 Compounds active against other diseases

This approach makes use of drugs already developed for other diseases that are also capable of antimalarial activity. The advantage that this approach offers is the fact that the huge funds put into antimalarial drug development are obviated because with the already developed compounds, their mechanisms of action have been developed for human indications and so development of antimalarials in this regard is considered inexpensive. This holds true for drugs whose initial production is considered

inexpensive however it is worth noting that production of drugs developed for diseases such as cancer is expensive thus by extension may be expensive to produce as antimalarials. Loyevsky and Gordeuk (2001) have documented antimalarial efficacy in iron chelators which are used to treat iron overload syndromes. Folate antagonists such as pyrimethamine and sulfadoxine, tetracylines and other antibiotics were developed for their antibacterial properties and were later found to be active against malaria parasites (Clough and Wilson, 2001). This approach as can be seen can provide inexpensive alternatives for the screening of available antimicrobial agents for new antimalarial drugs.

2.6 Hybrid molecules

This is a relatively new strategy of chemotherapy that is based on covalent linking of drugs into a single hybrid molecule resulting in molecules that tend to be more effective than the isolated components. Hybrid molecules have been described as chemical entities with two or more structural domains having different biological functions, though the different entities do not necessarily act on the same target (Meunier, 2008).

According to Morphy and Rankovic (2005), hybrid molecules can be classified into four main categories namely:

i. **Conjugates** in which a distinct linker group that is not found in either one of the pharmacophores is used to separate the two pharmacophores.

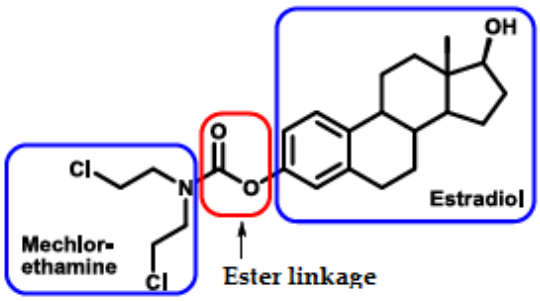


Figure 0.6: Example of a conjugate hybrid of the cancer drug estramustine (Gediya and Njar, 2009).

ii. **Cleavage conjugates** in which the linker that joins the two pharmacophores is designed such that it can be metabolically cleaved, releasing the individual components to act on their respective targets.

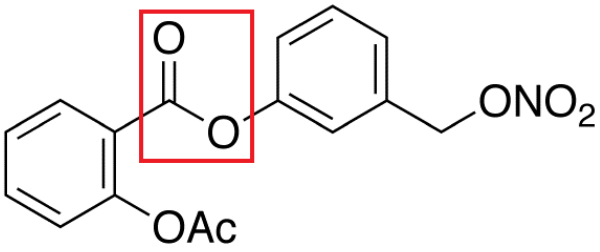


Figure 0.7: NO-Asprin which contains a cleavable ester linker in the red outlined box (Lazzarato *et al*., 2008)

iii. **Fused hybrid** in which the framework of the pharmacophores is essentially touching due to the reduced size of linker used.

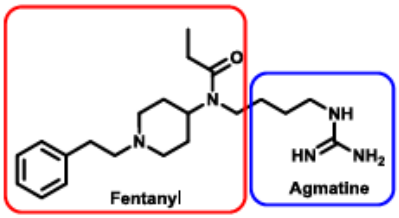


Figure 0.8: Fused hybrid of fentanyl-agmatine (Dardonville *et al.*, 2006)

iv. **Merged hybrid** in which commonalities between the two pharmacophores is used to merge the pharmacophores thus giving rise to a smaller and simpler molecule.

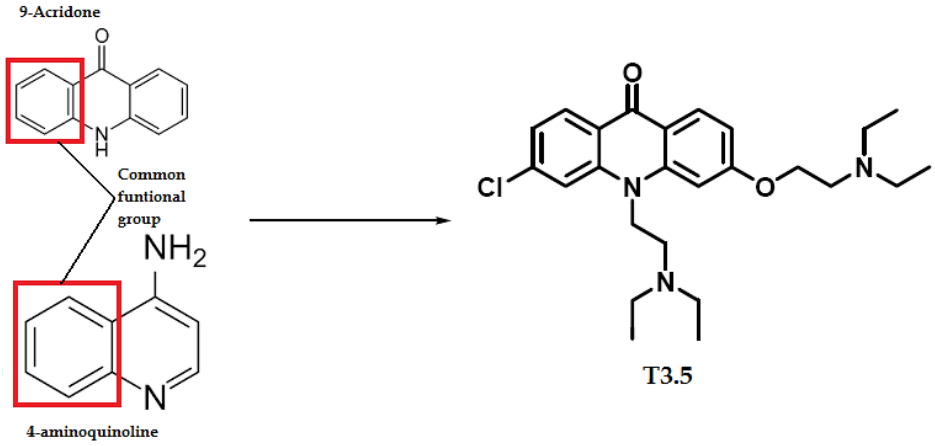


Figure 0.9: A merged hybrid of a dual-function acridone T3.5 designed by taking the commonalities of the 4-aminoquinoline and chemosensitizer 9-acridone (Kelly *et al.*, 2009).

Interest in the design of hybrid molecules with dual mode of action is said to have stemmed from the anticancer agent bleomycin (Ding *et al.*, 1990). Bleomycin was originally isolated as an antibiotic from *Streptomyces verticillus* and was found to have three distinct structural domains: one for DNA binding, a second for metal binding and a third containing carbohydrates. In essence, demonstrating the design by a

microorganism of a hybrid molecule containing three structural entities with three different biological roles (Umezawa, 1974). Another cancer drug estramustine which was developed by Jonsson and Hogberg (1971), demonstrated favourable results in patients with advanced carcinoma of prostate thus making it the first ever drug approved by the Food and Drug Administration for the treatment of prostate cancer in 1981 (D'Amico, 2014). Estramustine sold under the brand name Emcyt® is the only hybrid drug used clinically for the treatment of prostate cancer it is composed of estradiol which is an estrogen linked by a carbamate ester bridge to nitrogen mustard which is an alkylating agent. Thus the drug delivers both hormonal and cytotoxic activity in a single molecule (Di Lorenzo, 2007).

The hybrid molecule approach of drug design is said to be a more convenient way of drug design as opposed to a multicomponent drug firstly because of the troubles of making a combination drug as presented by Frantz (2006), such as those of different solubilities of the two drugs that are bound to modify the blood stream uptake of the drug and having to fine tune a formulation to ensure that the blood level of the two drugs are the same in the same tablet. Secondly the pharmacokinetics of a hybrid molecule is more predictable and thirdly and most importantly is the possibility of using the uptake capacities of one motif to boost the bioavailability of the second entity. However as a disadvantage highlighted by Muregi and Ishih (2010), it is difficult to adjust the ratio of activities at different targets.

2.6.1 Hybrid drugs for malaria

Through rational drug design approach, hybrid molecules have emerged as a beneficial tool against malaria. Some of the molecules that have emerged from this approach are trioxaquines that are defined as chimeric molecules consisting of a trioxane motif covalently linked to a 4-amoinoquinoline moiety (Dechy-Cabaret *et al*., 2000). The activity of artemisinin and its derivatives is attributed to the 1,2,4-trioxane group responsible for the production of radicals that damage the parasite, thus the dual activity in trioxaquines results from the alkylating activity of the trioxane moiety (Robert and Meunier, 1998; Robert *et al*., 2001) and the aminoquinoline entity which inhibits beta-hematin formation (Egan and Marques, 1999).

The first series of trioxaquines synthesized (Figure 2.10) showed high *in vitro* activity against chloroquine-resistant human isolates of *P. falciparum* with IC_{50} values at 72 hrs being 21 nM, 60 nM and 17 nM for DU1102, DU1106 and DU1108 respectively compared to 116 nM for chloroquine (Dechy-Cabaret *et al*., 2000). Further studies on DU1102 (Basco *et al*., 2001) were able to demonstrate its high activity against Cameroonian isolates of *P. falciparum* giving a mean IC_{50} of 43 nM. Encouraged by the high *in vitro* activity of DU1102, a series of second generation trioxaquines were synthesized in an effort to increase antimalarial activity (Dechy-Cabaret, 2004) out of which DU1302 (Figure 2.10) showed efficient activity *in vitro* against highly resistant *P. falciparum* strains (IC_{50} 5-19 nM) and was able to clear parasitaemia completely without recrudescence at an intraperitoneal dose of 20 $mgkg^{-1}d^{-1}$. DU1302 also demonstrated its

activity on gametocytes which are the mosquito transmissible forms of the parasite activity which is not observed in chloroquine. However despite its good *in vitro* and *in vivo* activity profile DU1302 has not been developed as a drug candidate because of its four stereoisomers (Benoit-Vical *et al*., 2007).

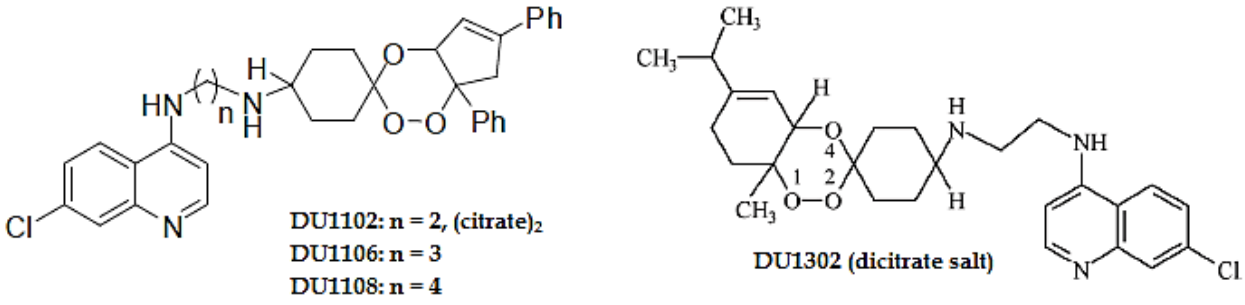


Figure 0.10: Structure of the first generation DU1102, DU1106 and DU1108 and second generation DU1302 antimalarial trioxaquines

The other set of molecules that have emerged from the hybrid molecule approach are reversed chloroquine molecules. These molecules consist of an aminoquinoline moiety covalently linked to a reversal agent/chemosensitizer termed reversed chloroquine (RCQ) molecule. The first of these molecules was synthesized by Burgess and colleagues (Burgess *et al*., 2006) and the RCQ molecule, showed high resistance subverting activity against CQ resistant Dd2 strain IC_{50} 5.3 nM compared to CQ's 102 nM and the low IC_{50} value of 2.9 nM exhibited by the RCQ molecule against CQ sensitive was attributed to the fact that the chemosensitizer was not detrimental to the activity of the CQ-like portion of the hybrid molecule. Other related molecules have been synthesized such as those by October *et al*. (2008) who made use of the chemistry

of 3,4-dihydropyrimidin-2(1H)-ones to construct RCQ molecules which exhibited excellent potency against CQ-sensitive and CQ resistant malaria strains this despite lacking the protonatable nitrogen of the classical chemosensitizer pharmacophore. The acridine skeleton has also been covalently linked to CQ in yet another attempt to synthesize a RCQ molecule (Kumar *et al*., 2010). Kelly *et al*. (2009) were able to make use of the acridone structure to engineer a new scaffold which incorporated a haem-targeting tricyclic mainframe with an ionizable side chain to promote accumulation in the digestive vacuole and a chemosensitization moiety into one molecule, giving rise to a dual function acridone active against CQ sensitive and resistant strains.

The other type of hybrid molecules are 4-aminoquinoline-pyrimidine based hybrids. Manohar and co-workers (2012) synthesized these series of hybrid molecules that consist of 4-aminoquinoline and pyrimidine pharmacophores connected by a flexible linear diaminoalkane linker. They found that the hybrid comprising of *N*-methyl piperazine as a substituent on the pyrimidine scaffold exhibited excellent *in vivo* antimalarial activity in *P. berghei* infected mice (Manohar *et al*., 2012).

Haem detoxification is seen to be of critical importance as an antimalarial drug target because for one it is not parasite encoded so it is immutable and most importantly haem detoxification that occurs during haemoglobin degradation is critical to parasite survival (Olliaro and Goldberg, 1995). Therefore in an effort to restore previously used affordable and effective antimalarial drugs a number of hybrid molecules that consist of

the quinoline pharmacophore and chemosensitizers have been synthesized. However, it is worth noting that a number of the chemosensitizers used such as the calcium channel blocker, verapamil (Martin *et al*., 1987), are pharmacologically active compounds with multisystem effects that result in a variety of undesirable side effects, also the concentrations needed to reverse clinical drug resistance for some of these agents can be toxic. Therefore the challenge remains to develop novel and inexpensive antimalarials one of the approaches gaining ground is hybrid molecules. Making use of already established chemical antimalarial scaffolds and covalently linking them to safe and inexpensive reversal agents such as nonylphenolethylate that not only reverse chloroquine resistance but also have intrinsic antimalarial activity (Crandall *et al*., 2000) could be an option worth exploring.

2.7 Chemosensitizers/ Resistance reversal agents

Resistance reversal agents or chemosensitizers are described by Martin *et al*. (1987) as compounds capable of reversing resistance and lowering the half maximal inhibitory concentration of drugs against resistant parasite strains. Drug resistance is generally accepted as an important cause of treatment failure for patients with neoplastic or infectious diseases. The dominant molecular mechanism that has been used to describe drug resistance is the action of drug efflux pumps that cause multidrug resistance (MDR) which was first suggested by Dano (1973). The concept of MDR has been extensively studied in cancer chemotherapy and is described as the ability of tumor cells that are exposed to a single cytotoxic agent to develop resistance to a broad range of structurally

and functionally unrelated drugs (Tredan *et al*., 2007). P-glycoprotein (Pgp) which is an ATP-Binding Cassette (ABC) coded by the human MDR1 gene has been implicated in the conferment of MDR and has been shown to act as a drug efflux pump to drugs like adriamycin, docetaxel and daunorubicin (Avendano and Mendez, 2002).

Current strategies to circumvent MDR have mainly focused on the inhibition of the product of the human MDR1 gene that is Pgp. The pharmacological reversal of Pgp mediated MDR *in vitro* and *in vivo* was first reported by Tsuro *et al*. (1981) who showed that verapamil a calcium channel blocker, enhanced the intracellular accumulation of vincristine, potentiating its antiproliferative activity in a multidrug-resistant murine leukemia cell line; this compound they named a chemosensitizer. This has since led to continued research on chemosensitizers such as cyclosporine and quinine which have been designed to inhibit Pgp protein both competitively and non-competitively (Leonard *et al*., 2003).

Phenothiazines have also been proven to sensitize resistant bacteria to the antibiotics to which they were initially resistant (Viveiros and Amaral, 2001; Amaral *et al*., 2011). In tuberculosis (TB) chemotherapy, MDR-TB infections have over the years progressed to extensively drug resistant (XDR-TB) status which has further evolved to totally drug resistant (TDR-TB) infections. The chemosensitizer thioridazine which is an old neuroleptic drug has been studied by Amaral and co-workers and has shown the capability to cure infected mice of an antibiotic susceptible and MDR TB infections and

when used in combination with antibiotics used for therapy of TB, would render the organism more susceptible (Amaral, 2012, Amaral; Viveiros, 2012).

2.7.1 Chemosensitizers in malaria

Chemosensitizers in malaria are compounds that are capable of reinstating antimalarial activity in CQR strains of *Plasmodium falciparum* in the presence of CQ. Thus the general assumption is that the RA inhibits the efflux of CQ from the digestive vacuole (DV) of *P. falciparum* thus allowing CQ to exert its antimalarial activity in the DV.

Verapamil was the first *P. falciparum* CQR chemosensitizer reported by Martin *et al*. (1987) who demonstrated how it reversed CQ resistance at the same concentration that is 1×10^{-6}M as that which was used to reverse resistance in MDR cultured neoplastic cells. This result provided the rationale for looking at similarities in acquisition of drug resistance in *Plasmodia* and neoplastic cells, suggesting that the *P. falciparum* MDR could be the same as the one described in cancer cells implying homologous proteins would be involved.

Following the development of a 3D QSAR pharmacophore model for CQR reversal by Bhattacharjee *et al*. (2002) from imipramine, desipramine and 15 of their analogues, it was found that an ideal CQ chemosensitizer should have two aromatic hydrophobic interaction sites linked by an aliphatic chain to a hydrogen bond acceptor preferably at a side chain nitrogen atom.

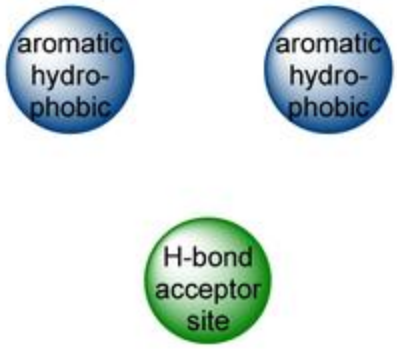


Figure 0.11: 3D QSAR pharmacophore model for CQR chemosensitizer reported by Bhattacharjee *et al*. (2002)

Many structurally and functionally diverse compounds have been identified and reported to demonstrate chemosensitizing activity against CQR *P. falciparum*. Some of the molecules capable of reversing *in vitro* MDR in *P. falciparum* are: calcium channel blockers, calmoduline inhibitors (antipsychotics), antidepressant drugs, histamine H_1-receptor antagonists, dihydroanthracenic derivatives and synthetic surfactants, natural alkaloids, antibiotics and antifungal drugs (Pradines *et al*., 2005).

