Section - I
Chapter 1

ANTIMALARIAL DRUGS, THEIR KNOWN MODE OF ACTIONS AND MECHANISMS OF DRUG RESISTANCE - A BRIEF REVIEW

Abstract: Brief historical review of antimalarial drug development and deployment shows that en masse drug deployment for chemoprophylaxis and monotherapy inevitably results in quick selection of drug resistance in Plasmodia. Drug combinations survive longer. The chemical structure, biological activity and mode of action of important antimalarials of the century have been discussed. Mechanisms by which parasites become resistant to dihydrofolate reductase (DHFR) inhibitors have been identified to specific point mutations in the DHFR gene of the parasite, thus altering the structure of the enzyme which avoids affinity binding of the drug. Advent of resistance to chloroquine, the most affordable low cost drug, is a cause of concern. Our inadequate knowledge about the subject is updated and highlighted for inclusion in future research agenda among other listed priorities. Old remedies like 'qinghaosu', quinine and other cinchona alkaloids have brought new hopes for the time being.

INTRODUCTION

Around the turn of this century, malariology enjoyed certain vogue, inspired largely by: a) the interest of colonial powers in places where malaria was rampant; b) the explorative expeditions to the tropical locations of the developing world; and c) the exciting discoveries of Ross, Ehrlich and others. The popularity and excitement in the study of malaria subsided for next few decades in favour of prokaryotic infectious diseases. Short supply of the main antimalarial drug of the time, quinine, was experienced during the two World Wars by the developed countries. This shortage in the meanwhile had instigated the efforts in malaria research leading, immediately after the Second World War, to two dazzling events which were to determine and dominate malarial strategy for next few decades. The first was the success of the residual insecticides in dramatically curtailing transmission of malaria in many countries and the second was the development of more than one synthetic antimalarial drugs which became available in rapid succession. Never before had man stockpiled such powerful arsenals to rid himself of malaria malady, so old an adversary. This led enthusiastically in 1955 to launching of malaria eradication programmes globally by the World Health Organization (WHO), one of the largest international efforts to improve the health in the developing world, but the initial enthusiasm slowly faded due to several technical as
well as socio-economic factors and this venture finally ended in 1970 when WHO decided to 'throw in the towel'. During this period support for further research into malaria had dried up throughout the world. In the meantime casualties inflicted to the U.S. forces in Vietnam War by drug-resistant parasites prompted the developed world to know more about the cure and prevention of malaria. This not only revived but intensified research and funding into several aspects of biology of parasitism of *Plasmodium* (causative agent of malaria) including molecular biology, immunology, vaccinology and chemotherapy. The sudden surge of research activity on plasmodia demanded ever increasing supplies of the parasite material, infecting man in particular, thus stressing an urgent need to tame the parasites in vitro. In 1976 Professor William Trager reported (Trager and Jensen 1976) the first successful continuous cultivation of erythrocytic stages of *P. falciparum* (causing malignant tertian human malaria) and this salutary breakthrough stimulated further research activity related to malaria parasites. It also provided a fresh impetus to monitoring of the emergence and spread of drug resistance in falciparum throughout the world. This article discusses the basic structure and biological activity of important antimalarial drugs used in the current century with special emphasis to chloroquine and DHFR inhibitors, their modes of action and mechanisms of resistance, analysis the causes leading to speedy selection of resistant parasites, suggests measures to impede the spread of resistance and future research strategies for development of new antimalarial drugs.

**Basic Chemical Structure of Antimalarials**

Known antimalarials have been variously grouped. They may be classified based on the following basic chemical structures (Figure 1.1):

**Pyrimidine derivatives:** The amino groups at the 2 and 4 positions of the pyrimidine forms the basis of synthetic antimalarials like pyrimethamine and other less popular compounds.

**Quinoline derivatives:** Fusion of a pyridine and a benzene ring gives rise to a
bicyclic quinoline ring. It forms the basis of a number of synthetic antimalarial compounds which are derivatives of the 4 or the 8 amino substituted quinoline. Chloroquine, a 4-aminoquinoline and primaquine, a 8-aminoquinoline are the two well known examples. Some of the natural cinchona alkaloids also have quinoline in their structure, quinine for instance is formed by complex substitution at the 4 position of the quinoline ring.

*Acridine derivatives:* Acridine is a polycyclic compound formed by the fusion of two benzene rings and pyridine. Mepacrine is an antimalarial drug which is a 9-amino acridine derivative belonging to this group. It is largely obsolete as an antimalarial for causing coloration of the skin and transient mental disturbances. *Non-cyclic derivatives:* A few groups of antimalarials posses non-cyclic basic structure and substitution at some positions with benzene ring derivatives. They are the biguanide and the sulfa compounds. The sulfa compounds may further be grouped into sulfones and sulfonamides. Proguanil is an antimalarial drug derived from biguanide. Sulfadoxine and dapsone are the derivatives obtained from the modification of sulfonamide and sulfone, respectively.

**Basic Biological Activity of Antimalarials**

Antimalarial drugs can also be classified based on their predominant site of action on the particular stage(s) in the life cycle of malarial parasite. There are four species of *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*) usually known to infect man. Malaria results when the infective stages of the parasite are injected into the bloodstream of man by an infected female *Anopheles* mosquito. The sporozoites immediately take shelter within hepatocytes of the host for further development. Maturation of all these intracellular protozoan parasites finally results in the release of pre-erythrocytic merozoites, almost simultaneously in *P. falciparum* and *P. malariae*, whereas in *P. vivax* and *P. ovale* some of the liver stage parasites may remain dormant for variable duration, for
months or years. These dormant stages are called hypnozoites, subsequently blossoming into pre-erythrocytic merozoites, causing relapse of malaria. The drugs which prevent the maturation of pre-erythrocytic stages are called tissue schizonticides and those acting on the hypnozoites are known as anti-relapse drugs. The pre-erythrocytic merozoites invade the red cells leading to the formation of daughter merozoites, by schizogony (asexual division), which on release invade the uninfected erythrocytes. The pathogenicity of malaria is chiefly due to synchronized burst of erythrocytic schizonts leading to malaria symptoms, namely fever, shivering, hemolytic anemia and sometimes (in infection with *P. falciparum*) to cerebral malaria and death. Blood schizontocides are toxic or lethal to intraerythrocytic parasites and thus relieve the host of malaria symptoms. Some of the merozoites on entering the erythrocytes start differentiating into the gametocytes (sexual forms) instead of undergoing usual asexual multiplication. The mature gametocytes will develop further only in the female *Anopheles*. The drugs which prevent the formation or differentiation of sexual forms are termed as gametocytocidal drugs. Male and female gametocytes form respective gametes which fertilize in the stomach of the insect vector to form the diploid zygote. The zygote continues its sporogonic development from oocyst to sporozoites. The compounds interfering with the sporogonic cycle are recognized as sporontocidal compounds, thus preventing the transmission of malaria. They have also been called anti-sporogonic chemicals.

**Mechanisms of Action and Resistance**

The main technical reasons for the failure of malaria eradication programme had been the development of resistance (exophilly) to residual insecticides by insect vectors and the emergence of drug resistance in parasites to the antimalarials. Improvements in malaria chemotherapy should result from better understanding of the mechanisms that govern drug action and drug resistance. The two criterions of chemical structure and biological activity of antimalarials on plasmo-
dia have been clubbed for convenience to discuss some of the known modes of action and resistance to antimalarial drugs.

1. Folate Pathway Blockers

A Mode of Action: Folate pathway blockers includes the antimetabolite drugs which interfere with folic acid cycle of the parasite, in one way or the other. Reduced folic acid derivatives are indispensable cofactors for de novo synthesis of pyrimidines in plasmodia. In the absence of any salvage pathway for the utilization of exogenous pyrimidines, depletion in the pool of reduced folic acid derivatives in the parasite milieu results in blocking of Plasmodium multiplication. The folate pathway blocker antimalarial drugs can be grouped into two broad categories, the dihydrofolate reductase (DHFR) inhibitors and the sulfa drugs which are structurally similar to para-aminobenzoic acid (PABA). Two commonly used DHFR inhibitor antimalarial drugs are pyrimethamine and proguanil whereas sulfonamides and sulfones are employed as antimalarials mostly in combination with pyrimethamine.

Proguanil was developed in Britain during the Second World War. While evaluating derivatives of pyrimidine, compounds of the chloroguanil series proved promising, opening the pyrimidine ring further simplified the structure to biguanide (Curd et al. 1945), which were to have good antiplasmodial activity. Thus proguanil (chloroguanide) was discovered. The efforts of British group in collaboration with American scientists further culminated in the development of pyrimethamine (Falco et al. 1951), first introduced in 1952 for routine human use. Both the drugs are employed for prophylaxis and suppression of malaria infection in man. They act on the dividing stages of the parasites but not on the dormant (hypnozoite) or growing sexual stages (developing gametocytes). They have the important properties of low toxicity. Pyrimethamine has added advantages of being effective in much smaller doses and longer half life with slow excretion rate compared to proguanil.
Proguanil and pyrimethamine act as antimalarial drugs by virtue of their great affinity for the plasmodial enzyme DHFR (Ferone et al. 1969). The enzyme gets inhibited following binding of the drug. DHFR catalyzes the conversion of dihydrofolate to tetrahydrofolate and occupies a key position in cellular biosynthesis of purines, pyrimidines and certain amino acids. DHFR is present both in the parasite and the host cells. The basis of selective action of these drugs is their differential (greater) binding to the plasmodial DHFR and the extraordinary sensitivity of this action to the nuclear division of the malaria parasite at the time of development of schizonts in erythrocytes and in the liver tissue (Schellenberg and Coatney 1967; Gutteridge and Trigg 1971; Inselburg and Banyal 1984; Janse et al. 1986). The affinity of *P. berghei* (rodent malaria parasite) DHFR for pyrimethamine is 2000 fold greater than the mouse erythrocyte enzyme (Ferone et al. 1969). Proguanil is not active against plasmodia in vitro but one of its metabolite, cycloguanil is the active intermediate having chemical structure somewhat similar to that of pyrimethamine (Carrington et al. 1951) (Figure 1.1). The activation of proguanil to cycloguanil is done by the mixed function oxidase system of the hepatic microsomes (Armstrong and Smith 1974). It is not known if pyrimethamine and cycloguanil bind to the same region of the plasmodial DHFR or occupy different sites.

Spectacular successes of sulfonamides as antibacterial drugs in 1930s led to the evaluation of their antiplasmodial action. In 1937 the antiplasmodial activity of some of the derivatives was recognized against human malaria (Anand 1979). The sulfonamides and sulfones exert activity against all actively dividing stages in the life cycle of plasmodia. Among the human plasmodia *P. falciparum* is more susceptible, whereas *P. vivax* is less so (Bruce-Chwatt et al. 1981). The earlier developed sulfa drugs acted slowly for short duration and the need for high and potentially toxic doses were responsible for their shelving, as also more reliable 4-aminoquinoline compounds became available. However, advent of resistance to 4-aminoquinolines has revived interest in sulfa drugs as they retain full activity.
against chloroquine resistant parasites (Peters 1990a) and these drugs act synergistically with the DHFR inhibitors (Bartelloni et al. 1967; Harinasuta et al. 1967). The mode of action of the sulfonamides and sulfones against plasmodia is considered analogous. Antimalarial sulfa compounds are structurally similar to para-aminobenzoic acid and the commonly accepted view is that these drugs are competitively antagonized by PABA and non-competitively by folic acid (Watkins et al. 1985). PABA is incorporated into the coenzyme folic acid; inhibition of folic acid synthesis by these drugs has been demonstrated and the enzyme involved was to be tetrahydropteric acid synthetase. Another study supports the view that altered permeability of the erythrocyte membrane might be responsible for action of sulfa drugs as dapsone has been demonstrated to accumulate at the surface of rat erythrocytes (Canedella and Jarrell 1970), leading to the reduced uptake of glucose in the erythrocytes and inhibition of glucose consumption by intraerythrocytic parasite.

