

MECHANISM OF ANTIMALARIAL ACTION OF DESFERRIOXAMINE B

A Thesis Presented to the Board of Graduate Studies of the University of Ghana, Legon, in Partial Fulfillment of the Requirement for the Degree of Master Of Philosophy (M.Phil.) in Biochemistry

By



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DECLARATION

I declare that except for references to other people's work, which have been duly acknowledged, this study is a result of my own research. I certify in addition that this thesis, either in whole or in part has not been previously presented for any degree and is not being concurrently submitted in candidature for any other degree.



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**Dedicated to my family,
Auntie,
Doris, Jeff, Suzie,
Sue
and
Aunt Emelia.**

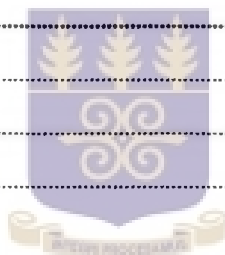


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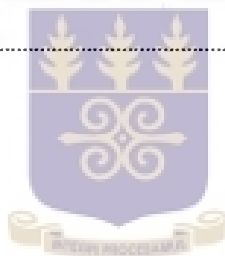
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LIST OF ABBREVIATIONS.

Abs	absolute value
CHQ	chloroquine
CRQ	<i>Cassia siamea</i>
DF	desferrioxamine B
Grp	group
IR	infra red
[x]	absolute value of x.
Val	value
PBS	phosphate-buffered saline
BHA	benzohydroxamic acid.
RA	rhodotorulic acid
AHA	aceto hydroxamic acid
T	time
Hrs	hours



ABSTRACT.

Desferrioxamine B was shown to exhibit potent antimalaria activity. A single bolus injection of 100mg of desferrioxamine B per kilogram body weight of mice reduced *Plasmodium berghei anka* parasitemia in CBA mice for 24 hours. Peripheral blood samples of desferrioxamine B-treated mice taken at 0, 3, 6, 12, 24 and 48 hours after drug administration show gross adverse ultrastructural changes in all developmental stages of the malaria parasite (trophozoites, schizonts and gametocytes) and a reduction in parasite numbers. The iron chelator was also found to function as an inhibitor of heme polymerisation by binding to and immobilising the central ferric ion of parasite-associated heme and hence disrupting heme detoxification into hemozoin.

It was concluded that desferrioxamine B is active against *Plasmodium berghei anka* and that iron chelation provides a new possible alternate strategy for the treatment of malaria.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW.

INTRODUCTION.

Malaria is unquestionably an important protozoan disease with a world wide high level of mortality and morbidity. It has been estimated that about two billion people live in areas exposed to malaria and 300 million individuals are infected every year (WHO, 1997). The transmission of malaria has increased in recent years in most countries and has re-emerged in countries where it was once eradicated or controlled (McNeely *et al*, 1998; Bruce-Chwatt, 1980). In Africa, south of the Sahara alone, over one million children die annually as a result of the disease (Kondrachine, 1997). Children, pregnant women and the immunologically naive are the most vulnerable making the disease a major public health problem, which must be addressed.



Malaria chemotherapy has come a long way since 1820 when quinine was first isolated from bark of the cinchona tree. In the eighteenth and nineteenth centuries, high and uncontrolled exploitation led to reduced natural and commercial plantation sources of the bark. There was also a threat to supplies of this useful drug during the First World War. These factors together with the high toxicity of quinine, led to intensive efforts to find synthetic replacements. This was accomplished in 1824 with the discovery of Pamaquine and later Mepacrine. Unfortunately, these drugs have also been found to be toxic and furthermore large doses are required to be effective. Therefore, they did not meet the requirements of being less toxic, fast-acting, and effective at lower concentrations.

The new class of antimalarials following these satisfied these conditions. These include the dihydrofolate reductase inhibitors, proguanil and pyrimethamine, and new quinoline containing drugs such as primaquine and chloroquine. Following its release, chloroquine became the drug of choice for the treatment of malaria and together with proguanil and pyrimethamine became the major drugs for prophylaxis. Primaquine was used solely for radical cure of infections with relapsing malarias caused by *P. malariae*, *P. ovale* and *P. vivax* (Wernsdorfer, 1981).

This happy state of affairs was not to last long with the emergence and rapid spread of parasite strains resistant to proguanil and pyrimethamine (Peterson *et al*, 1990; Wernsdorfer, 1981). This was followed shortly by the appearance of chloroquine resistant parasite species in East Africa (Bruce-Chwatt, 1980), South East Asia and Latin America (WHO, 1984; Nguyen-Dinh, 1978), gradually spreading globally. Strains resistant to combinations of these drugs have also been identified in East Asia and Africa (Desjardines *et al*, 1979; Peterson *et al*, 1990).

The appearance and rapid spread of these resistant species has resulted in a global increase in the incidence and transmission of malaria. This worldwide resurgence of malaria underscore the urgent need for new and more effective therapeutic agents.

It is therefore gratifying to note that siderophores such as Desferrioxamine B, have been found to have anti-malaria activity (Gordeuk *et al*, 1993; Hershko *et al*, 1992; Scott *e: al*, 1990) and may be of clinical significance in the treatment of malaria (Scheibel and Adler, 1980).

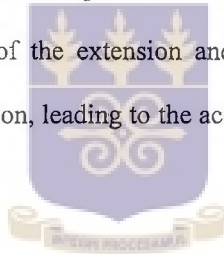
Siderophores are low molecular weight iron chelating substances, that are excreted by aerobic fungi and bacteria in iron limiting environments (Hershko *et al*, 1992; Raymond *et al*, 1984; Cotton *et al*, 1980). Iron is required in virtually all living organisms, for a wide variety of metabolic processes such as DNA replication and cell division (Weinberg, 1974). However, ferric iron at physiological pH is insoluble due to the formation of ferric hydroxide. Microorganisms, in a bid to acquire this critically important element, have evolved Siderophores. They are produced by these organisms and excreted into the external environment to solubilise and assimilate iron. Iron chelators have a high and low affinity for ferric and ferrous iron respectively. They also have a low affinity for other metal ions. This selective affinity for ferric iron may have an important role in the use of iron chelators as antimalarials.



Several different iron chelators have been identified (Raymond *et al*, 1984; Neilands and Ratledge, 1980). However, the iron-chelating drug Desferrioxamine B is the only iron chelator presently available for clinical use. It has been used for over three decades as an essential drug for the management of transfusional iron overload (Hershko, 1992) and also for the removal of iron from the body upon acute iron poisoning (Raymond *et al*, 1984; Anderson, 1981). However, its potential as an antimalaria agent has just begun to emerge. Murray *et al*, (1980) noted that in areas of hyperendemic *P. falciparum* infections, patients with clinical iron deficiency exhibited an attenuated incidence and severity of the disease. When such patients were fed an iron-replete diet, many exhibited sudden recrudescence of previously smouldering malaria infections. Laboratory observations also showed that several iron-binding substances including desferrioxamine

B, desferrithiocin, lactoferrin and desferricrocin block the replication of *P. falciparum* and other malaria parasite strains *in vitro* (Hepner *et al*, 1988).

As a potential drug in the treatment of malaria, the mechanism of action of siderophores, including desferrioxamine B, at the cellular level is unclear (Scott *et al*, 1990). They have been reported to function by chelating iron from a “pool” in the parasites’ compartment, rather than the chelation of intra- or extra-erythrocytic iron (Scott *et al*, 1990). However, the nature of the chelatable iron is not clear (Scott *et al*, 1990; Hepner *et al*, 1988). It has been suggested however that, the iron in this pool could be from hemoglobin (Scott *et al*, 1990). This suggests that iron chelators may work by binding to the iron atom in hemoglobin or by binding to the iron atom in hemozoin. The net result in either case is the prevention of the extension and formation of hemozoin thereby interfering with heme polymerisation, leading to the accumulation of heme which is toxic to the parasite.



JUSTIFICATION.

This study involves the use of murine models to determine the mode of action of desferrioxamine B in treatment and prophylaxis of malaria. A detailed understanding of the mechanism of action of siderophore mediated antimalaria activity would facilitate the design of new drugs for better management of malaria.

MAIN OBJECTIVES OF STUDY.

- a) To investigate the effect of desferrioxamine B on *Plasmodium berghei anka* growth in CBA mice.
- b) To determine whether the anti-malarial properties of desferrioxamine B is due to its inhibition of heme polymerisation.
- c) To determine whether this inhibition is as a result of binding of desferrioxamine B to the heme iron present in hematin and β -hematin.
- d) To determine any structural changes in the parasite as observed under light microscopy.



LITERATURE REVIEW

MALARIA: THE CAUSATIVE ORGANISM.

Malaria is caused by the intracellular microorganism belonging to the phylum Protozoa, subphylum Sporozoa, class Telesporida and of subclass Coccidiorhiza. These parasites are of the family Plasmodiidae and genus Plasmodium (Wernsdorfer, 1980)

There are over one hundred species of malaria parasites infecting a wide spectrum of vertebrate host species among which birds and mammals are the most widely infected. Malaria parasites vary greatly in host specificity, pattern of growth and pathogenicity. For example, *P. rodhaini* is specific for apes and gorillas, *P. berghei*, *P. yoelii* and *P. vinckei* are found to infect rodents. The owl monkey can be infected by human malaria parasites.

Human malaria.

Four species of malaria parasites are recognised as responsible for causing the disease in man. These are *P. malariae* which causes mild quartan malaria with a schizogonic periodicity of 72 hours, *P. vivax* causes benign tertian malaria of 48 hour periodicity, *P. ovale*; causes tertian malaria also of 48 hour periodicity and *P. falciparum* causing malignant tertian malaria also with a periodicity of 48 hours. *P. falciparum* causes the most fatal of all human malaria which is a supreme killer in endemic regions.

Distribution.

P. vivax is the most widely distributed species. It is naturally prevalent in many temperate as well as tropical and sub-tropical zones. *P. falciparum* is the commonest in the tropics and sub-tropics although it may occur in some areas with temperate climate. *P. malariae* is patchily present over the same range as *P. falciparum* but much less common. *P. ovale* is found chiefly in tropical Africa, but also occasionally in the West Pacific (Bruce-Chwatt, 1980).

MODE OF TRANSMISSION.

Natural transmission of human malaria occurs through exposure to the bites of infective female Anopheles mosquitoes. The source of infection is nearly always a human subject whether a sick person or a symptomless carrier, although transmission of *P. malariae* from infected chimpanzees to humans are known to occur. Accidental transmission, although infrequent, may occur as a result of blood transfusion when a donor harbours the parasite. Drug addicts may also transmit malaria by sharing used needles. Finally, congenital infection of the newborn from an infected mother is also known to occur, but this mode of transmission is rare.

THE ARTHROPOD HOST.

Only the female anopheles mosquito can transmit human malaria. This is because males of the species feed exclusively on nectar and fruit juices while the female feeds primarily on blood (Stone *et al*, 1985). Of the 400 species of anopheles identified, only about 60 are important as vectors of malaria under natural conditions. Factors that determine

whether a particular species of Anopheles is an important vector of malaria in a given locality include its preference of feeding on man to feeding on other vertebrates, the mean longevity of the local population of that species and its density in the area. Consequently, these vectors of malaria have definite distribution over large areas and an important vector in one region may be less important in another region (Wernsdorfer, 1980). For example in Africa, the main vectors are *A. gambiae*, *A. funestus* and *A. rufipes* whilst in southern Mexico, the Caribbean islands and fringes of the South American coast, the most important vectors include *A. darlingi*, *A. aquasalis*, and *A. Cruzi* (Ahmed, 1989).

LIFE CYCLE OF THE MALARIA PARASITE.

Malaria parasites employ a complex life cycle, alternating between an arthropod secondary host and a vertebrate primary host (Figure 1-1). This cycle, consists of an endogenous asexual cycle that occurs in man and an exogenous sexual cycle that takes place in female anopheles mosquitoes.

Asexual cycle.

With the bite of an infected female mosquito, sporozoites are injected and reach the blood stream. Within an hour they disappear from the blood, most of them having been destroyed by the immune system. The remaining sporozoites enter the parenchyma cells of the liver, where, during the next 10-14 days they undergo a pre-erythrocytic stage of development and multiplication (schizogony) to form merozoites. At the end of this stage, the liver cells rupture and the merozoites are released into the blood. These infect the red blood cells and form motile intracellular parasites named trophozoites.

Development and multiplication of the plasmodia within these cells constitute the erythrocytic stage.

Following mitotic replication of its nucleus, the trophozoites in an erythrocyte develop through schizogony to form schizonts, which grow and mature into merozoites. The merozoites grow and multiply until the red cell rupture, releasing the contents of the cell including the merozoites into the blood. The rupture of the red cell and the release of red cell contents into the blood is associated with chills, vomiting and hot and cold spells which are the symptoms of malaria.

Sexual cycle.

Some merozoites on entering the red cell differentiate into male and female forms called gametocytes, which can only complete the life cycle when taken up by a female mosquito.

The cycle in the mosquito involves fertilisation of the female gametocyte by the male gametocyte with the formation of a zygote, which develops into an oocyst (sporocysts).

A further stage of division and multiplication occurs leading to rupture of the sporocysts with release of sporozoites, which then migrate to the mosquito salivary glands and enter another human host with the mosquito's bite.

In certain forms of malaria (*P. vivax* and *P. ovale*) infections, some sporozoites on entering the liver cells develop into hypnozoites, which are resting forms of the parasite.

These can be reactivated to continue an exo-erythrocytic cycle of multiplication leading to another bout of infection even in the absence of introduction of fresh parasites. Relapses of malaria are likely to occur for these forms that have the dormant hypnozoites since they can emerge after an interval of several weeks or months to restart the infection.

Synthesis of pyrimidines.

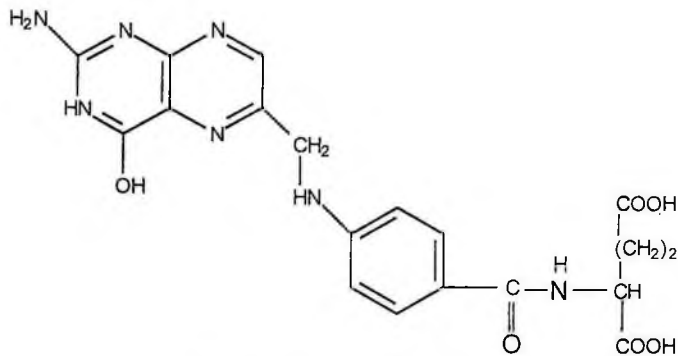
Exogenous pyrimidines, in contrast, cannot be taken up by most species (Conklin *et al*, 1973). Even if taken up, the parasite cannot use most of the cytosine, uracil and thymidine bases present in the host system due to a lack of the presence of the appropriate kinases. For example, *P. chabaudi* lacks thymidine kinase and hence cannot phosphorylate and use pre-existing thymidine (Walter *et al*, 1974). The parasite therefore relies on an obligatory *de novo* synthesis of pyrimidines (Elford *et al*, 1995).

Mammals in contrast, are prototrophic and able to synthesise both purine and pyrimidine nucleotides *de novo* as well as incorporate pre-formed bases when available. Their survival therefore, is not dependent upon the absorption of these compounds.

Folate synthesis.

During the *de novo* synthesis of thymidine in the parasite, N⁵, N¹⁰ methylene-tetrahydrofolate is required to transfer one carbon (methyl) groups to deoxyuridylate to form thymidylate, a necessary precursor to thymidine formation. However, the malaria parasite can only use a negligible amount of pre-formed folate and hence it relies on obligatory *de novo* synthesis of dihydrofolate from pteridine, para-aminobenzoate (PABA) and glutamate. This process requires the presence of the enzyme dihydropteroate synthase which catalyses the formation of dihydropteroic acid from PABA and pteridine.

In contrast to the parasite, humans cannot synthesise folate by this process and must obtain it as a vitamin in the diet (Martins *et al*, 1987). This important difference in folate syntheses between host and parasite, present a point for antimalarials to exert their effect.



Dihydrofolic Acid

Figure 1-2: Structure of Dihydrofolic Acid.

In order for folate to serve as a carrier, dihydrofolate (a reduced form of dihydrofolic acid, Figure 1-2) must be reduced to tetrahydrofolate by the enzyme dihydrofolate reductase (DHFR) which uses NADPH as a hydride donor. This enzyme is a small monomeric protein containing no disulphide bonds and present in both host and parasite cells in different isozymes. The presence of these different isozymes present an opportunity for the use of antimalarials that would selectively inhibit parasite DHFR enzymes. This is because the different isozymes of this enzyme have varying degrees of susceptibility to various (antimalarial) drugs. The parasite enzymes are more susceptible than human enzymes (Zubay, 1983).

Breakdown of hemoglobin and metabolism of amino acids.

The malaria parasite, like all other living cells, require proteins for growth, development and repair. This is particularly true for the intra-erythrocytic stages where the highest amount of growth and development occurs. However, the parasite has a limited capacity to synthesise all the amino acids it requires *de novo* (Goldberg *et al*, 1990).

It is worthy of note that the parasite matures within the red blood cell in which hemoglobin is the single most important cytosolic protein. It can therefore, obtain those amino acids it cannot synthesise either by digestion of red cell hemoglobin or from free amino acids occurring in host plasma (Homewood and Neare, 1980; Goldberg *et al*, 1990; Chou and Fitch, 1992).