2.7.1.1 Calcium Channel blockers

Verapamil (VRP) is a classical chemosensitizer that has proven useful in resistance reversal to chloroquine. The well-known target of verapamil is presumed to be the membranes because of the lipophilic side chains (Figure 2.11) found on the drug hence its action as an inhibitor of the slow channel of Ca^{2+} transport across the membranes (Sica and Prisant, 2007). It has been proposed in a study by Lakshmanan *et al*. (2005) that mutations in the *Pf*CRT protein especially the replacement of lysine (K) with

threonine (T) at position 76 may play a key role in the mode of action of verapamil. These findings were also backed by another independent study by Martin *et al*. (2009) in which they found that VRP inhibited the transport of CQ by *Pf*CRT showing IC_{50} of 30 ± 3µM. Adovelande and co-workers reported the synergistic interaction of verapamil and fantofarone in reversing CQ resistance in highly CQ resistant strain FcB1 (Adovelande *et al*., 1998). Ch'ng *et al*., (2013), made use of a direct means to rapidly screen for chemosensitizing compounds using a fluorescent-tagged CQ molecule that enabled them to find seven novel chemosensitizers out of which they found L703606 and mibefradil to be considerably more potent than the classical chemosensitizers VRP and desipramine (DSP). They found that unlike for VRP and DSP which required concentrations of about 291 nM and 152 nM respectively to half the IC_{50} of CQ in K1 strain, L703606 and mibefradil required only 98 nM and 38 nM respectively.

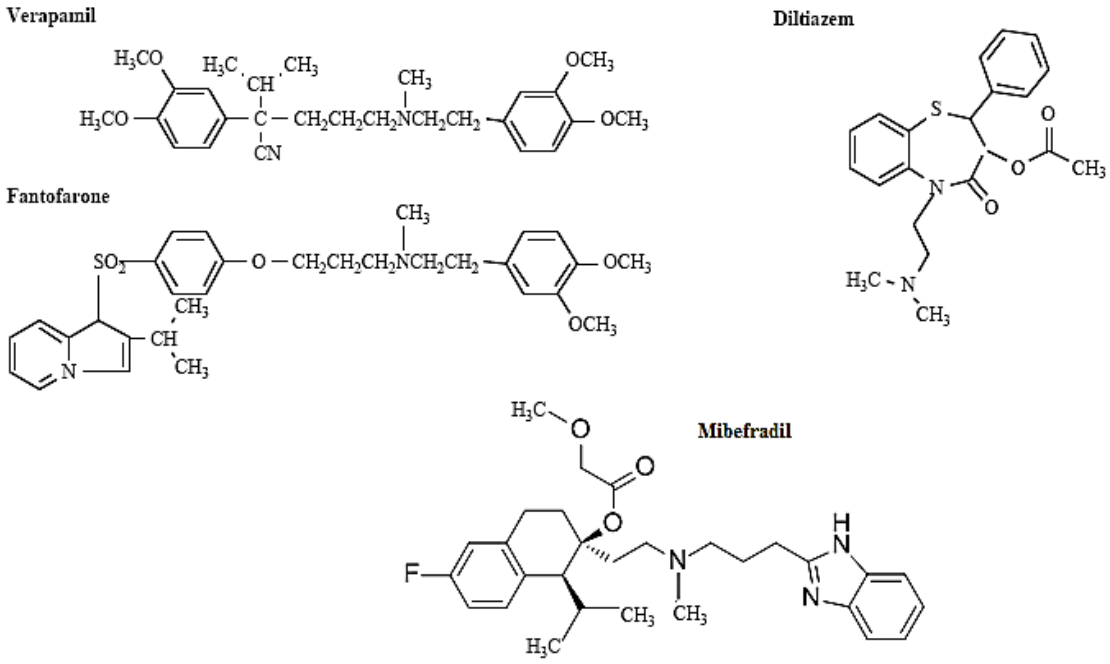


Figure 0.12: Chemical structures of some calcium channel blockers

2.7.1.2 Calmodulin inhibitors/Phenothiazines

Phenothiazines are a group of tranquilizing drugs with antipsychotic actions and they are thought to act by blocking dopaminergic transmission within the brain. Phenothiazines such as chlorpromazine, trifluoperazine, prochlorperazine are said to enhance the potency of CQ against *P. falciparum* CQ resistant strains *in vitro* (Guan *et al*., 2002; Kalkanidis *et al*., 2002; Menez *et al*., 2002). The likely manner through which phenothiazines are proposed to exert their CQ resistance reversal activity is by interacting with P-glycoprotein (Pgh1) in *Plasmodium falciparum* (Reed *et al.*, 2000). Combination of chlorpromazine or prochlorperazine with CQ confirmed the reversal effect of these drugs on CQ resistance this after complete cure was obtained in *Aotus*

monkeys infected with CQ resistant *P. falciparum* strains (Kyle *et al*., 1993). In another study (Miki *et al*., 1992) daily injections of chlorpromazine or trifluoperazine were also able to reverse the resistance to CQ in *P. chabaudi* resistant parasites.

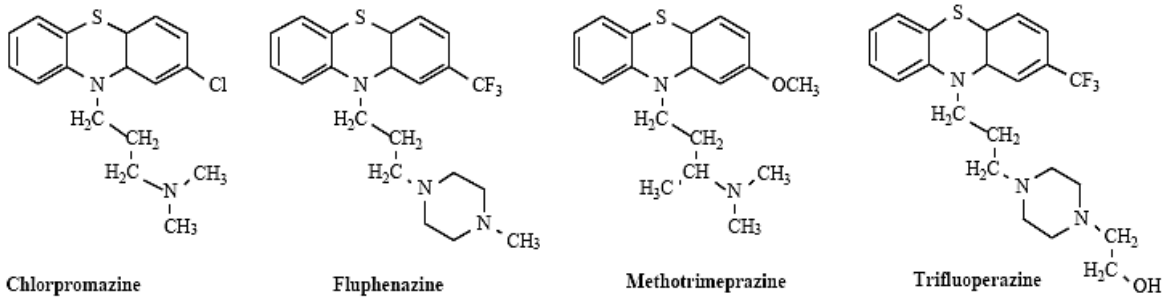


Figure 0.13: Chemical structure of some calmodulin inhibitors (phenothiazines)

2.7.1.3 Tricyclic Antidepressants

These are a class of antidepressant medications that function by increasing the levels of norepinephrine and serotonin consequently blocking the action of acetylcholine. Bhattacharjee *et al*. (2002) have been able to show that aminoalkyl substitution at the N5 position in the heterocyclic moiety of the tricyclic antidepressants and secondary and tertiary aliphatic aminoalkyl nitrogen separated from the intracyclic nitrogen (N5) by two or three carbon chains, are required for the resistance reversal activity. In the first report of activity of a tricyclic antidepressant demonstrating CQ reversal activity, Bitonti *et al*. (1988) showed that the combination of desipramine with CQ was able to reverse the resistance which is associated with increased CQ accumulation in the parasite in *Aotus* monkeys infected with CQR *P. falciparum* strains. In their study, Taylor *et al*. (2000) were also able to demonstrate how CQ sensitivity was restored to varying extents

in vitro in the CQR *P. falciparum* strain RSA11 using the antidepressants amitriptyline, citalopram, oxaprotiline and nomifensine reducing the IC_{50} of CQ from 360 nM to as low as 11 nM in the presence of the mentioned antidepressants.

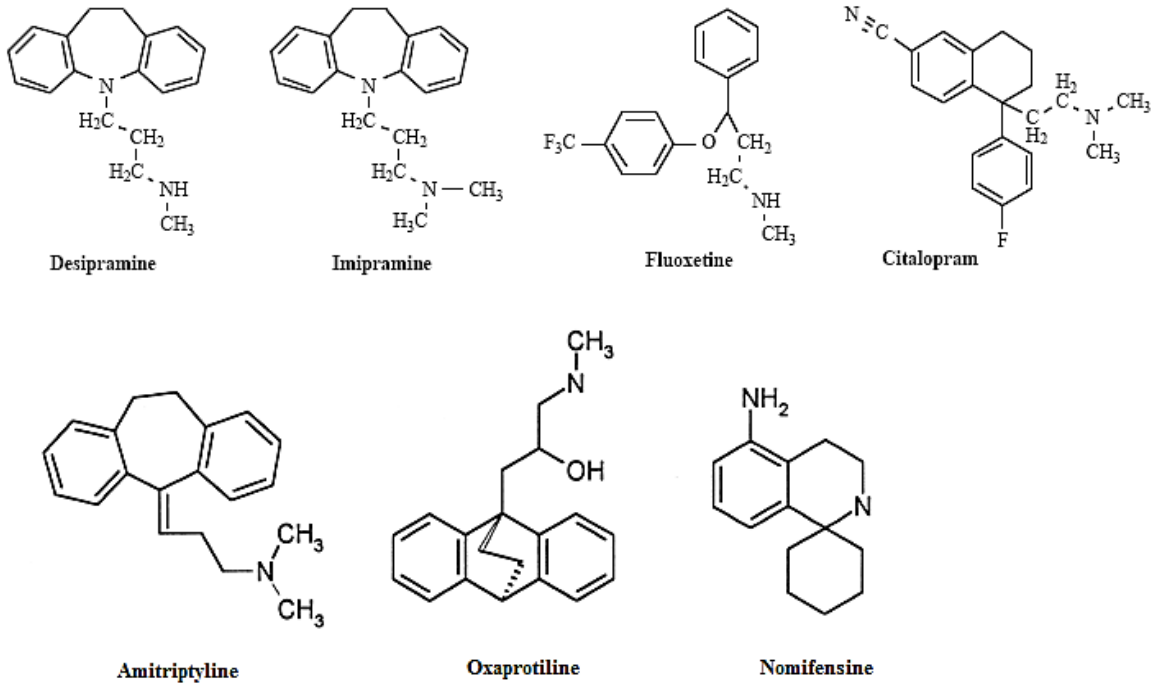


Figure 0.14: Chemical structure of some antidepressants

2.7.1.4 Antihistaminic drugs

Tricyclic histamine (H-1) receptor antagonists are drugs that competitively inhibit the action of histamine on tissues containing H_1 receptor. Some of the antihistaminic drugs that have shown the ability to reverse CQ resistance in *P. falciparum in vitro* include cyproheptadine, ketotifen, azatadine and promethazine (Basco *et al*., 1991). Moreover, CQR reversal was observed using these compounds in *P. berghei* and *P. yoelii* strains *in vivo* as reported by Singh and Puri, (2000a) and Oduola *et al*. (2004). Oduola and co-

workers (2004) evaluated the effects of promethazine on CQ resistant *P. falciparum* and found that the activity of CQ against CQ resistant parasites when combined with promethazine *in vitro* and *in vivo* was enhanced by 32-92 and 20-58%, respectively. *Aotus* monkeys in their study infected with CQR *P. falciparum* were completely cured following treatment with CQ and promethazine (Oduola *et al*., 1998). Further studies have gone to show synergy in halofantrine efficacy when administered in combination with cyproheptadine or ketotifen in halofantrine–resistant *P. yoelii* strains (Singh and Puri, 2000b).

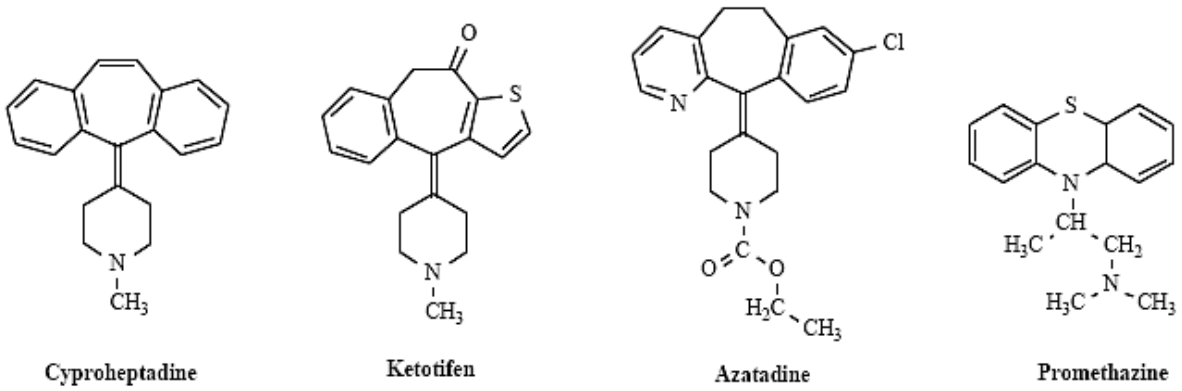


Figure 0.15: Chemical structures of some histamine (H-1) receptor antagonists

2.7.1.5 Synthetic 9,10-Dihydroethanoanthracene Derivatives

These are compounds derived from the polycyclic aromatic hydrocarbon anthracene. Among the several known isomers of dihydroanthracene, the 9,10 derivative is the most common. Millet *et al*. 2004 were able to assess the ability of four 9,10-dihydroethanoanthracene derivatives (BG920, BG932, BG958, and BG996), as well as verapamil and promethazine, to reverse chloroquine resistance against 24 chloroquine

resistant and 10 chloroquine-susceptible strains of *Plasmodium falciparum* from different countries. The 9,10-dihydroethanoanthracene derivatives showed increased chloroquine susceptibility only in chloroquine resistant isolates. Further to this they also found in a previous study (Alibert *et al*., 2002) that the 9,10-dihydroethanoanthracenes exerted more than 80% of *in vitro* reversal of CQ resistance at 1 µM and their IC_{50} values were statistically better than those of VRP or promethazine.

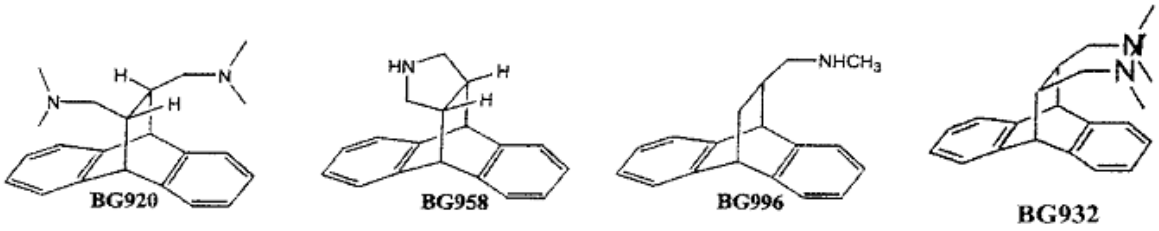


Figure 0.16: Chemical structures of some 9,10-dihydroanthracenes

2.7.1.6 Synthetic Surfactants

Nonylphenolethoxylates (NPE) are synthetic surfactants that have been used as wetting agents and as intestinal permeability enhancers to improve oral drug delivery (Swenson *et al*., 1994) they have also been shown to reverse CQR of *P. falciparum in vitro.* Crandall *et al*. (2000) established that when NPEs and chloroquine are used in combination they have synergistic effects that made them potent antimalarials. In addition, the maximum synergistic effect between an NPE and chloroquine was observed with an NPE having an average ethoxylate (EO) content of 30 EO units.

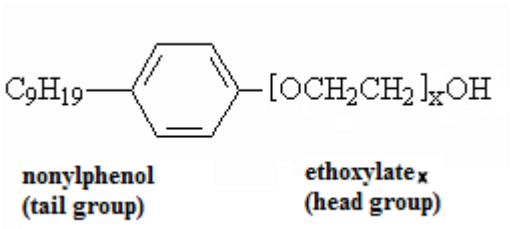


Figure 0.17: Generalized formula of a nonylphenol ethoxylate, x is the ethoxylate units

The chemosensitizer that was used in this study was probenecid (Figure 2.18). Probenecid is a uricosuric drug that increases uric acid excretion in the urine primarily used in treating gout and hyperuricemia. It does not belong to either one of the chemosensitizer groups mentioned above though as shown in (Figure 2.18), it does contain the essential components of an antimalarial chemosensitizer as pointed out by (Bhattacharjee *et al*., 2002). Nzila *et al*. (2003) assessed the chemosensitization of *Plasmodium falciparum* by probenecid *in vitro* and found that probenecid was able to increase the sensitivity of a highly resistant parasite isolate to the antifolates: pyrimethamine, sulfadoxine, chlorcycloguanil, and dapsone by seven-, five-, three-, and three fold, respectively. They also found that when probenecid was tested with chloroquine, it selectively sensitized the CQ resistant isolate to chloroquine unlike for the antifolates where it sensitized both the folate sensitive and resistant parasites. In a subsequent study they tested the effect of probenecid and verapamil in chemosensitizing *Plasmodium falciparum* to 14 antimalarials using the multidrug-resistant strain V1S and the drug-sensitive 3D7 and found that whereas verapamil chemosensitized V1S to quinine and chloroquine, probenecid profoundly chemosensitized V1S to piperaquine. A

result that led them to suggest the use of probenecid to increase piperaquine efficacy *in vivo* (Masseno *et al*., 2009).

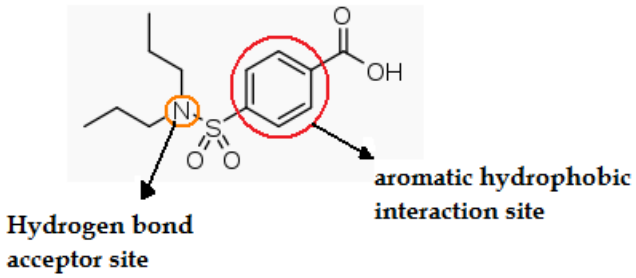


Figure 0.18: Structure of probenecid

Haem detoxification is seen to be of critical importance as an antimalarial drug target because for one it is not parasite encoded so it is immutable and most importantly haem detoxification that occurs during haemoglobin degradation is critical to parasite survival (Olliaro and Goldberg, 1995). Therefore in an effort to restore previously used affordable and effective antimalarial drugs a number of hybrid molecules that consist of the quinoline pharmacophore and chemosensitizers have been synthesized. However, it is worth noting that a number of the chemosensitizers used such as the calcium channel blocker, verapamil (Martin *et al*., 1987), are pharmacologically active compounds with multisystem effects that result in a variety of undesirable side effects, also the concentrations needed to reverse clinical drug resistance for some of these agents can be toxic.