**B. Mechanism of Resistance:** The use of DHFR inhibitors as antimalarials has been curtailed because of rapid development of resistance by the parasites. Shortly after the release of each drug, prophylactic and therapeutic failures against *Plasmodium* infections were reported (Peters 1970) from various places. Resistance to DHFR inhibitors by several strains of *Plasmodium* has subsequently become a major problem throughout the world. The ease with which resistance to DHFR inhibitors developed both in the field and laboratory suggested that single mutation in the *Plasmodium* DHFR segment of the genome might be enough to render these drugs ineffective (Bishop 1962; Diggens et al. 1970; Peters 1987). The experimental evidence as to the genetic basis of resistance came from the analysis of a cross between pyrimethamine resistant and sensitive lines of rodent malaria resulting in inheritance patterns compatible with the involvement of just a single gene locus (Walliker et al 1975, 1976; Knowlesi et al. 1981). Kinetic studies further revealed that DHFR from resistant *Plasmodium* binds less tightly to py-
rimethamine, as there was marked increase in inhibition constant ($K_i$) with respect to this drug (Ferone 1970; Sirawaraporn and Yuthavong 1984; Mc Cutchan et al. 1984; Walter 1986; Dieckman and Jung 1986), strongly suggesting the altered structure of the enzyme DHFR in resistant parasites. An important feature of \textit{P. falciparum} DHFR is that the activity resides within a bifunctional protein together with thymidylate synthetase (TS) (Garrett et al. 1984). Another novel property of the DHFR from resistant parasites is that it retains its normal enzymatic function but at the same time inhibits binding of the drug. First complete sequence of DHFR-TS was published in 1987 (Bzik et al. 1987). There work showed that as in \textit{Leishmania} there is a single intronless open reading frame that includes a junction region linking the DHFR and TS domains. The DHFR domain extends from the N-terminus to amino acid residue 225 approximately, while the TS domain occupies roughly the final 290 amino acids of the protein leading to the C-terminus at residue 607. The junction regions thus extends from about residue 225 to residue 320. Many of the or postulated active site residues in both DHFR and TS are found to be conserved in the \textit{P.falciparum} molecule (Bzik et al. 1987; Cowman et al. 1988). The DHFR-TS gene has been shown to reside on the fourth fastest chromosome in several strains (Peterson et al, 1988; Cowman et al., 1988; Snewin et al., 1989; Tanaka et al., 1990). Ever since, structure of the DHFR gene from number of resistant and sensitive falciparum clones/isolates have been compared by sequencing. This rapid accumulation of sequencing data has been made possible by in vitro amplification of DHFR region of the genome using polymerase chain reaction (PCR). Analysis of sequence data of natural resistance in diverse number of geographical distinct isolates shows that a single point mutation (resulting in Ser. 108 to Asn. 108) in the DHFR active site is perfectly linked to pyrimethamine resistance (Table 1.1). Two ancillary mutations Asn. 51 to Ile. 51 and Cys. 59 to Arg. 59 were found to be associated with increased level of resistance in association with Asn. 108 (Cowman et al. 1988; Peterson et al. 1988; Snewin et al. 1989; Tanaka et al.
1990). Experimentally induced resistance in *P. falciparum* using mutagens in vitro (Banyal and Inselburg 1986) showed that amino acid changes at entirely different sites than that seen in naturally resistant pyrimethamine parasites (Inselburg et al. 1988; Tanaka et al. 1990). This clearly shows that there are many sites in the DHFR gene where point mutations can alter the structure of the enzyme, preventing or retarding drug binding, without compromising with catalytic functions. More studies are needed to ascertain the extent of polymorphism of these sites in diverse geographical isolates.

The finding that specific changes in DHFR base pairs are linked to pyrimethamine resistance indicated that point mutations might also be responsible for resistance to cycloguanil. Sequence data analysis of cycloguanil resistant DHFR revealed that parasites with paired Ser. 108 to Thr. 108 and Ala. 16 to Val. 16 mutations were found to be resistant to cycloguanil but showed only a slight decrease in sensitivity to pyrimethamine. Parasites refractory to both the DHFR inhibitors had three mutations in common Cys. 59 to Arg. 59, Ser. 108 to Asn. 108 and Ile. 164 to Leu. 164 (Foote et al. 1990; Peterson et al. 1990). Identification of specific mutations in DHFR segment of genome opens up the possibility of mutation specific oligonucleotide probes, using PCR, capable of differentiating sensitive and resistant parasites (Zolg et al., 1990).

Resistance to sulfas has not been widely recorded with human plasmodia. For many years these drugs were exclusively used in combination with DHFR inhibitors mainly pyrimethamine. Of late there have been reports of emergence of resistance to these combinations from Brazil and Southeast Asia in *P. falciparum* (Brockelman and Tan Ariya 1982). There is, of course, little doubt that plasmodia resistant to the combination also are resistant to sulfas alone.

2. **Quinoline-Containing Antimalarials (QCA)** They essentially include blood schizonticides (except for primaquine). Cinchona alkaloids (quinine) and other 4-
quinolinemethanol (mefloquine); chloroquine (4-aminooquinoline), primaquine (8-aminooquinoline), are some of the drugs dealt in this section.

**Cinchona Alkaloids:**

Quinine, one of the major alkaloid components of cinchona bark, has saved mankind from ravages of dreaded malaria disease for more than 350 years and continues to cure malaria, any malaria even today! It acts on the multiplying asexual stages of the parasite but has no lethal effect on the exoerythrocytic stages, it is gametocytocidal for *P. vivax* and *P. malariae* but toxic only to the developing gametocytes of *P. falciparum*. Quinine toxicity to malaria parasite is based on concentrative uptake of the drug in infected erythrocytes leading to cessation of motility and haemozoin formation. It stands apart, in an era, when life cycles (usefulness) of drugs are mostly expressed in decades rather than centuries. Indiscriminate use of this drug shall definitely lead to selection of resistant strains of *Plasmodium*, as had been the case in the past with other drugs. We cannot afford this to happen now, at the time when quinine seems to be the only effective drug against the multi-drug-resistant (polyresistant) malaria strains. Time demands the strategies to prolong and preserve the antimalarial efficacy of cinchona alkaloids and derivatives. The question is how and why this drug has survived as an effective antimalarial for so long. If that is answered then perhaps one might be able to suggest ways to delay the emergence of resistance to this drug. Mostly crude bark was used for preparation of powder and infusion. As we know now that this extract from 'bark to bottle' contained not only quinine but other alkaloids as well in varying amounts, some of them even more potent than quinine. Even the 'pure' quinine salts, as quinine sulphate, available contained other cinchona alkaloids (Smit, 1987) to the extent of 16% in 1890, 8.5% in 1970 and 6% in 1986 (Figure 1.2). Clearly the alkaloid mixture used in the past for treatment, survived in retaining the antiplasmodium virtues for centuries. Even modern medicine
advocates drug combination over monotherapy to prolong the life span of the drugs (Peters 1990c). Quinine is not the most effective of the four major alkaloids found in cinchona bark. In vitro also some of the cinchona alkaloids and synthetic derivatives are more potent than quinine (Nair and Bhasin 1993). It has reached its unique position because it just happened to be the first to be isolated and used in 'pure' form. Perhaps the solution to the malaria problem even today can be rediscovered where it was first found - in the bark of cinchona tree. Of late, increasing failure rates of old and new synthetic drugs has again drawn the attention of research workers to the oldest natural antimalarial drug.

Mefloquine (4-quinolinemethanol):

Mefloquine may be considered the third generation quinolinemethanols. The first generation is represented by cinchona alkaloids, the second (less known) by the compounds in which the cinchona side chain was modified with introduction of 2-phenyl substituent and the third by mefloquine in which the 2-phenyl moiety is replaced by a trifluromethyl group (Figure 1.1.). Each generation of quinolinemethanols brought an improvement in the therapeutic properties of the representative drugs. Mefloquine is well-tolerated, effective in single dose against P. falciparum, as its half life is for about 14 days in man, with cure rate of 100%. It can be used as suppressive prophylactic drug for both vivax and falciparum malaria. It does not have tissue schizonticidal activity and thus cannot produce radical cure with relapsing malaria infections. It was first synthesized in 1971 and improved method of synthesis patented in 1978 (Sweeny, 1984). Parasites become quickly resistant to the drug specially chloroquine resistant ones. Thus it has a limited utility for mass use as a single drug for malaria treatment. However, it can be beneficially employed in drug combinations with caution.

Chloroquine and other 4-aminoquinolines:
Presence of quinoline ring in the two antimalarials, quinine and pamaquine, was known to the Germans by 1930. This prompted them also to explore several derivatives of 4-aminoquinolines, resulting in the final selection of chloroquine and sontaqueine for further trials in North Africa. Sontaquine was preferred over chloroquine by the Germans for being less toxic. Supplies of this drug together with German research data were captured by United States forces in Tunis which stimulated further studies in USA on these and other 4-aminoquinolines (Coatney, 1963; Peters, 1987). Chloroquine was found by US workers to be the most effective among them and less toxic on volunteers than any other. So the drug which was synthesized in 1934 in Germany was rediscovered in USA in 1946. Chloroquine and other 4-aminoquinolines have been found to be highly effective against the asexual blood stages of all four species of malaria (prior to appearance of resistant strains). They produce rapid clinical cure and are excellent suppressive agents. Parasitemia disappears within 48-72 hours following standard therapeutic regimen. They are gametocytocidal agents against all human malaria parasites except mature \textit{P. falciparum} gametocytes. Since release this inexpensive drug has been widely used both for treatment and prophylaxis. Its consumption had been progressively increasing for instance 265,052 kg base was actually consumed in 1978 and the demand rose to estimated 351,229 kg base in 1985, representing respectively 177 million and 234 million adult therapeutic doses (WHO, 1984). By far the most enormously consumed synthetic antimalarial drug in the history of mankind. Naturally, therefore, the most extensively studied antimalarial at the same time surprisingly our knowledge about the mode of action of this drug is far from complete. One wonders if this deplorable situation is due to complexity of the subject matter or absence of appropriate technology to resolve the knots or simply lack of more inquisitive minds to join the endeavour to unfold the specific mechanism of drug action. Whatever might be the case, it calls for periodical update about this riddle. If we knew the exact mode of action then per-
haps one could tackle more rationally the evolution of resistance to these drugs in the parasites and design effective strategies to combat or contain the ever increasing menace of resistance to chloroquine. We will restrict our deliberation to some of the recent discoveries in this field as reviewed by Ginsburg and Krugliak (Ginsburg and krugliak, 1992).

**Concentration of QCA in Parasitized Erythrocytes:**

QCA have variety of biological effects on plasmodia depending upon their concentrations. Chloroquine concentration of $10^{-7}$ M, for instance, is sufficient to bind ferriprotoporphyrin IX (FP) and form toxic complex; concentration of $10^{-6}$ M or greater inhibits lysosomal functions and concentration of $10^{-4}$ M or greater permit drug binding to the major cellular constituents and, presumably, prevent their functioning (Mc Chesney et al 1984). Most of the discussion on QCA relates to chloroquine.

**Inhibition of Lysosomal Functions:**

QCA are amphiphillic weak bases and driven freely across the membranes towards the acid digestive vacuoles of the malaria infected erythrocytes (Aikawa 1972). Extent of accumulation of the drug is dependent on the pH gradient of the lysosomal vacuole and the extracellular medium (Yayon et al. 1984). Once inside the acid vacuoles the QCA bases become protonated and their free permeability across the membranes is reduced by several folds (De Duve et al. 1974). The protonation of the influxed bases in the lysosomal compartment causes temporary alkalinization and is counteracted by the vacuolar proton pump (Geary et al. 1986). Alkalinization inhibits metabolism in the lysosomes and decline in the pH gradient decreases the drug influx. It had, therefore, been postulated that drug accumulation causes increase in the vacuolar pH resulting in inhibition of the vacuolar enzymes. Protease inhibitor, leupeptin, causes reversible cessation of digestion in the parasite whereas impedance of digestive process due to QCA is nonre-
versible (Rosenthal et al, 1988). But there is insignificant change in the pH of lysosomal vacuoles at the pharmacological level of QCA to cause any inhibition of lysosomal functions, as has been demonstrated (Ginsburg et al, 1989).

Interaction of QCA with Ferriprotoporphyrin IX:

Intraerythrocytic parasite consumes internalized hemoglobin by proteolysis in the lysosomes. Toxic residue FP or oxidized heme so formed is polymerized by the parasite into a crystalline nontoxic substance called haemozoin or malaria pigment. It has been proposed that chloroquine interferes with the formation of nontoxic compound by complexing with FP, thus lethally poisoning the parasite (Fitch 1983; Fitch 1986). These complexes not only contribute to inhibition of digestion through their effect on the parasite's acid cysteine protease but also can disrupt integrity of vesicle membranes resulting in lysis (Orjih et al. 1981; Fitch et al. 1982). However, QCA treated parasitized cells do not show either morphological or biochemical disruption of the vacuolar membranes which corroborates the finding that lytic effect of the complexes is prevented by the serum proteins (Zhang and Hempelmann, 1987). Also the failure to demonstrate presence of QCA in the necessary amounts are incompatible with this suggestion. This does not rule out the possible role of QCA-FP complexes in the antimalarial action. In fact, since they can be formed only in malaria-infected cells, they are good candidates for accounting for the specific antimalarial actions. Inhibition of heme polymerase by QCA has been recently implicated as mode of action (Slater and Cerami, 1992), also QCA preventing the release of iron from the acidified host cell cytosol, a possible source of this trace element for the parasite has also been proposed (Ginsburg and Krugliak, 1992).

Intercalation of QCA with DNA:

Chloroquine, quinine but not mefloquine have been found to inhibit DNA
synthesis by intercalating with DNA but at concentrations four orders above the pharmacological levels of the drugs. Another serious flaw with this hypothesis is that chloroquine has higher affinity to GC nucleotides (Cohen and Yielding, 1965) but plasmodia DNAs are AT rich (Weber, 1988), it is difficult to account for selective action of chloroquine against plasmodia.