Part of the parasite's requirement of amino acids, especially methionine, isoleucine (which is absent in hemoglobin), and glutamine is satisfied exogenously from host plasma (Goldberg *et al*, 1990; Elford *et al*, 1995) and to facilitate this, parasitised red cells are made more permeable to these and other amino acids so that much higher amounts would enter by diffusion or by active transport using membrane bound carriers (Sherman and Tanigoshi, 1971).

In the actively growing trophozoite, the parasite greedily ingests and degrades most of the host erythrocytic hemoglobin. This catabolic process is achieved through an efficient, ordered degradative pathway that takes place in the food vacuoles. Ingestion of host hemoglobin is done by means of the cytostome, which is a specialised structure, developed across the double membrane between the outer membrane of the parasite and the inner membrane of the host's red cell. Here, hemoglobin-containing vesicles are pinched off the cytostome and travel to the digestive vacuoles where proteolytic enzymes break it down to the constituent amino acids, peptides, heme containing compounds and free heme (Goldberg *et al*, 1990; Goldberg, 1992; Slater *et al*, 1991).

The free heme produced is potentially toxic to biological membranes (Goldberg *et al*, 1990; Matiney *et al*, 1996; Basilico *et al*, 1998). It has the ability to catalyse formation of oxygen-derived free radical species that can interact with cellular membranes and cytoplasmic constituents. It also accumulates in normal and thalassemic erythrocytes as membrane-bound hemichromes. This behaviour of free heme may lead to a modification of the functional integrity of the cells and their membranes and also damage various other cellular constituents such as enzymes especially, those involved in DNA synthesis and repair (Rice-Evans and Baysal, 1987). Therefore in all living cells, free heme must be removed or detoxified immediately it is formed.

In the mammalian host, toxic heme produced during the normal hemoglobin break down is detoxified by heme oxygenase, an enzyme which catalyses the breakdown of free heme to biliverdin leading ultimately to the re-use of heme iron for synthesis of new hemoglobin or for storage in ferritin (Zubay, 1983).

In contrast to the host, a parasite specific heme polymerisation activity occurring in the food vacuole detoxifies the free heme by cross-linking the heme monomers to form hemozoin or malaria pigment. This process is catalysed by a novel heme polymerase enzyme found to occur exclusively in the parasite (Slater and Cerami, 1992; Chou and Fitch, 1992).

Hemozoin is an insoluble crystalline material consisting mainly of 65% protein and peptides, 16% ferriprotoporphyrin-IX also called hemozoin, 6% carbohydrate and trace

amounts of lipid and nucleic acids. The overwhelming majority of the protein component is a mixture of native and denatured host globin, non-covalently associated with the metalloporphyrin. The carbohydrate groups also seem to come from non-enzymatic glycosylation of hemoglobin (Goldie *et al*, 1990). The most important component seems to be polymerised hematin.

The structure of hemozoin (figure 1-3) comprises a polymer of ferriprotoporphyrin-IX (heme) molecules linked between the central ferric ion of one heme and a carboxylate side-group oxygen of another (Slater and Cerami, 1992).

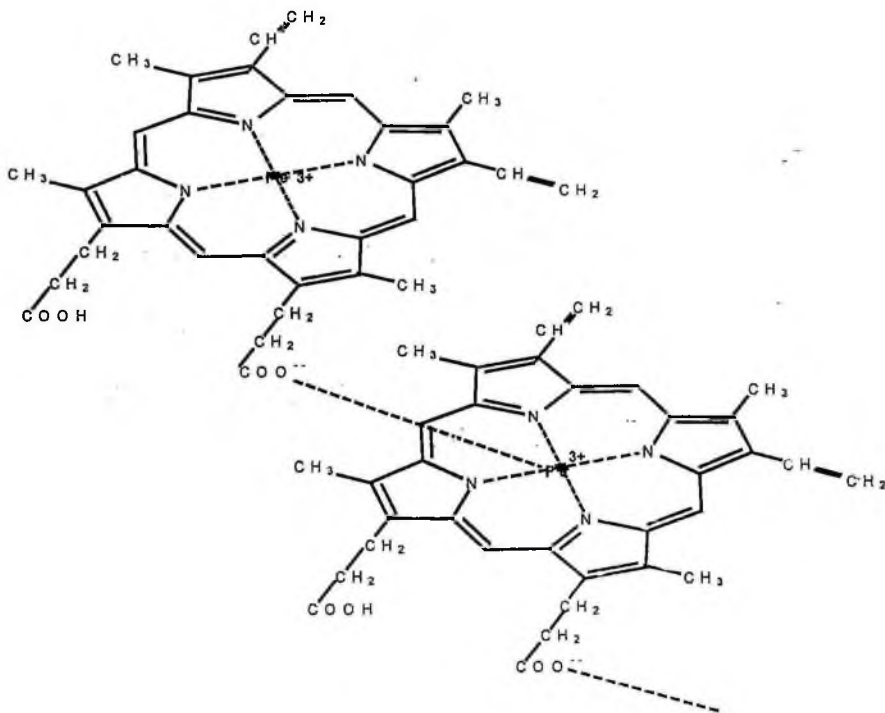


Figure 1-3: Structure of Hemozoin.

This unique heme polymerising activity and differences in nucleic acid synthesis between the parasite and the human host, present researchers with useful points at which to attack and kill the malaria parasite.

DIAGNOSIS

The presence of malaria infection is empirically established on finding parasites in blood films. However, semi-immune individuals living in endemic regions may have pre-patent concentrations of parasites without showing the disease. The presence of malaria is confirmed when a Giemsa stained thin blood smear obtained from a finger prick or the ear lobe of a subject and observed under oil immersion in a light microscope shows the presence of parasitised erythrocytes that present themselves as blue dots. Thick Giemsa stained films and serological tests may also be used in diagnosis.

TREATMENT.

Presently, malaria is treated mostly by chemotherapy. Chemical agents are used to eliminate both blood and tissue forms of malaria causing plasmodia once their presence have been established. The most widely used drugs include chloroquine, amodiaquine and quinine. Most of these chemotherapeutic agents are aimed at eliminating erythrocytic forms of the disease since these forms present the easiest targets.

BIOCLASSIFICATION OF ANTIMALARIAL AGENTS.

The different stages in the life cycle of malaria parasites show different susceptibilities to the different antimalarial agents presently available. On this basis, antimalaria

compounds can be classified generally as schizontocidal, gametocidal and sporontocidal depending upon the stage of the parasite most affected by the drug.

Schizontocidal drugs attack malaria parasites in the red blood cell, preventing or terminating the clinical attack. Examples include chloroquine, amodiaquine and quinine. The majority of antimalaria drugs are schizontocidal and are used in clinical treatment of the disease.

Tissue schizontocidal drugs act on all the exo-erythrocytic forms, especially the pre-erythrocytic forms in the liver, thereby, preventing invasion of the red cell. Drugs in this category include proguanil and pyrimethamine. They are used mainly in prophylaxis but can also be used to effect radical cure of relapsing malaria.

Gametocidal drugs destroy all sexual forms of the parasite. Some of these drugs have a pronounced anti relapse effect and are extensively used for radical cure of malaria. Primaquine is one such drug.

Sporontocidal drugs prevent or inhibit formation of oocysts and sporozoites in the stomach wall of a gamete-carrying mosquito. Sporontocidally active drugs include proguanil and primaquine.

CLASSIFICATION OF ANTIMALARIALS BASED ON THEIR CHEMICAL STRUCTURE.

Generally, antimalarials can be divided into groups based on their chemical structure and reactive groups in relation to their mode of action and biological activity (Bruce-Chwat, 1980). This system, classifies antimalarials as 4-aminoquinolines, 8-aminoquinolines, aminoacridines, cinchona alkaloids, biguanides, diaminopyrimidines, sulphonamides, sesquiterpene lactones, phenanthrene methanols and other compounds such as iron chelators (mainly desferrioxamine B) and herbal drugs.

4-Aminoquinolines such as chloroquine, amodiaquine and mefloquine (figure 1-4) have a quinoline ring with an active side chain, which is a substituted anilino group. This side chain is linked to the carbon atom at the 4th position of the quinoline ring.

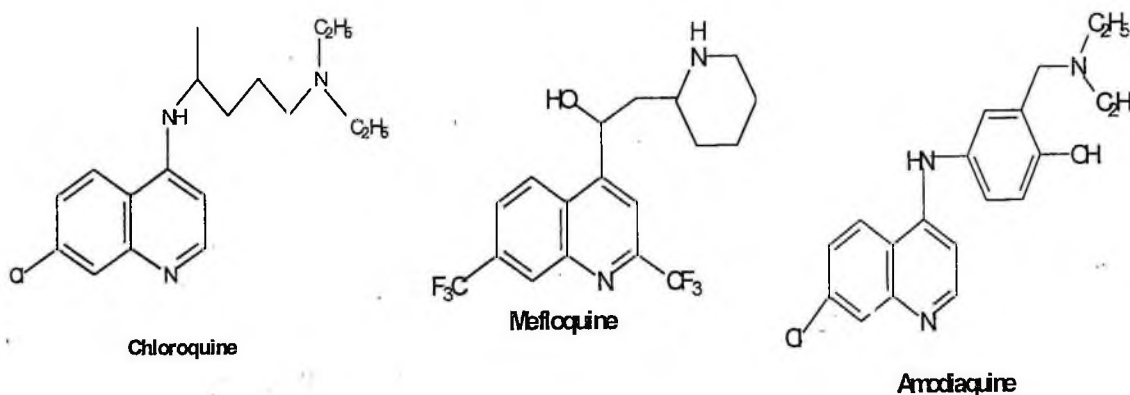
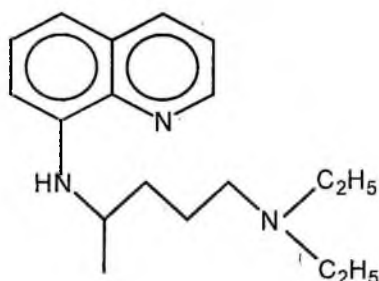


Figure 1-4: Structures of Some 4-aminoquinolines.

These drugs, are potent and rapid blood schizontocides, effective against all species of malaria parasites.

8-Aminoquinolines: The most important member of this group is primaquine (figure 5). Others include pamaquine and quinocide. The other drugs in this category are too toxic to the host for routine antimalarial use although they are active as blood schizontocides (Bruce-Chwat, 1980). They have an alkylamino chain attached to the carbon at position 8 in the quinoline ring.



Pamaquine

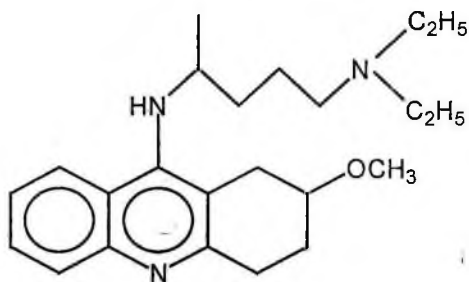
Figure 1-5: An 8-Aminoquinoline

Compounds of this group have an additional ability to inhibit parasite's mitochondrial respiration and this is thought to form one facet of their antimalaria action (Bitonti *et al*, 1988)

Primaquine and the other closely related compounds prevent relapses and hence could be used to effect radical cure since they destroy hypnozoites that persist in the liver. They are also effective against gametocytes but not against forms of the parasite in circulating blood. Because they are poor blood schizontocides, 8-aminoquinolines are used mainly in prophylaxis and in combination with other antimalaria drugs for treatment.



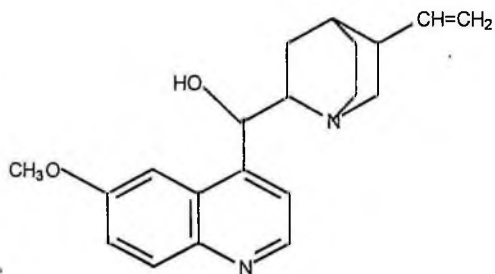
9-Aminoacridines: e.g. mepacrine (figure 1-6). These have the acridine ring. Mepacrine is not for routine use and considered an obsolete drug because of its high toxicity (Bruce-Chwatt, 1986).



Mepacrine

Figure 1-6: An example of 9-aminoacridines

The cinchona alkaloids: The four most important alkaloids from the cinchona bark are quinine (figure 1-7), quinidine, cinchonine and cinchonidine. Quinine and quinidine are the most important as far as antimalaria activity is concerned. It has a complex structure involving the basic quinoline ring.

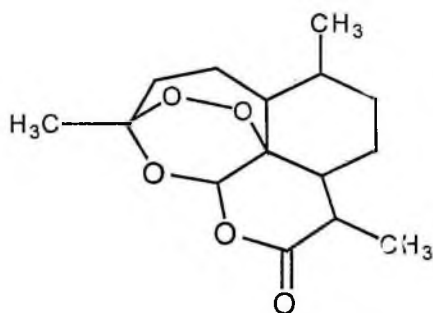


Quinine

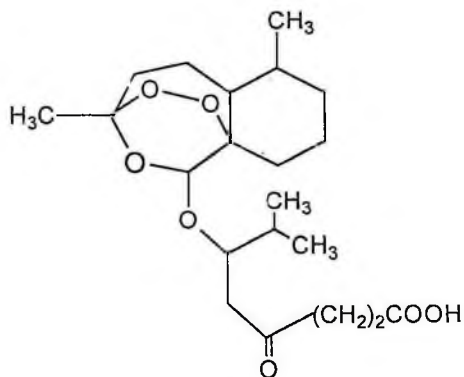
Figure 1-7: A Cinchona alkaloid.

It is highly active against blood schizonts and hence effective for clinical use as an emergency antimalarial. It is also effective against chloroquine resistant parasites.

Sesquiterpene lactones (figure 1-8): Artemisinin is the parent of this new group. The important derivatives include artemether, arteether and artesunate. These antimalarial drugs were isolated from the herb *Artemisia annua* (The Quingaosu Antimalarial Coordinating Research Group, 1979). They have a complex structure with an endoperoxide group which is essential for their antimalarial activity, destruction of which leads to a loss of activity (Meshnick, 1994). They have a broad window antimalaria effect, being potent against all forms of the parasite from rings to mature trophozoites. They are also known to be potent blood schizontocides, effective even against multiple drug resistant species of *P. falciparum* (Cook, 1996).



Artemisinin



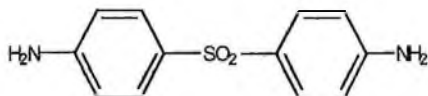
Artesunate

Figure 1-8: Structures of some sesquiterpenes.

The presence of the trioxane structure suggests that it is a potent oxidising agent (Hamburger and Hostettman, 1991). It had been observed that oxidising agents with this structure adversely affect parasite membranes by the generation of free radicals. The artemisinin-derived free radicals then form covalent bonds with parasite membrane structures leading to alkylation and destruction of these membranes (Cook, 1996).

Sulphones and sulphonamides: Antimicrobial compounds such as sulphones (figure 1-9) and sulphonamides (figure 1-10) interfere with coenzyme (folate) metabolism. This occurs at the dihydropteroate synthase reaction. These compounds could also be classified as antimalarials since they inhibit the growth and reproduction of malaria parasites.

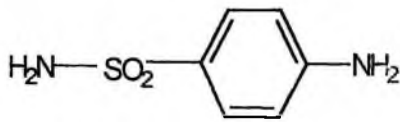
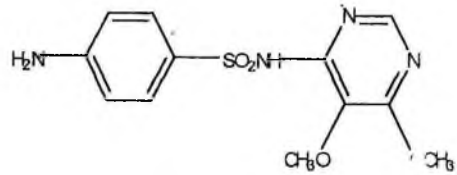
Sulphones are represented by the diamino-diphenyl sulphone (dapsone).



Diphenyl sulphone (Dapsone)

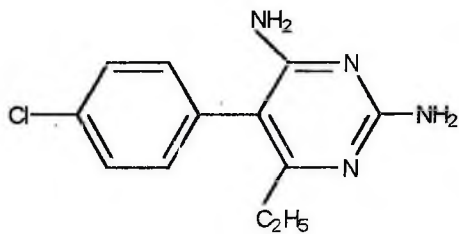
Figure 1-9: A Sulphone.

The base of all the sulphonamides is sulphanilamide. There are a large number of derivatives of sulphonamides of which sulphadiazane is the first and most successful sulphur drug to be used in malaria treatment. The most active sulphonamides are those in which the SO_2NH_2 group has been replaced with a bulky heterocyclic ring such as in sulphadoxine.

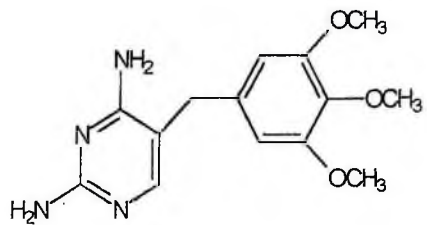
**Sulphanilamide****Sulphadoxine****Figure 1-10: Structure of some Sulphonamides.**

The sulphones and sulphonamides are usually used in combination with the antifolate antimalarials such as proguanil, and pyrimethamine in treatment. They are active against sporozoites and primary exo-erythrocytic forms of the parasites. They also act on blood forms of the parasites and this action is similar to that of the antifolates. However, they are slow acting and a combination with pyrimethamine increases their therapeutic action greatly (Bruce-Chwatt, 1986). Such combination formulation include Fansidar® (pyrimethamine and sulphonamide) and Fansimef® (mefloquine, pyrimethamine and sulphonamide).