Therefore the challenge remains to develop novel and inexpensive antimalarials. The approach of covalently linking two distinct chemical moieties with different modes of action is fast gaining ground in antimalarial drug development. Following the promising results of resistance reversal of quinoline drugs such as CQ when used in combination with chemosensitizers the use of already established chemical antimalarial scaffolds covalently linked to safe and inexpensive reversal agents could be an option worth exploring in yet another attempt to restore quinoline drug efficacy.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals, glassware and study site

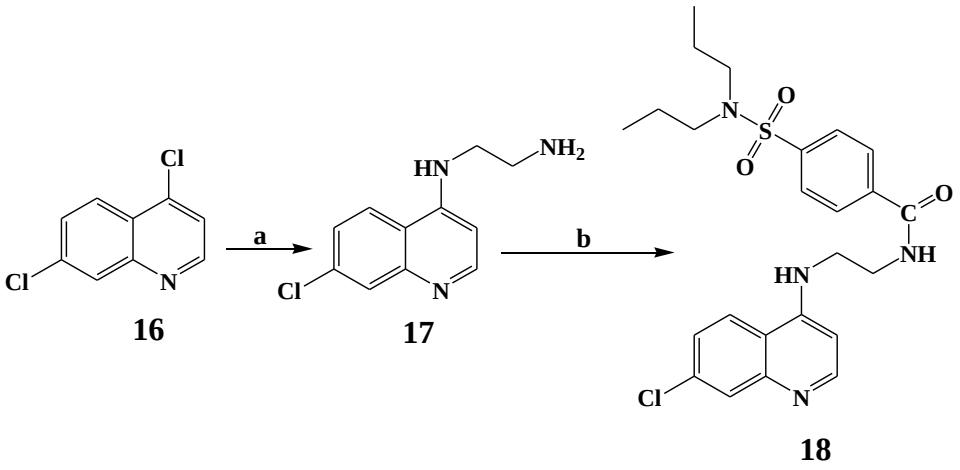
The test drugs, probenecid, chloroquine, 4,7-dichloroquinoline and all other chemicals and reagents were purchased from Sigma Chemical Co., Dorset, United Kingdom. The glassware apparatus used were washed, rinsed with distilled water, and dried in an oven at 110°C. They were allowed to cool and then rinsed with absolute ethanol. The study was carried out in the Centre for Traditional Medicine and Drug Research (CTMDR) and the animal facility at KEMRI.

3.2 Study design

This was a laboratory based experimental study.

3.3 Synthetic design of quinoline hybrid molecule

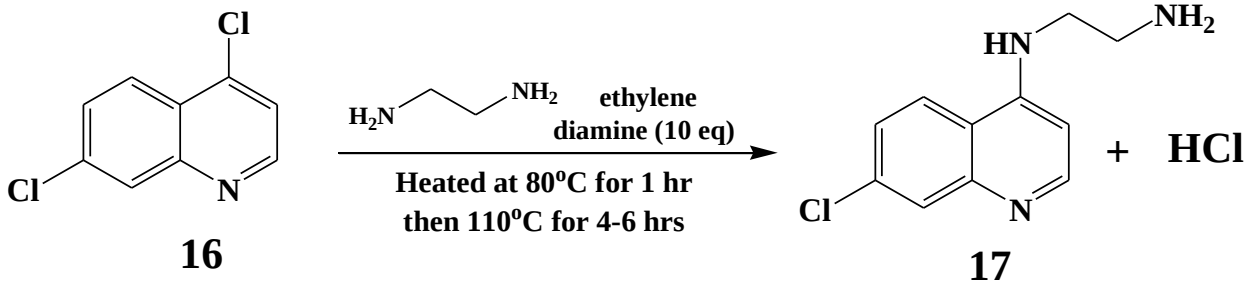
The synthetic design was as illustrated in Scheme 0.1. Generally, it involved introduction of a linker to 4,7-dichloroquinoline and subsequent coupling of the resulting molecule to probenecid (Chollet *et al*., 2009).



Scheme 0.1: Synthetic strategy for quinoline chemosensitizer linked molecule. a) 10 equivalent (eq) ethylene diamine, 80°C 1hr then 100-110°C for 4-6 hrs; b) 1.0 eq, 2 mmol probenecid, 1.5 eq EDC, 1.5 eq HOBt, 3.0 eq DIPEA, 2.5 eq of (17), CH_2Cl_2, 0°C to room temperature stirring for 24 hrs.

3.3.1 Introduction of a linker

A mixture of 4,7-dichloroquinoline (**16**) (1eq, 1.1883 g, 6 mmol) and ethylene diamine (10eq, 4.00 mL, 60 mmol) was refluxed at 80°C for 1 hr without stirring and then 100-110°C for 4-6 hrs with stirring then cooled to room temperature after which the reaction was quenched using 30 mL dichloromethane (DCM). The organic layer was successively washed with 5% NaOH (30 ml), water and finally with brine. The organic layer was dried over anhydrous $MgSO_4$ and the solvent removed under reduced pressure in a rotary evaporator to afford the intermediate, N^1-(7-chloroquinolin-4-yl)-ethane-1,2-diamine (**17**) (Antinarelli *et al*., 2012).

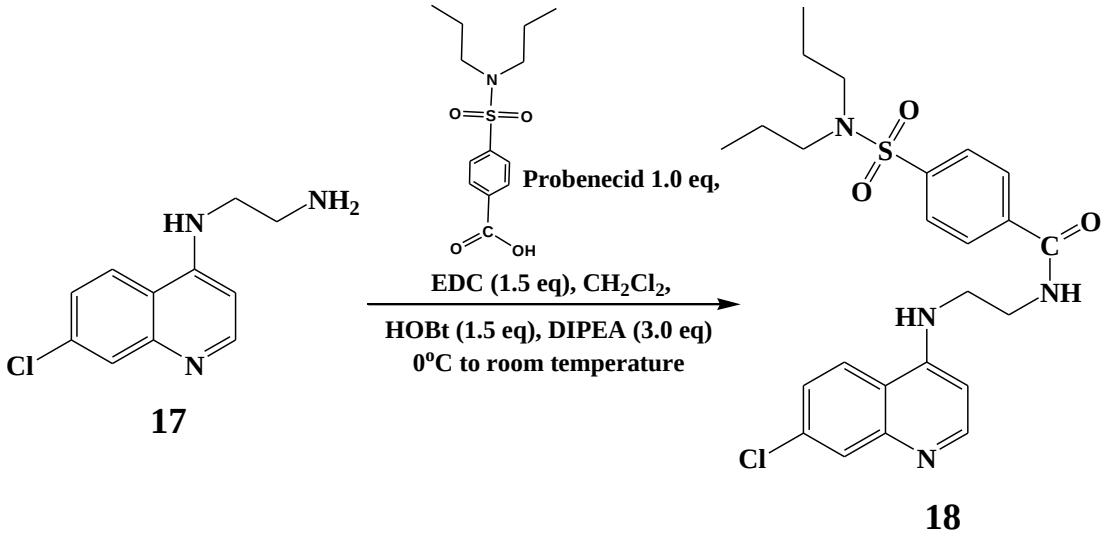


Scheme 0.2: Introduction of a linker

3.3.2 Coupling procedure

Probenecid (1.0 eq, 0.5707g) was dissolved in dichloromethane (10 mL), and then 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (1.5 eq, 0.5751g), 1-hydroxy-benzotriazole (HOBt) (1.5 eq, 0.4054g) and diisopropylethylamine (DIPEA) (3.0 eq, 1.05 ml) was added and the mixture stirred at 0°C in a round bottomed flask. To this mixture was added N^1-(7-chloroquinolin-4-yl)-ethane-1,2-diamine (**17**) (5.0 mmol) and the reaction stirred to room temperature overnight. After completion of the reaction, reaction mixture was quenched with saturated $NaHCO_3$. The organic phase was separated and the aqueous phase back extracted with DCM (20mL × 3). The combined organic layers were dried using anhydrous $MgSO_4$, filtered and solvent removed *in vacuo*. Purification was carried out by flash chromatography using silica gel column (ethyl acetate: 10% ammonia in methanol 7:1) yielding the desired chemosensitizer-quinoline linked molecule N-{2-[(7-chloroquinolin-4-yl)amino]ethyl}-4-(dipropylsulfamoyl) benzamide (**18**). The reaction was optimized by using other solvents

like toluene to dissolve the probenecid instead of DCM and other coupling agents like CDI instead of EDC.



Scheme 0.3: Coupling N^1-(7-chloroquinolin-4-yl)-ethane-1,2-diamine to probenecid

NMR spectra for both ^{13}C and ^{1}H was analyzed using characteristic chemical shifts in ppm while for IR characteristic peaks in wavenumbers generated were used. In mass spectra, the peaks for the molecular ion and major fragments were analyzed.

3.4 General instrumentation

Infrared spectra were obtained in potassium bromide pellets using IR-840 Shimadzu spectrometer. The ^{1}H and ^{13}C NMR analysis were done using mercury Vx Bruker NMR spectrometer operating at 200 MHz for ^{1}H and 100 MHz for ^{13}C NMR and a modified Bruker Avance 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR in deuterated dimethyl sulfoxide with TMS as the internal standard. The splitting pattern abbreviations are as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), and m

(multiplet). An electron impact and fast atom bombardment mass measurement was recorded on a mass spectrometer machine (VG–12–250). Thin layer chromatography (TLC) was performed using pre-coated silica gel 60 F-254 glass plates (Merck). The solvent system used was ethyl acetate: 10% ammonia in methanol 3.5:0.5. Silica gel 5-40 μm mesh (Merck, Germany) was used for column chromatography with a solvent system of ethyl acetate:10% ammonia in methanol 7:1. The solvents were redistilled to ensure purity.

3.5 Bioassays of the hybrid drug

3.5.1 In vitro assays

3.5.1.1 Drug solutions

Stock solution of the hybrid drug was prepared with sterile water (deionized and autoclaved) and filter-sterilized through 0.22 μm filters under laminar hood. Stock solutions of reference drugs CQ, probenecid as well as 4,7-dichloroquinoline was similarly prepared in sterile water. Dissolution of the drugs which was insoluble in water was enhanced by first dissolving them in dimethylsulfoxide (solvent concentration <0.02%) (Elueze *et al*., 1996). The test samples were prepared as a 2 mg ml^{-1} stock solution and sonicated to enhance solubility then stored at 4°C. Further dilutions were prepared on the day of biological assays. All the drug solutions were stored at 4°C for later use.

3.5.1.2 *Plasmodium falciparum* cultures

Laboratory-adapted *P. falciparum* cultures of the international reference isolate 3D7 (CQ–sensitive) and W2 (CQ–resistant) were used in this study. The strains have been cultured and maintained at the Malaria Laboratories of Kenya Medical Research Institute (KEMRI), Nairobi. The culture medium, a variation of that described by Trager and Jensen (1976), consisted of RPMI 1640 supplemented with 10% human serum, 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) and 25 mM $NaHCO_3$. Human type O^+ erythrocytes (<28 days old) served as host cells and the cultures were incubated at 37°C in an atmosphere of 3% CO_2, 5% O_2 and 92% N_2 obtained from BOC Nairobi, Kenya.

3.5.1.3 Evaluation of antiplasmodial activity of the hybrid drug

Aliquots (25 µl) of the culture medium were added to all the wells of a 96-well flat-bottom micro-culture plate. The hybrid drug solution in volumes of 25 µl was added in triplicate, to the first wells and a Titertek motorized hand diluter (Flow Laboratories, Uxbridge, United Kingdom) was used to make serial two-fold dilutions of each sample over a 64-fold concentration range. The highest concentration for the hybrid of 10 µg $ml^{-1}$was serially diluted 2-fold in complete medium to give seven doubling concentrations with the lowest concentration being 1.5625 µg ml^{-1}. The same dilution technique was used for the reference drug CQ (highest concentration: 0.05µg ml^{-1} for CQS-3D7 and 0.2µg ml^{-1} for CQR-W2) , 4,7-dichloroquinoline and probenecid whose highest concentration was 100 µg ml^{-1} each. The *in vitro* semi-automated micro-dilution

assay technique that measures the ability of the drugs to inhibit the incorporation of [G-^{3}H] hypoxanthine into the malaria parasite was used to assess the hybrid drug's antiplasmodial activity (Desjardins *et al*., 1979; Le Bras and Deloron, 1983). Briefly, a suspension (200 µl, 1.5% v/v) of parasitized erythrocytes (0.4% parasitaemia) in culture medium and growth rate (>3-fold per 48 h) were added to all test wells. Drug free wells of both parasitized and non-parasitized erythrocytes were also included as controls. The plates were incubated at 37°C in an airtight gas chamber under micro-aerobic environment of 3% CO_2, 5% O_2 and 92% N_2. After 48 h each well was pulsed with 25 µl of culture medium containing 0.5 µCi of [^{3}H] hypoxanthine and the plates were incubated for a further 18 h. The contents of each well was harvested onto glass fiber filters, washed thoroughly with distilled water, and dried, and the radioactivity (in counts per minute, cpm) was measured by liquid scintillation counter.

The concentration causing 50% inhibition of radioisotope incorporation (IC_{50}) was determined by interpolation after logarithmic transformation of both concentration and cpm values using the formula,

IC_{50} = antilog (log X_1 + (($logY_{50}$-log Y_1)($logX_2$-$logX_1$))/($logY_2$-$logY_1$))

Where, Y_{50} is the mid-point,

X_1, X_2 are lower and upper concentrations, respectively,

Y_1, Y_2 are the corresponding cpm values for the data points above and below the cpm mid-points (Sixsmith *et al*., 1984).

3.5.1.4 Combination studies of the individual precursors of the hybrid drug

To compare the antiplasmodial activity of the hybrid drug (10 µg ml^{-1}) with that of the combination of its precursors, in the ratio of 1:1 (mixture of 50 µg ml^{-1} of 4,7-dichloroquinoline and 50 µg ml^{-1} of probenecid). Subsequent serial dilutions and semi-automated micro-dilution assays as outlined in section 3.4.1.3 above were done. The cpm values for the wells of hybrid drug and for the drugs in combination at a given concentration were used to determine percentage *in vitro* inhibition of parasite proliferation, relative to drug-free parasitized erythrocyte wells.

3.5.2 *In vivo* assays

3.5.2.1 Parasite, host and ethics

Plasmodium berghei-ANKA isolates (wild type and lumefantrine-resistant) were used for this study. These parasites were maintained in a frozen state (−80°C) at KEMRI Centre for Traditional Medicine and Drug Research (CTMDR). The parasites were inoculated intraperitoneally (ip) into a female outbred Swiss albino mouse, the donor mouse to the experimental mice.

The day of infection was denoted as day zero post infection (pi) and all experiments were done using this revived parasite. At day 5 pi, the donor mouse parasitaemia was assessed microscopically (Olympus BX50F4, Olympus Optical Co., LTD., Japan) at 1000× magnification by examining Giemsa stained thin tail-vein blood smears. The

mouse was later sedated, bled via cardiac puncture and blood collected in heparinized tubes. The parasitaemia was adjusted downwards using phosphate saline glucose (PSG) buffer and each of the test Swiss albino mice 6 weeks old (weight 20±2 g) was inoculated ip with approximately $1x10^7$ parasitized erythrocytes in volumes of 0.2 ml (Muregi *et al*., 2008). The inoculated mice were then randomized into group of 5, housed in cages and maintained in the animal facility on commercial rodent pellets and water *ad libitum*.

This work was fully approved by KEMRI-Scientific and Ethics Review Unit (SERU) approval number KEMRI/SERU/CTMDR/017/3131 (Appendix 1) and all experiments conducted in accordance to the Institute's guide for the care and use of laboratory animals. All mice that were deemed to have completed their intended use were euthanized using CO_2 in the course of experiments, placed in biohazard disposable autoclave bags and stored at -20°C before incineration to destroy all infectious agents and to avoid environmental contamination during incineration.

3.5.2.2 Drug solvent

On the day of administration, the drugs were freshly prepared by dissolving them in a solvent consisting of 70% Tween-80 (d = 1.08 g ml^{-1}) and 30% ethanol (d = 0.81 g ml^{-1}) and subsequently diluted 10-fold with double distilled water (to result in a solution of 7% Tween-80 and 3% ethanol concentration).

3.5.2.3 Antiplasmodial evaluation of the hybrid drug against lumefantrine resistant *P. berghei* in mice

A *P. berghei* ANKA lumefantrine (LM) resistant strain, selected by increasing doses of LM, maintained in mice under constant drug pressure (LM 63.55 mg/kg) (Kiboi *et al*., 2009) and stored at −80°C was defrosted and used to inoculate mice. The mice were inoculated with 1 x 10^7 parasitized RBCs using blood parasites from a donor with parasites resistant to 63.55 mg/kg of LM. The 4-day suppressive test was performed as described by Peters *et al*., (1975) using the LM-resistant *P. berghei* ANKA blood parasites, with some modifications. Briefly, the blood parasites were maintained through weekly blood passages in mice. For the experiments, groups of 20–30 mice were inoculated with 1 × 10^7 infected erythrocytes. Approximately three hours later, they were randomly distributed into groups of five mice per cage, which were treated daily orally using cannula for four consecutive days. All the freshly prepared compounds were administered orally at doses of 64 mg/kg for LM, 50 mg/kg for hybrid and 114 mg/kg for the combination of LM and hybrid. The control mice received the drug solvent. On days 4, 7 and 9 after parasite inoculation, blood was taken from the tail of each mouse and used to prepare thin smears, which were methanol-fixed, Giemsa-stained and examined microscopically to determine parasitaemia. Percentage chemosuppression (parasite reduction) was calculated with mean parasitaemia of day 4 using the formula described in literature (Tona *et al*., 2001).