Mechanisms of Chloroquine Resistance:

Advent of chloroquine resistance is one of the principal factors for the present global resurgence of malaria. Resistance in the parasites may develop either due to reduced accumulation of the drug or because of alterations in the drug target-site of the parasite. Drug target has not yet been identified but there is ample evidence that resistant strains of *P. falciparum* accumulate less chloroquine (Krogstad and Schlesinger, 1987). Decreased activity of vacuolar proton pump (Ginsburg and Krugliak, 1989) has been implicated in reduced accumulation of the drug in lysosomes and not due to the activity of any efflux pump. Acidotropic chloroquine is driven to lysosomes by pH gradient maintained by hydrogen pumping into the vacuole, slowing of the pump activity causes lowering of pH gradient which in turn leads to reduced concentration of the drug in resistant parasites. Drug resistance is associated with amplification of multiple drug-resistant (*mdr*) genes and their products in cancer cells (Endicott and Ling 1989). By analogy with this situation two *mdr* genes have been identified in *P. falciparum* (Wilson et al. 1989) and one of them *Pfmdr1* is markedly homologous to human *mdr* gene (Foote et al., 1989). In cancer cells *mdr* gene products are involved in efflux of the accumulated drug but in malaria the existence of similar *mdr* vacuolar pumps seems doubtful at present, *mdr* products might be involved in imparting resistance by some other mechanism, also amplification of these genes have not been found in all resistant falciparum strains. Sequencing of *Pfmdr1* gene showed that resistant isolates differ by one to four nucleotides from chloroquine sensitive genes.
in some strains but were same in others (Foote et al. 1990).

**Resistance Reversers**:

Compounds like verapamil (Martin et al 1987), diltiazem, vinblastine (Ginsburg and Krugliak, 1992) etc. are known to reverse drug resistance in cancer cells. They are similarly effective in reversing chloroquine resistance in resistant falciparum, mostly in vitro. A psychotropic agent, desipramine, is effective in reversing falciparum resistance at well tolerated dose in *Aotus* monkeys (Peters 1990a). Similarly cyproheptadine and several other related antihistaminic agents have shown the ability to reverse chloroquine resistance against *P. falciparum* both in vitro and in vivo. However, clinical applications against malaria is still far from infancy.

**Primaquine (8-aminoquinoline)**:

Most widely used tissue schizontocide and antirelapse drug (for vivax and ovale infections) since its introduction in 1940s. Its gametocytocidal and sporontocidal virtues are important operationally, since a single dose might reduce the transmission of drug-sensitive and resistant falciparum malaria. Clinically ineffective against asexual blood stages. Although primaquine is the safest available 8-aminoquinoline it does have significant toxic effect on individuals with glucose-6-phosphate dehydrogenase deficiency. Primaquine inhibits parasite mitochondrial respiration and this is probably the basis of its action. Not enough is known about its exact mode of action and resistance status to this drug.

3. **Artemisinin**: *Artemisia annua* a herb of family Compositae has been used in malaria therapy in China for over 1000 years but its active antimalarial component, artemisinin or 'qinghaosu' was not isolated and characterized until 1972 by the Chinese scientists. Artemisinin and a derivative, artemether, are capable of curing patients with severe malaria resistant to most other drugs. Artemether
has been found to be more effective in preventing deaths from malaria than intravenous quinine - the traditional treatment for severe malaria. It is safe and fast acting blood schizonticide, a potential life-saving advantage in severely ill patients. Some of its derivatives have recently been shown to possess better schizontocidal and gametocytocidal properties in vitro (Mehra and Bhasin, 1993). Artemisinin has the structure of sesquiterpene lactone with an internal peroxide linkage. The peroxide moiety appears to be indispensable for chemotherapeutic activity. The definitive mode of action of this series of drugs is still not known and resistance to these compounds can be produced in vitro (Inselburg 1985). No doubt this old remedy brings new hope for the time being if deployed judiciously.

Factors contributing to the selection of drug resistant parasites

In the following section some of the factors which are likely to play an important role in the emergence and propagation of drug resistance in malarial parasite are considered.

Mass drug administration: Most forms of mass drug administration employ the medicament at subcurative doses, thus leading to the selection of less sensitive or resistant parasites. This is of limited importance while malaria transmission is interrupted or reduced to a very low level (Wernsdorfer and Kouznetsov 1980). However, if drug pressure is exerted whilst transmission is intensive and unabated, selection and subsequent propagation will be rapidly effected. This effect is most marked under conditions of universal drug pressure as is exerted by the use of medicated salt (Verdrager 1986 a,b).

Inadequate treatment: A strong correlation between the immune status and drug response in areas with intensive malaria transmission has been observed especially in children of different age groups. For many years chloroquine was administered as a single dose of 10 mg/kg body weight for providing radical cure in semi-immune subjects for instance in tropical Africa (WHO 1986). When infants and children not sufficiently immune were treated with the same dose the drug failed
to provide radical cure. The dose was thus subcurative and therefore conducive to the selection of resistant parasites (Gbarry et al. 1988; Ramanamirija et al. 1985).

**Vectorial factors:** Studies in Central and West Africa indicate that the risk of contracting chloroquine resistant *P.falciparum* is directly correlated to vector density and sporozoite rate (Brandicourt and Gentilini 1987). The propagation of chloroquine resistant *P. falciparum* was quite slow in the areas of moderate and low transmission where sensitive *P. falciparum* continues to prevail in spite of an early onset of chloroquine resistance in the hyper-endemic lowland regions of Madagascar (Le-Bras et al. 1987).

**Population mobility:** Migration has been identified as a major contributory factor to the occurrence and spread of drug resistance (Verdrager 1986 a,b). Migration is an important determinant in the transportation of the infection to unaffected areas. Intensity of migration in association with a different intensity of drug use may also cause major differences in the prevalence of resistant infections within relatively small geographic areas as was shown in the example of two population strata in Amazonian Brazil (Kremsner et al. 1989).

**Biological advantage of resistant parasites over sensitive ones:** Chloroquine resistant *P. falciparum* differs in certain biological characteristics from chloroquine sensitive populations. It has proved to be more infective to *Anopheles stephensi* and also to produce large number of oocysts: an effect also observed in *A. dirus* (Sucharit et al. 1977). Chloroquine may enhance the infectivity of chloroquine resistant plasmodia to anophelines. This has been shown in *P. berghei / A. stephensi* model (Ramkaran and Peters 1969). A marked decrease in sensitivity to chloroquine has been observed in *P. falciparum* isolates after continuous growth for long periods in vitro culture in the absence of drug pressure (Le Bras et al. 1980).

**Lessons from Past:**

History of drug resistance shows that whenever a new antimalarial was deployed *en masse* for monotherapy and chemoprophylaxis resistance to the drug
Figure 1.1 Chemical structures of antimalarial drugs
appeared quickly. Life span of drug combinations had been longer. Misuse of drugs has also contributed to the fast selection of resistant parasites, which should be prevented as far as possible. Antimalarials should only be deployed as one of the tools in an integrated malaria control programme. 'Think globally and act locally' should be the guiding principle for any malaria control programme.

Future Agenda:

The current grim situation is a cause of concern to malaria epidemiologists, indicating the urgent need to identify and develop alternate compounds capable of curing resistant malaria. If there was more information on relationship between in vitro antimalarial sensitivity and in vivo response to treatment, dose regimens of quinine could be improved. Therapeutic concentrations of quinine need to be defined in different countries where sensitivity of \textit{P. falciparum} to the cinchona alkaloids varies. Quinine should not be used alone. One of the most inexpensive ways of searching new lead antimalarial compounds for drug development would be to screen the drugs which have been developed for other diseases and evaluate their antimalarial potentials, either alone or in combination with other drugs. Searching of novel drugs empirically is expensive in time and resources. Rational drug design is feasible if modes of action of drugs is known, so its highly desirable to demonstrate how the proven drugs function at molecular level. Discovery of the molecular mechanisms causing resistance is of utmost importance in order to impede, reverse or contain the advent of resistance. Search for antirelapse and transmission blocking drugs should be a priority. New drug combinations should be explored to which emergence of resistance is extremely difficult, this strategy might bear fruits quickly, specially search for synergistic combinations. Efforts should continue for the search of less toxic 'resistance reverser' combinations. Ability of some oligos to revert DHFR resistant mutants to sensitive lines in vitro by site directed mutagenesis should be tried as a first step.
Drugs toxic to plasmodia and capable of evoking immunogenic responses against parasite will be of special interest. Potent new antimalarial combinations to be developed should be inexpensive, nontoxic and administered under strict prescription to limit their misuse. There should be a separate class of drugs for prophylaxis. Proper drug deployment, marketing and monitoring strategies will have to be worked out.
Table 1.1  Positions of amino acid residues that are different in pyrimethamine and cycloguanil sensitive or resistant isolates in the DHFR segment of the *P. falciparum* genome*.

<table>
<thead>
<tr>
<th>SUSCEPTIBILITY OF THE ISOLATE</th>
<th>AMINO ACID RESIDUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrimethamine</td>
<td>Cycloguanil</td>
</tr>
</tbody>
</table>

S = sensitive , R = resistant, HR = highly resistant

* compiled from the literature
Figure 1.2 Composition of 'quinine' from past to present
Chapter 2

SUSCEPTIBILITY OF PLASMODIUM FALCIPARUM ISOLATE AND A CLONE TO A FEW ANTIMALARIAL DRUGS IN VITRO

Abstract: In vitro susceptibility of a local Plasmodium falciparum isolate together with one of its fastest multiplying clone to some known natural antimalarial drugs (quinine and artemisinin) and commonly used synthetic drugs (chloroquine, amodiaquine, pyrimethamine and cycloguanil) has been performed, using the modified 48 hour test method. Erythrocytic stages of the parasites have been found to be resistant to chloroquine and sensitive to all other natural and synthetic drugs evaluated. Advantages of in vitro bioassay methods for drug susceptibility testing has been discussed. Growth rates of the parasites have been found to be comparable to those of the parasites maintained in cultures in non-endemic areas, implying that serum supplement (obtained locally) employed in the medium for cultivation has no adverse effect on growth and multiplication of the parasites.

INTRODUCTION

The efficacy of drugs against falciparum infections has traditionally been evaluated by observing the clearance of parasites from the blood stream following administration of a drug in a subject. If the parasitemia is cleared with no subsequent recrudescence the drug is considered effective and the infecting strain regarded sensitive to the administered drug. It is obvious from this type of experimental data that some strains of parasites are more sensitive than others to the drug. Marked differences in susceptibility are sometimes observed, particularly in strains of diverse geographical locations. However, with the emergence of chloroquine-resistant strains of falciparum in 1960s, need for defining drug resistance in malaria and standardizing procedures for assessing the varied responses of P. falciparum to chloroquine and other antimalarials was strongly felt. Drug resistance in malaria is defined by WHO (1967) as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject". Although this definition can logically be extended to include all plasmodial stages, it has generally been restricted to describe the drug susceptibility of asexual forms, presumably because this stage in the life cycle of the parasite produces acute clinical symptoms observed during
the course of malaria infection. Evaluation of drug resistance procedures in vivo was proposed by WHO first in 1967 and then slightly modified in 1973 (WHO, 1967, 1973). The obvious problems associated with in vivo determination of the drug susceptibility of *P. falciparum* has led to the development of simple in vitro tests which can be easily performed both in laboratory and field conditions (Rieckmann and Lopez Antunano 1971). The original in vitro microtest method has been the most successfully applied diagnostic test for chloroquine-resistance and was designated by the WHO as a standard test in 1979 (WHO 1979). Procedure of Trager and Jensen (1976) for in vitro cultivation of falciparum stages has given further impetus to modify and develop methods for in vitro determination of susceptibility not only to chloroquine but also to other antimalarial drugs (Nguyen-Dinh and Payne 1980). The results of in vitro assessment in most cases show excellent concordance with the results of their assessment with in vivo system. In vitro method has superseded the in vivo procedure for assessment of drug susceptibility in some situations. Evaluation of the drug susceptibility of *P. falciparum* is now not only confined to those locations where drug resistance is a problem but test are also carried out in all endemic areas where falciparum infections exist. Such susceptibility profile of local strains to antimalarials provide a baseline data against which subsequent changes in the susceptibility of parasites can be measured. Susceptibility status of local strains of *P. falciparum* to new drugs should also be determined in areas where these drugs might be used in future. Malaria is acknowledged as an exclusively local phenomenon and any control programme has to be meticulously designed taking into consideration the susceptibility profile and dynamics of resistance (if known) of the local falciparum fauna to the antimalarial drugs. Over the last few years we have established in vitro cultures of several *P. falciparum* isolates originating from Delhi. Clones have been derived from some of these isolates. Susceptibility profile of some isolates and clones has been determined in vitro to commonly used antimalarial drugs. In
this section sensitivity of an isolate and a clone, which have been employed in all
the subsequent work of the present study, to some antimalarials has been described.
In vitro cultivation procedures and their advantages in drug susceptibility testing
have been discussed.