Diaminopyrimidines:-These are characterised by the presence of a triazine or pyrimidine ring with two diamino groups attached to it. Examples include pyrimethamine and trimethoprim (figure 1-11). These attack the parasite in the blood and in the liver (Bruce-Chwatt, 1986).



Pyrimethamine



Trimethoprim

Figure 1-11: Structure of some Diaminopyrimidines.

Biguanides: Represented by proguanil, this class of antimalarials is characterised by the presence of a biguanide chain attached at one end to a chlorophenyl (diaminobenzyl) ring. Proguanil is a pro drug that undergoes oxidative ring closure in the liver through metabolism by the cytochrome P450 family (Cyp 2C19) to cycloguanil (figure 1-12), which is the active antimalarial (Bruce-Chwatt, 1986).

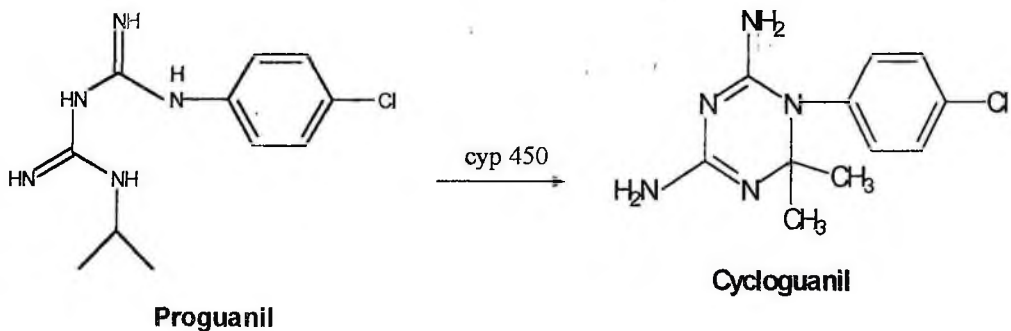
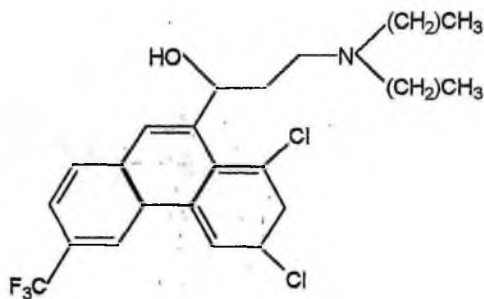


Figure 1-12: Proguanil and Cycloguanil.

Phenanthrene methanols: The most promising drug of phenanthrene methanol origin is halofantrine (figure 1-13). It is active against the erythrocytic stages of all species of malaria parasites but not against exo-erythrocytic form (Bruce-Chwatt, 1986).



Halofantrine

Figure 1-13: Structure of a Phenanthrene Methanol.

Other compounds that have been found to act against human malaria parasites include iron chelators such as desferrioxamine B and many substances of plant origin whose active principles and modes of action are yet to be elucidated.

MODE OF ACTION OF COMMON ANTIMALARIALS.

Dihydrofolate reductase inhibitors. These destroy the malaria parasite by interfering with the metabolism of folate. Folate is involved in reactions that require one carbon transfers. Such reactions are important in the synthesis of nucleic acids and also in cell division in living systems.

These drugs selectively bind to the enzyme dihydrofolate reductase of the parasite and prevent the conversion of dihydrofolate to tetrahydrofolate. Because they are competitive

inhibitors, they are structural analogs of folate. They include pyrimethamine, trimethoprim, and cycloguanil.

These drugs therefore, prevent one-carbon transfers by inhibiting formation of tetrahydrofolate and S-adenosine methionine (SAM) and eventually prevent DNA synthesis in the parasite (Bruce-Chwatt, 1986).

Dihydropteroate synthase inhibitors. These drugs also act by blocking the synthesis of the coenzyme tetrahydrofolate, which is needed either directly or indirectly for many methylation reactions, and other one carbon transfers at different biochemical sites.

They compete with para-amino benzoate thereby inhibiting the enzyme dihydropteroate synthase that catalyses the formation of dihydropteroate by the conjugation of PABA and pteridine pyrophosphate (figure 1-14).

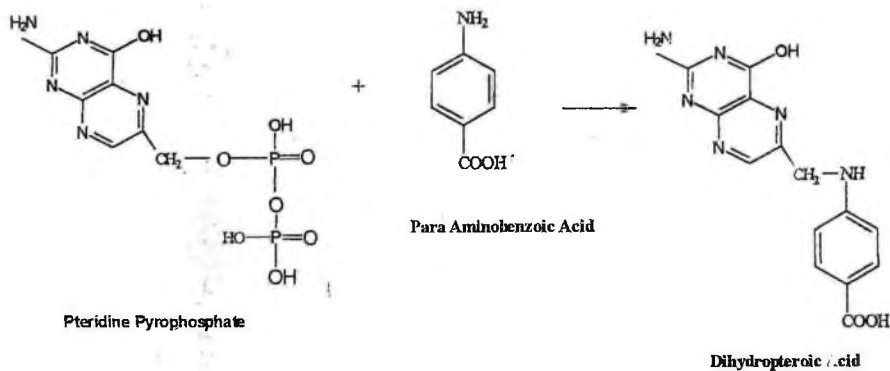


Figure 1-14: Formation of Dihydropteroic Acid.

They also combine with pteridine to form spurious dihydropteroate compounds that cannot be combined with glutamate to form dihydrofolate. They are structural analogues of PABA. All the sulphur drugs such as sulphadoxine, dapsone and sulphadiazine can be

placed in this category.

Administration of a combination of dihydrofolate reductase and dihydrothioate inhibitors in the treatment of malaria leads to reciprocal potentiation of each other's antimalaria activity. This is achieved by a sequential blockade of those two enzymes involved in the same pathway of folate synthesis at different points.

Heme polymerase inhibitors.

The quinoline-containing antimalarials block heme polymerisation by inhibiting the heme-polymerising enzyme, heme polymerase. This leads to accumulation of toxic ferriprotoporphyrin IX that kills off the parasite (Slater and Cerami, 1992).

Evidence provided by Slater and Cerami (1992) seems to suggest that alternate explanations of drug action such as for example, the assertion that quinoline containing antimalaria drugs directly affect lysosome function or that they intercalate into DNA and interrupt gene expression, are unlikely.

MALARIA CONTROL.

Malaria control and eradication involves interruption of the transmission chain, reduction of the incidence and transmission of the disease and elimination of the reservoir of infected cases. Since the anopheline mosquito is the principal vector, it plays an important role in transmission of malaria and its removal could spell a break in the life cycle. Therefore, malaria control includes measures that are aimed at killing both adult and larval forms of the vector. These include:

Blocking man-vector contact.

The screening of doors, windows and other openings of human habitations during the main biting periods of the vector prevent mosquitoes from feeding on man, which is from sunset to sunrise. The use of bednets impregnated with insecticides are especially effective in repelling and preventing mosquito bites (Binka, 1997). In addition, the use of protective apparel that leaves little skin uncovered, in combination with the use of insect repellents are effective in warding off mosquitoes.

Insecticide spraying.

Application of insecticides such as the organophosphates (Fenthion and Malathion (C-isopropoxyphenyl)) chlorinated hydrocarbons, such as dichloro-diphenyl-trichloroethane or DDT, hexachlorocyclohexane (HCH), dieldrin and aldrin and the carbamates such as propoxur (O-isopropoxyphenyl methylcarbamate) and carbaryl (naphthyl methylcarbamate) produced a major breakthrough in the fight against the malaria vector. When the WHO begun its malaria eradication programmes in 1957, the use of these residual insecticides to control the vector featured prominently. However, resistance in mosquitoes against the organophosphates and organochlorines and the persistence of these insecticides in the environment caused health problems, which led the WHO to discontinue their use. Some malaria vectors such as *Anopheles arabiensis*, *A. gambiae* and *A. funestus* have also developed widespread resistance to dieldrin and DDT. This has necessitated the use of new pyrethrum based insecticides, such as permethrin and pyrethroid for malaria control. Resistance to these insecticides have not yet been reported (Manson-Bahr, *et al*, 1987).

Removal of breeding sites.

Engineering or mechanical methods aimed at the removal of mosquito breeding places such as swamps, marshes burrow pits, rain and seepage pools by methods which include draining, flushing and trimming of canals and open drains all lead to malaria control. Also, removal of floatage and vegetation from canals and rivers also prevent mosquitoes from multiplying in these bodies (Bruce-Chwatt, 1986), hence a good measure for the control of the vector.

Larviciding.

This involves the spread of substance of hydrocarbon origin, usually petroleum oils, over pools of water to form continuous thin films on the surface thereby preventing the development of mosquito larvae in these pools. Among the most commonly used larvicides are Paris green (copper aceto-arsenate) and Temphos (O,O,O'-phenylle e phosphate). Larviciding as a control measure is only effective when the surfaces of the breeding ponds are not very extensive (WHO, 1992).

Biological control.

Biological means involving the use of larvae eating fish such as *Gambusia* sp., *Tilapia* sp. and *Northobranchus* sp. have also been used successfully to control mosquito populations in places where the ponds are stable enough to support growth of the fish.

By the use of a combination of any of these methods, the mosquito population can be controlled and consequently, the incidence of the disease (Wernsdorfer, 1980).

DRUG RESISTANCE IN MALARIA PARASITES.

Resistance to antimalarial drugs probably causes the most important interference with the control of the disease. Drug resistance is the ability of a parasite strain to survive and/or multiply in the presence of drugs to which they were hitherto susceptible (WHO, 1973). It must be noted however, that antimalarial drugs directed against pathogenic plasmodia show marked stage-specific and often also, species-specific action. Implicit in this observation is the fact that natural population of parasites consist of individuals showing different susceptibilities to a given antimalarial drug (Wernsdorfer, 1981).

Mechanisms of resistance.

Resistant mutants survive either by a modification of the mechanisms that transport the drug into the parasite or elimination of the drug from the parasite. Either way, there is a reduction in the amount of the drug that gets into the immediate environs of the parasite. *P. falciparum* strains resistant to chloroquine, for example, have been reported to possess membrane transporters that increase the excretion of the drug (Wernsdorfer, 1981).

Resistance to the anti-folate antimalarials arise from a modification of the dihydrofolate reductase gene leading to the production of mutant enzymes with reduced affinity for these drugs. Other parasite strains are known to use alternative pathways therefore reducing the importance of the pathways inhibited by the drug. Here, the salvage pathway for folate synthesis that allows the parasite to use pre-formed folate assumes prominence when the dihydrofolate reductase (DHFR) enzyme is inhibited (Cook, 1996).

Resistance appears to stem from spontaneous mutations in the chromosomes which are genetically expressed at multiple loci on the genes (Wernsdorfer, 1981). Differential

resistance of *P. falciparum* to the DHFR-inhibitors pyrimethamine and cycloquanil for example, result from specific mutations in the sequence of bases that code for the DHF enzyme. A single point mutation at position 108 changes Ser-108 (coded for by the bases AGC) to Asn-108 (AAC) in the DHFR active site cavity. This change confers a high level of resistance to pyrimethamine with only a small decrease in cycloquanil susceptibility. By contrast, the paired mutations of Ser-108 (AGC) to Thr -108 (ACC) and Ala-16 (GCA) to Val-16 (GTA) cause cycloquanil resistance with only a small change in pyrimethamine response (Gyang *et al*, 1992). Selection of resistant species is effected under drug pressure, which prevails under conditions such as prophylaxis and the administration of inadequate doses. These conditions are responsible for the occurrence and spread of resistant species (Wernsdorfer, 1981).

Control of resistance.

Possible measures to combat drug resistance include limiting the use of antimalaria drugs to reduce drug pressure, radical cure of microscopically diagnosed cases and the use of drug combinations (Wernsdorfer, 1981). Various combinations of antimalaria drugs have been employed to combat resistant stains. The use of complementary drug combinations enables the drugs involved to attack the parasite at different stages in the life cycle. The use of a combination of chloroquine and pyrimethamine in the treatment and prevention of (vivax) malaria takes advantage of both the gametocidal action of primaquine and the schizontocidal action of chloroquine. Additive drug combinations involving the use of two or more antimalarials that act at the same stage in the life cycle is also effective. The most frequently use additive combination is the use of quinine and tetracycline in the therapy of multiple-drug resistant *P. falciparum* infections. Potentiating antimalarial

drugs reinforce the action of each other and the combination is active against parasites at all stages of the cycle. An example is the use of sulphones or sulphonamides with pyrimethamine (Fansidar).

The ability of some calcium-channel antagonists such as Verapamil to inhibit the enhanced efflux of chloroquine in resistant phenotypes thereby re-establishing sensitivity also offers an excellent opportunity to overcoming chloroquine resistance.

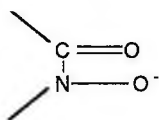
Finally, the development and use of new drugs such as iron chelators also offer new opportunities to overcome resistance and fight the disease.

SIDEROPHORES.

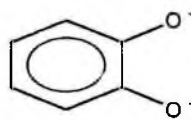
Structural features.

Siderophores are flexible organic molecules with two or more electronegative groups that form stable co-ordinate-covalent bonds with cationic ferric atoms. There is considerable diversity in siderophore structure and chemistry. The common feature of siderophores, is the use of electronegative oxygen-donor-type ligands as functional groups in the formation of complexes with ferric ion (Cotton and Wilkinson, 1980). The greater the number of these ligands present in a given siderophore, the more stable the metal-chelator complex and the more effective the siderophore in chelating iron (Raymond *et al*, 1984).

Iron chelators are classified as hydroxamate, catecholate or mixed function siderophores on the basis of the type of functional groups used in the chelation process. In hydroxamate siderophores, the ligating groups contain the oxygen atoms of hydroxamic acid. Catecholate siderophores make use of catechol, and the mixed function types employ a mixture of the two (figure 1-15).



Hydroxamate functional group



Catecholate functional group

Figure 1-15: The two main chelating groups used by siderophores.

Catecholate siderophores

These contain dihydroxybenzoyl groups, which are used in forming very stable complexes with Fe^{3+} . These stable complexes have formation constants of about 10^{52} . Bacteria, fungi and micro-algae are the main catecholate siderophores producers. Examples include such cyclic siderophores as enterobactin and linear iron chelators such as agrobactin and parabactin (figure 1-16).

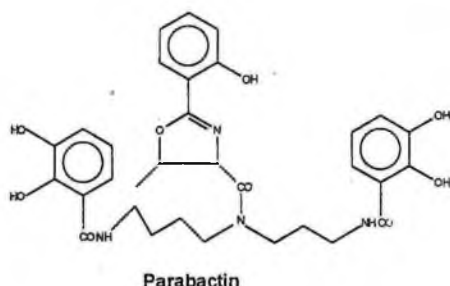


Figure 1-16: An example of a catecholate siderophore.

Hydroxamate siderophores.

A large number of the siderophores that have been characterised are hydroxamate. These are produced by fungi and include the ferrichromes. Ferrichromes contain cyclic hexapeptides in which three successive amino acid residues (mainly ornithine) have side chains that end in hydroxamate groups. Each hydroxamate group donates two oxygen atoms, which form co-ordinate bonds with Fe^{3+} . Six oxygen atoms are thus made available in the ferrichrome molecule to satisfy the six-coordination valence of ferric iron which then forms a trischelate octahedral complex. Other hydroxamate siderophore include the rhodoturlic acids (figure 1-17), the mycobactins, the fusarinines and the

ferrioxamines to which desferrioxamine B belongs (Neilands, 1981; Adjimani *et al*, 1987).

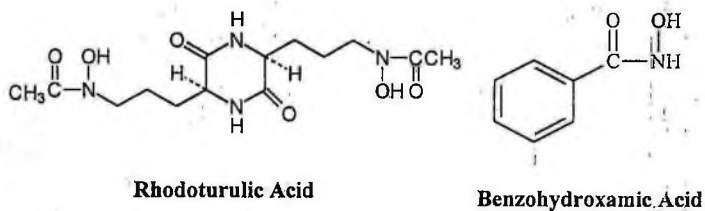


Figure 1-17: Some hydroxamate siderophores.

DEFERRIOXAMINE B.

Desferrioxamine B (figure 1-18) is a colourless crystalline substance produced by *Streptomyces pilosus* and used in the management of transfusional iron overload (Hershko, 1992; Raymond *et al*, 1984; Zevin *et al*, 1992). It consists of a chain of three hydroxamic acid groups terminating in the free amino acid L-ornithine (Neilands, 1980). This free amino acid enables the molecule form salts with inorganic compounds such as sodium sulphate yielding water soluble desferrioxamine mesylate (Desferal®).

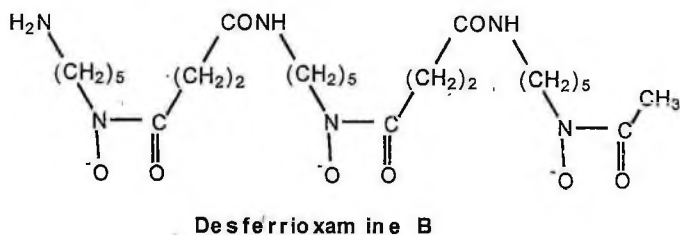


Figure 1-18: Structure of desferrioxamine B.

The siderophore binds ferric iron avidly but binds ferrous iron and other essential trace metals poorly.

