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Characterization of drug resistant isolates of *Plasmodium falciparum*

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fulfilment of the requirements of the degree of
Master of Science

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ABSTRACT

Plasmodium falciparum is a protozoan parasite and the causative agent of the most lethal form of malaria, a major disease in the tropical world. Chloroquine has been very effective in treatment of this disease, however the emergence of chloroquine-resistant strains in most geographical regions where malaria is endemic has made difficult the control of malaria. In addition, resistance to other antimalarials has been observed in these regions. The objective of this study was to determine the molecular mechanisms of multidrug resistance in *P. falciparum*. We have selected *in vitro* a *P. falciparum* strain resistant to actinomycin D from a parental drug sensitive clone, 3D7. Interestengly, we found that the actinomycin D resistant clone is less sensitive to chloroquine and mefloquine (antimalarial drugs) and rhodamine123. Comparison between parental 3D7 and resistant *P. falciparum* did not show differences in the copy number or level of expression of *pfmdr1* previously implicated in chloroquine or mefloquine resistance. Furthermore, to identify if other P-glycoprotein homologues are involved in resistance, we used oligonucleotide primers to conserved sequences in ABC domains. An ABC protein, a homologue to the subunit 4, of the 26S proteasome complex has been cloned. To determine if this gene was involved in resistance to actinomycin D, a Northern blot was done. Surprisingly it was found a decreased in the expression of this gene in the resistant cell line, 3D7R/actD2, in comparison with its parental cell line, 3D7. Studies are in progress to determine the role of the PFS4 subunit in the resistance phenotype of 3D7R/actD2.

Abrégé

Le protozoaire *Plasmodium falciparum* est le parasite responsable de la malaria sous sa forme la plus grave, une maladie d'importance majeur dans les pays tropicaux. Le traitement à la chloroquine de cette maladie, bien qu'efficace dans le passé, se trouve présentement freiné par l'émergence de souches résistantes dans plusieurs régions où le paludisme est endémique. Dans ces même régions, on observe aussi une résistance accrue aux autres drogues antipaludiques. Cette étude tente de déterminer les mécanismes moléculaires impliqués dans le phénomène de résistance aux drogues chez *P. falciparum*. Nous avons sélectionné *in vitro* une souche de *P. falciparum* résistante à l'actinomycine D à partir d'un clone susceptible (3D7). Nous avons observé que la lignée résistante ainsi sélectionnée, appelée 3D7R/actD2, était moins sensible à l'effet de drogues antipaludiques tel que la chloroquine et la mefloquine, ainsi qu'à la drogue rhodamine 123. Une comparaison entre la souche-mère 3D7 qui est susceptible et sa dérivée résistante 3D7R/actD2 n'indique aucune différence au niveau du nombre de copies ou de l'expression de *pfmdr*1, un gène connu pour son rôle dans le phénomène de résistance à la chloroquine et à la mefloquine. Afin de déterminer si d'autres homologues de la P-glycoprotéine sont impliqués dans la résistance, nous avons utilisé des oligonucléotides pouvant hybrider aux regions conservées des domaines ABC. Une protéine ABC, homologue à la sous-unité 4 du complexe protéosome 26S, a été clonée. Nous avons étudié l'expression au niveau de l'ARN du gène codant pour cette protéine afin de déterminer s'il est impliqué dans la résistance à l'actinomycine D. Nous avons observé une expression moins élevée chez la souche résistante 3D7R/actD2 que chez la souche susceptible 3D7. Nous poursuivons l'étude du rôle de la sous-unité PFS4 dans la resistance de la lignée 3D7R/actD2.

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STATEMENT OF CONTRIBUTION

The experimental work reported herein (**Manuscript I** and **Manuscript II**) was performed by myself under the guidance of Dr. Elias Georges, with the exception of the cytotoxicity assays that were performed by Zhi Liu and Abrahem Abrahem.

STATEMENT OF ORIGINALITY

The following aspects described in this thesis are considered original contributions to knowledge:

MANUSCRIPT I:

This was the first report demonstrating the selection of a clone of *P. falciparum* that showed decrease sensitivity to actinomycin D, chloroquine, mefloquine and rhodamine 123. Given the differences in the structures and functions of these drugs, our results suggest that 3D7R/actD2 is multidrug resistant. Moreover, the resistance phenotype of 3D7R/actD2 does not involve changes in *pfmdr*1 copy number, mutations or increased expression.