MATERIALS AND METHODS

Biological materials

Parasites: The isolate FCD-4 employed in the present study was procured locally
from a malaria infected patient reporting to a malaria clinic of malaria research
centre (MRC), Delhi. About 2 ml of blood was drawn aseptically, with the consent
of patient infected with *P. falciparum*, by venous puncture into a sterile heparin-
ized vial. The blood sample was transported to the laboratory for in vitro cultiva-
tion of erythrocytic stages within an hour. Soon after, the cultures were initiated
in vitro using candle-jar method of Trager and Jensen (1976). Once the isolate got
established in culture, clones were derived from it using limiting dilution method
(Rosario 1981). Several clones were realized and a few characterized with regard
to their multiplication ability in vitro. A clone F-56, one of the fastest growing
clone obtained was chosen for further studies.

Red blood cells and serum: Human erythrocytes and serum used for cultivation
of parasites were purchased from commercial blood banks. All blood products
used, were certified to be free from malaria, hepatitis, HIV etc. A unit of blood
(about 350 ml) obtained in acid citrate dextrose or citrate phosphate dextrose was
dispensed aseptically in 50 ml aliquots and stored at 4 °C. The stored blood was
used upto four weeks. Erythrocytes of A+ blood group were used in all studies.
For serum, 2-3 units of AB+ blood at a time were collected without any anticoagu-
lant. It was stored at 4 °C atleast overnight to retrieve maximum amount of
serum. The serum was harvested by centrifugation of this stored coagulated blood
and distributed in 40 ml aliquots in sterile flasks. These serum samples were
kept at -20 °C till further use. Prior to use the stored serum was thawed and heat inactivated by keeping it for 30 min at 56 °C.

Reagents

Medium: For one litre of medium 10.4 g of powdered RPMI-1640 with L-glutamine but without bicarbonate (obtained from Sigma) was dissolved in 900 ml of glass redistilled water. To this 5.94 g of HEPES buffer and 40 mg of gentamicin were added (RP-C). The above components were thoroughly mixed with the help of a magnetic stirrer and the final volume adjusted to 960 ml. This medium was immediately filter sterilized through 0.45µ presterilized millipore filter under negative pressure, aseptically in a flow hood. The sterilized medium was dispensed in 100 ml volumes into sterile screw cap bottles for storage in refrigerator. Each bottle was numbered and dated. The pH of the buffered medium was made to 7.2 just before use by adding sterilized 5% sodium bicarbonate solution at the rate of 4.2 ml per 96 ml (RP+C) medium. The RP+C medium was supplemented with 10% (v/v) heat inactivated serum to be used as complete medium (RPS) for cultivation. Complete medium could be stored at 4 °C upto one week in closed bottles. The RP-C was consumed within four to five weeks.

Cryoprotectant: It was prepared by adding 70 ml glycerol to 180 ml of 4.2% sorbitol in 0.9% NaCl and filter sterilized to be used as cryoprotectant solution for freeze-storing of parasites.

Thioglycollate medium: Thioglycollate medium, 29.8 g (powder) was mixed in 1 litre of glass distilled water and heated slowly so as to dissolve it completely. This solution was dispensed in 10 ml volumes into glass tubes with cotton plugs and autoclaved at 15 lbs for 20 min. Tubes were stored at 4 °C for subsequent testing of bacterial contamination in medium or blood products.

Drug solutions The stock solutions concentration and methods of preparation of the various drugs employed in this study are tabulated below.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Source</th>
<th>Concentration of Stock</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>Sigma Chemicals</td>
<td>40 mg/ml base</td>
<td>Water</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>Sigma Chemicals</td>
<td>$10^{-2}$ M</td>
<td>Water</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>Sigma Chemicals</td>
<td>$10^{-2}$ M</td>
<td>Lactic acid</td>
</tr>
<tr>
<td>Cycloguanil</td>
<td>ICI Pharmaceuticals*</td>
<td>$10^{-2}$ M</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Quinine</td>
<td>WHO*</td>
<td>10 mg/ml</td>
<td>Sulfuric acid</td>
</tr>
<tr>
<td>Artemisinin</td>
<td>WHO*</td>
<td>1 mg/ml</td>
<td>DMSO</td>
</tr>
</tbody>
</table>

* gifts from these agencies

Each of these stock drug solutions was filter sterilized through 0.2 mm cellulose acetate millipore sterile disposable syringe filter and stored at -20 °C. Test or working solutions were prepared by appropriately diluting the stock solutions with complete medium (RPS). All the test solutions were kept at 4 °C.

**PROCEDURES**

**Maintenance of *P. falciparum* parasites in vitro**

*Sterility test:* All media, blood and glassware must be sterile for culture work. The glasswares were autoclaved and baked. The sterility of the glassware was ensured by the pressure and heat indicator tapes. To test the sterility of media and blood products, 1ml of test solution was mixed with the sterile thioglycollate medium. The tube was incubated at 37 °C for 4 days. If thioglycollate solution remained clear, the tested product was considered free of bacterial contamination.

*Red cell preparation* (washed erythrocytes): Human erythrocytes obtained from blood bank were centrifuged at 2500 rpm for 5 min. Supernatant and buffy coat were aspirated out. The pellet was resuspended in 5-10 volume of RP+C medium and spun again to remove any residual anticoagulant. The cells were washed twice this way and finally the pellet of red cells was resuspended in an equal volume of complete medium (RPS) to give 50% cell suspension.

*Cultivation of parasites in vitro:* Parasites were cultivated using the Petridish candle jar method of Trager and Jensen(1976). The parasite material was drawn into a sterile syringe from a flow vessel, in which stock cultures of parasites were
routinely maintained in the laboratory (Trager 1979). The material was transferred to a graduated centrifuge tube and centrifuged at 2000 rpm for 10 min. Supernatant was discarded and packed cells were resuspended in equal volume of complete medium. Thin blood smear was made from this 50% infected cell suspension. The slide was fixed in methanol and stained in 5% Giemsa's stain prepared in 0.067 M phosphate buffer. The slides were air dried and microscopically examined under oil immersion magnification (1000 X). Differential parasite count was made to determine the extent of dilution required, if any. Parasite material was diluted with 50% suspension of non-infected erythrocytes. Cultures were initiated with less than 1% of infected erythrocytes. Appropriately diluted parasite material of 50% cell suspension, 2 ml of which was mixed with 10 ml of complete medium (RPS) so as to give a final suspension or hematocrit of 8%. This material (about 12 ml) was dispensed in a glass Petridish (10 cm in diameter). The Petridish was placed in a desiccator equipped with a stopcock. The gas phase was generated by burning a white candle in the desiccator jar holding the Petridishes until the flame extinguished; the stopcock was closed immediately. The candle jar, holding parasite material in Petridishes, was placed at 37 °C in an incubator. The medium was removed daily by gently tipping the Petridish and aspirating off the medium. All this was done under sterile conditions in a flowhood. The dishes were then replaced in the candle jar. Culture growth was routinely monitored every 48 hour from thin blood smears stained in Giemsa solution. Differential parasite count was made by counting at least 5000 erythrocytes. If parasitemia was found to be more than 5% subcultures were prepared.

**Cryopreservation:** Cultured parasite material was periodically stored in liquid nitrogen for recultivation. Parasites were cryopreserved by a modified method of Rowe et al. (1968). Cryopreservation of parasite material was done when number of parasites per 100 red cells exceeded 5 in a Petridish and majority of para-
sites were in ring stage. The parasite material was transferred to a graduated centrifuge tube and centrifuged at 1500 rpm for 10 min. Supernatant was discarded and the packed cells mixed gently with an equal volume of cryoprotectant and 1ml each of this suspension was transferred into sterile, screw-capped plastic cryovials. These vials were immediately dipped in liquid nitrogen and stored in liquid nitrogen cryocan.

Resuscitation of parasites: Cryopreserved parasites were revived by bringing the cryovial from liquid nitrogen to a 37 °C water bath (momentarily loosening the cap to vent any compressed nitrogen). On thawing, contents of the vial were transferred to a centrifuge tube, spun at 1500 rpm for 5 min, supernatant discarded and packed cells suspended in an equal volume of 3.5% sterile sodium chloride solution. The saline suspension of cells was recentrifuged to discard the saline, packed cells washed twice with complete medium and finally made to 50% cell suspension with complete medium. To this, equal volume of freshly washed uninfected 50% suspension of erythrocytes was mixed. The mixture was made to 4% hematocrit by adding complete medium supplemented with 15% heat inactivated pooled serum in a glass Petridish (5 cm diameter) and parasites grown by candle jar method.

Susceptibility test method

Erythrocytic schizontocidal activity of each compound at graded concentrations was evaluated in vitro using the modified 48 hr test method of Nguyen-Dinh and Payne (1980). The parasite material was drawn from stock continuous culture, centrifuged and pelleted cells suspended in 2.5 volume of 5 % sorbitol and incubated at room temperature for 7 min. Mostly the ring stage parasites survive this treatment. The sorbitol treated parasites were washed thrice with RP+C medium. The pellet was finally resuspended in an equal volume of complete medium to give 50% suspension of infected erythrocytes. This infected material was appropriately diluted with 50% washed, uninfected erythrocytes so as to get
the final parasitemia of less than 1% (zero hour) to be used as seeding material for susceptibility tests. Aliquots of 20 μl of this seed material was added to each of the series of wells, in 24 well sterile plastic tissue culture plates, holding 480 μl of complete medium with or without drug. The final erythrocyte suspension in these experimental wells was thus 2%. The experiment was done in duplicate for each drug concentration. After thoroughly shaking the multiwell plates to ensure resuspended and uniform settling of erythrocytes, the plates were placed in candle jar and incubated at 37 °C. After 24 hours of incubation, medium in each well was replaced with or without drug. Drug was included at same concentration as initially and plates returned to candle jar. Cultures were under continuous drug pressure for 48 hour, at the end of this period a thin blood film from each of the well holding control and drug treated parasite material was made. Slides were stained with 5% Giemsa solution and number of parasites counted by enumerating at least 5000 erythrocytes. Percentage inhibition of parasites in relation to control was calculated. MIC, IC_{50} and IC_{90} values were determined from semilog plot of percentage inhibition against drug concentration.

RESULTS

In vitro cultivation of *P. falciparum*

A vial each of cryopreserved material of *P. falciparum* isolate FCD-4 and clone F-56 was retrieved from the liquid nitrogen storage container to initiate in vitro cultures of these parasites separately. Once the parasites started multiplying to an extent of at least four fold in an erythrocytic cycle (from starting parasitemia of less than 1%) they were further maintained in a routine medium (RP+C) supplemented with 10% serum (RPS). This usually took not more than 6 days of culture, if properly cryopreserved material was used for resuscitation. Freeze preserved material when initiated into culture retained synchronous multiplication for first one or two schizogonic cycles and subsequently grew asynchronously. An
erythrocytic cycle is of about 48 hours duration. During this period intraerythrocytic parasites undergo morphological changes leading to the formation of ring, trophozoite and schizont stages. Rupture of a schizont releases merozoites, the only extracellular form, which reinitiates the erythrocytic cycle on invading an uninfected red cell. A representative Giemsa stained thin blood film preparation from cultured parasite material showing various asexual and sexual stages is presented in Figure 2.1. The ring stage is the youngest intraerythrocytic stage of the parasite occupying approximately one fifth of the red cell area. Host red cells at this stage were least modified with regard to morphology or membrane permeability. A typical 'signet' ring stage occupied first 18-24 hours of an erythrocytic cycle before being transformed into the trophozoite stage. A growing trophozoite could be seen as a small round mass of blue cytoplasm with one red chromatin dot or area and a small clump of brown pigment. The more mature or nucleus dividing stage is called schizont which looks like a large trophozoite containing more than one chromatin dot. Each nucleus with cytoplasm surrounding it is called merozoite, may be seen in a schizont. Fully formed merozoites released from a mature schizont can be seen in the Figure 2.2. Occasionally developing sexual stages, called gametocytes and often multiple infection of red cell can also be found in cultures. A typical distribution of different erythrocytic stages in a culture started from ring stages after first and second schizogonic cycle is depicted in Table 2.1.

**Table 2.1.** A differential parasite count made at the end of first and second schizogonic cycles of in vitro cultivated parasites in multiwell plates with starting hematocrit of 4%, showing typical profile of erythrocytic stages in the cultures.