Desferrioxamine B easily penetrates and interacts with a number of cell types including red blood cells (Pippard *et al*, 1982), liver, spleen, kidney and brain cells (Octave *et al*, 1983). It is also able to react with iron located in these cells (Hershko, 1992). The compound is readily catabolised *in vivo* and has a very short metabolic half life which is estimated to be about one hour however, it is estimated to be distributed over 65% of the body (Hershko, 1992). This rapid body-wide distribution is very useful in the treatment of malaria.

The desferrioxamine B molecule has a high affinity for ferric (Fe^{3+}) iron and it combines with the ion at a molar ratio of 1:1 to form ferrioxamine B (figure 1-19) with stability constants of 10^{31} . The molecule is wrapped around the Fe^{3+} nucleus encasing it in an envelope of organic material. As a result of this change in configuration following interaction with iron, the siderophore becomes an extremely stable compound, resistant to enzymatic degradation and incapable of penetrating cells. It therefore, accumulates in the intracellular space (Hershko, 1992).

larger the size of iron stored in hemosiderin and ferritin, the higher the amount of iron excreted in the urine following desferrioxamine B treatment. The nature of this proposed iron pool, however, is not known (Hershko and Peto, 1988, Hershko, 1992).

It has been observed that, the antimalarial activity of the drug is directly proportional to its ability to enter parasitised red cells (Cotton *and* Wilkinson, 1980). Parasite uptake of desferrioxamine B is also a prerequisite for its antimalarial activity (Scott *et al*, 1990).

Hence, it could be concluded that, chelation of serum iron is unimportant and that, the antimalarial effect is exerted mainly in the erythrocytes. It follows therefore that, either desferrioxamine B may be chelating iron that is essential to the malaria parasite and making it unavailable for growth and reproduction or it may work by interfering with the process of heme polymerisation thereby releasing free heme that may be toxic to the parasite.

Toxicity and side effects.

Effective as desferrioxamine B is in the treatment of iron overload and malaria, the drug is poorly absorbed when administered orally (Bertram, 1992; Hershko, 1992; Hepner, *et al*, 1988; Raymond, *et al*, 1984) necessitating its intravenous or intramuscular administration.

Although it is relatively non-toxic when administered in low doses (Hershko, 1992), high dose and long term desferrioxamine B therapy is associated with such conditions as vision and hearing loss, impairment of growth in children and breathing difficulties (Bertram, 1992). Therefore, new orally effective, relatively non-toxic inexpensive iron chelators are required if their use as antimalarials is to be accepted.

CHAPTER 2

MATERIALS AND METHODS.

MATERIALS.

CHEMICALS.

Desferal® (Desferrioxamine mesylate) was obtained from CIBA-GEIGY Limited, Switzerland. Chloroquine injection from TROGE MEDICA GmbH, Germany. Giemsa: Gurr improved stain, sodium chloride, sodium hydroxide, ethanol and diethyl ether from BDH, England. Trisodium citrate from HOPKIN & WILLIAMS, England and hematin form SIGMA, USA.

EQUIPMENT.

Denley Wellscan, DENLEY INSTRUMENTS LTD, England. Kobuta microfuge, KOBUTA, Japan. Shimadzu double-beam spectrophotometer UV-190 and Shimadzu infrared spectrophotometer IR-450 from SHIMADZU CORPORATION, Japan.

PLASMODIUM BERGHEI.

Plasmodium berghei anka was obtained from NMIMR, Ghana.

ANIMALS.

CBA mice from BOM, Denmark.

METHOD.

IN VIVO EXPERIMENTS.

Culture of *Plasmodium berghei* anka in CBA mice.

Thin blood smears were made from the tails of three 12-day-old BALB.C mice infected with *P. berghei* (Anka strain) and allowed to dry. The dried blood smears were fixed with methanol, air dried and stained with a 1:10 dilution of Giemsa stain in phosphate buffer for 15 minutes. The stained films were then washed with a gentle flow of water and parasitemia determined. The mouse with the highest parasitemia ($\geq 86\%$) was anaesthetised with diethyl ether and a total of 0.7ml of blood drawn by cardiac puncture. This blood obtained from the BALB C mouse was used to culture the malaria parasites in the CBA mice used in the study.

1.4ml of sterile phosphate buffered saline (PBS) was added to the blood and centrifuged at 5000g for 20mins and the supernatant discarded. Washing was repeated four more times and the pellet (containing both parasitised and non-parasitised erythrocyte) resuspended in 0.7ml of sterile PBS. 20 μ l this of washed suspension was added to 4.0ml of sterile PBS to attain a dilution of 1:200 μ l. A Neubaur cell counting chamber (hemocytometer) was completely filled with the diluted erythrocyte suspension and the cell concentration determined.

$$\begin{aligned} \text{Number of RBCs per 5 central squares} &= 197 \\ \text{Number of RBCs per central 25 squares} &= 197 \times 5 \end{aligned}$$

$$\text{Number of RBCs /field of 5 central squares} = \frac{\text{No. of RBCs in a field} \times \text{Dilution factor}}{0.1\mu\text{l}}$$

Where $0.1\mu\text{l}$ is the total volume of fluid occupied by a field of 5 central squares

$$\text{Number of RBCs /ml of blood} = \frac{197 \times 5 \times 200 \times 1000\mu\text{l/ml}}{5 \times 0.1\mu\text{l}}$$

$$= 3.94 \times 10^8 \text{ RBCs/ml}$$

$$\text{Therefore number. of RBCs /100}\mu\text{l of blood} = 0.394 \times 10^8$$

But percentage parasitemia of Balb C mouse was 86.74%

$$\therefore \text{parasitised RBCs per } 100\mu\text{l of blood} = \frac{86.74 \times 0.394 \times 10^8}{100}$$

$$= 0.342 \times 10^8 \text{ parasites.}$$

This means that 34.2×10^6 parasites are contained in $100\mu\text{l}$ of blood.

$\therefore 1 \times 10^6$ parasites are contained in $2.9\mu\text{l}$ of blood.

17.4 microlitres of this parasitised blood was made up to $1200\mu\text{l}$ with phosphate buffered saline. $200\mu\text{l}$ of this dilution containing 10^6 parasitised RBCs was injected intraperitoneally into each of five CBA mice. Parasitemia levels were determined on the third day after passage and subsequently, every other day for 14 days after which time the average parasitemia was in excess of 30%. The passage was continued for six consecutive times to establish the *P. b Anka* in the CBA mice.

One hundred 6-8 week old CBA mice each weighing between 21-23g (average weight of 22g) were housed in groups of between 5 and 15 in stainless-steel cages with stainless steel grid lids and fed ad lib with RNM3 food, and tap water from plastic bottles. Animals were sacrificed by diethyl ether anaesthesia and blood samples taken via cardiac puncture and/or from the tail.

Fixation, staining and determination of parasitemia.

Blood samples taken from the tail were made into thin uniform smears on microscope slides of dimension 76 x 26mm, allowed to dry and then fixed with methanol and air dried. The fixed films were then stained with 10% Giemsa for 15 minutes, washed under running tap water, dried and examined microscopically under oil immersion. Parasitemia was determined as a percentage of the ratio of the number of parasitised erythrocytes to a total number of 1000 erythrocytes.

Inoculation of CBA mice and administration of antimalarial compounds.

Eighty 6-8 week old female CBA mice (of average weight of 22g) were inoculated intraperitoneally with 10^6 *P. berghei* infected RBCs and parasitemia levels monitored daily. The mice were put into 3 groups of 15 mice each and the rate of growth of parasites monitored daily. On the 13th day, average parasitemia was in excess of 28%. The first group was given an intraperitoneal injection of 100 μ l of PBS only (placebo or control group), the second 100 μ l of chloroquine (5mg/kg body weight) and the third 100 μ l desferrioxamine B (100mg/kg body weight). Blood samples were collected from three mice from each group via cardiac puncture at times 0, 3, 6, 12, 24, 36 and 48 hours post injection. Thin smears were made on microscope slides for parasitemia determination. Samples were also taken for histological analysis.

IN VITRO* EXPERIMENTS.*INHIBITION OF HEME POLYMERISATION.**

One hundred microlitres of a solution containing 4mM solution of hematin dissolved in 0.1MNaOH, was distributed in a 96-well round-bottomed microplate to achieve a concentration of 0.4 μ mol/well. 50 μ L of 200mM solution of desferrioxamine B was added to each of triplicate wells to give a desferrioxamine to heme ration of 2.5:1. 50 μ L of chloroquine concentrations of 4 μ mol/well and 10 μ mol/well were also added in triplicate to other test wells. 50 μ L of ddH₂O was added to control wells and hematin polymerisation initiated by the addition of 50 μ L of 16M acetic acid (containing 0.8mmol) at a final pH of 3 and the suspension incubated at 37°C for 24hours to allow complete polymerisation. Plates were then spun at 3300g for 15 min. and the supernatant of unpolymerised hematin collected (fraction I). The remaining pellet was resuspended in 99.9% DMSO to remove unreacted hematin. The plates were again centrifuged at 3300g for 15min and the DMSO soluble fraction (fraction II) was collected. The pellet in each well consisting of pure β -hematin was dissolved in 200 μ L of 0.1MNaOH (fraction III). 150 μ L aliquot of each fraction was transferred to new plates and a two-fold serial dilution performed. The amount of hematin present in each fraction was determined spectrophotometrically at 405nm using a microtitre plate reader (Denley Wellscan).

A standard curve of unpolymerised hematin dissolved in 0.1MNaOH was used to calculate the amount of hematin present in each fraction.

This procedure of measuring the extent of heme polymerisation was repeated using other iron chelators including benzohydroxamic acid, rhodoturulic acid and acetohydroxamic acid in place of desferrioxamine B.

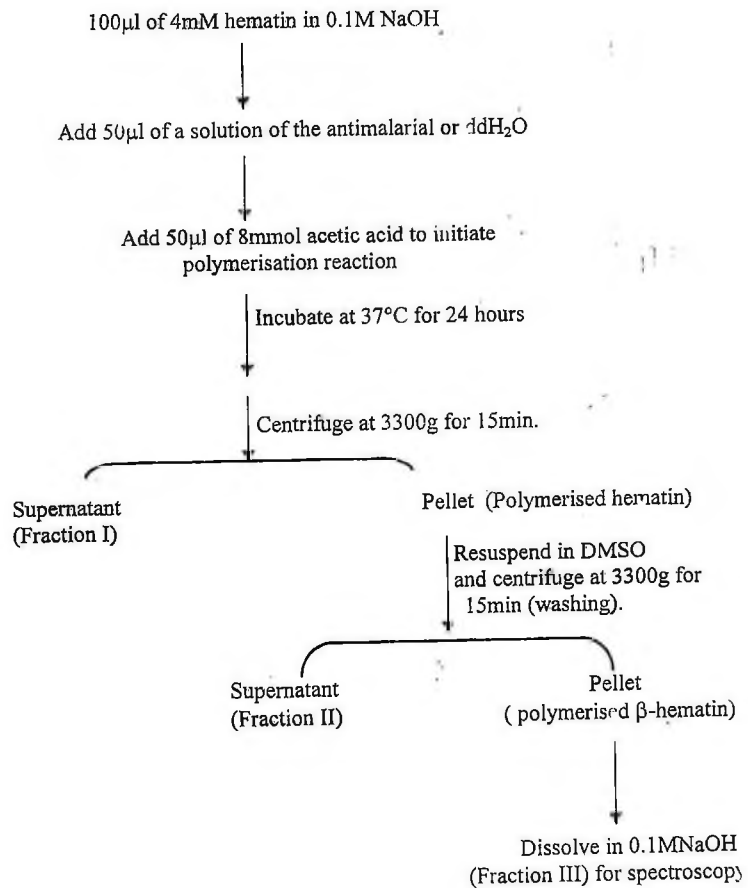


Figure 2-1: Flow diagram showing the sequence of events in the hematin polymerisation experiments.

Spectroscopic determination of binding.

Solutions of hematin (4mM) , desferrioxamine B (4mM) and chloroquine dissolved in NaOH were analysed spectrophotometrically in the UV-visible range (800-110nm). Spectra for hematin-desferrioxamine B and hematin-chloroquine combinations at drug:hematin ratios of 1:1 were also analysed spectrophotometrically in the UV-visible region.

Infrared scan of desferrioxamine B and hematin were run separately. Combinations of the two compounds were also run. The hematin-desferrioxamine B reaction was carried out in 0.1M NaOH on microplates and the plates left uncovered overnight to allow the aqueous NaOH to evaporate. These were then dissolved in 10 μ l of DMSO for the (infrared) IR analysis. The IR spectra were obtained using KBr discs.

STATISTICAL ANALYSIS.

Mean percentage parasitemia were expressed as the average of percentage parasitemia \pm standard deviation of three separate determinations and the student's t-test was used to analyse the significance of all data to within 95% confidence levels.

CHAPTER 3

RESULTS.

HEMATOLOGICAL EXPERIMENTS.

Plasmodium Berghei. Anka Cultures.

The growth of *Plasmodium berghei anka* strain in CBA mice was not found to be sigmoidal (figure 3-1) as seen in the growth pattern of most microbial systems under optimum conditions (Goodsel, 1996). There was an initial establishment period during which growth was slow (day 0 to 10). This was followed by a rapid acceleration phase after day 10.

The period (between day 10 and day 21) within which plasmodia growth was exponential was chosen for the efficacy test as parasites in this phase are the most amenable to physical and chemical changes in their environment (Prescot *et al*, 1993). In addition, this period in the growth of parasites provides a good approximation of non-severe malaria in living systems.

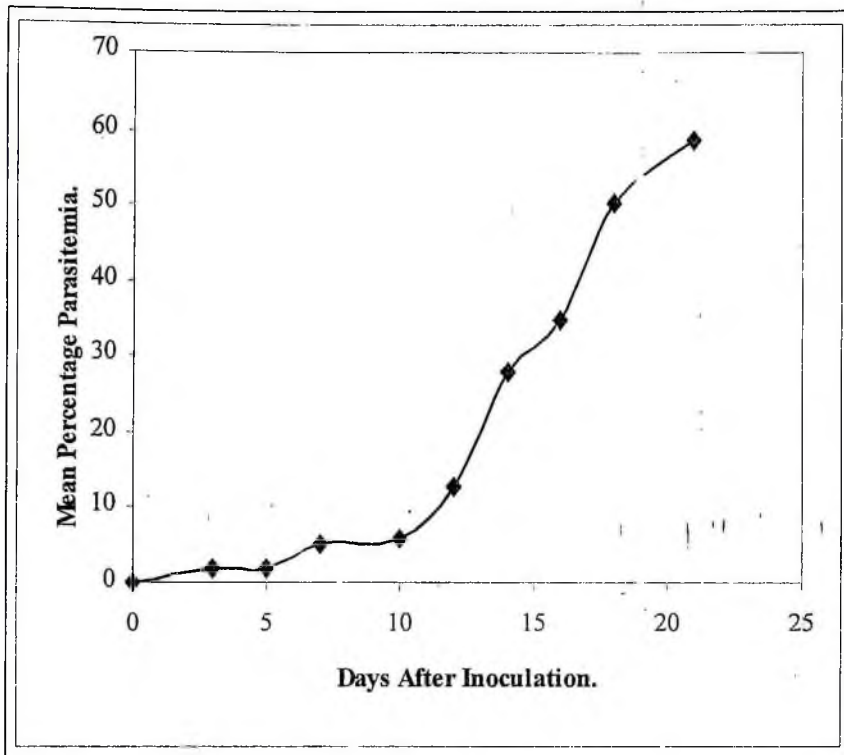


Figure 3-1: Growth pattern of *P. b. anka* in CBA mice.

All but a few of the parasite-treated experimental mice survived after day 21. The percentage of the infected mice that survived reduced rapidly from a value of 96% at 18 days after infection to fewer than 3% after 21 days of infection with the malaria parasites. This trend seems to be the result of increased burden of parasites. The mice at this time had a mean parasitemia of about 60% of the total red cell count. At this level of parasitemia, mice were sluggish, had dull eyes and rough coats. Most of them died shortly after exhibiting these symptoms.

Figure 3-2 shows the effect of injection of 100mg of desferrioxamine per kg body weight or 5mg of chloroquine per kg body weight on peripheral parasite growth in *P. berghei* anka infected CBA mice. Mice in the control group were given phosphate buffered saline alone. All the drugs were constituted in PBS.

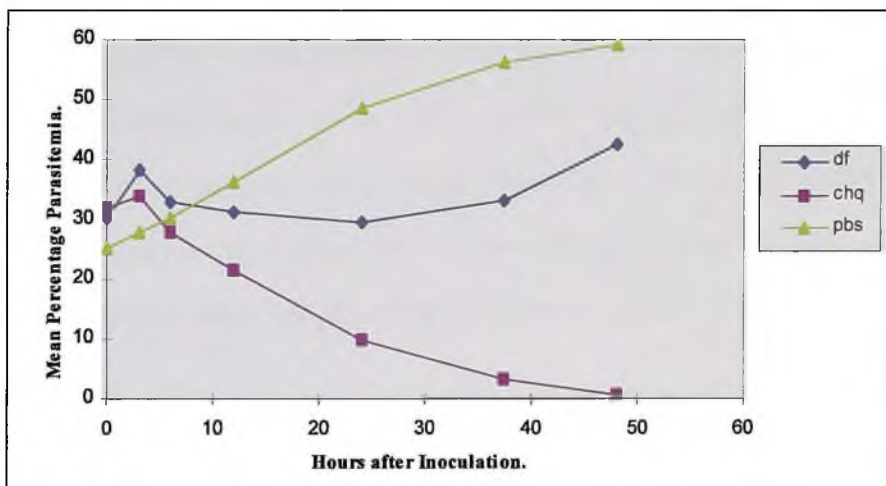


Figure 3-2: Effect of Desferrioxamine B and Chloroquine on *Plasmodium berghei* anka growth in CBA Mice. Peripheral parasite count in mice treated with desferrioxamine B and chloroquine compared to untreated controls given only phosphate buffered saline.