MANUSCRIPT II:

This was the first report demonstrating the presence of a gene in *P. falciparum* , PFS4, with a very high homology to the S4 subunit of the 26S proteasome complex. This gene is expressed mainly during the ring stage of the parasite and our preliminary results suggest for the first time that this gene may be involved in resistance.

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MANUSCRIPT I

MANUSCRIPT II

LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ActD	ActinomycinD
Asn	Asparagine
Asp	Aspartic acid
BP	Base pair
CFTR	Cystic Fibrosis Membrane Regulator
CHO	Chinese hamster ovary
CQ	Chloroquine
CQS	Chloroquine sensitive
CQR	Chloroquine resistant
Cyst	Cysteine
DHFR	Dyhidrofolate resitant gene
Edta	ethylenediaminetetra acetate
Fig	Figure
HIV	Human immunodeficiency
IC50	50 percent inhibitory concentration
kB	Kilobases
kDa	Kilodaltons
MCP	Multi-catalytic protease
MDR	Multidrug resistance
MQ	Mefloquine

MIC	Minimal inhibitory concentration
MRP	Multidrug resistant associated protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P. falciparum	Plasmodium falciparum
P-gp	P-glycoprotein
RBC	Red blood cells
Ser	Seryne
VRP	Verapamil

INTRODUCTION

The most severe form of human malaria is caused by *Plasmodium falciparum* (*P. falciparum*) and it is commonly treated with quinine analogues (e.g. chloroquine). However, a major obstacle in the control of malaria has been the development of drug resistance to chloroquine in many areas of the world. In addition, an increasing number of chloroquine resistant malaria isolates show cross resistance to other antimalarial drugs (Draper *et al*, 1989).

It is presently believed that chloroquine resistance in *P. falciparum* shares a similar phenotype with multidrug resistant tumour cells that over express P-glycoprotein (P-gp) (Karner *et al* 1985, Hamada and Tsuruo, 1986). P-glycoprotein is the product of the MDR1 gene in human cells. Resistance to high concentrations of drugs is accompanied by an amplification of MDR1 and a simultaneous increase in the levels of the protein. P-gp mediates the efflux of drugs via an energy dependent mechanism. The drug efflux function of P-gp was shown to be reversed with various agents collectively known as chemosensitizers (Georges *et al.*, review, 1990). There are similarities between the MDR phenotype and chloroquine resistance in malaria: Chloroquine resistance in malaria is associated with a higher efflux of the drug in comparison to sensitive isolates (Krogstad *et al*, 1987). Also resistance can be reversed *in vitro* by chemosensitizers such as verapamil (Martin *et al*, 1987).

A P-gp homolog (*pfmdr1*) has been cloned from chloroquine resistant isolates of *P. falciparum* (Foote *et al*, 1989). The plasmodial *pfmdr1* gene encodes the Pgh1 protein, a member of the large ATP binding cassette protein gene family which includes the mammalian MDR genes, the CFTR gene and others (Hyde *et al,* 1990). However, it has not been possible to show how *pfmdr1* mediates chloroquine drug resistance in *P. falciparum*. In addition, other studies have excluded the role of *pfmdr1* in chloroquine drug resistance. (Barnes *et al,* 1992).

Although the role of *pmdr1* in drug resistance is controversial, experimental data suggests that changes in another unknown gene may be required for the expression of chloroquine drug resistance. Thus, in an effort to further understand the mechanism of chloroquine resistance or multidrug resistance in *P. falciparum*, we set out to select and characterize the changes in actinomy

cin D resistant *P. falciparum*. Given the capacity of actinomycin D to induce P-gp expression in tumour cells, we speculated that actinomycin D could also induce the expression of a membrane transporter in *P. falciparum* that could mediates drug resistance.

LITERATURE REVIEW

1. MALARIA

1.1. Epidemiology

Malaria is a mosquito borne infection caused by a protozoa of the genus *Plasmodium*. Man is commonly infected by four species of the parasite: *P. vivax, P. ovale, P. malariae* and *P. falciparum*. The latter is the most pathogenic and prevalent of the human malarias. It has been estimated that over one half of the world's population lives in endemic areas of malaria, specifically in tropical and subtropical areas of America, Africa and Asia (Katz *et al.*, 1988). More than 200 million cases and at least one million consequent deaths are estimated to occur annually with nearly 300 million people infected by the parasite (WHO, 1995). All age groups are susceptible to malaria infection; however, the pediatric population is most affected (Reyes Romero *et al.*, 1988).--

Infection with malaria is generally acquired when an infected female of the anopheles mosquito bites and sucks blood, injecting salivary fluids into the host's wound. It is also possible to acquire the infection as a result of blood transfusions or by transplacental infection (congenital paludism).