<table>
<thead>
<tr>
<th>Schizogonic cycle</th>
<th>R*</th>
<th>T</th>
<th>S</th>
<th>TP</th>
<th>%P</th>
<th>R</th>
<th>T</th>
<th>S</th>
<th>TP</th>
<th>%P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hour</td>
<td>36</td>
<td>0</td>
<td>0</td>
<td>36</td>
<td>0.72</td>
<td>32</td>
<td>0</td>
<td>0</td>
<td>32</td>
<td>0.64</td>
</tr>
<tr>
<td>First</td>
<td>110</td>
<td>18</td>
<td>29</td>
<td>157</td>
<td>3.14</td>
<td>138</td>
<td>26</td>
<td>27</td>
<td>191</td>
<td>3.82</td>
</tr>
<tr>
<td>Second</td>
<td>259</td>
<td>48</td>
<td>86</td>
<td>393</td>
<td>7.86</td>
<td>414</td>
<td>87</td>
<td>111</td>
<td>612</td>
<td>12.24</td>
</tr>
</tbody>
</table>

* R= rings, T= trophozoites, S= schizonts, TP= total parasites, %P= % parasitemia
Usually a parasite produces by asexual multiplication (schizogony) 8 to 24 merozoites. But only some of these merozoites succeed in invading and multiplying further. This determines the rate of multiplication and to monitor the growth of parasites, thin smears were made after every 48 hours for two consecutive cycles from each of the well holding either FCD-4 or F-56 culture material. Parasitemia was enumerated from the stained slides by counting about 5000 erythrocytes. Multiplication factor (MF) and parasite multiplication rate (PMR) were determined using the following formula (Brokelman et al. 1985):

For the first schizogonic cycle $MF = \frac{N \text{ (day 2)}}{N \text{ (day 0)}}$

where $N$ is the number of parasites per 5000 erythrocytes on the day indicated.

Thus the $MF$ from day 2 to day 4 was $= \frac{N \text{ (day 4)}}{N \text{ (day 2)}}$

Because multiplication is exponential (theoretically for first few cycles), a logarithmic transformation was used as follows:

$$\log MF = \log N \text{ (day 4)} - \log N \text{ (day 2)}$$

Log $MF$ has been referred as the parasite multiplication rate (PMR). Results regarding $MF$ and PMR for the isolate and clone are presented in Table 2.2.

**Table 2.2** In vitro parasite multiplication as determined by multiplication factor (MF) and parasite multiplication rate (PMR) in multiwell tissue culture plates. (refer to the text for explanation of MF and PMR)

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Erythrocytic schizogonic cycle</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First</td>
<td>MF</td>
<td>PMR</td>
</tr>
<tr>
<td>FCD-4</td>
<td>4.36</td>
<td>0.64</td>
<td>2.5</td>
</tr>
<tr>
<td>F-56</td>
<td>5.96</td>
<td>0.78</td>
<td>3.2</td>
</tr>
</tbody>
</table>
Parasites were periodically cryopreserved for subsequent use from culture plates having more than 7-8% parasitemia with majority of parasites in the ring stage. This type of freeze-stored material revived fast, mostly within 4 to 6 days of resuscitation cultures could be grown in a routine medium supplemented with 10% serum.

**In vitro susceptibility to antimalarials**

The response of the parent isolate FCD-4 and one of its derived clone F-56 to the various antimalarials evaluated in vitro were as follows:

*Chloroquine:* Figure 2.3 shows the drug dose response for chloroquine in the isolate FCD-4 and clone F-56 after 48 hours of incubation. The IC$_{50}$ and IC$_{90}$ values of chloroquine were found to be 0.018 μg/ml and 0.07 μg/ml for FCD-4 and 0.015 μg/ml and 0.027 μg/ml for clone F-56.

*Amodiaquine:* Susceptibility of the parasites to various concentrations of amodiaquine is illustrated in Figure 2.4. Interpolation of the drug dose response curve to determine concentrations that caused 50% and 90% inhibition of parasite growth gave values of $6.8 \times 10^{-9}$ M and $5.4 \times 10^{-8}$ M for FCD-4 and $1.7 \times 10^{-9}$ M and $7.4 \times 10^{-8}$ M for clone F-56. The clone was found to be slightly more sensitive to this drug since complete inhibition of clonal parasites was achieved at a concentration of $10^{-8}$ M whereas in the isolate it was observed at $10^{-7}$ M of drug concentration.

*Pyrimethamine:* Sensitivity of the isolate FCD-4 and clone F-56 to graded concentrations of pyrimethamine in vitro is presented in Figure 2.5. Drug concentration of $10^{-8}$ M or more uniformly affected the morphological appearance of schizonts, as seen from 96 hours Giemsa stained blood films in both the parent isolate FCD-4 and clone F-56. Schizonts showed fragmented chromatin dots of unequal size scattered over a large area. Normal merozoite formation was prevented resulting in the inhibition of parasite multiplication. In control wells 7 to 8 fold increase in parasitemia was observed by the end of 96 hours. The IC$_{50}$ and IC$_{90}$
values of the drug derived from the drug dose response curve were $1.5 \times 10^{-9}$ M and $4.6 \times 10^{-9}$ M for FCD-4 and $1.5 \times 10^{-9}$ M and $3.6 \times 10^{-9}$ M for F-56. Apparently lactic acid used for drug preparation at concentrations present in the test solutions had no effect on parasite multiplication. This confirms the finding of Richards and Maples (1979).

Cycloguanil: Parasite counts made at the end of 96 hours of the experiment revealed that multiplication of the parent isolate FCD-4 and clone F-56 was completely inhibited in vitro at $5 \times 10^{-9}$ M and $5 \times 10^{-10}$ M concentration of cycloguanil. Percent inhibition of parasite growth in relation to control was plotted against log concentration of drug to determine IC$_{50}$ and IC$_{90}$ values. The isolate FCD-4 and clone F-56 showed marked sensitivity to cycloguanil. The IC$_{50}$ and IC$_{90}$ values derived from the drug dose response curve (Figure 2.6) were $8.4 \times 10^{-11}$ M and $6.8 \times 10^{-10}$ M for FCD-4 and $7.2 \times 10^{-11}$ M and $2.7 \times 10^{-10}$ M for the clone F-56. Inhibitory effect of the drug on parasite growth was observed even at very low concentrations. The morphological appearance of cycloguanil sensitive parasites was very similar to that observed in the case of pyrimethamine. These abnormal parasites appeared enlarged and contained fragmented red staining nuclear material. Good growth of parasite was observed in control wells at the end of 96 hours.

Quinine: The effect of quinine on erythrocytic stages of the clone F-56 and isolate FCD-4 in vitro is illustrated in Figure 2.7. The IC$_{50}$, IC$_{90}$ and MIC values of the drug for the parasites derived from the drug response curve are 0.02 $\mu$g/ml, 0.052 $\mu$g/ml and 0.06 $\mu$g/ml for clone F-56 and 0.01 $\mu$g/ml, 0.047 $\mu$g/ml and 0.06 $\mu$g/ml for the isolate FCD-4.

Artemisinin: The response of clone F-56 to graded concentrations of artemisinin is presented in Table 2.3. Complete inhibition of erythrocytic multiplication was obtained at a concentration of 10 ng/ml of the drug. The concentrations of the drug that caused 50% and 90% inhibition of parasite growth with respect to control derived from the tabulated data (Table 2.3) are 0.0019 $\mu$g/ml and 0.0058 $\mu$g/ml
respectively.

**Table 2.3** In vitro susceptibility of a *P. falciparum* clone, F-56 to artemisinin. The parasites were under drug pressure for 48 hours. Starting parasitemia was less than 1% and hematocrit 4%. Slides were enumerated for parasitemia at the end of 48 hours.

<table>
<thead>
<tr>
<th>Drug concentration (μg/ml)</th>
<th>% parasitemia</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>2.66</td>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.005</td>
<td>0.37</td>
<td>86.09</td>
</tr>
<tr>
<td>0.001</td>
<td>2.07</td>
<td>22.18</td>
</tr>
<tr>
<td>0.0005</td>
<td>2.37</td>
<td>10.9</td>
</tr>
<tr>
<td>0.0001</td>
<td>2.71</td>
<td>0</td>
</tr>
</tbody>
</table>

**DISCUSSION**

One of the most widely used applications based on continuous cultivation of erythrocytic stages of falciparum malaria (Trager and Jensen 1976) has been the in vitro determination of susceptibility of parasites to various antimalarial drugs around the world, more so in endemic locations. The popularity of these methods is obviously because of several advantages they offer over in vivo procedures. The 'standard' WHO Field Test (1973) for instance which is used to determine the susceptibility of *P. falciparum* infection in the subject requires 3 days of drug administration and 7 days of follow up period to ascertain the sensitivity of infecting parasites. Low levels of resistance can not be detected by this in vivo procedure.
Modified 'extended' Field Test requiring 28 days of follow up is employed to determine the gradation from sensitive (S) to R I, R II or R III (high) level of resistance to chloroquine. Prolong follow up period with precautions for preventing any reinfection during this duration are limiting factors to carry out this test, particularly in endemic locations. The in vitro methods of determining the sensitivity of an isolate require much less time and can be easily completed in 2 to 3 days using very little blood from the infected person. The response of falciparum infection to drug treatment may differ from one individual to another depending upon the immune status of the individual (Powell et al. 1964; Waliker and Lopez Antunano 1968). The hosts immune and other factors do not affect the outcome of results in vitro evaluation, thus permitting a more accurate evaluation of the intrinsic inhibitory properties of the drug on the parasites. However, serum supplement used, specially those procured from endemic regions, might interfere with correct assessment of the inhibitory properties of the drug, as serum from various endemic regions are known to contain antibodies capable of retarding the growth of cultures in vitro (Chulay et al. 1981; Jensen et al. 1982). To overcome this possibility each serum unit should be screened for the presence of malaria specific antibodies or the serum used should be heat inactivated to nullify the influence of these serum factors on the cultures. All through the present study the latter option was employed. This minimized the interference of serum factors in drug response studies of the parasite. Another drawback with in vivo evaluation is concerning uncertainty of complete absorption and retention of the drug in the subject. Assessment of complete uptake and presence of the correct amount of the drug in the blood of infected person requires use of time consuming, elaborate methods like HPLC. Applications of in vitro techniques have thus greatly improved the overall efficiency and precision of drug sensitivity tests. It is now possible to process and evaluate large number of compounds on different strains of falciparum simultaneously, thus providing broader range of information with respect to susceptibil-
ity to various drugs. This has been made possible also because these techniques are relatively inexpensive compared to experimental animal model systems which require more space, equipment and manpower. In vitro cultures have made it possible to study clonal populations of parasites and this will contribute towards better understanding of genetic analysis of drug action and considerably facilitate molecular/biochemical studies on the mechanism of drug action. In vitro systems permit study of structural-activity relationship of compounds with great precision and relative ease, thus allowing the selection of lead compounds with greatest intrinsic antimalarial activity very rapidly. Evaluation of potential drug interaction for synergism, antagonism or indifference has been made simpler by in vitro methods, enabling the selection of favourable combinations. By employing multi-drug resistant strains in culture it is now possible to look for compounds which are not cross-resistant with existing antimalarial drugs. Finally it is possible in vitro studies to evaluate parent compounds and known or putative metabolites of the parent compounds separately, especially since only small quantities of each are needed. There are also important limitations associated with the use of in vitro methods. The most frequently cited limitation is the inability to detect the potential antimalarial activity of those compounds which require in vivo metabolism to an active state, for example the antimalarial drug proguanil which is inactive in vitro system. Similarly certain compounds which require some form of host interaction such as enhancement of host immunity may prove to be inactive when assessed in vitro. Another limiting characteristic of in vitro method is the artificial time course chosen for drug exposure, which is usually at a constant level of concentration for a relatively short period of time. Certain drugs which have been identified as effective antimalarials may ultimately prove to be toxic to the host or might have very little safety margin. Many compounds will also prove to be insoluble in the media used for culturing the parasites in vitro. Others will be subject to artifacts peculiar to in vitro systems such as adherence to the glass or plas-
tic dishes in which the experiments are carried out. The above considerations lead to the conclusion that though the availability of in vitro culture techniques is important for antimalarial drug research, they are neither a substitute nor a replacement for in vivo or basic biochemical methods. A well-designed drug development programme must carefully integrate these varied approaches, recognising their respective advantages, limitations and their mutual potential contributions towards the goal of identifying and characterising useful new antimalarial drugs.

Parasite multiplication factor in our study was comparable with the isolates and clones being cultivated elsewhere even in non-endemic areas. There were no crisis forms observed in cultures which have been reported from some holoendemic regions. The presence of malaria specific antibodies in the blood have been implicated for the presence of crisis form (Reese et al. 1981; Jensen et al. 1982, 1984). There had been no excessive conversion rate of asexual forms to sexual stages in the culture as has been the case in most endemic regions employing hyperimmune serum (Jensen et al. 1984). All these observations peculiar to parasite growth and multiplication in the malaria endemic regions are contrary to the observations made in our cultures which confirm that the blood component used by us for cultivation were free from most of the factors involved in generating these characteristics. Oduola and coworkers (1992) have also found that plasma from semi-immune persons is suitable for continuous cultivation and drug susceptibility testing of *P. falciparum*.