Percentage chemosuppression = [(mean parasitaemia in the negative control group - parasitaemia in the test group)/mean parasitaemia in the negative control group] x 100

3.5.3 Cytotoxicity Test

The cell viability was determined by the MTT (3- (4,5-dimethyltrazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay method according to Mosmann (1983). Vero cells were grown in Eagle's minimum essential medium (MEM) (GIBCO, Grand Island, N.Y.) supplemented with 5% fetal bovine serum (FBS) in 25 ml cell culture flasks and incubated at 37°C in 5% CO_2. Upon attainment of confluence, the cells were seeded with 5×10^4 cells/well in 96-well plates and incubated under the same environment. The drug samples were solubilized in MEM and DMSO (0.02%, v/v) and after 24 h, culture medium was replaced by the solution of fresh MEM containing the drugs at different concentrations (in μg/mL: 1, 10, 100, 1000), followed by 24 hours of further incubation. The MTT (2.0 mg/mL) was added, followed by incubation at 37°C in an atmosphere of 5% CO2 for 4 hours. The cells in the micro-culture wells were detached by trypsinization and the number of viable cells determined by MTT bioassay.The optical density was determined at 570 nm and 630 nm to measure the signal and background, respectively. The cell viability was expressed as a percentage of the control absorbance in the untreated cells after subtracting the appropriate background. Inhibition data was plotted as a dose-response curve, from which CC_{50} (concentration required to cause visible alterations in 50% of intact cells) was determined by linear regression.

3.5.4 *In vivo* assay for the determination of acute toxicity

This was carried out along OECD Test Guideline 423 (Acute Toxic Class Method, adopted on 17th Dec. 2001). Briefly, female Swiss albino mice, 8-12 weeks old, nulliparous and non-pregnant were used. Four fixed dose levels of 5, 50, 300 and 2000mg/kg body weight were used and dosing was stepwise using 3 animals per step. The time interval between treatment groups was determined by the onset duration and severity of toxic signs such as changes in skin and fur, eyes and mucous membranes, tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. Animals were observed individually at least once during the first 30 minutes, periodically during the first 24 hours with special attention given during the first 4 hours and daily thereafter, for a total of 14 days, except where they needed to be removed from the study and humanely killed for animal welfare reasons or were found dead. Animals dying or sacrificed as a result of experimentation were pooled in a bio-hazard container and stored at -20°C before being incinerated. The ones that were still alive at the end of the experiments were euthanized using carbon dioxide (CO_2) and incinerated.

3.6 Data Analysis

Data for *in vitro* drug assays were transferred into graphic programme (Microsoft Excel 2007) and results expressed as the drug concentration required for 50% inhibition of (G-^{3}H) hypoxanthine incorporation into parasite nucleic acid using non-linear regression analysis of the dose-response curve. For *in vivo* data, percentage parasitaemia was recorded in Excel (Microsoft) and expressed as the mean±standard error of mean (SEM).

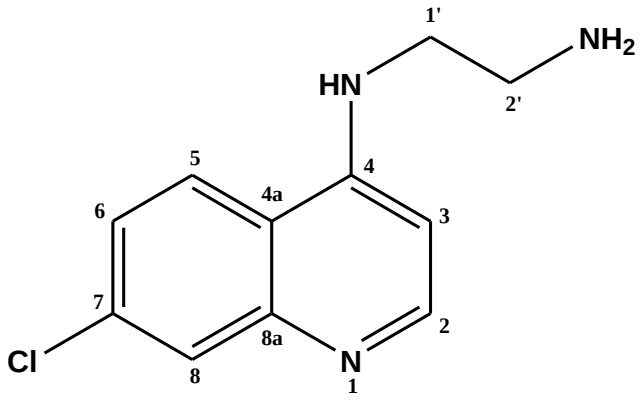
CHAPTER FOUR

RESULTS

4.1 Synthesis of hybrid molecule

4.1.1 Structural confirmation of N^1-(7-chloroquinolin-4-yl)-ethane-1,2-diamine (Compound 17)

The reaction of 4,7-dichloroquinoline and ethylene diamine which has been previously described was used to synthesize the intermediate compound **17**. This reaction afforded an off white powder at 75% yield. Thin layer chromatography was used to compare the product with a reference sample than had previously been synthesised.



17

Figure 0.1: Structure of intermediate compound 17

The chemical shifts of the quinoline pharmacophore linked to ethylene diamine were identified from the ^{1}H-NMR spectrum of the final hybrid compound **18** (Appendix 4). The proton chemical shifts are summarised in Table 4.1 below:

Table 0.1: ^{1}H -NMR (100 MHz) of compound 17 in DMSO-d6

Position	Chemical Shift (ppm)	Coupling Constant *J* (Hz)	Multiplicity	Integral
1’	3.52	5.2	t	2[H]
2’	3.57	6.3	t	2[H]
2	8.42	5.4	d	1[H]
3	6.63	5.5	d	1[H]
5	7.79	2.1	d	1[H]
6	7.45	8.9,2.1	dd	1[H]
8	8.21	9	d	1[H]

d = doublet, dd = doublet of doublet and t = triplet

Similarly, from the ^{13}C-NMR and DEPT spectrum of the hybrid molecule **18** (Appendix 6 and 7, respectively), the 11 carbon peaks that constitute compound **17** were identified and summarized in Table 4.2 below.

Table 0.2: ^{13}C-NMR Spectrum (100 MHz) data of compound 17 in DMSO-d6

Position	Chemical Shift (ppm)	DEPT
1’	41.7	CH_2
2’	38.0	CH_2
2	151.8	CH

3	98.6	CH
4	149.0	C_q
4a	117.4	C_q
5	127.5	CH
6	124.1	CH
7	133.4	C_q
8	123.9	CH
8a	150.0	C_q

C_q = Quaternary Carbon

4.1.2 Structural confirmation of N-{2-[(7-chloroquinolin-4-yl)amino]ethyl}-4-(dipropylsulfamoyl)benzamide (Compound 18)

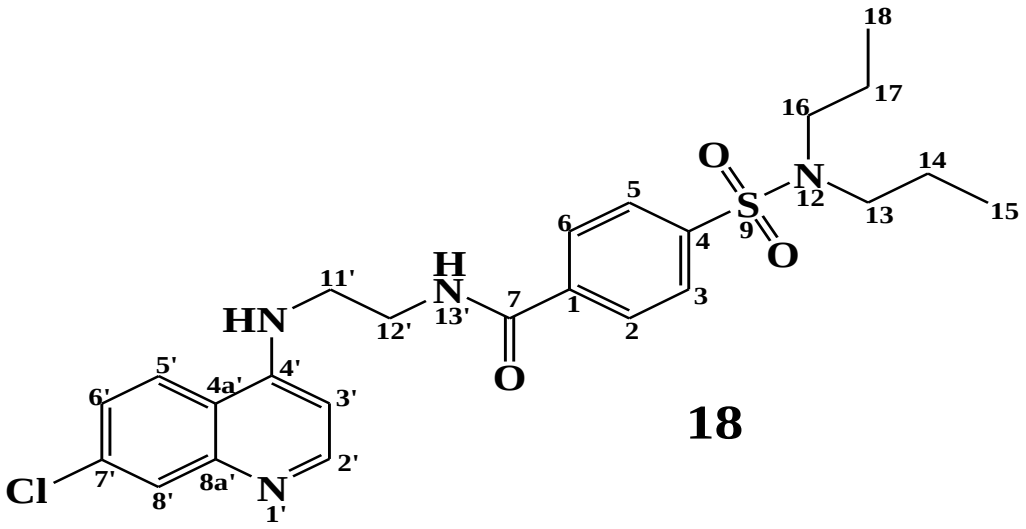
Compound **18** was isolated as a pale white powder {0.4663g, 48 % yield, Rf 0.5, SiO_2, 7:1 EtOAc:MeOH, with 10% NH_3 in MeOH}. This yield was a result of optimization of the coupling reagents and solvents as shown in Table 4.3. The yields obtained from other coupling reagents during optimization reactions of compound **17** to **18** are also given in Table 4.3.

Table 0.3: Yield of compound 18 using various coupling reagents

Entry	Coupling Reagent	Compound 17	Probenecid	DIPEA	Solvent	Yield (%)
1	EDC	1.0 eq	1.2 eq	2.5 eq	Water	5.8
2	EDC	1.0 eq	1.2 eq	2.5 eq	Toluene	16.1
3	EDC	1.0 eq	1.2 eq	2.5 eq	Dichloromethane	3.4
4	EDC	1.0 eq	1.2 eq	2.5 eq	Dichloromethane	10.3
5	CDI	1.0 eq	1.2 eq	2.5 eq	Dichloromethane	12.8
6	EDC	1.0 eq	1.2 eq	2.5 eq	Dichloromethane	14.8
7	CDI	1.0 eq	1.2 eq	2.5 eq	Toluene	14.4
8	EDC	1.0 eq	1.2 eq	2.5 eq	Xylene	14.6
9	EDC	2.5 eq	1.0 eq	1.0 eq	Dichloromethane	26.8
10	EDC	1.0 eq	2.5 eq	3.0 eq	Dichloromethane	17.5
11	**EDC**	**2.5 eq**	**1.0 eq**	**3.0 eq**	**Dichloromethane**	**48**

Compound **18** which was a product of covalently linking N-(7-chloro-4-quinolyl)-1,2-diaminoethane ($C_{11}H_{12}ClN_3$) (compound **17**) with 4-(dipropylsulfamoyl) benzoic acid/probenecid ($C_{13}H_{19}NO_4S$), was identified as N-{2-[(7-chloro-4-quinolyl)amino]ethyl}-4-(dipropylsufamoyl) benzamide with a molecular formula of $C_{24}H_{29}ClN_4O_3S$ by fast atom bombardment mass spectrum (Appendix 2) that showed a

molecular ion peak at *m/z* 489.2 amu with a relative abundance of 26% against a calculated mass of 489.03 amu.



18

Figure 0.2: Structure of intermediate compound 18

The IR spectrum (Appendix 3) showed characteristic bands at V_{max}: 1643 cm^{-1} for the C = O stretch, 3348.42 cm^{-1} for the secondary amide N-H stretch and 1610.56 cm^{-1} for the N-H bend.

There were 13 peaks identified from the ^{1}H-NMR spectrum (Appendix 4) for compound **18** with chemical shifts between 0.5 and 9 ppm. A look at compound **18** gives an indication of aliphatic protons belonging to the dipropyl group that are in a chemically equivalent environment as such, the methyl protons were observed as one peak at δ = 0.80 ppm (t, *J* = 7.4 Hz, 6H, H-15/18) and the methylene protons of the two propyl tethers were observed at δ = 1.46 ppm (sextet, *J* =7.4 Hz, 4H, H-14/17) and δ = 3.04 ppm (t, *J* = 7.6 Hz, 4H, H-13/16). Two peaks that were assigned to the methylene

protons of the ethylene diamine linker were observed at δ = 3.52 ppm (t, *J* = 5.2 Hz, 2H, H-11") and δ = 3.57 ppm (t, *J* = 6.3 Hz, 2H, H-12").

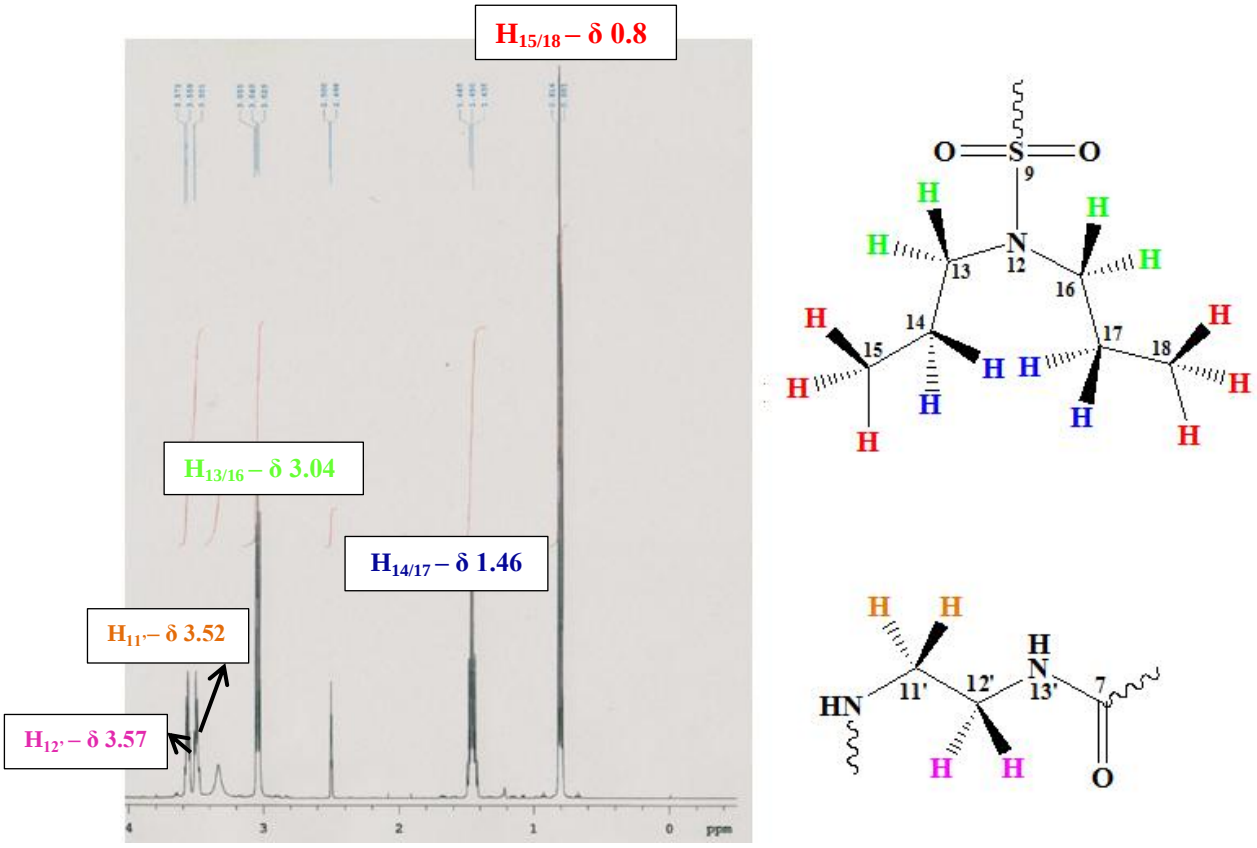


Figure 0.3: Snapshot of ^{1}H NMR spectrum showing the aliphatic protons of compound 18.

Seven different peaks were observed in the aromatic region (Figure 0.4) that is: δ = 6.63 ppm (d, *J* = 5.5 Hz, 1H, H-3'), δ = 7.45 ppm (dd, *J* = 8.9, 2.1Hz, 1H, H-6'), δ = 7.79 ppm (d, *J* = 2.1 Hz, 1H, H-5'), δ = 7.90 ppm (dd, *J* = 6.7,1.9 Hz, 2H, H-3/5), δ = 8.02 ppm (dd, *J* = 6.7,1.9 Hz, 2H, H-2/6), δ = 8.21 ppm (d, *J* = 9 Hz, 1H, H-8') and δ = 8.42 ppm (d, *J* =5.4Hz, 1H, H-2').

The amide N-H proton was also observed furthest downfield of the TMS signal at δ = 8.93 ppm. The individual resonances, the number of protons associated with each resonance (the integral), the splitting pattern and the associated coupling constants from the ^{1}H-NMR spectrum are summarized in Table 4.4.

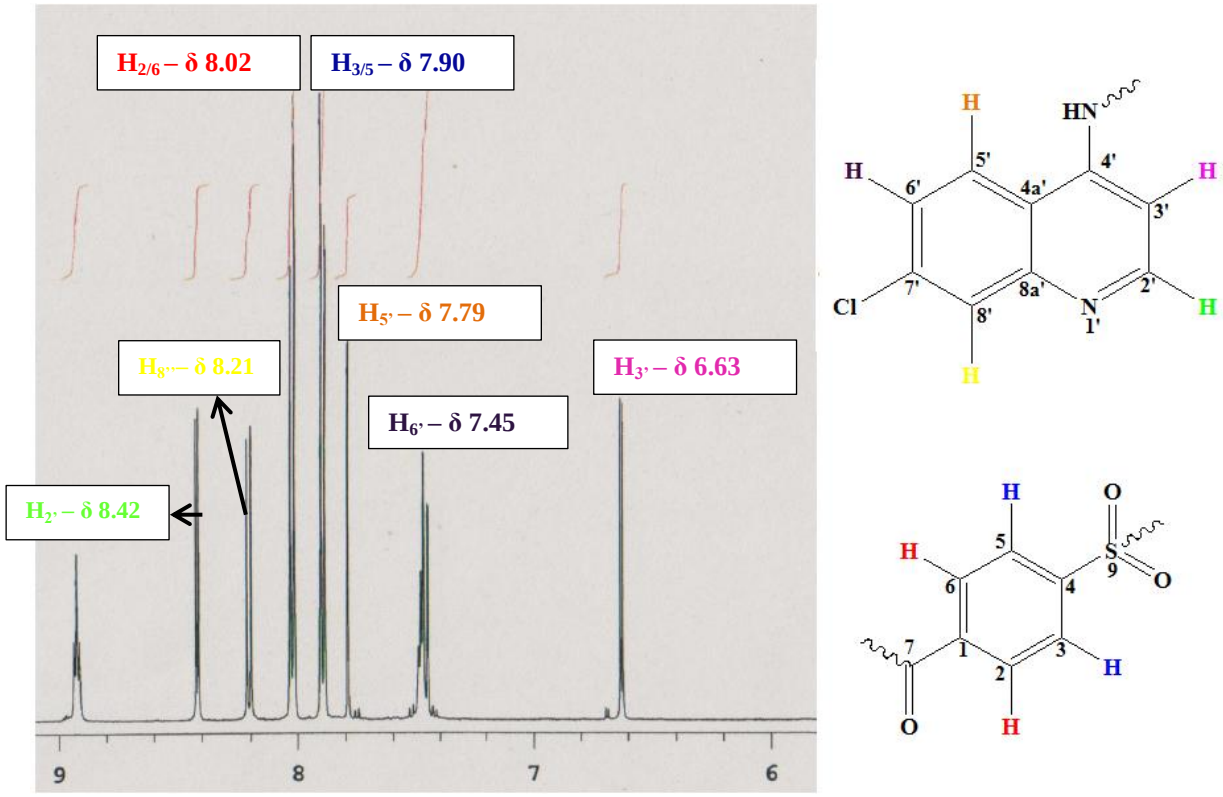


Figure 0.4: Snapshot of ^{1}H NMR spectrum showing protons in the aromatic region of compound 18

Table 0.4: ^{1}H-NMR (100 MHz) chemical shifts of compound 18 in DMSO-d6

Chemical Shift (ppm)	Multiplicity	Coupling Constant	Hydrogen Position	Integration
0.80	t	J = 7.4	H – 15/18	6 H
1.46	Sextet	J = 7.4	H – 14/17	4 H
3.04	t	J = 7.6	H – 13/16	4 H
3.52	t	J = 5.2	H – 11′	2 H
3.57	t	J = 6.3	H – 12′	2 H
6.63	d	J = 5.5	H – 3′	1 H
7.45	dd	J = 8.9, 2.1	H – 6′	1 H
7.79	d	J = 2.1	H – 5′	1 H
7.90	dd	J = 6.7, 1.9	H – 3/5	2 H
8.02	dd	J = 6.7, 1.9	H – 2/6	2 H
8.21	d	J = 9.0	H – 8′	1 H
8.42	d	J = 5.4	H – 2′	1 H
8.93	-	-	H – 13′	-

d = doublet, dd = doublet of doublet, t = triplet

The ^{13}C NMR spectrum of compound **18** (Appendix 6) exhibited 24 carbon signals as 2 methyls, 6 methylenes, 9 methines and 7 quartenary carbons (Table 4.5). Among the 24 carbon signals in compound **18** was a carbonyl groups (δ 165.6, C-7), and fifteen aromatic carbons (δ 151.8-98.6, C-1, C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-4', C-4a', C-5', C-6', C-7', C-8', C-8a').