1.2. Life cycle of the parasite

The biology of the parasite consists of two stages, the sexual and the asexual.The asexual stage in man develops first in the liver and then in the circulating red blood cells (RBCs); the sexual stage occurs in the mosquito (Katz *et al.*, 1988).

The asexual stage is initiated when an infected female mosquito takes a blood meal injecting sporozoites into the wound of the host. This motile form of the parasite infects parenchymal cells of the liver. Inside the liver, the parasites begin asexual division (schizogony) to

produce merozoites. This process of development and multiplication is called extraerythrocytic schizogony.

After six to fourteen days merozoites are released from the hepatocyte to invade susceptible red blood cells. Within the erythrocyte, the merozoite undergoes the erythrocytic schizogony (fig. 1).

Attachment of the merozoites to the erythrocyte membrane involves interactions with several protein receptors (Zucker *et al.* 1993). Following adhesion, the RBC undergoes a rapid and marked deformation leading to the endocytosis of the parasite. Once inside the RBC, the parasite grows to form a ring-like early trophozoite. The formation of the schizont marks the asexual division and the second stage of the parasite. The asexual cycle is completed when the erythrocyte is ruptured and the merozoites are released to invade other RBCs. During parasite development in the host some merozoites will be transformed into microgametocytes (male) and macrogametocytes (female). When gametocytes are ingested by an appropriate mosquito vector the sexual stage occurs. Male gametocytes initiate exflagelation, a rapid process that produces microgametes that can fertilise the macrogametes. The resulting zygotes elongate into diploid vermiform ookinetes, which penetrate the gut wall and come to lie under the basement membrane. The parasites then transform into oocysts. Development of sporozoites follows, leading to the production of several thousands sporozoites. The sporozoites mature within 10-14 days and escape from the oocyst to invade the salivary glands. A new cycle begins when the mosquito takes a blood meal from its host.

1.3. Pathogenesis

The pathogenic process occurs only during the erythrocytic cycle. The rupture of erythrocyte and release of pyrogens causes fever, chills and sweating associated with the disease. Cerebral malaria, a severe consequence of *P. falciparum* infection, is caused by the blockage of cerebral capillaries by infected deformed erythrocyte, which adhere to the endothelium. Other manifestations of malaria infection include, anaemia and bone marrow depression. In some cases, the damage of erythrocyte by intravascular hemolysis is greater than that caused by the rupture of the infected red blood cells alone.