In the desferrioxamine-treated group, parasitemia increased from an initial value of 30% to a maximum of 38%, three hours after treatment. The parasite count then reduced from this peak level to 32.53% six hours after treatment. Parasitemia levels remained almost

unchanged at 32% until the 24th hour after which the levels increased again to 42.76% by the 48th hour.

Peak parasitemia of 34% was observed in mice treated with 5mg of chloroquine per kg body weight by the third hour from an initial 32% level. Parasitemia levels then fell to a negligible value of 0.5% by 48 hours into the experiment. Within this same period, percentage parasitemia in mice in the untreated group increased steadily to a peak of 57% in 48hours.

Until hour 6, there was no statistically significant difference between percentage parasitemia in both treated and untreated control mice ($p < 14$). However, at 12 hours and after, differences in parasite counts in chloroquine treated mice became significant ($p = 14.76$). Significant differences in parasitemia levels between desferrioxamine B treated mice and the control was observed after 24 hours of treatment ($p < 16.32$). These significant differences were maintained from this point onwards (Table 3-1).

Two of the mice receiving desferrioxamine B died 6 and 12 hours after treatment was started. These deaths were presumed to be the result of drug toxicity since the mice looked extremely sluggish and had stunted growth (lower weights). This was in contrast to the fact that none of the mice in the other groups (chloroquine and phosphate buffered saline treated) suffered any such adverse outward symptoms and fate.

No significant differences were observed between desferrioxamine B treated and control mice (phosphate buffered saline treated) until 24 hours after drug administration.

Table 3-1: Growth of *Plasmodium berghei anka* in Chloroquine Treated Mice Compared to Desferrioxamine B Treated Mice.

Time after injection (hours)	Mean percentage parasitemia.		Statistical difference
	CHQ treated group	df treated group	
0	32.00 ± 5.38	30.36 ± 11.93	NS
3	33.94 ± 9.22	38.49 ± 2.89	NS
6	27.86 ± 4.09	32.84 ± 9.22	NS
12	21.72 ± 8.73	33.23 ± 13.70	NS
24	9.89 ± 3.57	32.53 ± 7.76	S
36	3.35 ± 1.20	39.54 ± 9.61	S
48	0.51 ± 0.07	42.76 ± 8.11	S

* Two-tailed test at 95% confidence interval.

S Significant

NS Not significant

A combination of desferrioxamine B and chloroquine has been reportedly used in the treatment of malaria (Thuma *et al*, 1998; Hersko *et al*, 1992; Raventous-Suarez *et al*, 1982). To study the effects of this combination therefore, 50mg of desferrioxamine B and 2.5mg of chloroquine per kg body weight of mice dissolved in phosphate buffered saline was administered. This drug combination injected intraperitoneally into *P. b. anka*-infected CBA mice produced the effects presented in figure 3-3 below.

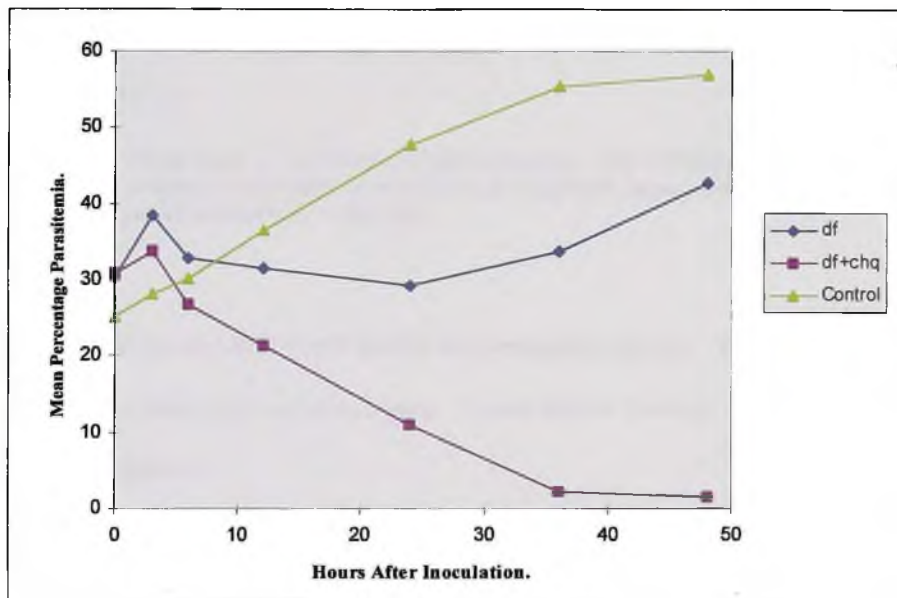


Figure 3-3: Peripheral parasite count in mice treated with a combination of chloroquine and desferrioxamine compared to desferrioxamine-treated and phosphate buffered saline-treated controls.

Here, percentage parasitemia increased from an initial level of 31%, to a peak value of 34% after three hours of treatment. It then reduced to a minimum value of 0.5% at hours 36 and 48.

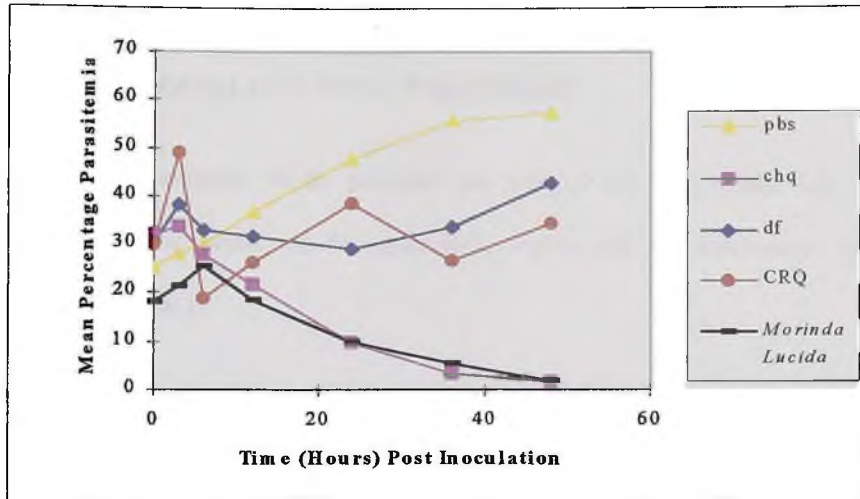


Figure 3-4: Comparison of the effect of desferrioxamine and chloroquine to extracts of the commonly used herbs *Morinda lucida* and *Cassia siamea* (CRQ) on the growth of malaria parasites in CBA mice.

The effect of extracts of *Morinda lucida* was particularly striking. The growth inhibition pattern was similar to that of chloroquine. *Cassia siamea* however, was not found to be particularly effective.

In the presence of chloroquine and *Morinda lucida*, parasite clearance was almost complete 38 hours after injection of drug whilst with desferrioxamine B only about half of the parasites present initially had been cleared.

**ULTRASTRUCTURAL CHANGES INDUCED IN *PLASMODIUM BERGHEI*
ANKA AS A RESULT OF DRUG TREATMENT.**

Study of the structure of the parasites and infected red cells would help identify the possible site of action of the antimalarial drugs and the mechanism of action of desferrioxamine B.

Under the light microscope, infected red blood cells were found to have enlarged to a size of about two times that of the uninfected cells. These cells were also puffy and exhibited gross morphological changes. Infected cells also had knobs spaced at regular intervals on their surfaces (figure 3-5).

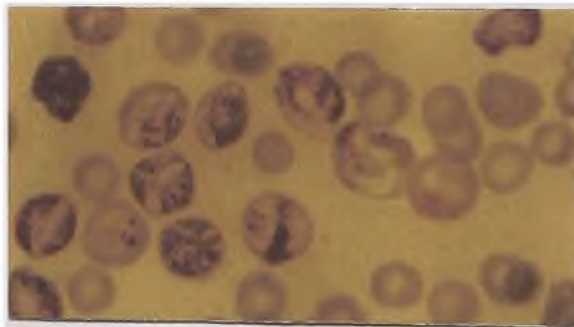


Figure 3-5: Non-infected red blood cells compared to infected red cells.

The predominant forms of parasites observed at the time treatment commenced were trophozoites (65% of infected cells) which were the matured ring-forms and schizonts (28% of infected cells), having up to 32 merozoites through the mitotic division of the trophozoites. Very few gametocytes were seen. Parasites were found to occupy up to 16% of the internal space of infected red cells. The presence of two or more parasites per red cell was not uncommon. At this stage, most parasites had thin staining cytoplasm containing granules with small and compact purple-staining nuclei.

Three hours after drug injection.

Very little changes, other than those observed at the time of drug injection, were observed in infected red blood cells of both desferrioxamine and chloroquine-treated mice. However, a small proportion of parasites in the chloroquine-treated group had enlarged nuclei with faint-staining cytoplasm. The proportion of young trophozoites in this group had also decreased significantly by 75%, results of this observation not shown.

Six hours after drug injection.

In the chloroquine-treated group, changes were seen in about 50% of the infected red cells. Parasite nuclei had increased in size with most becoming oval-shaped. Most cytoplasm had also become enlarged, discontinuous and pale, but these observations were not clearly shown by the photographs presented.

In the desferrioxamine B treated group, the proportion of trophozoites had decreased. Both trophozoites and schizonts observed to have lost some of their cytoplasm whilst the rest had become enlarged and thin staining was difficult to see.

Meanwhile in the control (PBS-treated group), the proportion of both young and old trophozoites had increased. The cytoplasm of the parasites had become darker with more prominent granules and rods. The changes had also become more widespread engulfing more previously uninfected red blood cells.

Twelve hours after drug injection.

Widespread morphological changes were observed after 12 hours. These include the observation of irregularly shaped gametocytes in the chloroquine-treated group. About 90% of the other forms had lost their cytoplasm and that of the rest could only be detected with difficulty. In the desferrioxamine B-treated group, there was little change in the morphology of the parasites except for the observation that, those changes observed after six hours had spread to cover more parasitised cells.

Twenty-four hours after drug injection.

Most of the parasites in the chloroquine-treated group were shrivelled (results of this observation not shown). All the trophozoites had completely disintegrated. The previously infected red cells had large amounts of purple staining Schüffer's stippling.

The proportion of trophozoites remained at the same level in the desferrioxamine B-treated mice. Structural and morphological damages were the same as those after twelve hours of treatment.

Control mice had increased parasitemia and of both male and female gametocytes occurred with a higher frequency.

Thirty-six hours after drug injection.

Parasites in the chloroquine-treated group had disintegrated completely. The cytoplasm, where observable, was thread-like and the nuclei were vesicled. Most of the red cells had also regained their normal sizes and even the proportion of red cells with Schüffer's dot had reduced.

Desferrioxamine B-treated cells show an increase in the proportion of young trophozoites in parasitised red cells. Some of the previously disintegrating cytoplasm had become continuous and had begun to pick up stain.

Forty-eight hours after drug injection.

All parasites in the chloroquine-treated group had been almost cleared. Most of the red cells had regained normal shapes and morphology, and only few parasitised cells show Schüffer's dots.

In the case desferrioxamine B-treated mice, a high proportion of trophozoites and schizonts were observed in the red blood cells at this point. Here, the red cells had also become enlarged again and more than one parasite could be observed in most of them compared to the control.

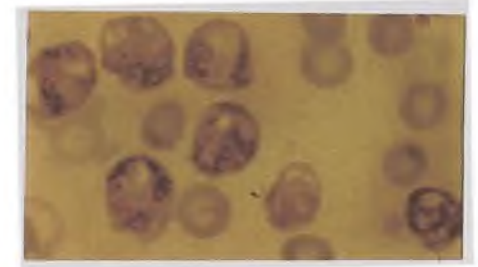
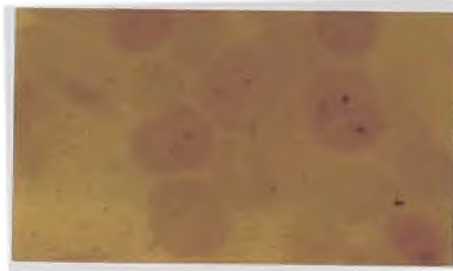
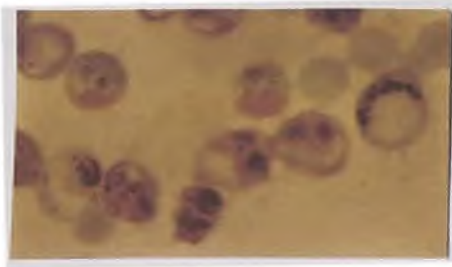
Figure 3-6 a:

Control

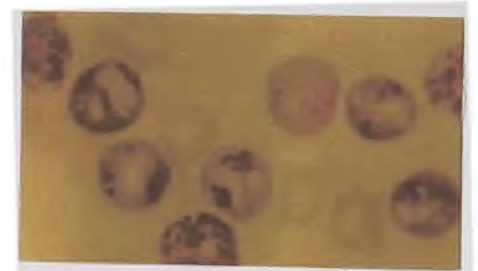
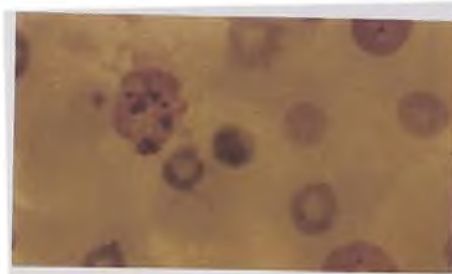
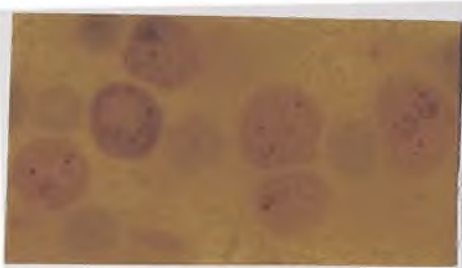
Chloroquine-treated mice

Desferrioxamine B-treated mice

T = 0 Hrs



T = 6 Hrs



T = 12 Hrs

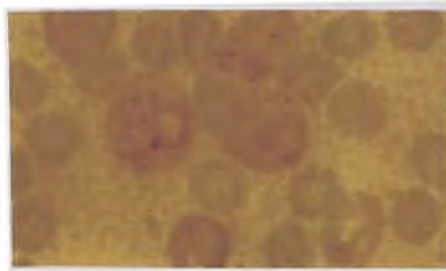
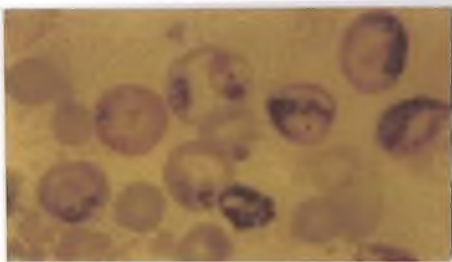


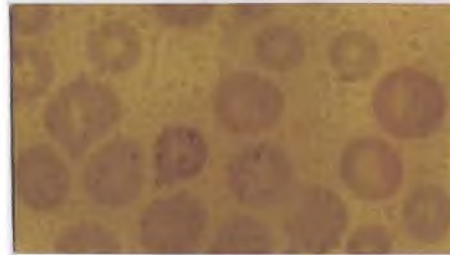
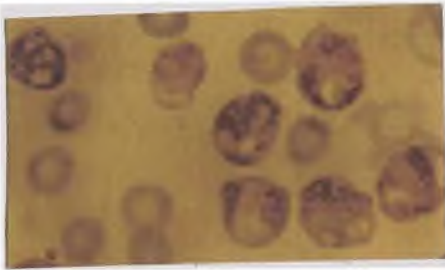
Figure 3-6 b:

Control

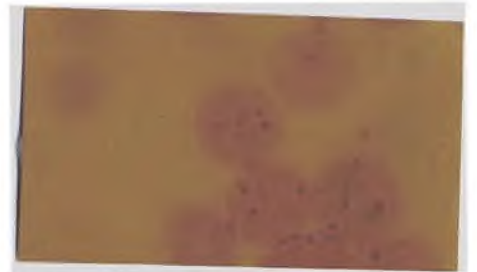
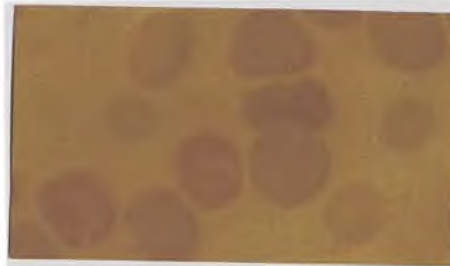
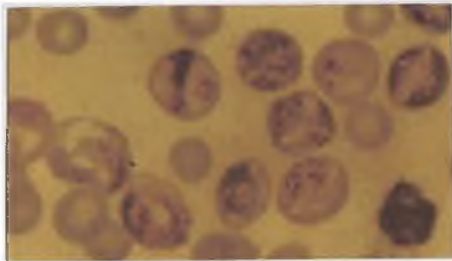
Chloroquine-treated mice

Desferrioxamine B-treated mice

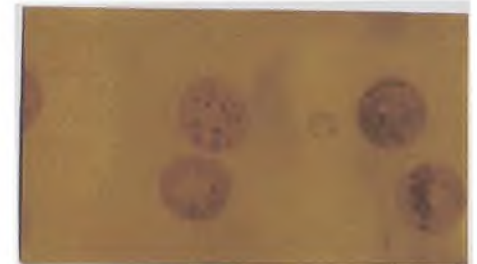
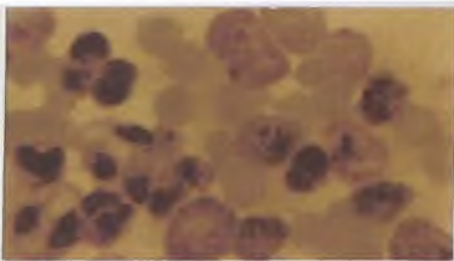
= 24 Hrs



= 36 Hrs



= 48 Hrs



INHIBITION OF HEME POLYMERIZATION.