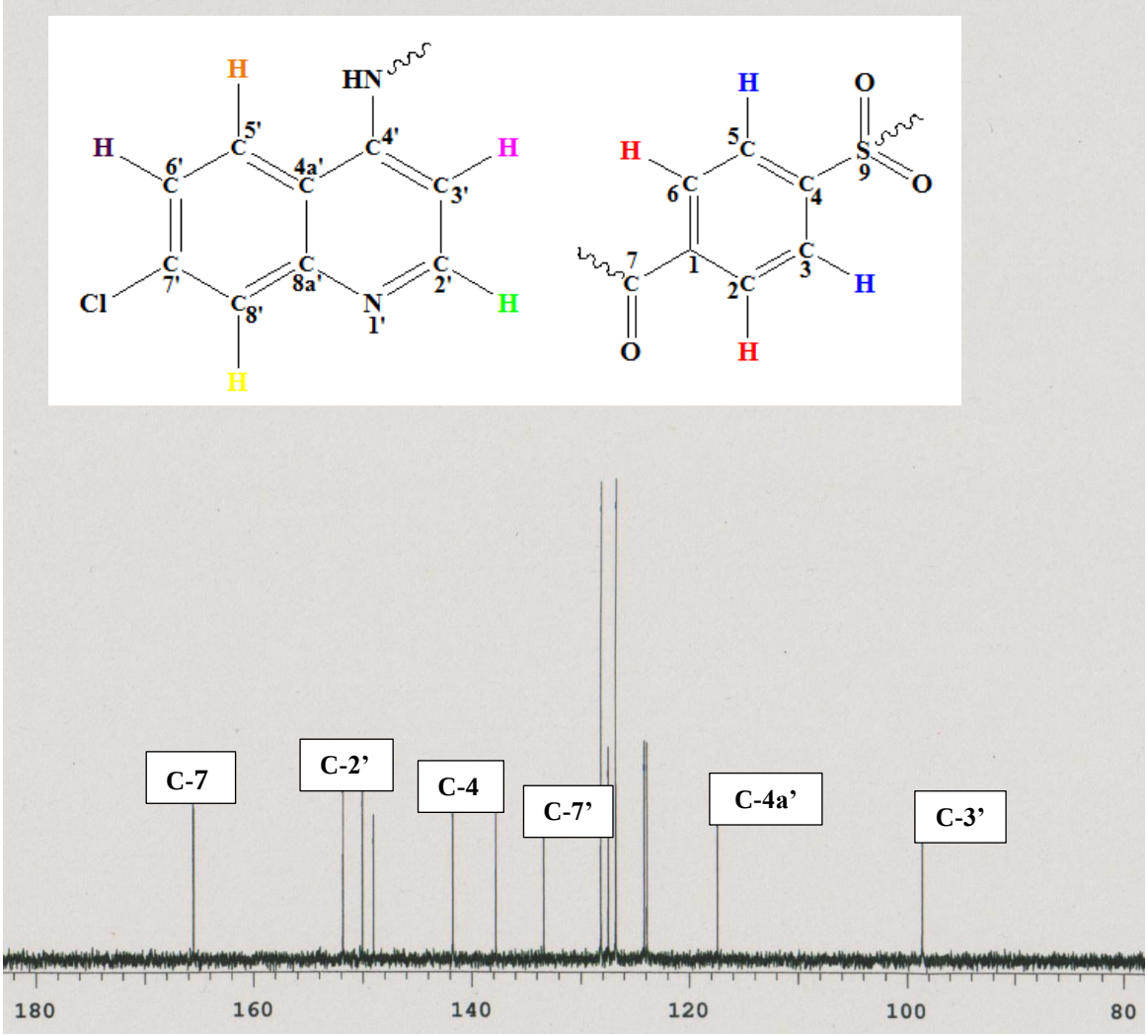


Figure 0.5: Snapshot of ^{13}C NMR spectrum showing the aromatic carbons of compound 18

The seven signals between δ 49.5-21.5 were assigned to the methylene carbons (C-13, C-14, C-16, C-17, C-11', C-12'). Signals from the two methyl groups were observed at *δ* 10.9 (C-15, C-18).

Further analysis of the DEPT spectrum (Appendix 7) in combination with the ^{13}C-NMR, aided in assigning carbon atoms in the molecule that had protons attached to them that is DEPT-90 for CH groups and DEPT-135 for CH_2 appearing in the negative phase/inverse peaks CH and CH_3 appearing in the positive phase. The carbon chemical shifts are summarized in Table 4.5 below:

Table 0.5: ^{13}C-NMR (100MHz) data of compound 18 in DMSO-d6

Chemical Shift (ppm)	Carbon Position	DEPT - 135	DEPT - 90
10.9	C- 15/18	CH_3	-
21.5	C – 14/17	CH_2	-
38.0	C - 12′	CH_2	-
41.7	C - 11′	CH_2	-
49.5	C – 13/16	CH_2	-
98.6	C - 3′	CH	CH
117.4	C – 4a′	C_q	C_q
123.9	C - 8′	CH	CH
124.1	C - 6′	CH	CH
126.8	C – 3/5	CH	CH
127.5	C – 5′	CH	CH
128.2	C – 2/6	CH	CH
133.4	C - 7′	C_q	C_q
137.8	C - 1	C_q	C_q
141.8	C - 4	C_q	C_q
149.0	C - 4′	C_q	C_q
150.1	C – 8a′	C_q	C_q
151.8	C - 2′	CH	CH
165.6	C - 7	C_q	C_q

C_q – Quaternary carbon

Using the HSQC spectrum (Appendix 8), it was possible to identify which protons were directly attached to each of the carbons in the hybrid compound **18**. Because the hybrid contains seven quaternary carbons (see the results of ^{13}C NMR and DEPT), the

connections observed were for only 12 carbons that is the five chemically equivalent carbons (δ = 128.2 ppm, C-2/6; δ = 126.8 ppm, C- 3/5; δ = 49.5 ppm, C- 13/16; δ = 21.5 ppm, C-14/17 and δ =10.9 ppm, C-15/18) and the other seven carbons with protons attached to them (δ = 151.8 ppm, C-2′; δ = 98.6 ppm, C-3′; δ = 127.5 ppm, C-5′; δ = 124.1 ppm, C-6′; δ = 123.9 ppm, C-8′; δ = 41.7 ppm, C-11′ and δ = 38.0 ppm, C-12′). Red (negative) peaks correspond to CH_2 groups, and in this case, two methylene carbons "connected" to two chemically equivalent protons (δ = 21.5 ppm, C-14/17 and δ = 49.5 ppm, C- 13/16), whereas the other two methylene carbons were connected to two chemically different protons (δ = 38.0 ppm, C-12′ and δ = 41.7 ppm, C-11′). Blue (positive) peaks correspond to both CH and CH_3 groups. The 12 "pieces" of the hybrid molecule which were identified from the HSQC spectrum are shown in Table 4.6 below:

Table 0.6: HSQC-NMR data for compound 18 in DMSO-d6

Carbon Chemical Shift (ppm)	Proton Chemical Shift (ppm)	Carbon – Hydrogen Coupling	Carbon Number
10.9	0.8	CH_3	C- 15/18
21.5	1.46	CH_2	C – 14/17
38.0	3.57	CH_2	C – 12′
41.7	3.52	CH_2	C - 11′
49.5	3.04	CH_2	C – 13/16
98.6	6.63	CH	C - 3′
117.4	-	C_q	C – 4a′
123.9	8.21	CH	C - 8′
124.1	7.45	CH	C - 6′
126.8	7.90	CH	C – 3/5
127.5	7.79	CH	C – 5′
128.2	8.02	CH	C – 2/6
133.4	-	C_q	C - 7′
137.8	-	C_q	C - 1′

141.8	-	C_q	C - 4
149.0	-	C_q	C - 4′
150.1	-	C_q	C – 8a′
151.8	8.42	CH	C - 2′
165.6	-	C_q	C - 7

C_q – Quaternary Carbon

The 2D H-H–COSY NMR spectrum (Appendix 5) showed two kinds of peaks: those on the diagonal, which had the same chemical shift in both dimensions, and the off-diagonal peaks which identified protons coupled to other protons via one bond, thus allowing identification of the pieces identified from HSQC (Appendix 8) to be joined into larger fragments of the molecule. For instance as illustrated in the COSY spectrum (Appendix 5), the proton at δ = 1.46 ppm H-14/17 on the diagonal peak is coupled to proton δ = 0.8 ppm H-15/18 and δ = 3.04 ppm H-13/16 on the off diagonal peak. Thus given that the proton δ = 0.8 ppm H-15/18 was identified as a methyl proton from DEPT and HSQC above, the protons identified would therefore be assigned to the dipropyl group in the hybrid molecule. A list of the most obvious one-bond correlations which were deduced from the COSY spectrum of the hybrid is given in the table below. The correlation Table 4.7 includes all the resonances which were identified in the HSQC table.

Table 0.7: H-H COSY NMR data for compound 18

Carbon Chemical Shift (ppm)	Diagonal Peak (ppm)	Off-Diagonal Peak (ppm)
10.9	0.8 (H – 15/18)	1.46 (H – 14/17)
21.5	1.46 (H - 14/17)	0.8 (H – 15/18), 3.04 (H – 13/16)
38.0	3.57 (H - 12′)	8.93 (N-H - 13′)
41.7	3.52 (H - 11′)	7.45 (H - 6′)

49.5	3.04 (H – 13/16)	1.46 (H – 14/17)
98.6	6.63 (H - 3′)	8.42 (H - 2′)
117.4	-	-
123.9	8.21 (H - 8′)	7.45 (H - 6′)
124.1	7.45 (H - 6′)	3.52 (H - 11′),7.79 (H - 5′), 8.21 (H - 8′)
126.8	7.90 (H – 3/5)	8.02 (H – 2/6)
127.5	7.79 (H - 5′)	7.45 (H - 6′)
128.2	8.02 (H – 2/6)	7.90 (H – 3/5)
133.4	-	-
137.8	-	-
141.8	-	-
149.0	-	-
150.1	-	-
151.8	8.42 (H -2′)	6.63 (H - 3′)
165.6	-	-

The HMBC spectrum (Appendix 9) was used to identify ^{13}C and ^{1}H resonances which were connected by either 2- or 3-bonds. The HMBC spectrum therefore enabled the joining together of the individual pieces of the molecule which had been determined from the HSQC spectrum in essence, completing the "jigsaw" by "connecting up" the quaternary carbons, which could not be assigned from H-H COSY spectrum. Thus, for instance the quaternary carbonyl carbon at δ = 165.5 ppm (C-7) showed a connection to the aromatic methine at δ = 8.02 ppm (H-2/6) and the amide hydrogen at δ = 8.93 ppm (N-H - 13′) from which this carbon was assigned C-7 via a 3-bond and 2-bond coupling respectively. Another quaternary carbon at δ = 133.4 (C-7′) correlated both to the methine protons at δ = 7.42 ppm (C-6′), δ = 7.79 ppm (C-5′) and δ = 8.21 ppm (C-8′), which thereby identified this carbon as C-7′ (C-Cl), involved in 2-bond couplings to protons at δ = 8.21 ppm and δ = 7.42 ppm and 3- bond coupling to proton at δ = 7.79

ppm. A summary of the correlation information available from the HMBC spectrum was given in the Table 0.8 below:

Table 0.8: HMBC-NMR data of compound 18

^{13}C Resonances (ppm)	Carbon Number	^{1}H resonances connected by 2-or 3- bonds
10.9	C- 15/18	1.46 (H – 14/17), 3.04 (H – 13/16)
21.5	C – 14/17	0.8 (H- 15/18), 3.04 (H – 13/16)
38.0	C – 12′	3.52 (H -11′), 8.93 (H - 13′)
41.7	C - 11′	3.57 (H - 12′)
49.5	C – 13/16	0.8 (H – 15/18), 1.46 (H – 14/17), 3.04 (H – 13/16)
98.6	C - 3′	7.45 (H - 6′), 8.42 (H - 2′)
117.4	C – 4a′	6.63 (H - 3′), 7.42 (H - 6′), 7.79 (H - 5′), 8.21 (H - 8′), 8.42 (H - 2′)
123.9	C - 8′	6.63 (H - 3′)
124.1	C - 6′	7.79 (H - 5′)
126.8	C – 3/5	7.79 (H - 5′), 7.9 (H – 3/5), 8.02 (H – 2/6)
127.5	C – 5′	7.42 (H - 6′), 8.21 (H - 8′)
128.2	C – 2/6	7.9 (H – 3/5), 8.02 (H – 2/6), 8.21 (H - 8′)
133.4	C - 7′	7.42 (H - 6′), 7.79 (H - 5′), 8.21 (H - 8′)
137.8	C - 1	7.9 (H – 3/5)
141.8	C - 4	8.02 (H – 2/6), 7.9 (H – 3/5)
149.0	C - 4′	7.79 (H - 5′), 8.21 (H - 8′), 8.42 (H - 2′)
150.1	C – 8a′	3.52 (H - 11′), 7.45 (H - 6′), 8.21 (H - 8′), 8.42 (H - 2′)
151.8	C - 2′	6.63 (H - 3′)
165.6	C - 7′	3.57 (H - 12′), 8.02 (H – 2/6), 8.93 (H - 13′)

4.2 Biological studies for the quinoline hybrid molecule

4.2.1 *In vitro* antiplasmodial assay

Antiplasmodial activity was evaluated using the 3D7-CQS and W2-CQR strains of *P. falciparum.* The IC_{50} values were calculated from experiments carried out in triplicate and the results are presented in Table 4.9.

The *in vitro* activity data suggests that linking the 4,7-DCQ to probenecid via an ethylene diamine linker resulted into the hybrid molecule (CQProb-H) having antiplasmodial activity (0.66 ± 0.06 - 3D7 and 0.61 ± 0.01 - W2). In comparison to the CQProb-H precursor molecules that is 4,7-dichloroquinoline, probenecid and the combination, CQProb-H showed better antiplasmodial activity.

Table 0.9: Antiplasmodial activity of quinoline-probenecid hybrid and its precursor molecules.

Molecule	Antiplasmodial Activity (IC_{50} µg/ml) [a]		Cytotoxicity (CC_{50} µg/ml)
	3D7	W2	
4,7-DCQ	> 100	> 100	NT
Probenecid	> 100	53.178	NT
4.7-DCQ+ Probenecid	> 100	67.446	NT
CQProb-H	0.66	0.61	40.87
CQ[b]	0.005	0.078	57.46

DCQ – Dichloroquinoline, CQ – Chloroquine, (IC_{50} – maximum concentration tested 100µg/ml for 4,7-DCQ, Probenecid and 4.7-DCQ+ Probenecid, 10µg/ml for hybrid, 0.05µg/ml for CQ-3D7 and 0.2µg/ml for CQ-W2), NT – Not Tested, NC – Not Cytotoxic (maximum concentration tested 1000µg/ml for hybrid and 100µg/ml for CQ).

[a] Values are means of triplicate samples

[b] Reference drug

4.2.2 Antiplasmodial tests against lumefantrine resistant *P. berghei* in mice

The efficacy of the hybrid molecule **18** was measured using the 4-day suppressive test using a model of LM–resistant *P. berghei* infection. For this study, Swiss albino mice were infected on day 0 and treated once daily for 4 days. Parasitaemia was measured on

day 4 and compared to those of control non-treated mice. As seen in Table 0.10, 50mg/kg of the hybrid alone suppressed day 4 parasitaemia by 8.69% whereas 64 mg/kg of LM and a combination of 64 mg/kg LM and 50 mg/kg hybrid molecule (114 mg/kg) gave a parasite suppression of 82.27 and 62.06%, respectively.