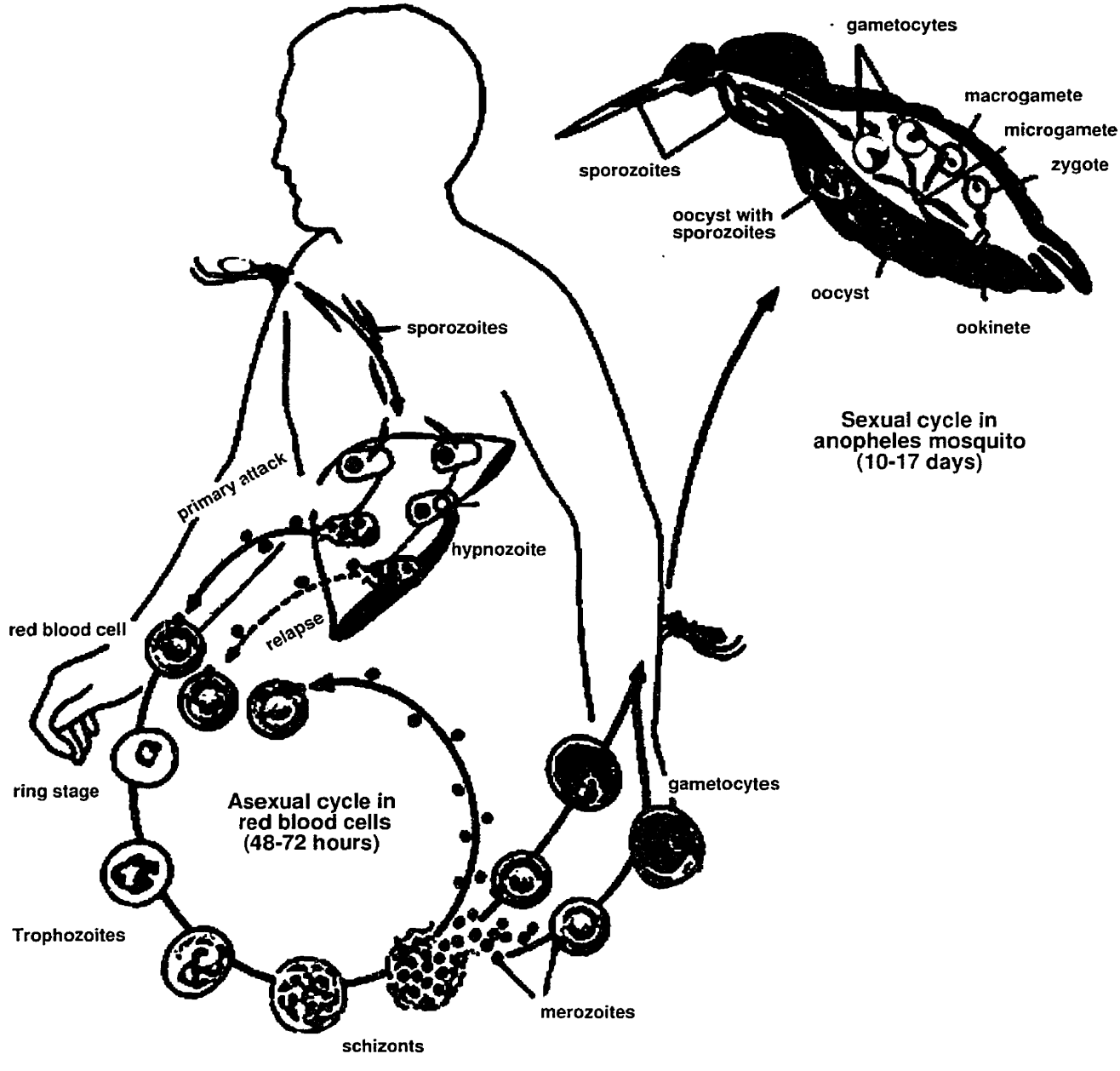


Fig. 1. Life cycle of human malaria parasites.

Chronic infection which can cause splenomegaly, is sometimes accompanied by hepatomegaly, portal hypertension, leukopenia and thrombocytopenia. The gastrointestinal tract can also be affected when the parasite invades the vessels. There are focal haemorrhages, edema and malabsorption (Miller *et al.*, 1994).

Some biological features of *P. falciparum* have been identified with its lethal potential (Wyler *et al.*, 1993). Firstable, the asexual blood stage parasites can invade erythrocytes of all ages, so there is no limit on the potential magnitude of parasitemia. One of the consequences of high parasitemia is severe hemolysis. In contrast, other species can develop only either in the youngest forms or in the oldest forms of erythrocytes.

Another feature of *P. falciparum* is that electron dense excrescences called knobs develop on the surface of RBCs containing mature forms of the parasites. These knobs mediate binding of the infected red blood cells to post capillary venules where the parasites finally mature. It has been proposed that parasites modify the surface of the cell to enhance its survival. As a result, micro vascular congestion occurs resulting in tissue hypoxia and subsequently organ failure. The brain, kidneys and lungs are specially susceptible (Miller *et al.*, 1994).

1.4. Clinical disease

The symptoms usually appear 10-15 days after the bite by an infected mosquito. At early stages, malaria infection manifests as a flu like prodrome including headache, myalgias, and fever. Malaria paroxysms usually develop within few days.

The classical pattern of clinical disease consists of paroxysms of chills and fever, lasting up to 6 hours, followed by sweating and a drop in temperature. Additional symptoms include nausea, anorexia and abdominal pain. Splenomegaly and hepatomegaly may be detected. The infection can be synchronized or asynchronized depending if parasites in all infected erythrocyte are at the same stage of maturation at any given time. For patients with synchronized infection, these symptoms occur within hours and are followed by diaphoresis, euphoria and a feeling of exhaustion. Paroxysm then return 48-72 hours later. For patients without synchronized infection, the symptoms and signs are not periodic. All forms of untreated malaria tend to become chronic,

repeated attacks are caused by recrudescencia or relapses (Wyler, 1993).