Heme was polymerised under acidic conditions. Concentrations of hematin in microplates were determined from a standard curve constructed using the absorbances of known concentrations of unpolymerised hematin dissolved in 200 μ L of 0.1M NaOH (Figure 3-7).

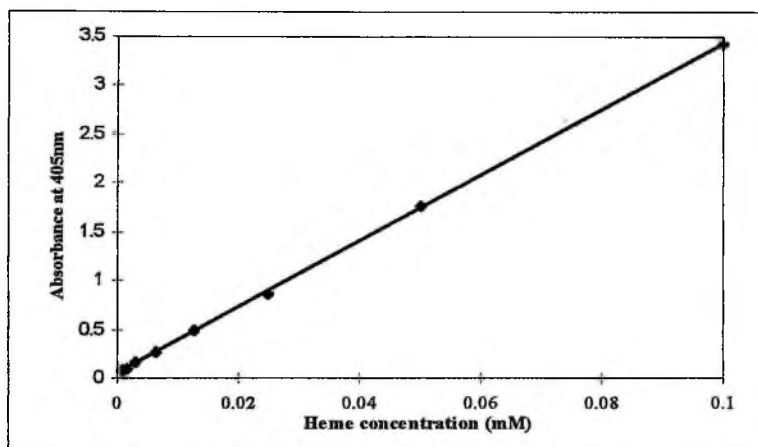


Figure 3-7: Hematin standard curve.

Both desferrioxamine B and chloroquine inhibited the polymerisation of heme into β -hematin. Inhibitions of 66.25%, 77.63% and 98.96% were observed for desferrioxamine and chloroquine (at 2mM and 5mM) respectively. There was no significant inhibition of heme polymerisation ($P < 0.05$) by citrate at a concentration ten times greater than that of desferrioxamine (table 3-2).

Table 3-2: Extent of Inhibition of Heme Polymerisation by the Various Compounds Tested.

Compound	Hematin Conc. (mM)			Mean* Hematin Conc. (mM)	Percentage Inhibition
Control	2.53×10^{-2}	2.53×10^{-2}	2.53×10^{-2}	$2.53 \times 10^{-2} \pm 0.0001$	0.00
Citrate (0.5M)	2.69×10^{-2}	2.69×10^{-2}	2.69×10^{-2}	$2.70 \times 10^{-2} \pm 0.0001$	6.72
DF (5mM)	8.60×10^{-3}	8.54×10^{-3}	8.54×10^{-3}	$8.54 \times 10^{-3} \pm 0.0000$	66.25
Chloroquine at (2mM)	5.66×10^{-3}	5.66×10^{-3}	5.66×10^{-3}	$5.66 \times 10^{-3} \pm 0.0001$	77.63
Chloroquine at (5mM)	2.63×10^{-4}	2.60×10^{-4}	2.63×10^{-4}	$2.63 \times 10^{-4} \pm 0.0000$	98.96

* Mean hematin concentration = hematin concentration \pm standard deviation of three separate determinations

There was a clear and significant inhibition of heme polymerisation when desferrioxamine B at (5mM) and chloroquine at 2mM and 5mM were used.

Although an iron chelator, citrate did not inhibit heme polymerisation to a significant extent ($P < 0.05$). Inhibition produced by desferrioxamine B at 5mM was not significantly different from that produced by 2mM chloroquine ($P = 0.004$) but significantly lower than that produced by 5mM chloroquine.

As compared to the control, percentage inhibition of heme polymerisation in the presence of desferrioxamine B and at the two concentrations of chloroquine were significant (table 3-3).

Table 3-3. Statistical Comparison in Percentage Inhibition of Heme Polymerisation Produced Chloroquine, Citrate and Desferrioxamine B.

Compound	Percentage inhibition	Critical Value*	P-Value	Difference
Citrate	6.72	0.05	0.037	NS
Df (5mM)	66.25	0.041	0.066	S
Chloroquine (2mM)	77.63	0.040	0.077	S
Chloroquine (5mM)	98.96	0.032	0.100	S

* One-tailed test at 95% confidence interval.

S significant

NS not significant

Other monodentate iron chelators namely acetohydroxamic acid, benzohydroxamic acid and rhodotorulic acid were also tested for their inhibitory effect on heme polymerisation.

Table 3-4: Inhibition of Heme Polymerisation Produced by Monodentate Iron Chelators.

Compound	Mean β -Hematin Concentration (mM)	Percentage Inhibition	Critical Value*	P-Value	Difference
Control	4.917×10^{-2}	0.00			
Acetohydroxamic acid	2.112×10^{-2}	57.05	0.014	0.066	S
Benzohydroxamic acid	2.443×10^{-2}	50.31	0.041	0.067	S
Rhodoturlic Acid	2.151×10^{-2}	56.25	0.032	0.100	S

* One-tailed test at 95% confidence Interval.

s significant

ns not significant

Benzohydroxamic acid and acetohydroxamic acid are monodentate and together with rhodoturlic acid, a bidentate iron chelator, proved to be very good inhibitors of heme polymerisation.

Table 3-5: Wavelengths of Maximum Absorption Exhibited by Hematin, Desferrioxamine B, Chloroquine and their Combinations from the UV-Visible spectra.

Compound*.	Wavelength of maximum absorption (nm).
Hematin	407
Desferrioxamine B	300
Chloroquine	315
Df + Hematin	370
Chloroquine + Hematin	402

* All compounds were prepared in 0.1MNaOH.

There was a large shift in the wavelength of maximum absorption (peak maximum) of hematin from 407nm to 370nm (a shift of 37nm towards wavelengths of higher energy) when desferrioxamine B was added to hematin. However, only a small shift in peak absorption was observed (from 407nm to 402nm, a shift of 5nm) in the hematin spectrum on addition of chloroquine to hematin (table 3-5).

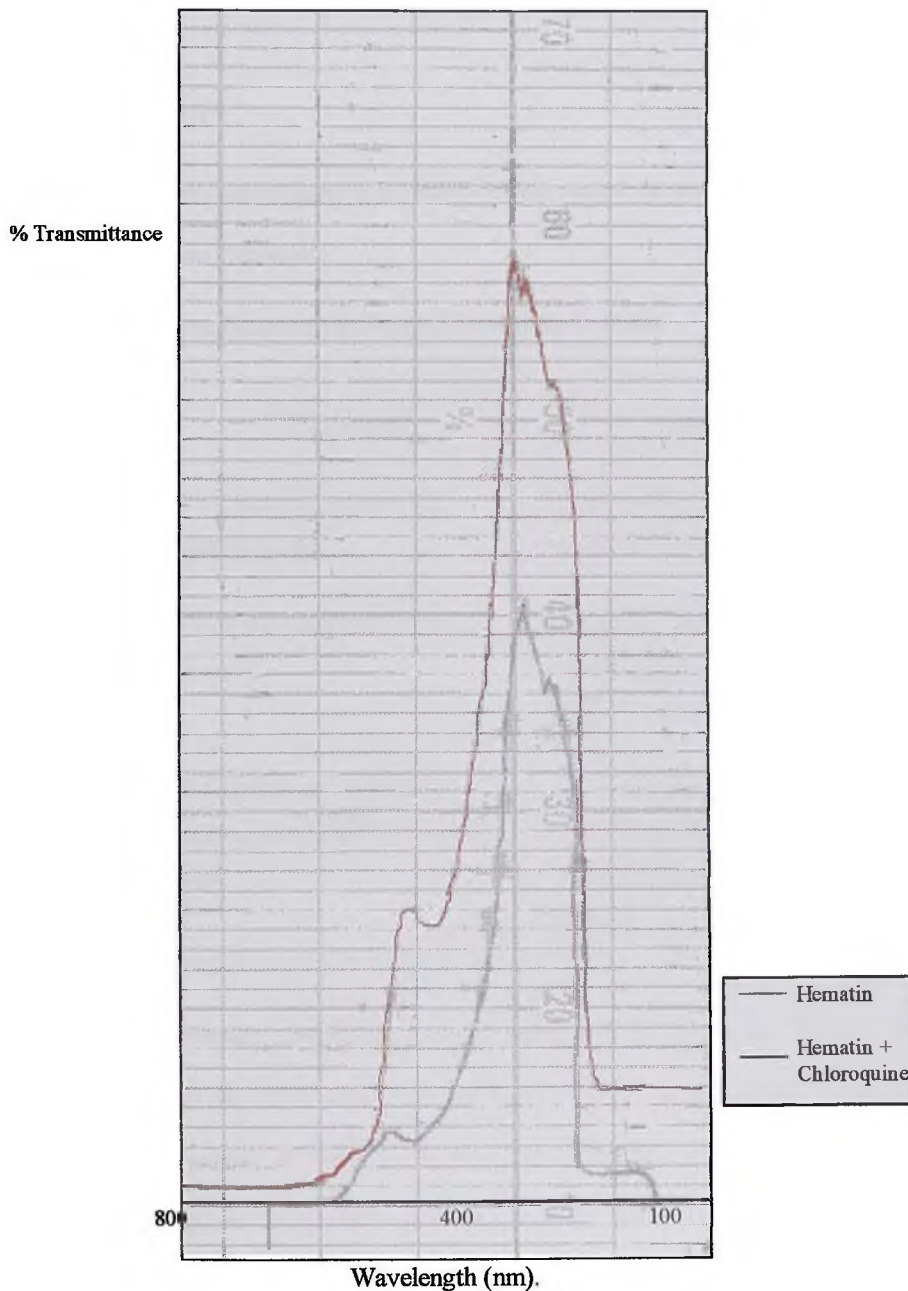


Figure 3-9: Electronic absorption spectra of hematin and a combination of hematin and chloroquine.

Infra red Spectroscopy.

From the infrared spectrum of figure 3-10, the following important peaks could be assigned to the hematin spectrum.

Table 3-6: Peaks Assignments in the Infrared Spectrum of Hematin.

Band (cm ⁻¹)	Assignment	Inference
1565 and 1250	C-O bending vibrations of COOH groups	Confirms the presence of free carboxyl groups in the complex
1226 (Broad)	Characteristic peak of free hematin	This shows the presence of free unpolymerised hematin in the NaOH solution.
3150 (Sharp)	O-H stretching vibration of basic groups directly bonded to iron	This band confirms the presence of hydroxyl groups directly bonded to the ferric iron of hematin

The hemozoin and hematin specific absorbance peaks usually present at 1665cm⁻¹ and 1211cm⁻¹ (Basilico *et al*, 1998) which are the result of C-O bending vibrations of free carboxyl groups appeared at 1565cm⁻¹ and 1250cm⁻¹ respectively. The broad peak centred at about 1226cm⁻¹ is another characteristic peak present in the spectra of free hematin (Blauer and Aikawa, 1995). The presence of a sharp peak at 3150cm⁻¹ could be attributed to O-H stretching vibration of basic groups directly bonded to iron.

The desferrioxamine B spectrum exhibited characteristic broad peaks centred at 1550cm^{-1} and 1200cm^{-1} . A peak at 2900cm^{-1} , the result of N-H stretching vibrations of amines which is another characteristic of desferrioxamine B was also observed confirming the presence of the compound.

On combining the two compounds, the broad peak observed at 1226cm^{-1} was diminished. In addition, the sharp peak at 3150cm^{-1} attributed to OH stretching did not appear at all.

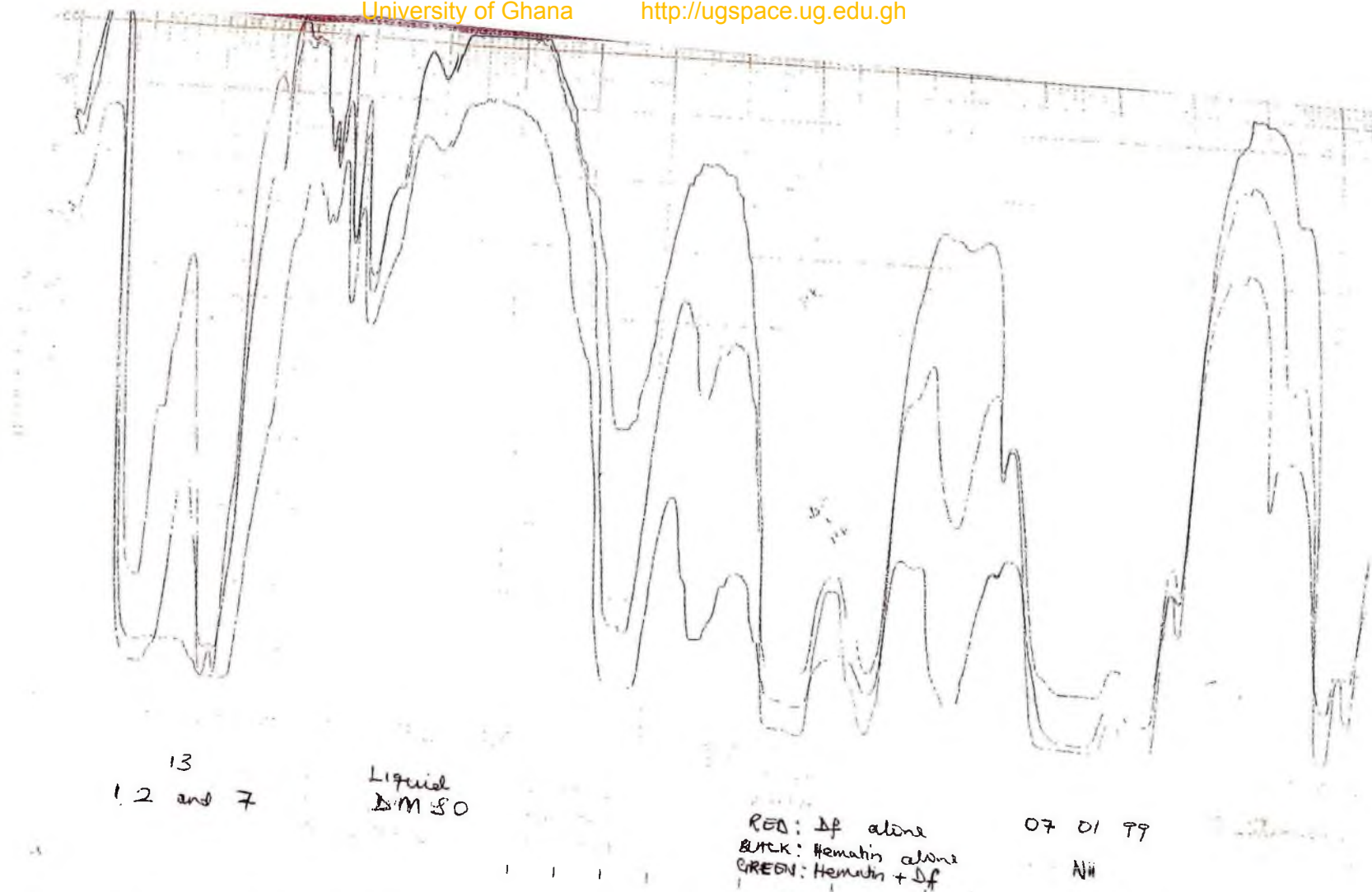


Figure 3-10: Infra red spectra of hematin alone, desferrioxamine alone and a combination of desferrioxamine and chloroquine.

CHAPTER 4

DISCUSSION AND CONCLUSIONS.

DISCUSSION.

The study described in this thesis was to determine the mechanism of the antimalarial action of desferrioxamine B using both animal (mice) models and *in vitro* methods including the determination of the extent of heme polymerisation and the morphological changes produced in malaria parasites as a result of the administration of desferrioxamine B.

Results from the study shows that iron chelation therapy with desferrioxamine B has antiplasmodial action against malaria parasite infection in CBA mice. Furthermore, the reduction in parasitemia achieved by the administration of a single bolus injection of 100mg of desferrioxamine per kg body weight of mice may last for up to 24hours (figure 3-2).

Parasitemia clearance in the presence of desferrioxamine B was relatively short-lived. The rise in parasitemia levels observed 24 hours after injection of desferrioxamine B could be attributed to the clearance of the drug from the blood after its injection. It has been noted that in contrast to ferrioxamine (the iron complex of desferrioxamine), desferrioxamine is effectively catabolised and rapidly cleared from the plasma (Hershko, 1992). The iron chelator has in addition to this, been observed to have high tubular excretion (Raymond *et al*, 1984). This leads to its early elimination from blood and cellular fluids (Hershko,

1992). This easy excretion could explain the relatively short duration of the antimalarial effects of single injections of desferrioxamine B as seen in the study.