Table 0.10: Antiplasmodial activity of LM and hybrid molecule against *P. berghei* evaluated as percentage reduction of parasitaemia 4 days after inoculation with LM-resistant *P berghei*.

Drug	Dosage mg/kg	Percentage reduction of parasitaemia (%)*		
		Day 4	Day 7	Day 9
Lumefantrine	64	82.27	58.11	47.61
Hybrid	50	8.69	8.55	14.46
LM + Hybrid	114	62.06	59.00	60.24

*Percentage reduction of parasitaemia calculated in relation to control non-treated mice (100% of parasite growth)

4.2.3 Acute toxicity study

The acute toxicity study was done stepwise using three mice at a time starting at a dose of 50 mg/kg body weight. The only visible effect that was noted was minimal lethargy 2 hours after dosing which may have been due to the drug solvent used to dissolve the drug however thereafter normal activity resumed and no deaths were reported for the 3 mice used. An increased dose of 150 mg/kg body weight resulted in 2 deaths by day 1 with the remaining mouse dying on day 2. The results are presented in Table 4.11 below.

Table 0.11: Acute toxicity of synthesized hybrid molecule.

CQProb-H Dose (mg/kg)	Number of Mice tested	Number of mice deaths
50	3	0
300	3	3

CHAPTER FIVE

DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussions

5.1.1 Synthesis of hybrid molecule

5.1.1.1 Structural confirmation of N^1-(7-chloroquinolin-4-yl)-ethane-1,2-diamine (Compound 17)

The reaction of 4,7-dichloroquinoline and ethylene diamine which has been previously described (Antinarelli *et al*., 2012; de Souza *et al*., 2011) was used to synthesize the intermediate compound **17**. This compound had previously been synthesized (Wamakima, 2015) and although characterization was not carried out distinctly, a look at the ^{1}H-NMR spectrum of the final hybrid compound **18** (Appendix 4) suggested the presence of the quinoline pharmacophore linked to ethylene diamine with values comparable to those reported by Manohar *et al*. (2015) as identified by the proton chemical shifts. Similarly, from the ^{13}C-NMR and DEPT spectrum of the hybrid molecule **18** (Appendix 6 and 7, respectively), the 11 carbon peaks that constitute compound **17** were identified.

5.1.1.2 Structural confirmation of N-{2-[(7-chloroquinolin-4-yl)amino]ethyl}-4-(dipropylsulfamoyl) benzamide (Compound 18)

In the optimization reaction, it was noted that the use of water as a solvent together with EDC coupling reagent gave the lowest yield (5.8%) possibly due to poor solvation of the reaction mixture. Further investigation on the solvent effect was carried out using EDC as the standard coupling reagent. Non polar aprotic solvents like toluene and xylene improved the yields slightly (16.2 and 14.6%, respectively). Use of dichloromethane on the other hand yielded the product in comparative yield (14.8%) but within a longer reaction time increased from 12 hours to 48 hours the product yield was increased to 48%. Change of coupling reagent from 1,1'-Carbonyldiimidazole (CDI) to EDC gave an improved yield of 12.8 to 14.8%. This may have been as a result of having limited water soluble urea by-product which was easily extracted leaving more of the desired product. The lower yield observed from using CDI was possibly as a result of having less acylimidazole which is formed from the reaction of the carboxyl group and CDI in the reaction mixture as such, when the amine group (compound 17) was added low conversion to an amide was observed. The 48% yield resulted from using EDC as the coupling reagent, HOBt as the additive and CH_2Cl_2 as the solvent.

There were 13 peaks identified from the ^{1}H-NMR spectrum (Appendix 4) for compound **18** with chemical shifts between 0.5 and 9 ppm. The ^{13}C NMR spectrum of compound **18** (Appendix 6) exhibited 24 carbon signals as 2 methyls, 6 methylenes, 9 methines and

7 quartenary carbons. The spectral data is partially consistent with literature data obtained for probenecid (Carradori *et al*., 2015) and quinoline (Manohar *et al*., 2015) moiety.

The benefits of chemosensitization intimated in drug resistant cancer lines as a means to enable cells to respond to otherwise resistant drug treatments (Slater *et al*., 1982; Tsuro, 1983; Ozols, 1985) have also been investigated for reversal of CQR in malaria parasites. Martin *et al*. (1987) and Bitonti *et al*. (1988) found that the ability to sensitize previously resistant parasite was achievable in the presence of calcium channel blocker verapamil. Focus has largely been placed on improving the structures of potential chemosensitizing agents for malaria (Ch'ng *et al*., 2013; Guan, 2002; Deane 2014) which have been tested mainly by co-administration with CQ and quinoline like drugs as an alternative therapeutic approach to chloroquine resistant *Plasmodium falciparum* (Bitonti *et al*., 1988; Basco *et al*., 1992; Gerena *et al*., 1992; Baso *et al*., 1994). More recently, hybrid molecules consisting of chemosensitizers covalently linked to CQ have been synthesized (Burgess *et al*., 2006) in a bid to improve both pharmacokinetic and pharmacodynamic properties of CQ. Subsequent studies on these compounds termed 'reversed chloroquine' have resulted in improved uptake and accumulation of CQ in the parasite's digestive vacuole and potency greater than CQ (Burgess *et al*., 2010).

In this study, the quinoline structural motif – 4,7-dichloroquinoline was hybridized to a chemosensitizer structural motif – probenecid making use of ethylene diamine as the

linker. This 2-step process afforded the successfully linked quinoline-chemosensitizer hybrid molecule described above. The choice to use quinoline as one of the pharmacophores was informed by its *sui generis* target that is haem detoxification (Zhang *et al*., 1999). This in essence allows for modification of the quinoline structural motif given that haem as a target is immutable to the CQ-resistant parasite and its detoxification during haemoglobin degradation is detrimental to parasite survival (O'Neill *et al*., 2005). Whereas the chemosensitizer probenecid was used following studies that have been able to show its capability to enhance the activity of chloroquine in chloroquine resistant parasite when administered as a combination (Nzila *et al*., 2003; Masseno *et al*., 2009).

5.1.2 Biological studies for the quinoline hybrid molecule

5.1.2.1 *In vitro* antiplasmodial assay

Antiplasmodial activity was evaluated using the 3D7-CQS and W2-CQR strains of *P. falciparum* according to the procedure reported by Trager and Jensen (1976). The 4,7-dichloroquinoline was used as the starting material in the synthesis of the hybrid molecule **18** however it is not an active antiplasmodial drug thus the reason why in both 3D7 and W2 strains the antiplasmodial activity was >100µg/ml. Probenecid which is a uricosuric drug that increases uric acid excretion in the urine is primarily used for the treatment of gout and hypertension. Therefore not surprising was the antiplasmodial activity >100µg/ml in 3D7 strain.

Probenecid was envisioned as inhibiting the *P. falciparum* chloroquine resistant transporter (CQRT) associated with CQ export from the DV in CQ^R parasite. When combined with 4,7-dichloroquinoline however, it possesses minimal antiplasmodial activity *in vitro* 67.446 µg/ml which did not translate *in vivo* as observed when the hybrid was combined with LM. Assuming that in this case (combination of LM with hybrid) there was delivery of the hybrid molecule to the DV one would expect a potentiating effect which was not observed, on the contrary, the percentage parasite suppression reduced. Moreover, unlike the other chemosensitizers discussed earlier, probenecid lacks the basic nitrogen atom required for a CQ chemosensitizer (Bhattacharjee *et al*., 2002) by virtue of the nitrogen atom being linked to a sulfonyl moiety which correspondingly reduces its basicity.

Nonetheless probenecid when combined with 4,7-dichloroquinoline showed activity of 67.446 ± 6.32 µg/ml whereas activity of 4,7-dichloroquinoline was found to be >100 µg/ml. This could imply the possibility of a potentiating effect of probenecid on 4,7-DCQ. Probenecid has previously been shown to increase the sensitivity of chloroquine-resistant isolate V1/S (Nzila *et al*., 2003), and so a similar mechanism could be inferred in this case as 7-chloro-4-alkylamino-quinoline present in the hybrid **18** does form part of the CQ pharmacophore. Another possible reason for the activity observed is that probenecid contains a sulphonamide moiety. Sulfonamides are well known competitive inhibitors of the enzyme dihydropteroate synthetase present within the folate biosynthetic pathway which catalyses the conversion of para-aminobenzoic acid

(PABA) to dihydropteroate, a key step in folate synthesis (Hitchings, 1973; Hong *et al*., 1995). Therefore given W2 *P*. *falciparum* strain presents resistance to sulfadoxine the structural difference could be the reason for activity observed in probenecid.

Probenecid is not a typical chemosensitizer like the calcium channel blockers, antidepressant drugs or antihistaminic drugs however it does have the essential components in its structure pointed out by Bhattacharjee *et al*. (2002) required for chemosensitization. Thus in the case of compound **18**, the 7-chloro-4-aminoquinoline nucleus which is obligatory for antiplasmodial activity, particularly, inhibition of β-hematin formation and accumulation of the drug at the target site (Kaschula *et al*., 2002; Cheruku *et al*., 2003; Egan, 2003), coupled with the presence ethylene diamine link essential for maintained activity against CQR strains of *P. falciparum* (Ridley *et al*., 1996; De *et al*., 1998) that was linked to probenecid could be said to be the contributing factor for increased antiplasmodial activity observed. Cytotoxicity of compound **18** was tested on Vero cell line and it was not cytotoxic with a CC_{50} of 40.87 µg/mL and the selectivity index of 67.

5.1.2.2 Antiplasmodial tests against lumefantrine resistant *P. berghei* in mice

The efficacy of the hybrid molecule **18** was measured using the 4-day suppressive test (Peters *et al*., 1975) using a model of LM–resistant *P. berghei* infection. The hybrid showed no suppression activity when compared to LM and even further combination of

the two drugs that is LM and the hybrid showed no improved suppression. The lack of parasite suppression by the hybrid molecule could be attributed possibly to the molecule being metabolically unstable *in vivo*, as 4,7-dichloroquinoline and probenecid were tethered via an ethylene diamine link. Consequently, metabolism of the molecule which was administered orally could have led to failed delivery of the hybrid molecule as a whole entity to the site of action – the parasite digestive vacuole. Additionally, unpublished *in vivo* data on the activity of 4,7-dichloroquinoline against *P. berghei* at a dose of 50 mg/kg resulted in percentage parasite suppression of 52.4%, further supporting the argument of minimal antiplasmodial activity from the precursor molecule – 4,7-dichloroquinoline.

5.1.2.3 Acute toxicity study

The acute toxicity study was done stepwise using three mice at a time starting at a dose of 50 mg/kg body weight. It was noted that 50 mg/kg body weight of the hybrid molecule was well tolerated by the mice over the period of observation and so it was the dose used to administer the hybrid molecule *in vivo*.

5.2 Conclusions

Despite having no activity *in vitro* and *in vivo* proof of concept of covalently linking two structural motifs into one molecule was achieved.

5.2.1 A novel quinoline hybrid molecule consisting of quinoline and probenecid scaffold was synthesized in a 2-step process. Ethylene diamine was used as the linker to facilitate hybridization via an amide bond.

5.2.2 *In vitro*, the hybrid molecule showed antiplasmodial activity of 0.66 ± 0.06 µg/ml in CQS -3D7 strain and 0.61 ± 0.01 µg/ml in CQR –W2 strain which was less active than the reference drug CQ (0.005 µg/ml, 0.078 µg/ml –W2).

5.2.3 Evaluation of antiplasmodial activity *in vivo*, went to show that the synthesized hybrid molecule could not counter CQ resistance as had earlier been hypothesized. In the LM-resistant *P. berghei* mouse malaria model, the hybrid molecule displayed no significant activity on its own (8.9% parasite suppression) and when combined with LM (62.06% parasite suppression) compared to the 82.27% parasite suppression obtained from LM.

5.2.4 The synthesized hybrid molecule gave a CC_{50} value of 40.87 µg/ml. The selectivity index of the hybrid molecule was 67 implying that the molecule was not cytotoxic. 50 mg/kg body weight was the dose concentration used to treat mice.

On the hypothesis, the results reported showed that the quinolone chemosensitizer hybrid was not able to reverse CQ/multidrug resistance thus failed to reject the null hypothesis.

5.3 Recommendations

5.3.1 The quinoline component of the hybrid molecule used was not an active antimalarial drug as such could have contributed to the lack of activity observed *in vivo*. As such active antimalarial drug pharmacophores such as those of quinine and amodiaquine could be used in the synthesis of the hybrid. Variation of both the linker (De *et al*., 1996; Hocart *et al*., 2011; Ridely *et al*., 1996) and possibly the reversal agent moiety (Carradori *et al*., 2015; Salahuddin *et al*., 2013) could be considered with the aim of making the compound more stable to metabolic cleavage and possibly improving the overall activity of the compound. More importantly, alternative reversal agents/ chemosensitizers could be sought that preferably possess considerably good antiplasmodial activity so as to give not only resistance reversal activity but also dual antiplasmodial activity to the hybrid molecule.

5.3.2 The antiplasmodial activity of the intermediate formed in the two step reaction could be before linking to another molecule to ensure that activity is present in the intermediate before hybridization can be carried out.

5.3.3 A chloroquine resistant strain of *P. berghei* or one bearing structural resemblance to the quinolone drug CQ such as amodiaquine or piperaquine could be used to carry out the *in vivo* study for better comparison of activity of synthesized molecule. Dosage forms of the hybrid molecule could be introduced to improve the bioavailability of the compound.

5.3.4 Ensure the newly synthesized compounds have activity lower than those of the reference drugs chloroquine and artemether lumefantrine for comparison purposes.

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APPENDICES

Appendix I: KEMRI Scientific Ethics Review Unit Approval Letter

KENYA MEDICAL RESEARCH INSTITUTE

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Email: director@kemri.org info@kemri.org Website: www.kemri.org

KEMRI/RES/7/3/1 **November 02, 2015**

TO: **FIONA OYATSI,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. PETER MWITARI,**
THE DIRECTOR, CTMDR,
NAIROBI

Forwarded 5/11/2015 for D/CTMDR

Dear Madam,

RE: **PROTOCOL NO. KEMRI/SERU/CTMDR/017/3131 (*RESUBMISSION*): SYNTHESIS OF NOVEL HYBRID MOLECULES FOR MANAGEMENT OF *PLASMODIUM FALCIPARUM (VERSION 1.2, 12TH OCTOBER 2015)***

Reference is made to your letter (not dated). KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study protocol on October 23, 2015.

This is to inform you that the Committee notes that the issues raised during the 242nd Committee C meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on 27th August 2015 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day **2nd November 2015** for a period of one year. Please note that authorization to conduct this study will automatically expire on **1st November, 2016.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **September 20, 2016**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

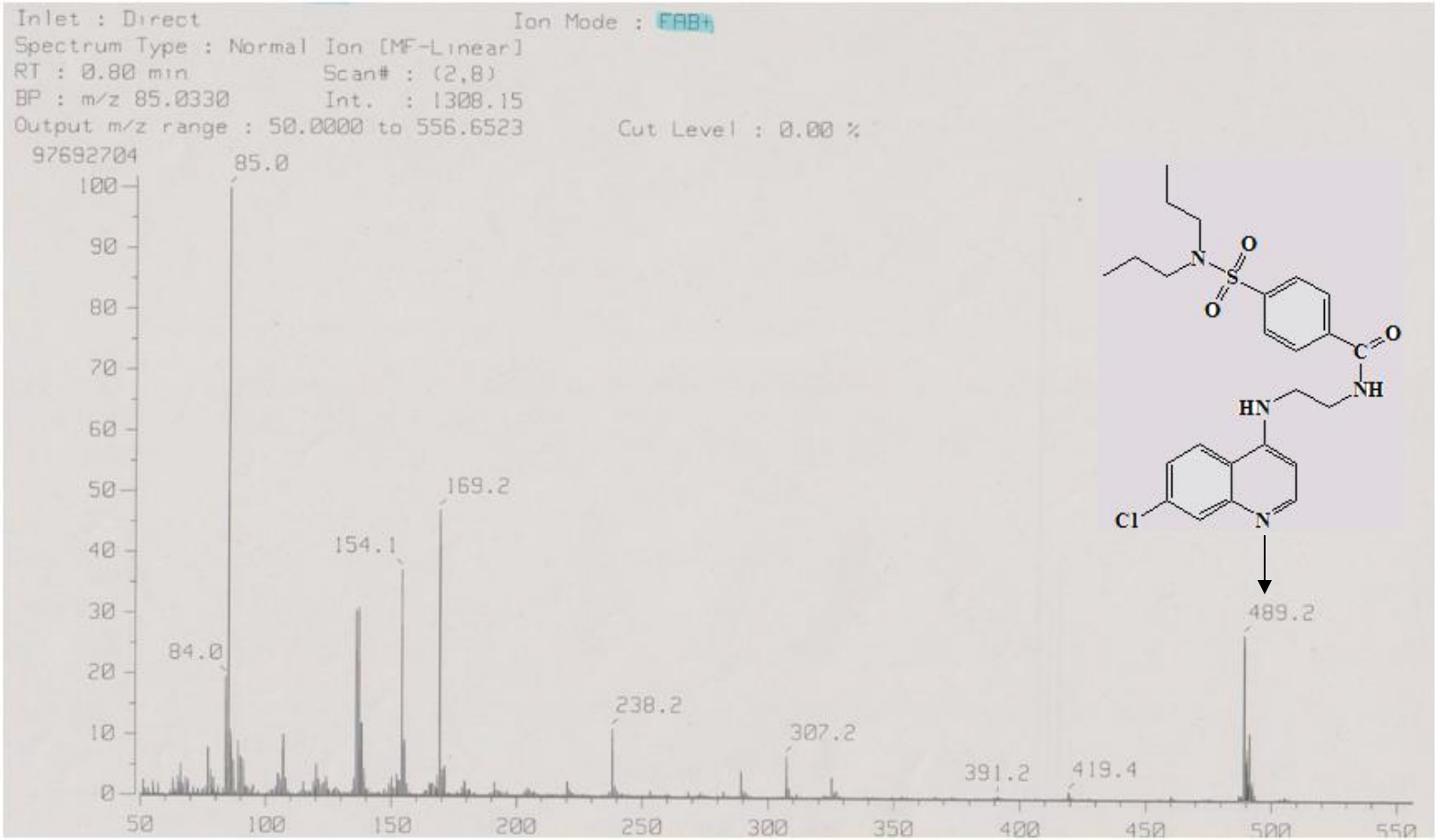
You may embark on the study.