Falciparum malaria in its early stages can not be distinguish from infection due to other species. However, *P. falciparum* infections can be associated with life threatening complications. Severe malaria is a multisystem disease affecting the central nervous system causing coma and convulsions.

1.5. Treatment

Once the diagnosis of malaria has been confirmed, the treatment will depend on several factors: The infecting specie of *Plasmodium*, parasite density and clinical status of the patient. Non-falciparum malaria rarely causes severe manifestations of the disease but *P. falciparum* infections can be severe when the patient is not treated promptly with an effective medication. The management should include drugs and also supportive care (Dourado *et al.*, 1994).

All infections with *P. vivax* , *P. ovale* , *P. malariae* and *P. falciparum* should be treated with chloroquine, 600 mg base for 2 days, followed by 300 mg base 6 hours later, then 300 mg base for 2 days. The pediatric dosage is 10 mg/kg orally followed by 5 mg/kg 6 hours later, then 5 mg/kg for 2 days (Zucker *et al.*, 1993).

Although traditional treatment of acute attack of malaria is with chloroquine administered orally, the development of chloroquine resistance has had a great impact in the epidemiology of malaria, increasing morbidity and mortality (Wernsdorfer *et al.*,1991).

In case of infection with chloroquine resistant *P. falciparum*, the treatment consists of quinine and tetracycline if the parasitemia is less than 5%, there are no complications and the patient can take oral medication (Zucker *et al.*, 1993). Infection by chloroquine resistant *P. falciparum* have also been treated with Pyrimethamine/sulfadoxine as a single dose. Mefloquine has been used as a single dose treatment regimen, but severe adverse reactions have been observed. Curdlan sulphate is a novel compound which has lower anticoagulant activity *in vivo* than heparin and other sulphated polysaccharides. This compound has been shown to inhibit infection by blocking the adsorption of the parasite to the surface of target cells (Havlik *et al.*, 1994). Artemisinin and its derivatives, alone or in combination with mefloquine have also been

used as an effective treatment of acute, uncomplicated *falciparum* malaria (Tran Tinh Hien *et al.*, 1994).

2. DRUG RESISTANCE IN MALARIA

Drug resistance in malaria is defined as the ability of a parasite strain to survive despite the administration of drug doses within the limits of tolerance of the subject (Looareesuwan *et al.*, 1992).

In the patient the level of resistance can vary from a low level of resistance with a loss of the effect of the treatment, to a high level of resistance at which the drug does not have any effect resulting in severe infections.

Drug resistance to several antimalarial compounds has been seen in many geographical regions where malaria is endemic (Wyler *et al.*, 1983). Infections with chloroquine resistant *P. falciparum* were first encountered in the late 1950s in both South America and South East Asia. Since then chloroquine resistant has spread to most regions where malaria is endemic. In addition, new antimalarials such as mefloquine and halofantrine are rapidly suffering from the same situation.

There are at least two different forms of drug resistance in malaria. The antifolate resistance results from specific point mutations of the dihydrofolate reductase (DHFR). Chloroquine resistance involves alterations in drug transport rather than changes at the site of action of the drug (Looareesuwan *et al.*, 1992).

2.1. Antifolate resistance

Resistance to antifolates appeared soon after the introduction of the drug. The mechanism of resistance to antifolates, pyremethamine, proguanil and cycloguanil in *P. falciparum* is due to mutations within the dihydrofolate resistant gene (DHFR). Several mutations have been identified and shown to render the enzyme less susceptible to inhibition by antifolates. The inhibition of DHFR with pyremethamine decreases the amount of tetrafolates cofactors and inhibits DNA

synthesis in the parasite. Comparison of the DHFR gene from pyremethamine sensitive and resistantparasites revealed a single amino acid change from Ser ^{108} or Thr ^{108} to Asn (Walliker, review, 1994). The combination of sulfadoxine with pyremethamine was shown to potentiate antimalarial activity. This combination was effective when it was introduced (Looareesuwan *et al.*, 1992) but over the years resistance has developed in many parts of the world. In addition, the results regarding the cause of pyremethamine resistance are consistent with the rapid development of resistance to this drug.The knowledge of the mechanism of pyremethamine resistance has led to the development of a Polymerase Chain Reaction (PCR) technique for the diagnosis of Pyrimethamine resistant parasites in patients.