Although the reduction in parasitemia achieved with desferrioxamine was modest, this noteworthy effect occurred almost immediately (3 hours) after injection of the drug into the experimental mice. This could be attributed to the rapid body-wide distribution of the drug (over 60% of the total body volume) and its easy access to the intracellular compartments (Hershko, 1992) including its availability in RBCs (Pollack *et al*, 1987).

Chloroquine has been used as a standard antimalarial and a first line drug for malaria control for a long time. Therefore, it was good that the antimalarial efficacy of desferrioxamine be compared to the effects of chloroquine under similar conditions. Five milligram per kilogram body weight of chloroquine was enough to induce almost complete clearance of parasites in the experimental mice. The drug continued to clear parasites from the blood 48 hours after the single injection used (figure 3-2).

It was evident that lower concentrations of chloroquine produced more dramatic reductions in percentage parasitemia of peripheral parasite than that produced by the higher concentrations of desferrioxamine B used. This could again be attributed to the easy catabolism of the drug as opposed to the long lasting properties of chloroquine which has been shown to maintain high cidal serum concentrations up to one week after a single injection (Cook, 1996). These observations suggest the usefulness of chloroquine as an antimalarial in conditions of little or no parasite resistance and iron chelation therapy as a supplement under these conditions (Kondrachine and Trigg, 1998).

The administration of desferrioxamine B with chloroquine has been used in the treatment of both cerebral (Thuma *et al*, 1998) and other forms of malaria (Traore *et al*, 1991; Gordeuk *et al*, 1993) in both children and adults. This combination has been reported to abate parasitemia more quickly than either chloroquine or desferrioxamine B when used alone and also at lower concentrations of both drugs than the amounts normally used when administered alone (Traore *et al*, 1991). The combination used in this study consisted of 2.5mg of chloroquine and 50mg of desferrioxamine B per kg body weight of mice. This drug combination was found to be effective in the clearance of parasitemia. Its parasite clearance characteristics were found to be similar to that of chloroquine (2mM) when administered alone at a concentration of 5mM (figures 3-2 and 3-3). A combination of desferrioxamine B and chloroquine therefore, proved to be more effective in the treatment of malaria at lower amounts of both the iron chelator and chloroquine (figure 3-3).

In both chloroquine and desferrioxamine B treated mice, there was progressive degeneration of parasite and parasite structures. Parasites in the chloroquine-treated group were unable to form hemozoin 3 hours after drug treatment (as shown by the structural changes observed under the light microscope in figure 3-6). Desferrioxamine-treated parasites began to exhibit this inability 6 hours after administration of the drug whilst mice in the control group did not show this impairment at all. This is evidence of damage to the ability of the growing parasite to detoxify heme into hemozoin in the presence of these antimalarial compounds.

In the desferrioxamine-treated group, adverse changes to parasite structures were most widespread six to twenty-four hours after drug injection. Here, most of the parasites at schizogony had disintegrated. Gametocytes appeared abnormal whilst most of the trophozoites had lost their cytoplasm and nuclei. These adverse changes however appeared to reverse thirty-six hours after injection of the drug. Regeneration of parasite structures and reversal of hemozoin loss coincided with the increase in parasitemia (figures 3-6 and figure 3-2). This may have occurred when the drug was completely metabolised and removed from circulation.

This result shows that after being effective for up to twenty-four hours, a single injection of desferrioxamine may be metabolised from the blood, reducing its effectiveness and allowing previously smouldering parasites to rejuvenate. To overcome this, a repeated dose of the drug would have been appropriate.

Compared to the effects of desferrioxamine, degeneration of parasite structures in chloroquine-treated mice appeared early (3 hours after drug injection) and was seen to proceed at a faster rate too. By the end of the experiment, all parasites in chloroquine-treated mice had disintegrated and previously infected red cells had regained their normal morphology.

These structural changes show desferrioxamine action, like that of chloroquine, is diffuse. Its effects likely to affect all important parasite structures including parasite nuclei and cytoplasm.

Desferrioxamine B, like chloroquine, was found to inhibit heme polymerisation *in vitro* (Table 3--2). The drug was found to prevent the polymerisation of heme to a significant extent (66.25%). This ability of the iron chelator to inhibit heme polymerisation *in vitro* seems to suggest that its suppression of malaria *in vivo* may be as a result of antiplasmodia action precipitated by the inhibition of hemozoin formation. This therefore seems to corroborate the work of Hershko, who proposed that, the antimalarial effect of desferrioxamine B is not as a result of a reduction in host iron status (Hershko *et al*, 1982). This inference contrasts sharply with the proposals of other researchers who postulate that the antimalarial action of the iron chelator is due to its sequestration of host iron (Golenser *et al*, 1997; Gordeuk *et al*, 1993) as well as iron required for parasite growth (Hepner *et al*, 1988).

Of all the compounds tested in the study, chloroquine was found to be the most powerful inhibitor of heme polymerisation. Inhibitions of 98.96% and 77.63% were observed for chloroquine concentrations of 5mM and 2mM respectively. Inhibition of heme polymerisation is in line with the current understanding of the mode of chloroquine action. Here, the antimalarial action of chloroquine is thought to be effected when the drug interferes with heme polymerisation into hemozoin either by inhibiting the parasite's heme polymerase enzyme (Slater and Cerami, 1992) or by binding to ferriprotoporphyrin IX groups (Margues *et al*, 1996; Sullivan *et al*, 1996; Moreau *et al*, 1982). Either way, chloroquine prevents the detoxification of poisonous heme groups that result from the parasite degradation of host hemoglobin leading ultimately to the death of the malaria parasites.

This degree of inhibition of the parasites by chloroquine is comparable to that produced by desferrioxamine B at a concentration of 5mM (table 3-2). This shows that lower amounts of chloroquine could produce antimalarial effects whereas higher concentrations of desferrioxamine are required to produce the same antimalarial effect. This observation confirms the continued usefulness and superiority of chloroquine as an antimalarial in conditions of little or no parasite resistance (Kondrachine and Trigg, 1998).

To inhibit heme polymerisation, the iron chelator must have access to and bind the central iron of heme. Therefore, it is thought that desferrioxamine B binds to this iron and makes it unavailable for extension into β -hematin (hemozoin).

Although citrate has some iron chelating properties (Raymond *et al*, 1984) it did not significantly inhibit heme polymerisation. It could only produce a negligible inhibition of 6.72%. This may be attributed to its lower affinity for iron (Raymond *et al*, 1984).

The other iron chelators namely acetohydroxamic acid, benzohydroxamic acid and rhodoturlic acid were however, good inhibitors (Table 3-3). Rhodoturlic acid forms a 2:3 chelate with ferric iron whilst benzohydroxamic acid and acetohydroxamic acid are in 3:1 chelate with iron. These chelators, produced inhibitions of 56.23%, 50.31% and 57.05% respectively, although they were relatively smaller in sizes. Therefore, the relatively high inhibition percentages observed were least expected. These high inhibition percentages were thought to be related to the small sizes of these chelators, which allow them more intimate access to the central iron of the heme in spite of the presence of the bulky tetra imine ring. This may explain the relatively high rates of inhibition of heme polymerisation as compared to bigger iron chelators like desferrioxamine B. As these

smaller iron chelators that have been reported to be less toxic to humans (Zevin *et al*, 1992), they have a potential use as antimalarials.

To investigate this theory of binding of iron and antimalaria activity of desferrioxamine B, UV-Visible and infrared spectroscopy were carried out on combinations of desferrioxamine/hematin and chloroquine/hematin to determine the associations that occur between them.

ELECTRONIC ABSORPTION (UV-VISIBLE) SPECTROSCOPY.

The number and types of atoms or groups disposed around a given central metal ion (e.g. Fe^{3+}) and the stereochemistry of the resulting complex are important in determining the stability, reactivity and the wavelength of maximum absorption (λ_{max}) exhibited by the compound (Jolly, 1976).

Any chemical change, such as a substitution, affecting either the central metal ion or the ligand shifts the wavelength of maximum absorption, increasing or decreasing the λ_{max} (Perrin, 1964, West *et al*, 1970).

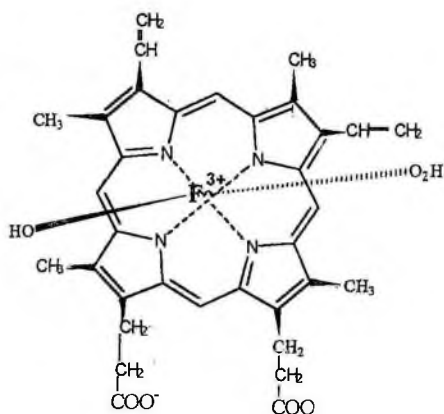


Figure 4-1: **Hematin in basic medium.**

Substitutions involving the introduction of unsaturated compounds or groups with a lone pair of electrons at the central ferric ion produce large downward shifts in the λ_{\max} (Lemberg and Legge, 1949).

Addition of desferrioxamine B to hematin in 0.1MNaOH at a ratio of 1:1 was observed to cause a large downward shift in λ_{\max} from 405nm to 370nm which is a shift of 35nm (chapter 3, figure 3-8). This large shift in wavelength appears to be the result of a substitution at the ferric ion involving a group or groups of atoms directly bonded to the central iron. Here, molecules of desferrioxamine are thought to replace these groups in these positions.

The binding groups of the drug contain three hydroxamate groups providing a total of six oxygen atoms and twelve lone pairs and negative charges (Raymond *et al*, 1984) that

come in direct contact with the central iron during the substitution. The presence of this large amount of electrons near the central iron atom could be responsible for the observed bathochromic shift.

In contrast to desferrioxamine B, the shift in the wavelength of maximum absorption of hematin observed on addition of chloroquine was small (402nm to 407nm) (figure 3-9). Appearance of the hematin peak at 402nm (instead of 405nm for hematin in basic medium) is indicative of chloroquine binding to hematin (Moreau *et al*, 1982).

However, from the small shift in the λ_{\max} observed, the binding appears to be at a site other than the central iron. This may be explained by the observation that substitutions at the carboxyl or any of the other side chains were expected to shift the λ_{\max} only slightly. This is because methyl groups, which discontinue the conjugation system of the ring (Kemp, 1987; Lemberg and Legge, 1949), separate the side chain groups from the iron-tetraimine complex.

Indeed, it has been observed that, the quinoline of chloroquine avidly binds to some of the groups (especially the carboxyl groups) in the side chain of the hematin molecule (Fitch, 1998; Sullivan *et al*, 1996; Moreau *et al*, 1982). The inability of chloroquine to bind to the central iron has been attributed to steric hindrance produced by the tetrapyrrole ring of the heme which prevents the bulky quinoline ring of chloroquine from approaching and binding to the central ion (Lemberg and Legge, 1949).

INFRA RED SPECTROSCOPY.

The changes induced by the addition of desferrioxamine B to hematin were confirmed by the infrared data presented in chapter 3 (table 3-6). The shifts in the characteristic bands and reduction in the strength of others suggest a substitution of ferric bonded hydroxyl groups with carbonyl groups. These results strongly indicate a substitution of the hydroxyl groups that were directly bonded to the iron of free hematin by carbonyl groups that come from desferrioxamine B.

These substitutions seemed to have resulted in removal of free hematin from solution as indicated by the loss of the characteristic peak of hematin in the infrared spectrum (figure 3-10) as a result of chelation and sequestration of the ferric ion by the iron chelator.

Although the drug binds to this central Fe^{3+} , it appears not to remove it from its position in the tetraimine macro-cyclic ligand (porphyrin ring).

The interactions between the drug and the heme used in this study, renders one ferric ion unavailable for further binding by other heme molecules in the process that would lead to extension of the hematin polymer. Binding of desferrioxamine B to the central ferric iron *in vivo*, would prevent extension of the hemozoin polymer, thereby disrupting the heme detoxification process in malaria parasites.

CONCLUSIONS.

The study confirms the antimalaria activity of desferrioxamine B and shows that the antimalaria activity is likely to be effected through the inhibition of heme polymerisation. The effects thereof, were observed on most of the important parasite structures and on all the various forms of the parasite (trophozoites, schizonts and gametocytes). The iron chelator was also shown to work by binding to the central ferric ion of heme.

RECOMMENDATIONS FOR FURTHER WORK.

It would be interesting to observe the ultrastructural effects of desferrioxamine B using the electron microscope.

Administration of a repeated dose of the iron chelator instead of a bolus injection as was used in this study, can also provide another line of investigation.

Further drug study should also examine the mechanism of chloroquine action in relation to the involvement of heme polymerase and binding of chloroquine to heme in the inhibition of heme polymerisation.

REFERENCES.

- Aджимани, J. P. and Emery, T. (1987). Iron uptake in *Mycelia sterilia* EP-76. *Journal Of Bacteriology*. **169**:3664-3668.
- Ahmed, K. (1989). Epidemiology of malaria in Ghana. *Ghana Medical Journal*. **23**:190-195.
- Basilico, N., Pagani, E., Monti, D., Ollisto, P. and Taramelli, D., (1998) A microtitre-based method for measuring the haem polymerisation inhibitory activity (HPIA) of antimalarial drugs. *Journal of Antimicrobial Chemotherapy*. **41**:1-6.
- Binka, F. Newton. (1997). Impact and determinants of permethrin impregnated bednets on child mortality in northern Ghana. *Inaugural dissertation presented to the University of Basel for Doctor of Philosophy degree*. Pages 58-103.
- Bitonti, A., Sjoersma, A., McCann, P. P., Kyle, D. E., Oduola, A. M. J., Rossan, R. N. and Davidson, D. E. Jr. (1988). Reversal of chloroquine resistance in malaria parasite *plasmodium falciparum* by desipramine. *Science* **242**:1301-1403
- Blauer, G. and Akkawi, M. (1995) β -heinin. *Biochem. Mol. Biol. In.t* **35**:231-235.
- Brase, C. H. and Brase, C. P. (1987). *Understanding statistics*. 3rd Edition. D. C. Heath and Company, Lexington. Pages 297-382.
- Bruce-Chwatt, L. (1980). *Chemotherapy of malaria*. WHO, Geneva. Pages 1-157
- Bruce-Chwatt, L. (1986). *Chemotherapy of malaria*. 2nd edition WHO, Geneva Pages 103-107.
- Chou, A. C. and Fitch, C. D., (1992). Heme polymerase: modulation by chloroquine treatment of a rodent malaria. *Life Sciences*. **51**: 2073-2078.
- Conklin, K. A., Chou, S. C., Siddiqui, W. A. and Schnell, J. V. (1973). DNA and RNA syntheses by intraerythrocytic stages of *Plasmodium knowlesi*. *J Protozool*. **20**:683-688.

Cook, G. (1996). *Tropical Diseases* 27th edition Saunders Publishing House New York. Pages 1131-1147.

Cotton, F. A. and Wilkinson, G., (1980). *Advanced Inorganic Chemistry; A Comprehensive Text*, 4th Edition. John-Wiley and Sons Inc., Toronto. Pages 1311-1328.

Desjardines, R. E., Canfield, C. J., Haynes, D. S. and Chuley, D. J. (1979). *Anti-Microbial Agents And Chemotherapy*. 16:710-718

Elford, B. C., Cowan, G. M. and Ferguson, S. J., (1995). Parasite-regulated membrane transport processes and metabolic control in malaria-infected erythrocytes. *Biochem. J.* **308**:361-374.

Fitch, C.D. (1998). Involvement of heme in the antimalarial action of chloroquine. *Trans. Am. Clin. Climatol. Assoc.* **109**:97-105

Goldberg, D. E. (1992). Plasmodial Hemoglobin Degradation: An Ordered Pathway in a Specialised Organelle. *Infectious Agents and Diseases*. **1**: 207-211.

Goldberg, D. E., Slater, A. F. G., Cerami, A. and Henderson, G. (1990). Hemoglobin degradation in the malaria parasite *Plasmodium falciparum*: An ordered process in a unique organelle. *Proc. Natl. Acad. Sci. USA*. **87**:2931-2935

Goldie, P., Roth, E. F., Oppenheim, J., Banderberg, J. P. (1990). Biochemical characterisation of *Plasmodium falciparum* hemozoin. *Am. J. Tro. Med. Hyg.* **43**: 584-596.

Golenser, J., Donb, A., Teonim, D., Tsafack, A., Nidim, O., Ponka, P., Eling, W. And Cabatchik, Z. I., (1997). The treatment of animal models of malaria with iron chelators by use of a novel polymeric device for slow drug release. *Journal Of Pharmacology And Experimental Therapy*. **283**: 1127-1135.

Goodsell, D. S., (1996). Dangers and Defences. *Our molecular nature the body's motors, machines and messages*. Springer-Verlag New York, Inc. Pages 110-119.

Gordeuk, V. R., Thuma, P. E., Brittenham, G. M., Zulu, S., Simwanza, G., M'hangu, A., Flesch, G. and Pary, D. (1992). Iron chelation with desferrioxaraine B in adults with asymptomatic *plasmodium falciparum* parasitemia. *Blood*. **79**: 308-312.

Gyang, F. N., Peterson, D. S. and Wellems, T. E. (1992). *Plasmodium falciparum*: rapid detection of dihydrofolate reductase mutations that confer resistance to cycloguanil and pyrimethamine. *Exp. Parasitol.* **74**: 470-472.