The first simple in vitro test for assessing the drug susceptibility of *P. falciparum* parasites drawn from venous blood of an infected individual was developed by Rieckmann et al (1968). The test relied on a basic observation that parasites could survive in vitro system for short duration of atleast 30 hours. This fact was exploited in measuring the extent to which maturation of ring forms to schizonts was inhibited after incubation of parasitized blood in various drug concentrations for a period of 24 hours. This short term culture system provided a quick and
reliable diagnostic tool to differentiate chloroquine resistant and sensitive isolates both in laboratory and field conditions (Rieckmann and Lopez Antunano 1971; Valera and Shute 1975; Palmer et al. 1976; Ebisawa et al. 1976; Sucharit et al. 1977). Usefulness of this method culminated in the development of a test kit for monitoring the sensitivity of *P. falciparum* isolates to chloroquine by the WHO (1979). Using this kit one could even assess the level of resistance ranging from sensitive to highly resistant R III forms of the parasites. This test has also been used for other antimalarials. The main shortcoming of this macro-method is that it requires about 10 ml of venous blood which is not readily given by the patient, more so if the subjects are children. Development of microtechnique using capillary blood specimen (Rieckmann et al. 1978), which can be obtained by a finger prick, has made the susceptibility test easier and can be performed by drawing little blood from even young children. Period of incubation in the micromethod can be extended to 48 hours without change of medium, so that one can even observe reinvasion of the merozoites (Rieckmann 1980; Yisunsri and Rieckmann 1980). This technique has replaced macro test specially in the field with the development of inexpensive battery operated out door incubators (Eastham and Rieckmann 1981). Considering the benefits of incubating parasites under drug pressure for longer duration in determining the susceptibility of falciparum isolates, Nguyen-Dinh and Trager (1980) described a new 48 hour test system. Infected red blood cells, either from cultures or from clinical specimens (Nguyen-Dinh et al. 1981) were diluted in a 50% suspension of fresh red blood cells in culture medium to obtain a parasite density of 0.1%-0.8%. The resulting parasitized erythrocyte suspension was then further diluted in culture medium, with or without drug added, to a 2% erythrocyte suspension. A similar test method of 72 hours has been described by Kramer and Siddiqui (1981) with 5% erythrocyte suspension. In the present study a modified 48 hour test method of Nguyen-Dinh and Payne (1980) has been employed in determining the susceptibility of para-
sites to antimalarial drugs in vitro, because this test method is reliable, reproducible and results are not modified by slight variations in starting parasitemia, erythrocyte suspension, blood group of the red cells used, age of the erythrocytes, inoculum size etc.

According to Nguyen-Dinh and Trager (1980) for a strain sensitive to chloroquine complete inhibition of growth occurs at 0.01 μg/ml and for a resistant strain at 0.1 μg/ml. From IC_{50} and IC_{90} values derived from the drug response curve it can be inferred that both the parent isolate and the clone evaluated are resistant to chloroquine, the isolate being more resistant since at a concentration of 0.06 μg/ml complete inhibition is observed in the clonal parasites whereas only 86% inhibition was observed in the parent isolate. In control wells in which parasites were incubated in drug free medium good growth was obtained with 4 to 5 fold increase in parasitemia in 48 hours, which is comparable with the growth observed by Nguyen-Dinh and Trager (1980). Amodiaquine and chloroquine are both derivatives of 4-amino-7-chloroquinoline and differ only in the nature of the group attached to the 4 amino position, amodiaquine has a substituted benzene ring at the position, while chloroquine has a branched chain aliphatic substituent. The parasites evaluated have been found to be sensitive to amodiaquine while extremely resistant to chloroquine. Similar observations have been made by other workers (Thaithong et al. 1983; Watkins et al 1984; Looareesuwan et al. 1985) in strains of *P. falciparum* from different geographical regions, and also it has been observed that isolates found to be resistant to chloroquine develop resistance to amodiaquine sooner or later (Draper et al. 1988). Response of parasites to both the DHFR inhibitors evaluated was found to be similar. This is perhaps expected since both pyrimethamine and cycloguanil are known to act by affinity binding to the dihydrofolate reductase of the parasite which occupies a position in the cellular biosynthesis of the malaria parasites (Ferone et al. 1969). Smalley and Brown (1982) estimated that parasites obtained from clinical samples growing in 10^{-7} M
pyrimethamine or more are likely to be resistant at least to some degree in 24 hour test. Concentration of $1 \times 10^{-8}$ M of pyrimethamine in a 48 hour test in vitro by Richards and Maples (1979) has been reported to be both inhibitory and cidal. As the morphological criterion has been used to determine the effect of the compound on *P. falciparum*, sometimes it becomes difficult to differentiate between the cidal and inhibitory effect on the parasites in a stained slide. In order to eliminate this ambiguity, the parasites were further cultivated for next 48 hours in drug free medium and percentage inhibition of parasites calculated at 96 hours. The IC$_{50}$ and IC$_{90}$ values determined both at the end of 48 and 96 hour experiments clearly indicate that isolate (FCD-4) and clone (F-56) tested are highly sensitive to pyrimethamine and cycloguanil. Information on the relationship between in vitro schizontocidal activity and in vivo response to treatment are lacking for quinine and artemisinin. Quinine is being used in India for emergency medicament of malaria but so far no reports have emerged regarding treatment failure with this drug, indicating the sensitivity of the parasites to quinine. Mean plasma level of 1.5 $\mu$g/ml of quinine base is reached in a person 4 hours after ingesting a single dose of 300 mg of quinine (Saggers et al. 1970). Complete inhibition of parasites were obtained at much lower concentrations, clearly indicating that parasites used were sensitive to quinine. This experiment has also generated a baseline data of these parasites for quinine susceptibility. Artemisinin has not been used for malaria treatment in India, therefore parasites are expected to be sensitive to this drug. The mean plasma level of 110 ng/ml attained at about 7 hours in humans taking the curative dose of 10 mg/kg body weight (Zhao and Zhang 1985, 1986) of artemisinin is much higher the concentration of the drug needed to prevent the multiplication of the parasites used in vitro, indicating that the parasites of the clone F-56 are sensitive to artemisinin and we have baseline values of susceptibility of this parasite line to a drug which may be used in future for malaria treatment.
Figure 2.1 A typical Giemsa stained thin blood film made from asynchronous culture showing various asexual erythrocytic stages (R= ring, T= trophozoite, S= schizont) and a sexual stage (G= gametocyte). (X 1200)

Figure 2.2 A Giemsa stained thin blood film depicting free merozoites released from a ruptured schizont.
Figure 2.3  Drug dose response curve for chloroquine in the *P. falciparum* isolate FCD-4 and cl F-56 after 48 hours of incubation in vitro (performed in 24-well sterile plastic tissue culture plate).
Figure 2.4  Response of the *P. falciparum* isolate FCD-4 and clone F-56 to various concentrations of amodiaquine in vitro.
Figure 2.5  Pyrimethamine sensitivity of the *P. falciparum* isolate FCD-4 and clone F-56 determined in vitro by the modified 48 hour test method.
Figure 2.6  Cycloguanil response curves of the *P. falciparum* isolate FCD-4 and clone F-56 in the modified 48 hour test method.
Figure 2.7  Sensitivity of erythrocytic stages of *P. falciparum* (FCD-4 and F-56) to quinine in vitro.
Chapter 3
CHEMOSENSITIVITY OF A CHLOROQUINE-RESISTANT P. FALCIPARUM ISOLATE TO QUININE-TYPE COMPOUNDS IN VITRO

Abstract: In vitro relative chemosensitivity of erythrocytic stages of the chloroquine resistant P. falciparum isolate to quinine-type compounds has been evaluated. Apart from natural cinchona alkaloids, quinine and quinidine, their hydrogen derivatives proved to be more potent erythrocytic schizontocidal compounds. Some drug combinations show extremely good antimalarial property in vitro. Going by the IC<sub>50</sub> values of the eight preparations tested the compounds can be arranged in following order with respect to their schizontocidal activity: quinidine/hydroquinidine ≥ hydroquinidine > quinine/hydroquinine ≥ quinidine > hydroquinine/quinine > apoquinine > epiquinine.

Clearly in depth investigations are required for exploiting the full antimalarial potentials of these cinchona derivatives or mixtures and other combinations.

INTRODUCTION

The property of cinchona bark to cure fevers of diverse origin was well known to the natives of South America, particularly to the Peruvian and the Bolivian (now Ecuador) Indians, much before the recorded historical date of 1630s when the first European was successfully treated (presumably of malaria) by the powdered bark of cinchona tree (Guerra 1977 a, b.). Causative agent of the disease itself was first discovered and identified by Laveran (1880). The power of hot infusion of the powdered bark has been widely exploited in the treatment of malaria since then. Ever increasing demand of this bark, which could easily be mistaken for that of other trees, especially in the dried up conditions led to frauds, speculative and hoarding tendencies among traders in nineteenth century (Gramiccia 1987). This generated interest in chemical analysis of the cinchona bark. The curative property of the bark was assigned to alkaloid components. The bark of cinchona trees contains a mixture of some 10 alkaloids, but most of them are not crystallizable (Bruce-Chwatt et al 1981). Quinine and cinchonine were the first two alkaloids isolated in crystalline form in 1820 by Pelletier and Caventou (Hofheinz and Merkli 1984). Quinine salt proved to be an excellent antimalarial drug. Although correct structure of quinine had already been suggested by 1908, synthesis of this compound was achieved by Woodward and Doering.
MATERIALS AND METHODS

Parasites  Locally collected *P. falciparum* isolate FCD-4, being routinely maintained in the laboratory using candle-jar method of Trager and Jensen (1976) was employed in this study. Isolation, establishment and maintenance in vitro of this isolate has been described in an earlier section of this chapter. The characterization of the isolate demonstrated that FCD-4 was resistant to chloroquine.

Drug solutions  Coded preparations of cinchona alkaloids and their derivatives or mixtures were kindly provided by Dr. P.I. Trigg of the World Health Organization. Detail chemical structure of these compounds is given in Figure 3.1. Identity of the eight preparations used in the present investigation is as follows:

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Quinine (base)</td>
<td>Q</td>
</tr>
<tr>
<td>2. Quinidine (base)</td>
<td>Qn</td>
</tr>
<tr>
<td>3. Apoquinine (base)</td>
<td>AQ</td>
</tr>
<tr>
<td>4. Hydroquinine (base)</td>
<td>HQ</td>
</tr>
<tr>
<td>5. Hydroquinidine (base)</td>
<td>HQn</td>
</tr>
<tr>
<td>6. Quinine/Hydroquinine 60:40 mixture (bases)</td>
<td>Q/HQ</td>
</tr>
<tr>
<td>7. Quinidine/Hydroquinidine 60:40 mixture (bases)</td>
<td>Qn/HQn</td>
</tr>
<tr>
<td>8. Epiquinine/Epiquinidine sulphate 60:40 mixture</td>
<td>EQ/EQn</td>
</tr>
</tbody>
</table>

Stock solution of each compound was made by dissolving 1000 mg of the substance in 61.6 ml of 0.1N sulphuric acid and diluted with glass distilled water to 100ml. Further dilutions of the stock solutions were made with HEPES buff-
RESULTS

Chemosensitivity of isolate FCD-4 to each compound was initially evaluated by determining the blood schizontocidal activity in duplicate at 1, 3, 6, 10, 60, 100, 300, 600, 1000 ng/ml concentrations of the compound. Minimum inhibitory concentration (MIC) required to completely eliminate parasites with EQ/EQn (mixture), AQ, Q, HQ was found to be 600, 100, 60, 30 ng/ml, respectively whereas other preparations effectively eliminated all parasites at concentration of 10 ng/ml or less. The results obtained are summarized in the Table 3.1.