Yours faithfully,

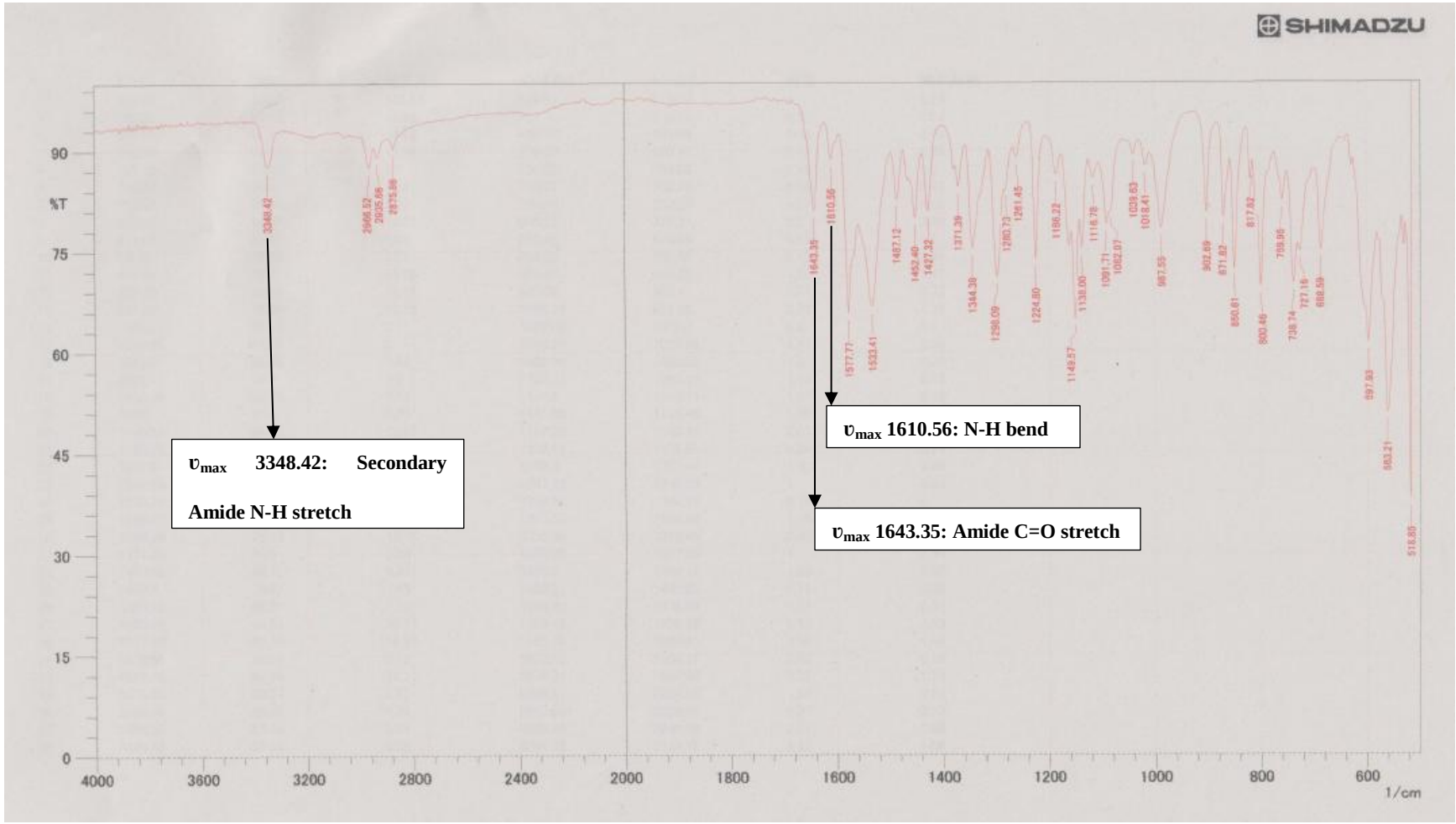
FOR:

PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

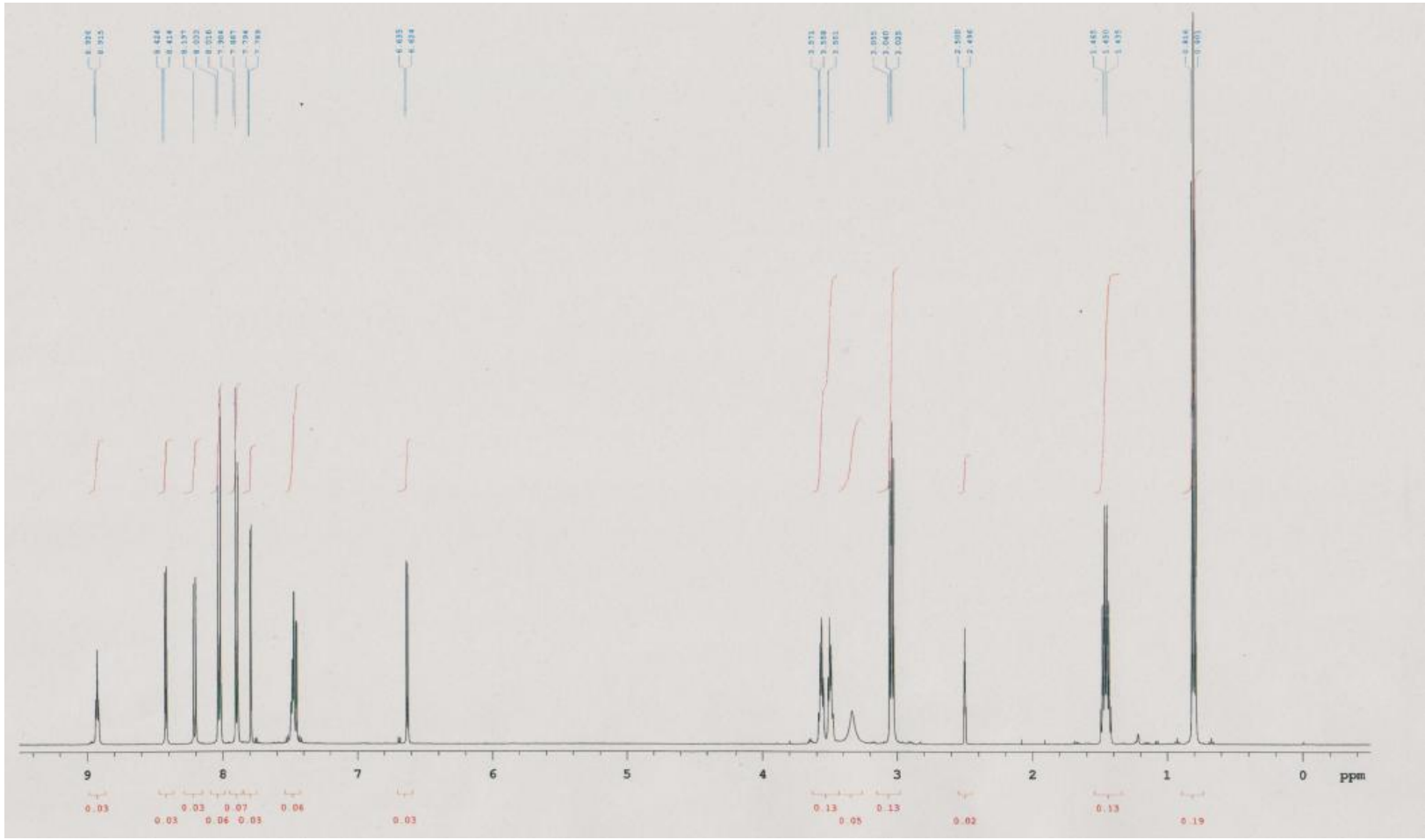
Appendix II: Mass spectrum of N-{2-[(7-chloroquinolin-4-yl)amino]ethyl}-4-(dipropylsulfamoyl)benzamide



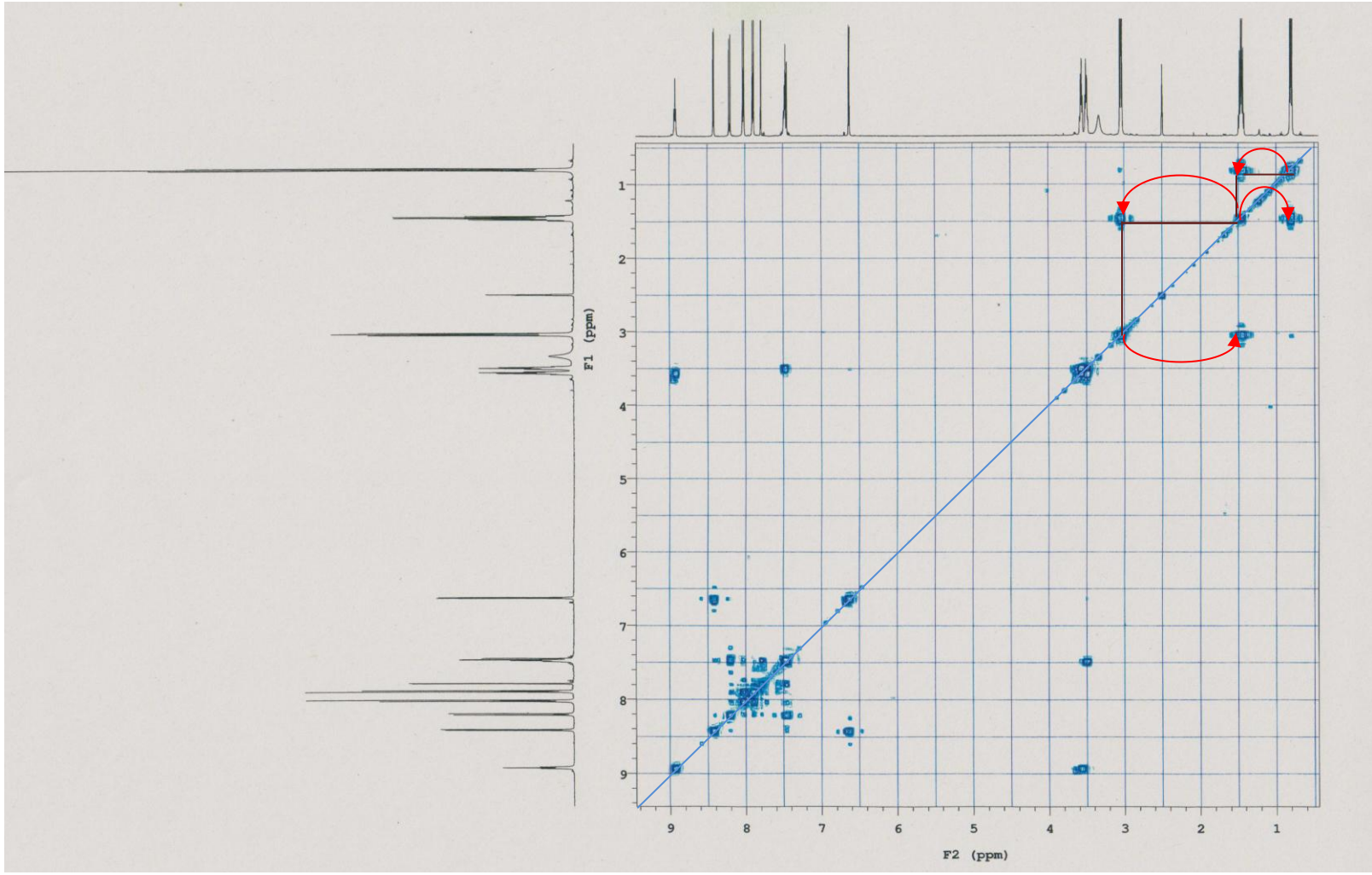
Appendix III: IR Spectrum of N-{2-[(7-chloroquinolin-4-yl)amino]ethyl}-4-(dipropylsulfamoyl)benzamide



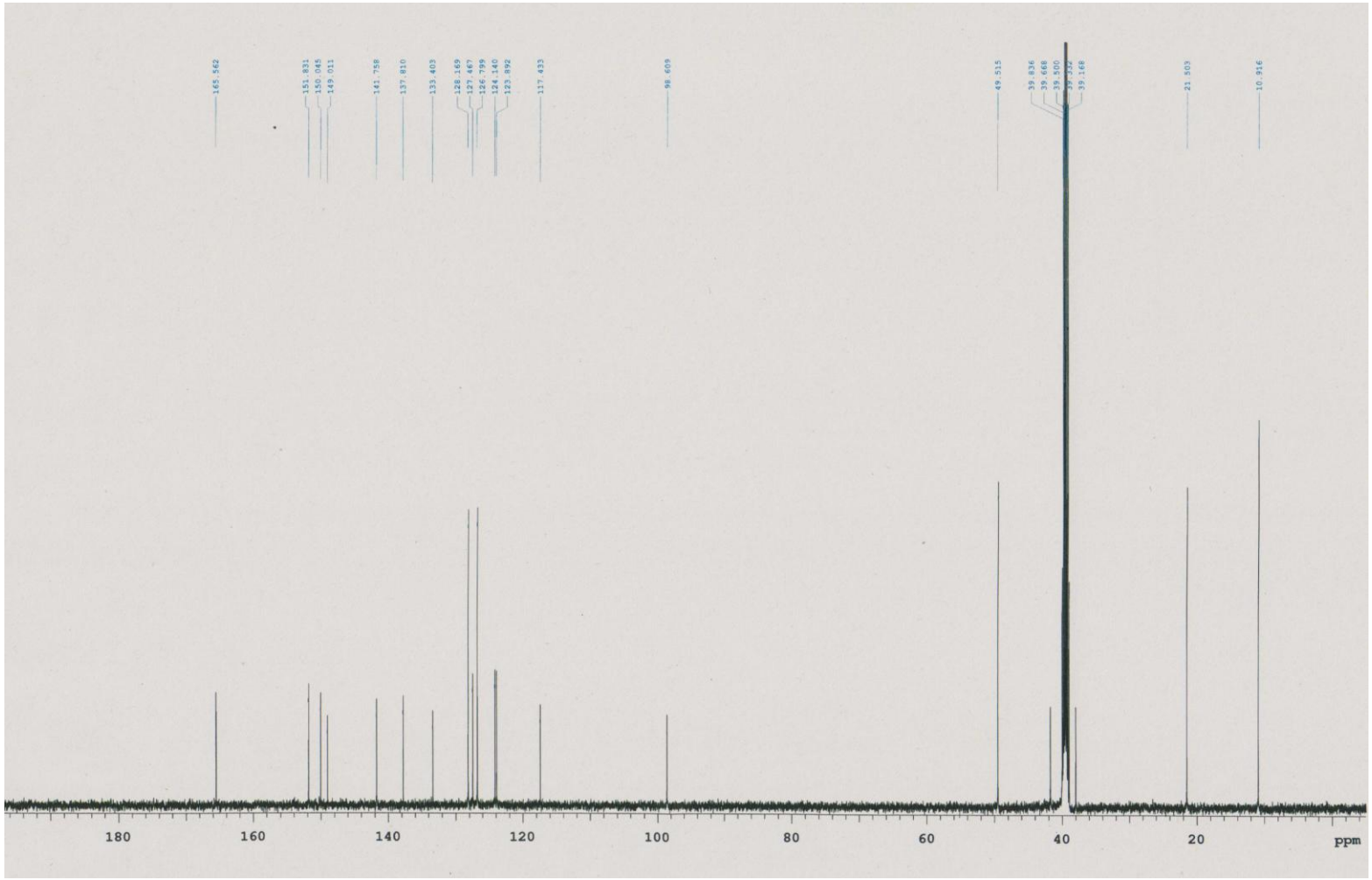
Appendix IV: ^{1}H NMR spectrum of N-{2-[(7-chloroquinolin-4-yl)amino]ethyl}-4-(dipropylsulfamoyl)benzamide