Gyang, F. N., Poole, B. and Trager, W. (1982). Peptidases from *plasmodium falciparum* cultured in vitro. *Molecular And Biochemical Parasitology.* **5**:286-273

Hamburger, M. and Hostettman, K., (1991). Bioactivity in plants:- the link between phytochemistry and medicine. *Phytochemistry* **30**: 3864-3874.

Hepner, D. G., Hallaway, P. E., Kontoghiores, G. J., and Eaton, J. W. (1988). Antimalarial properties of orally effective iron chelators. *Blood.* **72**:358-361

Hershko, C. (1992). Iron chelators in medicine. *Molecular Aspects of Medicine.* **13**:113-165.

Hershko, C. and Peto, T. E. (1988). Desferrioxamine inhibition of malaria is independent of host iron status. *Journal of Experimental Medicine.* **168**:375-387.

Hershko, C., Gordeuk V. R., Thuma, P. E., Theanachio, E. N., Spira, D. T., Hider, R. C., Peto, T. E. A. and Brittenham, G. M. (1992). The antimalarial effect of iron chelators: studies in animal models and in humans with mild falciparum malaria. *J. Inorg. Biochem.* **47**: 267-277.

Homewood, C. A. and Neame, K. D. (1980). Biochemistry of Malarial Parasites in *Malaria Epidemiology, Chemotherapy Morphology and Metabolism.* Edited by Krier, J. P. **1**: 345-405.

Jenson, J. B. and Tragger, W. (1977). *P. falciparum* culture; use of outdated erythrocytes and description of the candle jar method. *J. Parasitol.* **63**:883-886

Jolly, William (1991). *Modern Inorganic Chemistry.* 2nd edition McGraw Hill Inc. Pages 25-27.

Kombilla, M, Doung, T. H., Duffillot, D., Koko, J., Guiyedi, V., Guiguen, C., Ferrer, A. and Richard-Lenoble, D. (1997). Light microscopic changes in *Plasmodium falciparum* from Gabonese children treated with artemether. *Am. J. Med. Hyg.,* **57**:643-645

Kondrachine, A. V. and Trigg, P. I. (1997). Global Overview Of Malaria. *Ind. J. Med. Res.* **106**: 39-52.

Kondrachine, A. V. and Trigg, P. I. (1998). Commentary: malaria control in the 1990s. *WHO Bulletin* **76**: 11-16.

Lemberg, R and Legge, J. W. (1949). *Hematin compounds and bile pigments; their constitution metabolism and function*. Interscience Publishers Ltd, London. Pages 3-17 and 137-169.

Manson-Bahr, P. E. C. and Bell, D. R. (1987). Manson's tropical diseases, 19th edition. Balliere Tindall. Pages 5-41.

Marques, H. M., Voster, K. and Gluzman, I. Y. (1996). The interaction of the heme-octapeptide, N-acetylmicroperoxidase-8 with antimalarial drugs: solution studies and modelling by molecular mechanics methods. *J. Inorg. Biochem.* **64**:7-23

Matiney, J. A., Cerami, A. and Slater, A. F. (1996). Inhibition of hemozoin formation in *plasmodium falciparum* trophozoite extracts by heme analogs: possible implication in the resistance to malaria conferred by the beta-thalassemia trait. *Mol. med.* **2**:236-246.

Meshnick, S. R. (1994). The mode of action of antimalarial endoperoxides. *Trans. Roy. Soc. Trop. Med. Hyg.* **88**:31-32.

Moreau, S, Perlym B. And Biguet, J. (1982). Interaction of chloroquine with ferriprotoporphyrin IX. Nuclear Magnetic resonance studies. *Biochimie* **64**:1015-1025

Neilands, J. B. and Ratledge, C., (1980). Microbial iron transport compounds. *Microbiol. Res.* **9**:565-573.

Neilands, J. B., (1981). Microbial iron compounds. *Ann. Rev. Biochem.* **50**:715-735.

Octave, J. N., Schneider, Y. J., Crichton, R. R. and Trouet, A. (1983). Iron mobilisation from cultured hepatocytes: effect of desferrioxamine B. *Biochemical Pharmacology.* **32**: 3413-3418

Perrin, D. D. (1964). Organic complexing reagents: structure, behaviour, and application to organic analysis. *Chemical Analysis*. **18**:15-17

Peters, Wallace (1980). Chemotherapy of malaria in *Malaria; epidemiology, chemotherapy, morphology and metabolism*. Edited by Kreier. Academic Press Inc. Sydney. **1**: 145-279.

Peters, Wallace, (1987) Chemotherapy and drug resistance. *Malaria*. Pages 102-212. Academic Press, Orlando

Peterson, S. D., Milhous, W. K. and Wellems, T. E. (1990). Molecular basis of differential resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum* malaria. *Proc. Natl Acad. Sci. USA*. **87**:3018-3022

Peto, T. E. A. and Thompson, J. L. (1986). A reappraisal of the effects of iron and desferrioxamine on the growth of *plasmodium falciparum* 'in vitro': the unimportance of serum iron. *Br. J. Haem.* **63**: 273-280.

Pippard, M. J., Johbson, D. K. and Finch, C. A. (1982). Hepatocyte iron kinetics in the rat explored with an iron chelator. *Br. J. Haem.* **52**:211-224.

Pollack, S., Rossan, R. N., Davidson, D. E., Escajadillo, A. (1987). Desferrioxamine suppresses *Plasmodium falciparum* in monkeys. *Proc. Soc. and Exp. Biol. Med.* **184**: 162-164.

Prescott, L. M., Harley, J. P. And Klein, D. A. (1993). *Microbiology*. 2nd Edition. Wm. C. Brown Publishers, Iowa. Pages 113-130.

Raymond, N., Muller, G. and Matzanke, B. F. (1984). Complexation of iron by siderophores; A review of their solution, structural chemistry and biological function. *Topics in current chemistry*. **23**:49-102

Rice-Evans, C. and Baysal, E. (1987). Iron-mediated oxidative stress in erythrocytes. *Biochemical Journal*. **244**:191-196.

Scott, D. M., Ranz, A., Kuypers, F. A., Lubin, B. H. and Meshnick, S. R. (1990). Parasite uptake of desferrioxamine: a prerequisite for antimalarial activity. *Br. J. Haem.* **75**:598-602.

Shanzer, A., Libman, J., Lytton, S. D., Glickstein, H. and Cabantchik, Z. I. (1991). Reversed siderophores act as antimalarial agents. *Proc. Natl Acad. Sci. USA*. **88**: 6585-6589.

Sherman, I. W. and Tanigoshi, L. (1971). Incorporation Of ¹⁴C-Amino -Acids By Malaria (*Plasmodium Lophurae*). III. Metabolic Fate Of Selected Amino-Acids. *International Journal of Biochemistry*. **2**: 41-48.

Slater, A. F. G. and Cerami, A. (1992). Inhibition By Chloroquine Of A Novel Heam Polymerase Enzyme Activity In Malaria Trophozoites. *Nature*. **355**: 167-169.

Stone, R. H, Cozens, A. B. and Ndu, F. O. C. (1985). New Biology for West African Schools. 2nd Edition Longman Group Ltd, Warrington. Pages 7-9.

Sullivan, D.J., Gluxman, I.V., Russel, D.G. and Goldberg, D.E. (1996). On the molecular mechanism of Chloroquine's antimalarial action. *Proc. Natl Acad. Sci. USA*. **93**:11865-11870.

The Quingausu Antimalaria Research co-ordination Group. (1979). Antimalarial studies of quinghaosu. *Chin. Med. J*. **92**:811-816

Theanacho, E. N., Sarel, S., Samuno, A., Avramocici-Grisaru, S., Sua, D. T. (1991). Growth Inhibition of *Plasmodium falciparum* Involving Carbon Centred Iron-Chelate Radical (L., X-)-Fe (III) Based on Pyridoxal-Betaine. A Novel Type of Antimalarials Active Against Chloroquine-Resistant Parasites. *Free Radical Research Communication*. **15**: 1-10.

Traore, O., Carnevale, P., Kapture-Noche, L., M'Bede, J., Desfontaine, M., Elion, J., Labie, D. and Nagel, R. L. (1991). Preliminary Report on the use of Desferrioxamine in the Treatment of *Plasmodium falciparum* Malaria. *Ame. J. Hem*. **37**: 206-208

Walter, R. D., Nordmeyer, J. P. and Konigk, E. (1974). NADP-specific glutamate dehydrogenase from *Plasmodium chabaudi*. *Zool. Phy. Chem*. **355**:495-500.

Weinberg, E. D., (1974). Iron and susceptibility to infectious diseases. *Science*. **184**:952-955.

Wellems, E. T., (1992). How chloroquine works. *Science* :**355**:108-109

Wernsdorfer, W. (1981). Drug-resistant malaria: The report of a meeting held in Kuala Lumpur, Malaysia 10-15 August 1981. UNDP/WHO/World Bank. Pages 59-79

West, W. S., Todd, W. R., Nason, H. S. and Breggen, J. T. V. (1970). *Textbook of Biochemistry*, 4th edition. The Macmillan Company, London. Pages 593-599.

WHO (1973). *Technical Report Series*. 529:3-17

WHO (1997). Bases for development of recommendation on protection for short-term travellers against malaria WHO/Mal/87.1040. Pages 3-23.

WHO Scientific Group (1984). *WHO Technical Report Series* 711:4-218

Zevin, S., Link, G., Grady, R. W., Hider, R. C., Peter, H. H. and Hershko, C. (1992). Origin and fate of iron metabolised by the 3-hydroxypyridin-4-one oral chelators: studies in hypertransfused rats by selective radioiron probes of reticuloendothelial and hepatocellular iron stores. *Blood*. 79:248-253.

Zubay, G. L., (1983). *Biochemistry*. Addison Wesley Pub Inc.; New York. Pages 227-228

APPENDICES

APPENDIX A

PREPARATION OF SOLUTIONS AND BUFFERS.

PREPARATION OF PHOSPHATE BUFFERED SALINE (PBS).

Phosphate buffered saline was prepared by dissolving 8.500g of NaCl and 1.096g of NaH_2PO_4 in distilled water and the pH brought up to 7.4 by adding drops of 1M NaOH. The mixture was made up to 1000 ml with more distilled water and mixed thoroughly. The solution was then made sterile by filtration through a 0.2 μM Millipore filter into sterile 50ml tubes in a sterile laminar flow hood. The PBS so prepared was stored at 4°C until used.

PREPARATION OF CHLOROQUINE.

3000 μL of 10mg/ml chloroquine was prepared from a stock of 64.5mg/ml chloroquine phosphate BP (containing 40mg/ml chloroquine base for intramuscular injection) by adding 2,250 μL of sterile PBS to 750 μL of chloroquine solution.

100 μl of this dilution was injected intraperitoneally into each member of group 2 mice which weigh 22g on the average. This produced an *in vivo* concentration of 5mg/Kg body weight

PREPARATION OF DEFERRIOXAMINE B

500mg of Deferal® (desferrioxamine mesylate) was dissolved in 2,500 μl of sterile PBS to yield a concentration of 200mg/ml. 100 μl of this solution was injected into each mouse of group three, also of average weight 22g to attain an *in vivo* concentration of 100mg/kg body weight.

APPENDIX B.**Table B-1: Growth Pattern of *Plasmodium Berghei Anka* in CBA Mice Treated with Desferrioxamine B Compared to Control Mice Given Phosphate Buffered Saline.**

Time (hours) after injection	Mean percentage parasitemia		Statistical test of significance*		
	control grp (pbs)	df treated grp	critical val	P-value	Diff
0	25.17 ± 4.74	30.36 ± 11.93	15.80	-5.19	NS
3	28.16 ± 3.53	38.49 ± 2.89	5.62	-10.33	NS
6	30.17 ± 8.13	32.84 ± 9.22	15.13	-2.67	NS
12	36.45 ± 5.96	33.23 ± 13.70	18.39	3.21	NS
24	47.78 ±	32.53 ± 7.76	7.24	16.32	S
36	51.54 ±	39.54 ± 9.61	11.95	12.00	S
48	57.15 ± 4.04	42.76 ± 8.11	11.36	14.39	S

* one-tailed test at 95% confidence interval

S significant

NS not significant

Diff difference

Table B-2: Growth Pattern of *Plasmodium Berghei Anka* in Chloroquine-Treated CBA Mice Compared to Control Mice Given Phosphate Buffered Saline.

Time (hours) after injection	Mean percentage parasitemia		Statistical test of significance.*		
	Control grp (pbs)	CHQ treated grp	Critical val	P-value	Diff.
0	25.17 ± 4.74	32.00 ± 5.38	8.82	-13.02	NS
3	28.16 ± 3.53	33.94 ± 9.22	12.17	-5.78	NS
6	30.17 ± 8.13	27.86 ± 4.09	11.20	2.31	NS
12	36.45 ± 5.96	21.72 ± 8.73	13.00	14.76	S
24	47.78 ± 86	9.89 ± 3.57	4.68	38.96	S
36	51.54 ± 1.39	3.35 ± 1.20	2.26	48.19	S
48	57.15 ± 4.04	0.51 ± 0.07	4.97	56.64	S

* One-tailed test at 95% confidence interval
 S significant
 NS not significant
 Diff difference

Table B-3: Growth Of *Plasmodium Berghei Anla* in Chloroquine Treated Mice Compared Directly to Desferrioxamine B Treated Mice.

Time (hours) post-injection	Mean percentage parasitemia		Statistical test of significance.*		
	CHQ treated grp	df treated grp	Critical value	P-value	Diff.
0	32.00 ± 5.38	30.36 ± 11.93	± 20.98	1.54	NS
3	33.94 ± 9.22	38.49 ± 2.89	± 15.49	-4.55	NS
6	27.86 ± 4.09	32.84 ± 9.22	± 16.17	-4.98	NS
12	21.72 ± 8.73	33.23 ± 13.70	± 26.04	-11.55	NS
24	9.89 ± 3.57	32.53 ± 7.76	± 10.86	-22.64	S
36	3.35 ± 1.20	39.54 ± 9.61	± 15.52	-36.19	S
48	0.51 ± 0.07	42.76 ± 8.11	± 13.00	-42.25	S

* two-tailed test at 95% confidence interval.
S significant
NS not significant
Diff difference

Table B-4: Growth of *Plasmodium berghei anka* in Chloroquine Treated Mice Compared to Desferrioxamine B Treated Mice.

Time (hours) post-injection	Mean percentage parasitemia		Statistical test of significance.*		
	CHQ-treated grp	df-treated grp	critical value	P-value	Diff
0	32.00 ± 5.38	30.36 ± 11.93	± 20.98	1.54	NS
3	33.94 ± 9.22	38.49 ± 2.89	± 15.49	-4.55	NS
6	27.86 ± 4.09	32.84 ± 9.22	± 16.17	-4.98	NS
12	21.72 ± 8.73	33.23 ± 13.70	± 26.04	-11.55	NS
24	9.89 ± 3.57	32.53 ± 7.76	± 10.86	-22.64	S
36	3.35 ± 1.20	39.54 ± 9.61	± 15.52	-36.19	S
48	0.51 ± 0.07	42.76 ± 8.11	± 13.00	-42.25	S

* Two-tailed test at 95% confidence interval.

S Significant

NS Not significant

APPENDIX C.**Table C-1: Hematin Calibration Curve.**

Hematin Conc. (mM)	Absorbance			Mean Absorbance
1.00×10^{-1}	3.377	3.473	3.428	3.4265
5.00×10^{-2}	1.780	1.731	1.775	1.7628
2.50×10^{-2}	0.848	0.864	0.878	0.8653
1.25×10^{-2}	0.481	0.521	0.497	0.5010
6.25×10^{-3}	0.262	0.273	0.277	0.2730
3.13×10^{-3}	0.171	0.173	0.181	0.1758
1.56×10^{-3}	0.063	0.088	0.112	0.0995
7.81×10^{-4}	0.090	0.086	0.081	0.0865

Table C-2: Absorbances and the Corresponding Hematin Concentrations in the Heme Polymerisation Inhibition Assay.

Compound	Absorbance			Hematin Conc. (mM)			Mean Hematin Conc. (mM)	Percent Inhibition
	Control	0.9040	0.9041	0.9039	2.53×10^{-2}	2.53×10^{-2}	2.53×10^{-2}	2.53×10^{-2}
Citrate (0.5M)	0.9610	0.9609	0.9609	2.69×10^{-2}	2.69×10^{-2}	2.69×10^{-2}	2.70×10^{-2}	6.72
DF (0.05M)	0.3370	0.3370	0.3370	8.60×10^{-3}	8.54×10^{-3}	8.54×10^{-3}	8.54×10^{-3}	66.25
Chloroquine at (0.02M)	0.2395	0.2396	0.2395	5.66×10^{-3}	5.66×10^{-3}	5.66×10^{-3}	5.66×10^{-3}	77.63
Chloroquine (0.05M)	0.0565	0.0564	0.0565	2.63×10^{-4}	2.60×10^{-4}	2.63×10^{-4}	2.63×10^{-4}	98.96

Percentage inhibition was calculated as follows:

$$\text{Percentage inhibition} = \frac{[\text{Hematin Conc.}_{(\text{control})} - \text{Hematin Conc.}_{(\text{test})}]}{\text{Conc.}_{(\text{control})}} \times 100\%$$

Where:

Hematin conc._(control) = Amount of hematin polymerised in control well.

Hematin conc._(test) = Amount of hematin produced in test well.