Table 3.1 Estimated MIC, IC_{90} and IC_{50} values of quinine and quinine-type compounds assayed in vitro against a chloroquine resistant isolate FCD-4 of *P. falciparum* in 24 well sterile plastic tissue culture plates with starting parasitemia of less than 1%, erythrocyte suspension of 2%. The parasites were under drug pressure for 48 hours.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>*IC_{50}</th>
<th>*IC_{90}</th>
<th>*MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinine</td>
<td>11</td>
<td>48</td>
<td>60</td>
</tr>
<tr>
<td>Quinidine</td>
<td>4</td>
<td>5.8</td>
<td>10</td>
</tr>
<tr>
<td>Apoquinine</td>
<td>13</td>
<td>78</td>
<td>100</td>
</tr>
<tr>
<td>Hydroquinine</td>
<td>5.6</td>
<td>9.4</td>
<td>30</td>
</tr>
<tr>
<td>Hydroquinidine</td>
<td>2.5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Quinine/Hydroquinine</td>
<td>3.9</td>
<td>8.4</td>
<td>10</td>
</tr>
<tr>
<td>Quinidine/Hydroquinidine</td>
<td>2.2</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Epiquinine/Epiquinidine sulphate</td>
<td>140</td>
<td>270</td>
<td>600</td>
</tr>
</tbody>
</table>

* Concentrations are in ng/ml.
tive than quinine at IC₅₀ level. Amount of quinidine needed to completely eliminate parasites in vitro is far below the non-toxic peak quinidine levels achievable in plasma of human subjects. Quinidine has greater systemic clearance and a larger total apparent volume of distribution compared with quinine (White 1987). Plasma concentrations during malaria treatment are therefore lower. In the slightly insensitive strains of *P. falciparum* to quinine, although mostly cases of the RI-type of resistance have been recorded (Cook 1986) quinidine has been rediscovered as more potent antimalarial than quinine. So far there has been no report of quinidine resistance either from field or laboratory studies. Information on the relationship between in vitro antimalarial sensitivity and in vivo response to treatment with quinidine is lacking. This information can greatly improve and define the dose regimens of quinidine in different parts of the world depending on the susceptibility of local strains, thus increasing the safety margin of this drug. Apart from natural cinchona alkaloids the synthetic hydroderivatives of quinidine, quinine and their combinations showed extremely good erythrocytic schizontocidal activity. Advantages of hydroquinine over quinine had also been observed by other workers (Giemsa and Werner 1914). The unnatural isomers of dihydroquinine and dihydroquinidine, the enantiomers or mirror image compounds, were shown by Brosi and colleagues (1971) to have same degree of activity as the natural isomers in *P. berghei* in mice. The four major alkaloids all possess significant blood schizontocidal activity but their relative potencies vary with the species of *Plasmodium* (Hofheinz and Merkli 1984). Differences in apparent activity of cinchona alkaloids in vivo are related to both pharmacokinetic factors in the host and pharmacodynamic factors in the parasite. But evaluation of chemosensitivity in vitro system eliminates host factors effectively. Quinine is evidently not the most effective cinchona alkaloids but has reached a unique position because it just happened to be the first to be crystallized and used in 'pure' form. Also because the activity of cinchona in vitro is high.
Figure 3.1 Chemical structures of quinine and quinidine-type compounds
Chapter 4

CURE WITH CISPLATIN OF MURINE MALARIA INFECTION AND INHIBITION IN VITRO OF A CHLOROQUINE RESISTANT P. FALCIPARUM ISOLATE

Abstract: Antiplasmodium properties of cisplatin (cis-platinum (II) diammine dichloride), a neoplastic drug, have been assessed using in vivo and in vitro model systems of malarial parasite. A well tolerated dose of 6 mg/kg body weight of the compound cured the mice infected with P. berghei and the amount of cisplatin required for in vitro inhibition (IC₅₀) of a chloroquine resistant falciparum isolate was lower than either chloroquine or quinine. Minimum inhibitory concentration (MIC) needed to prevent the multiplication of asexual blood parasites in vitro was 30 ng/ml. Late ring and trophozoite stages of erythrocytic cycle were most susceptible whereas schizont and early ring stages were least sensitive to the toxic effect of cisplatin. Smaller multiple doses had been the most effective in curing malaria in mice than a single large dose. In a few cases, mice treated with a single intraperitoneal large dose of 6 mg/kg body weight, there was a delay in appearance of parasitemia but most of them recovered completely but slowly. This compound exerts its toxicity mainly by randomly damaging and cross-linking DNA strands as has been shown by Southern hybridization using a synthetic oligonucleotide probe, which is a repeat sequence in the falciparum genome. The report clearly demonstrates the antimalarial potentials of this compound and suggests a closer evaluation of this and other related compounds, specially in combination with antimalarial drugs to probe their synergistic properties.

INTRODUCTION

Species of Plasmodium are the causative agents of malaria in man and some other vertebrates. P. berghei is responsible for murine malaria in mice and P. falciparum causes 'malignant tertian' human malaria. Appearance and speedy spread of resistance in P. falciparum to common synthetic antimalarial drugs like chloroquine (Peters 1990b; Bruce-Chwatt et al. 1981), pyrimethamine (Peters 1984; 1990b), proguanil (Chaudhuri and Chaudhuri 1949; Field and Ederson 1949; Peters 1990b), used for treatment are showing an increasing rate of curative failures (Peters 1984; Wernsdorfer 1991). The current grim situation is thus a cause of concern to malaria epidemiologists, indicating the urgent need to identify and develop alternate compounds capable of curing resistant malaria. Malaria pathogenicity can be diminished by administration of drugs which inhibit active multiplication of asexual erythrocytic parasites and cure can be achieved by effectively eliminating the blood forms from the host. The parasite clearance may be achieved either by lethal action of the compound used for treatment and/or by its...
ability to evoke a stronger immune response against the parasite. Cisplatin has twin properties of being employed for neoplastic treatment (Einhorn and Williams 1979; Rosenberg 1984) and immunomodulation (Page et al. 1977; Kleinerman and Zwelling 1982). Cisplatin is a coordination compound of bivalent platinum and was the first inorganic antineoplastic agent to enter into clinical investigations around 1972 (Rosenberg 1973). Some of the known properties or salient features of cisplatin have been enumerated as under:

1. The drug is active against a wide variety of tumors (Rosenberg 1973, 1980)
2. Inactivates transforming DNA and viruses (Munchausen 1974; Shooter et al. 1972)
3. Induces filamentous growth and causes prophage induction in lysogenic strains of *E. coli* bacteria (Rosenberg 1971, 1973)
4. The drug causes chromosomal abnormalities and toxicity (Vandenberg and Roberts 1975)
5. Supresses graft rejection against H2 histocompatibility barrier in mice (Khan et al. 1972)
6. Activates the immune system of the host (Rosenberg 1985)
7. Selectively inhibits DNA synthesis (Harder and Rosenberg 1970; Howley and Gale 1970)
8. Causes mutagenesis (Monti-Bragdin et al. 1975; Lecointe et al. 1977)

To our knowledge no work has been reported on the effect of this compound on malaria parasites. These considerations prompted us to assess antiplasmodial properties of cisplatin in vitro and in vivo. Further, in order to understand the mode of action of this compound, studies on stage-specific susceptibility of erythrocytic parasites in vitro and interaction of cisplatin with intact or restriction enzyme digested *P. falciparum* DNA have been carried out. The later experiments
were performed with a view to look for changes, if any, in the number and size of restriction fragments generated, following incubation of *P. falciparum* DNA with cisplatin, on a Southern blot using *P. falciparum* specific oligonucleotide probe.

**MATERIALS AND METHODS**

**Parasites** Rodent malaria parasite *P. berghei* was obtained from National Institute of Communicable Diseases, Delhi, India and maintained in Swiss albino mice of 30-40 g each, by weekly mechanical passage of infected erythrocytes drawn from a donor mouse by cardiac prick into a sterile, heparinized syringe. The biology of these parasites has been reviewed by Killick-Kendrik (1978). Chloroquine resistant *P. falciparum* isolate FCD-4 of human origin which has been described in earlier section, was the other parasite used in this study.

**Stock solution of cisplatin** The stock solution of cis-platinum (II) diamine dichloride, obtained from Sigma Chemicals, USA, was prepared by dissolving 1 mg of the compound in 60% dimethylsulfoxide (DMSO). Further dilutions were made with buffered RPMI-1640 culture medium.

**Collection of parasitized blood from infected donor mouse**

The strain of *P. berghei* was propagated in Swiss-albino mice by weekly mechanical passage of infected erythrocytes. The parasitized blood was drawn from an infected donor mouse via cardiac puncture. The infected blood was centrifuged at 2000 rpm for 10 min at 4 °C. The buffy coat and supernatant containing plasma were discarded. Packed cells were washed twice in 3.5 volumes of sterile 0.85% NaCl. This parasitized blood was used for passaging in mice and the course of infection followed by examining Giemsa stained blood films made each day from the tail blood.

**Inoculation of test animals**

Infected blood collected from the donor mouse usually contained about 70% parasitemia. The infected erythrocytes were washed with sterile normal saline as
described above. The number of cells per 0.05 ml of infected blood was calculated using Neubauer cell counting chamber. Percentage parasitemia was determined from Giemsa stained thin blood films. Each mice was inoculated with 0.05 ml of infected blood containing $10^7$ to $10^8$ parasites. Infected mice were kept in an air-conditioned room (24 °C - 26 °C). Mice were fed *ad libitum* with commercial pellet and water supplemented with 0.01% p-aminobenzoic acid.

**Erythrocytic schizontocidal activity**

*In vivo susceptibility of P. berghei*: Curative property of cisplatin was assessed by comparing the survival times of mice untreated or treated with cisplatin receiving same amount of parasite inoculum in the form of infected erythrocytes obtained from a donor mouse. Test mice were taken in groups of five. Preliminary experiments were conducted in batches consisting of three mice each to arrive at the optimum tolerable dose of cisplatin effective in curing the mice. These preliminary doses ranged from 2-12 mg/kg body weight. Each member of the test group was injected with $10^8$ parasitized erythrocytes, inoculated intraperitoneally. The cisplatin was given intraperitoneally but eight hours later either as a single dose or in four equally divided doses, at 4 hour intervals each in 1 ml saline solution. Appropriate sets of control mice were maintained. The development of parasitemia was monitored by making Giemsa stained blood films from the cut tail vein of the experimental and control animals every 24 hours for a period of 60 days.

*In vitro susceptibility of P. falciparum*: Erythrocytic schizontocidal activity of cisplatin on *P. falciparum* was determined by exposing parasites in triplicates to graded concentrations of cisplatin using the modified 48 hour test method (Nguyen-Dinh and Payne 1980). The experiments were conducted in 24-well tissue culture plates by setting up microcultures with eight different concentrations, ranging from 1 to 1000 ng/ml. The parasite material for experiments was ob-
tained from stock cultures and subjected to sorbitol lysis (Lambros and Vanderberg 1979), to get synchronized ring stages. The parasitemia of the 50% cell suspension of this synchronized material was adjusted to less than 1% with uninfected freshly washed erythrocytes. Aliquot of 20 μl of the suspension was added into series of wells of the test plate, each holding 480 μl of complete medium with or without drug, yielding final cell suspension of 2%. Loaded test plates were incubated at 37 °C in a candle-jar for 96 hours with daily change of medium. The drug was included in experimental wells for first two days only. Blood smears were made at the end of 48 and 96 hours, stained with Giemsa. Minimum of 5000 erythrocytes were enumerated to determine the parasitemia. Percentage reduction of parasitemia in relation to control was calculated. Fifty percent and 90% inhibitory concentrations (IC₅₀ and IC₉₀, respectively) were extrapolated from semi-log plot of varying concentrations of cisplatin against percentage inhibition of growth.

**In vitro stage-specific sensitivity:**

To investigate the preferential toxicity to any specific stage of *P. falciparum* in vitro, asynchronous culture material was subjected to triple sorbitol treatment at 0, 40 and 47 hours interval, to obtain synchronized parasites of 0-7 hours old (Lambros and Vanderberg 1979). These synchronized parasites were in early ring stage for another 12 hours, for next 12-24 hours of development in late ring or early trophozoite stage, 24-36 hours old in late trophozoite stage and during subsequent 36-48 hours of growth in schizont stage of the erythrocytic schizogonic cycle. The synchronized stages were subjected to drug pressure for 12 hours each, the respective period of their residence in the specific stage. The experiments were conducted in triplicates in multiwell plates and slides made at the end of schizogonic cycle. The toxicity of cisplatin to ring, trophozoite and schizont infected stages was determined by calculating the percentage inhibition of parasitemia in drug treated parasites to that in drug free controls. The extent of syn-
chronization of parasites achieved is depicted elsewhere.

**Platination of DNA**

Binding of cisplatin to *P. falciparum* DNA, extracted from erythrocytic stages (Tungpradabkul and Panyim 1985) was studied by incubating 4 μg of DNA at 37 °C for 24 hours with 50 or 100 ng of cisplatin in total volume of 10 μl. The incubated material was then digested with restriction enzyme *Hind* III at 37 °C for 2 hours (Sambrook et al. 1989). In another set of experiment *Hind* III digested DNA material was incubated with cisplatin. Similar experiments were performed using *Taq* I. These digested samples were size fractionated on a 0.7% agarose gel along with control samples of uncleaved or restriction enzyme cleaved DNA not incubated with cisplatin. The gel was stained with ethidium bromide and visualized under UV before the material was transferred on to a nylon membrane by Southern blotting. The membrane was then hybridized with γ-32P end labelled 21-mer synthetic oligonucleotide, which is known to be a repetitive sequence in *P. falciparum* genome (Mucenski et al. 1986), at 42 °C using stringent washing conditions. The methods employed for the extraction of DNA, Southern transfer and hybridization have been described in the following chapters. The sequence of the oligomer used to probe the membrane was as follows:

5'-AGGTCTTAACTTGACTAACAT-3'

**RESULTS**

The periodicity of blood schizogony of the rodent malaria parasite *P. berghei* was a quotidian cycle i.e., 22-25 hours. The mortality rate of *P. berghei* in the strain of white mice used in the present study was 100%, if untreated. The mortality curve was bimodal. During the first 6 days of infection, mature erythrocytes were invaded, and the mouse died in a condition of shock. If the animal survived the first phase, many reticulocytes then occurred in the blood and were preferen-
tially invaded, anemia increased and the animal died later in a condition of an-oxia. Parasites were observed in tail blood films of test animals within 24 hours after inoculation of infected blood. As seen in Figure 4.1 a & b the characteristic course of infection was highly asynchronous. Blood films showed the presence of all the different asexual blood stages of the parasite namely rings, trophozoites and schizonts at the same time. Reticulocytes were frequently infected with multiple parasites than mature erythrocytes. Upto 10 parasites per reticulocyte were observed. The intraerythrocytic ring forms showed red staining chromatin dot with a very small area of blue stained cytoplasm. The trophozoites were recognizable by the presence of chromatin dot with enlarged cytoplasm containing faint brown-yellowish spots of haematin granules. Mature schizonts were characterized by 12-20 distinct merozoites and a dark brown-yellowish dot of clustered haematin. These schizonts in due course became more fragile and released their merozoites, resulting in groups of free merozoites accompanied by a free pigment cluster (Figure 4.1 b). Platelets and white blood corpuscles are seen in Figure 4.2.