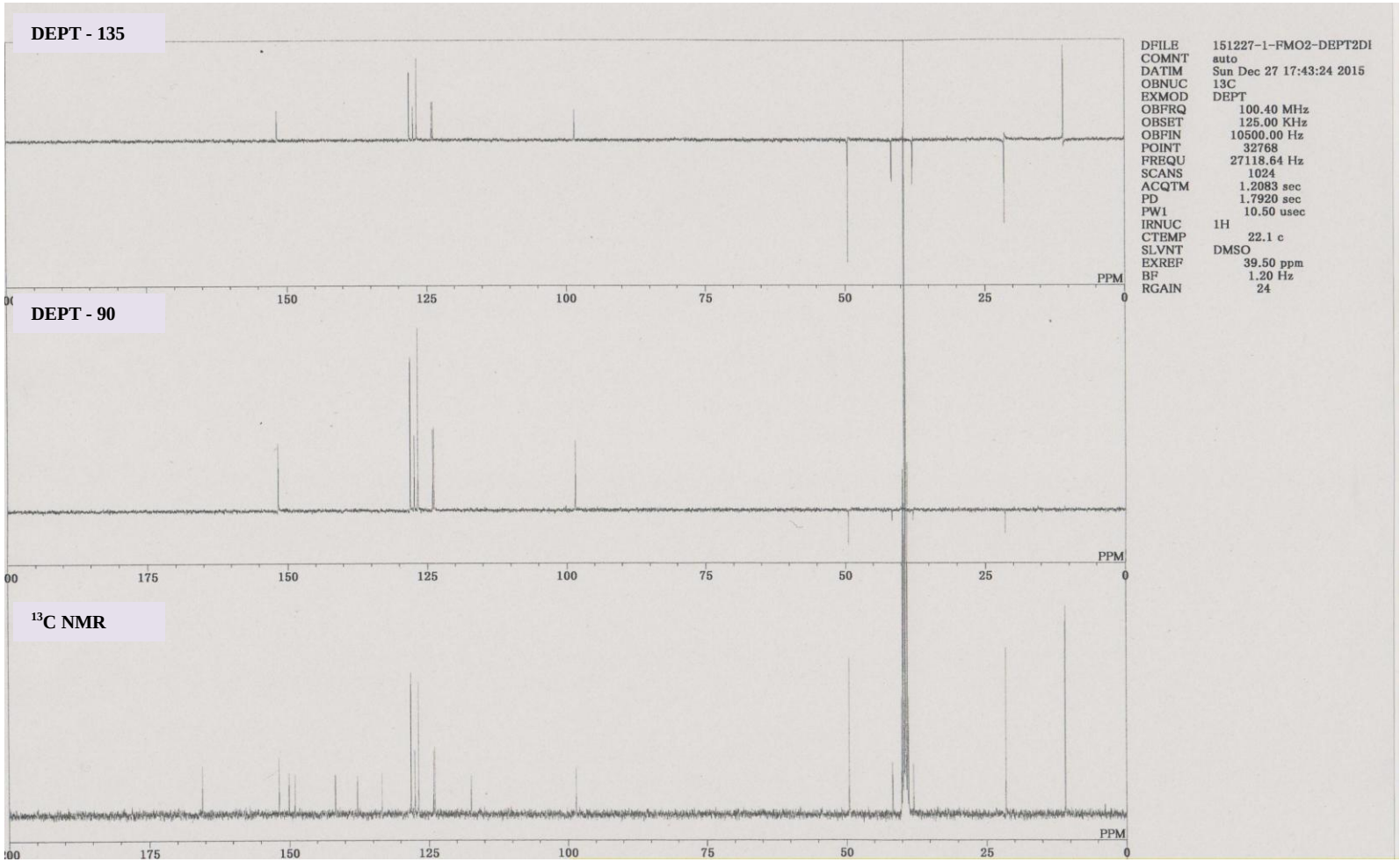
Appendix V: H-H COSY Spectrum of N-{2-[(7-chloroquinolin-4-yl)amino]ethyl}-4-(dipropylsulfamoyl)benzamide



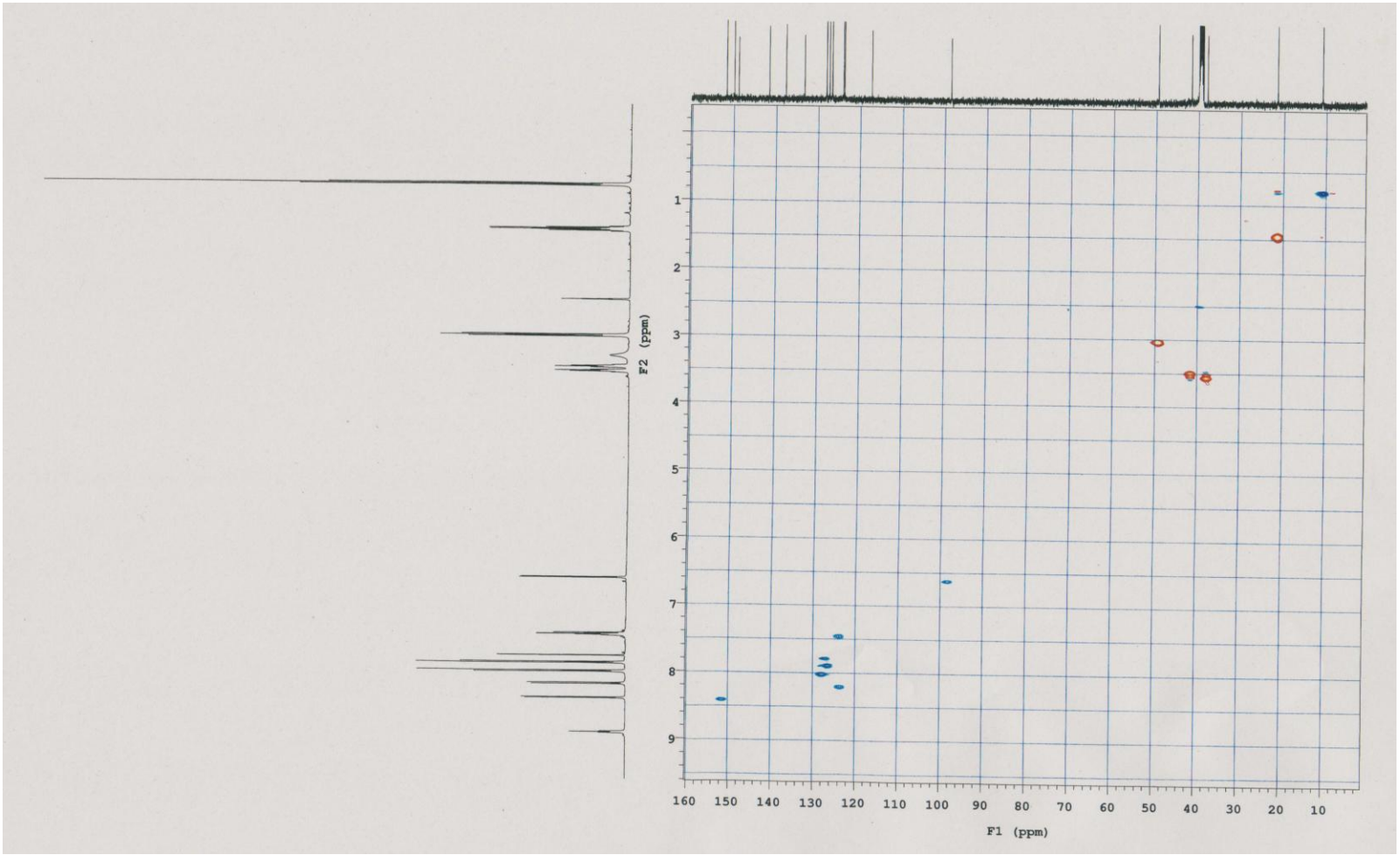
Appendix VI: ^{13}C Spectrum of N-{2-[(7-chloroquinolin-4-yl)amino]ethyl}-4-(dipropylsulfamoyl)benzamide



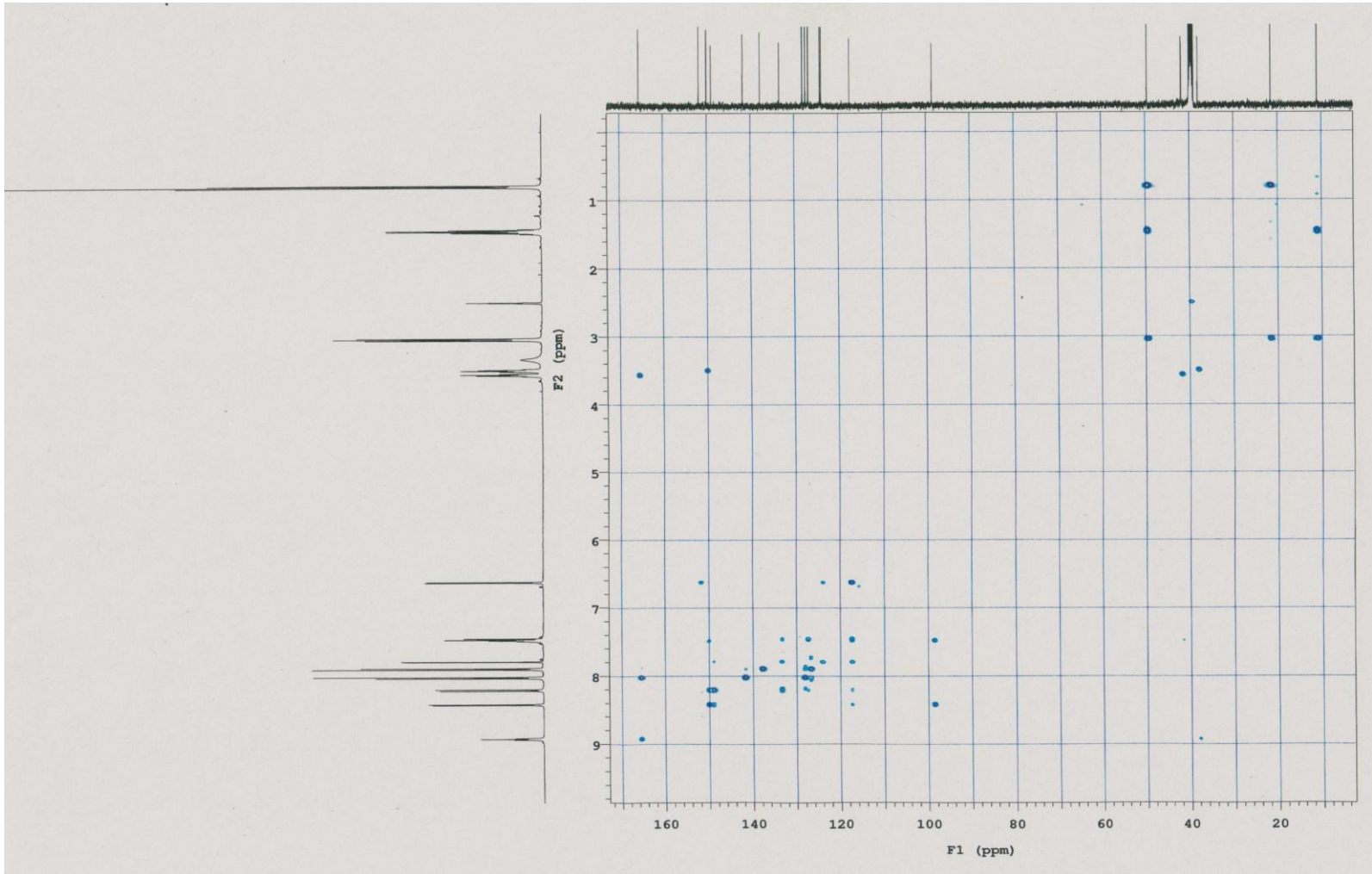
Appendix VII: DEPT 90 and 135 Spectrum of N-{2-[(7-chloroquinolin-4-yl)amino]ethyl}-4-(dipropylsulfamoyl)benzamide



Appendix VIII: HSQC Spectrum of N-{2-[(7-chloroquinolin-4-yl)amino]ethyl}-4-(dipropylsulfamoyl)benzamide



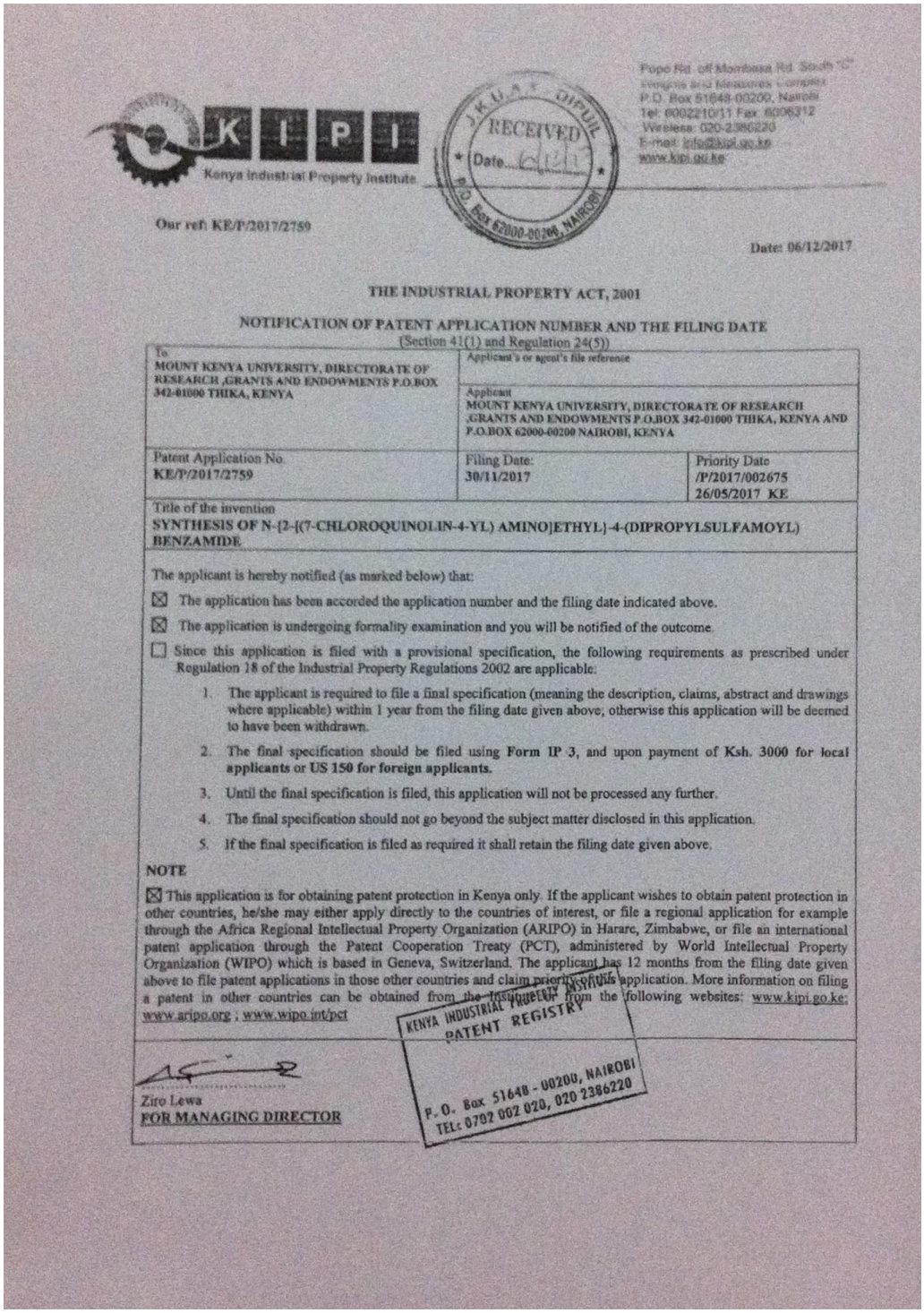
Appendix IX: HMBC Spectrum of N-{2-[(7-chloroquinolin-4-yl)amino]ethyl}-4-(dipropylsulfamoyl)benzamide



Appendix X: Journal publication

Oyatsi, F.M., Ng'ang'a, J.K., Gathirwa, J.W. and Kirira, P.G. (2017), Synthesis of Quinoline-Chemosensitizer Hybrid Molecule for the Management of *Plasmodium falciparum*: *International Journal of Engineering Development (IJEDR); ISSN: 2321-9939. pp* ***5(4)*** *1538-1542*

Appendix XI: Certificate of patent filing



Kenya Industrial Property Institute

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J.K.U.A.T. OFFICE RECEIVED Date 6/12/17 P.O. Box 62000-00200, NAIROBI

Our ref: KE/P/2017/2759

Date: 06/12/2017

THE INDUSTRIAL PROPERTY ACT, 2001

NOTIFICATION OF PATENT APPLICATION NUMBER AND THE FILING DATE

(Section 41(1) and Regulation 24(5))

To	Applicant's or agent's file reference	
MOUNT KENYA UNIVERSITY, DIRECTORATE OF RESEARCH ,GRANTS AND ENDOWMENTS P.O.BOX 342-01000 THIKA, KENYA	Applicant MOUNT KENYA UNIVERSITY, DIRECTORATE OF RESEARCH ,GRANTS AND ENDOWMENTS P.O.BOX 342-01000 THIKA, KENYA AND P.O.BOX 62000-00200 NAIROBI, KENYA	
Patent Application No. KE/P/2017/2759	Filing Date: 30/11/2017	Priority Date /P/2017/002675 26/05/2017 KE

Title of the invention

SYNTHESIS OF N-{2-[(7-CHLOROQUINOLIN-4-YL) AMINO]ETHYL}-4-(DIPROPYLSULFAMOYL) BENZAMIDE

The applicant is hereby notified (as marked below) that:

☒ The application has been accorded the application number and the filing date indicated above.

☒ The application is undergoing formality examination and you will be notified of the outcome.

☐ Since this application is filed with a provisional specification, the following requirements as prescribed under Regulation 18 of the Industrial Property Regulations 2002 are applicable:

1. The applicant is required to file a final specification (meaning the description, claims, abstract and drawings where applicable) within 1 year from the filing date given above; otherwise this application will be deemed to have been withdrawn.
2. The final specification should be filed using **Form IP 3**, and upon payment of **Ksh. 3000 for local applicants** or **US 150 for foreign applicants.**
3. Until the final specification is filed, this application will not be processed any further.
4. The final specification should not go beyond the subject matter disclosed in this application.
5. If the final specification is filed as required it shall retain the filing date given above.

NOTE

☒ This application is for obtaining patent protection in Kenya only. If the applicant wishes to obtain patent protection in other countries, he/she may either apply directly to the countries of interest, or file a regional application for example through the Africa Regional Intellectual Property Organization (ARIPO) in Harare, Zimbabwe, or file an international patent application through the Patent Cooperation Treaty (PCT), administered by World Intellectual Property Organization (WIPO) which is based in Geneva, Switzerland. The applicant has 12 months from the filing date given above to file patent applications in those other countries and claim priority of this application. More information on filing a patent in other countries can be obtained from the Institute or from the following websites: www.kipi.go.ke; www.aripo.org ; www.wipo.int/pct

KENYA INDUSTRIAL PROPERTY INSTITUTE PATENT REGISTRY

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Ziro Lewa
FOR MANAGING DIRECTOR