Ability of cisplatin to clear *P. berghei* infection from mice is presented in Table 4. Total dose of 6 mg/kg body weight given in four equally divided intraperitoneal injections at four hour intervals effectively cured the infected mice and no parasitemia could be observed for sixty days in any of the treated mice. The same dose when given as a single intraperitoneal injection finally cured 80% of the infected mice. Initially, however, in three out of five of these test animals parasite appearance was delayed by 11 days compared to controls and parasite population increased subsequently leading to death of one of the mice whereas in the other two mice appearance of high parasitemia was followed by slow decline with final disappearance of the parasites after 14 days. Four week onwards from the start of the experiment no parasite could be observed in these two surviving mice till day sixty. In all the control animals, not receiving treatment with cisplatin, healthy parasites appeared in circulation within 24 hours of receiving the infective inocu-
lum and they died in less than 10 days due to high parasitemia.

Table 4.1  Ability of cisplatin to cure *P. berghei* infected mice. Each of the mice received an intraperitonial innoculum of 1x10⁸ parasites. The treated animals were injected intraperitoneally with cisplatin 8 hr later.

<table>
<thead>
<tr>
<th>Infected mice</th>
<th>Untreated</th>
<th>Treated with cisplatin</th>
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<tbody>
<tr>
<td>Dose</td>
<td>nil</td>
<td>6mg/kg body weight</td>
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<tr>
<td></td>
<td></td>
<td>as a single dose</td>
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<tr>
<td></td>
<td></td>
<td>6mg/kg body weight</td>
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<td></td>
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<td>in four equally divided</td>
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<td></td>
<td></td>
<td>doses at 4 hr intervals</td>
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<tr>
<td>Mice showing</td>
<td>5/5</td>
<td>3/5</td>
</tr>
<tr>
<td>parasitemia</td>
<td></td>
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<tr>
<td>within two</td>
<td></td>
<td></td>
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<tr>
<td>weeks of</td>
<td></td>
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<td>innoculation</td>
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</tr>
<tr>
<td>Mice</td>
<td>0/5</td>
<td>4/5</td>
</tr>
<tr>
<td>surviving</td>
<td></td>
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<tr>
<td>till 60 days</td>
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<td>following</td>
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<td>innoculation</td>
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Inhibitory effect of cisplatin on multiplication of the synchronized cultures of *P. falciparum* in vitro are presented in Figure 4.3. Complete inhibition of the multiplication was achieved by minimum amount of 30 ng/ml of cisplatin present in culture medium for a duration of 48 hours. From the dose response curve IC₅₀ and IC₉₀ values determined after 48 hours were found to be 6.2 and 24 ng/ml, respectively and 3.8 and 26 ng/ml, respectively after 96 hours experiment. Abnormal parasites were observed from drug treated wells at the end of 48 hours, showing different degree of vacuolization or pale staining, whereas normal healthy parasites multiplied to about six fold in control wells in a schizogonic cycle. Most of the abnormal parasites in treated wells had degenerated or lysed by 96 hours.
The stage specific toxicity of cisplatin is given in Figure 4.4. Schizont stages were least susceptible to the toxic effect of the compound followed by young rings whereas trophozoites and old rings were most susceptible. The schizont stages exposed to 30 ng/ml of cisplatin in the medium resulted in only 36% growth inhibition.

Autoradiographs of the restriction enzyme digested falciparum DNA incubated with or without cisplatin and probed with a synthetic repetitive oligonucleotide are shown in Figures 4.5 & 4.6. No hybridization band was observed in the lanes containing uncut DNA or digested DNA incubated with cisplatin whereas control lanes showed the clear presence of one or several hybridization bands.

DISCUSSION

One of the most inexpensive ways of searching new lead antimalarial compounds for drug development could be to screen the drugs which have been developed for other diseases and evaluate their antimalarial potentials, either alone or in combination with other known antimalarial drugs. Cisplatin is a drug with high potency in treatment of a few human malignancies (Rosenberg 1985; Hosokawa et al. 1982; Volge et al. 1982) and is a broad spectrum anticancer drug in animals (Rosenberg 1985; Welsh 1971). It is believed that the drug basically enhances the immunogenicity of the tumor cells by exposing new antigenic sites on the tumor cell surface and this evokes a host of offending immune reactions directed against the tumors. This assumption is based on the following observations: a) Swiss white mice cured of advanced Sarcoma-180 tumors with therapeutic dose of cisplatin completely rejected all further transplants for 11 subsequent months because of high level of immunity generated against the tumor lasting for this period (Rosenberg 1973, 1985). b) In the Swiss white mice cisplatin treatment produced nearly absolute cure of advanced Sarcoma-180 tumors with a single intraperitoneal injection on day eight but administration of hydrocortisone, an
immunodepressent, on a day prior to the injection of cisplatin produces only 40% cure by eighth day (Conran and Rosenberg 1972). *P. falciparum* also possesses genes with affinities to multiple drug-resistance (mdr) genes of human cancer cells (Bradely et al. 1988; Foote et al. 1989) and this particular factor provided a strong clue that some anticancer drugs may possess antiplasmodial properties. The results presented in this report amply demonstrate the antiplasmodial potential of cisplatin. Apparently non-pathogenic dose of this compound cured the malaria infected mice and in vitro inhibited multiplication of a chloroquine resistant falciparum isolate at a low concentration included in the medium.

Toxicity to the host is an important consideration of any candidate compound with potential of being employed as a drug. The amount of cisplatin used in this study to cure malaria infection in mice is well tolerated by the host. The dose of 6 mg/kg body weight given either as a single intraperitoneal push-in injection or in four equally divided doses at 4 hour intervals with saline did not cause any mortality in control mice. Other cancer workers have also reported that therapeutic dose of 7 mg/kg body weight of cisplatin causes less than 10% mortality in mice and as a single intraperitoneal injection, the administered LD$_{50}$ dose is 13 to 14 mg of cisplatin/kg body weight (Rosenberg 1985). Slow infusion of cisplatin gives better results in cancer treatments (Rosenberg 1985) and also kidney toxicity is ameliorated with saline hydration (Hayes et al. 1977). Present report also shows that 1.5 mg/kg body weight dose of cisplatin 4 times at 4 hour intervals with saline is far more superior than a single dose of 6 mg/kg in treatment of mice malaria. This is perhaps due to longer retention of higher level of cisplatin in the body for extended period of time, as half life of cisplatin in mice has been reported to be only 20 hours and an erythrocytic schizogonic cycle of mice malaria is of 24 hour duration. In the two mice, which received single 6 mg/kg body weight dose of cisplatin, appearance of parasitemia was followed by slow but complete recovery, this could be due to immunostimulation property of cisplatin which has been clearly
demonstrated in several reports (Kleinerman et al. 1980; Kleinerman and Zwelling 1982; Bahadur et al. 1984). Cisplatin has trypanocidal activity in mice (Wysor 1982). The preliminary result in this study clearly demonstrates the antiplasmodial properties of cisplatin both in vitro and in vivo systems. Therefore, it would be worthwhile to investigate the antimalarial properties of this and other related platinum complexes with regard to their ability to cure polyresistant *Plasmodium* species. Probing additivity and synergism properties in combination with other antimalarial drugs having different mode of actions should also not be ignored, but all this needs further serious investigations.

Amount of cisplatin required in vitro to achieve 50% inhibition of parasitemia in isolate FCD-4 was found to be less than either chloroquine (IC$_{50}$ = 18 ng/ml, our unpublished data) or quinine (IC$_{50}$ = 11 ng/ml) (Nair and Bhasin 1993). Not much difference in IC$_{50}$ and IC$_{90}$ values calculated from the slides made at the end of 48 or 96 hours of the experiment suggests that there was an insignificant delayed mortality of the parasites due to cisplatin toxicity. Most of its lethal effect is exhibited within an erythrocytic schizogenic cycle.

Each of the synchronized asexual erythrocytic stages were subjected in vitro to uniform drug pressure of 12 hours with differential outcome with regard to parasite survival and multiplication. Intraerythrocytic merozoites in the schizont and the early rings are the least susceptible to toxic effects of cisplatin, perhaps in these stages no active DNA synthesis takes place (Inselburg and Banyal 1984) and the parasite plasma membrane is most intact thus retarding free entry of the compound into the parasite milieu. Cisplatin is known to prevent the multiplication of cancer cells by randomly inter- and intralinking of DNA strands of these dividing cells (Cohen et al. 1979). The most damaging outcome, therefore, are seen in the early and late trophozoite stages of malaria parasite, known to be actively involved in DNA synthesis (Inselburg and Banyal 1984).

Molecular basis of cisplatin toxicity has been attributed to lesions in DNA
molecules of cancer cells leading to inter- and intrastrand DNA cross-links, fi-
nally resulting in cell death (Cohen et al 1979; Zwelling and Kohn 1979). In vitro
binding of cisplatin can occur with any base but prefered order of binding is
G>A>C>T (Rahn et al. 1980). We explored the interaction of cisplatin with
_P. falciparum_ DNA, by incubating it with intact DNA and digesting it later with
restriction enzymes and by incubating predigested DNA with cisplatin. When these
samples were run on agarose gel and stained with ethidium bromide, no fluores-
cence was observed in lanes containing samples incubated with cisplatin, showing
that cisplatin prevents intercalation of ethidium bromide with DNA. Following
blotting of these samples on the nylon membrane and on probing with radiola-
belled 21-mer repeat sequence, no hybridization in these lanes are visible. This
may be due to inter- and intrastrand cross linking of DNA by cisplatin, thus pre-
venting the specific binding of the probe to complementary sequences on the DNA
which is clearly seen in the form of distinct bands in control lanes of the autorad-
iograph. Thus mode of action of this compound on _Plasmodium_ may be similar to
the reported mechanism of action on neoplastic cells.

Figure 4.1 a. A Giemsa stained thin blood film from *P. berghei* infected mouse showing different asexual stages of the parasite namely ring (R), trophozoite (T) and schizont (S).

Figure 4.1 b. A Giemsa stained thin blood film from *P. berghei* infected mouse showing multiple infection of reticulocytes, a cluster of free merozoites and pigment.
Figure 4.2  A Giemsa stained thin blood film from *P. berghei* infected mouse showing parasites, platelets (P) and WBC (W).
Figure 4.3 In vitro growth inhibition response of asexual erythrocytic stages of *P. falciparum* to different concentrations of cisplatin in 48 hour test. Slides were made at the end of 48 and 96 hours. Starting synchronized parasitemia was less than 1%. 
Figure 4.4 In vitro growth inhibition of different asexual erythrocytic stages of *P. falciparum* to various concentrations in a 48 hour test. Slides were made at the end of 48 hours. Starting parasitemia in each case was less than 1%.
Figure 4.5  An autoradiogram showing hybridization bands of *P. falciparum* DNA with species-specific synthetic $\gamma^{32}$P labelled 21-mer oligonucleotide probe. The DNA samples (4 µg in each lane except lane 3, which was blank) were size fractionated on 0.7% agarose gel and Southern transferred on to nylon membrane for hybridization.

Lane 1. Undigested *P. falciparum* DNA
2. DNA digested with *Hind III*
3. Kept blank
4. DNA incubated with DMSO prior to digestion with *Hind III*
5. DNA digested with *Hind III* and incubated with DMSO
6. DNA incubated with 50 ng of cisplatin
7. DNA digested with *Hind III* and incubated with 50 ng of cisplatin
8. DNA incubated with 100 ng of cisplatin and later digested with *Hind III*
9. DNA digested with *Hind III* and incubated with 100 ng of cisplatin
Figure 4.6 An autoradiogram showing hybridization bands of *P. falciparum* DNA with species-specific synthetic γ ${}^{32}$P labelled 21-mer oligonucleotide probe. The DNA samples (4 μg in each lane except lane 3, which was blank) were size fractionated on 0.7% agarose gel and Southern transferred on to nylon membrane for hybridization.

Lane 1. *P. falciparum* DNA incubated with 50 ng of cisplatin and then digested and then digested with *Taq* I

2. DNA digested with *Taq* I

3. DNA digested with *Taq* I and then incubated with 50 ng of cisplatin

4. DNA incubated with DMSO prior to digestion with *Taq* I