2.2. Chloroquine resistance

In contrast to antifolates, little is known about chloroquine drug resistance in *P. falciparum*. Chloroquine has been one of the most successful antimicrobial agents produced and also the most widely used for the treatment of malaria. Unfortunately, the spread of resistance to chloroquine over the last thirty years has made difficult to control the disease. Furthermore, chloroquine mode of action and its mechanism of resistance are not well understood.

It has been suggested (Cowman and Foote, review, 1990) that ferriprotoporphyrin IX, a product of the haem degradation pathway, is targeted by chloroquine. Haemoglobin degradation by the parasite generates large amounts of free heme that is toxic, therefore it has to be detoxified by heme polymerase. Chloroquine is thought to inhibit the heme polymerase in the digestive vacuole of the parasite.

Others have cited the lysosomotropic effect of chloroquine to explain its mode of action. Chloroquine, a weak base, is believed to cross the cell membrane and concentrate in the acidic vesicles of the parasite. Once in the acidic vesicles, chloroquine gets protonated and thus can not diffuse out of the food vacuole. The accumulation of chloroquine in the food vacuole inhibits the growth of the parasite by raising the pH. This rise in pH inhibits the activity of the proteolytic enzymes and starves the parasite (Krogstad *et al.*, 1985).

It has been known for some time that chloroquine resistant parasites accumulate lower amounts of chloroquine than wild type parasites (Yayon *et al.*, 1985; Krogstad *et al.* 1987) . However, the latter observation have not been consistent. Recently, the uptake of chloroquine in both chloroquine resistant and sensitive parasites was shown to be similar; while the efflux of chloroquine in resistant parasites was 40-50 fold faster (Krogstad *et al.*, 1992; Slater *et al.*, 1993). In mammalian cells, chloroquine diffuses across the membrane and concentrate in the acidic vesicles due to the pH gradient across the endosomal membranes (Ross *et al.*, 1981). Nevertheless, the level of chloroquine accumulation in acidic vesicles in mammalian cells is much lower than that found in both susceptible and resistant parasites. This discrepancy suggests that the parasite has a high affinity chloroquine binding site not present in endosomes of mammalian cells.

Given the latter mode of action of chloroquine, it was speculated that resistance to chloroquine in *P. falciparum* may be associated with changes in the activity of a proton pump found in the food vacuole. Karcz and co-workers (1993), have cloned and characterized a gene , VAP-A , that encodes a polypeptide of 611 amino acids which shows approximately 60% identity to the ATPase A subunits from different species. However, sequence analysis of the coding region of this gene from two chloroquine sensitive and three chloroquine resistant isolates did not show changes that correlate with chloroquine resistance. Therefore, it was concluded that chloroquine resistance is unlikely to be due to a defect in vacuolar acidification involving the VAP-A gene.

Krogstad *et al.* (1992), found that chloroquine efflux is inhibited reversibly by either the removal of glucose, a metabolizable substrate, or by the ATPase inhibitor vanadate, suggesting that chloroquine efflux is an energy dependent process that requires the generation and hydrolysis of ATP.

2.3. Efficacy of other antimalarial

2.3.1. Mefloquine resistance

Mefloquine is a quinine like alcohol, it was develop in response to the emergence of chloroquine resistance (Schmidt *et al.*, 1978). Mefloquine shares structural similarities with the

quinoline antimalarials, quinine and chloroquine (Volkman *et al.*, 1993). Initial comparative studies for mefloquine showed that the susceptibility rate was 98-100%. Nevertheless, resistance to mefloquine was reported in Thailand in 1982.The recent cure rates with mefloquine reported from Thailand have declined to 41% (Lim *et al.*, 1996). However, mefloquine resistance appears to be different from chloroquine resistance. It has been shown that penfluridol increases the susceptibility of mefloquine resistant strains, while verapamil which reverses chloroquine resistance has no effect. On the other hand, penfluridol does not modulate chloroquine resistance (Mockenhaupt review, 1995).

Mefloquine resistance is often associated with resistance to halofantrine and quinine (Webster, 1985). For example, mefloquine resistant isolates from regions where halofantrine has not been used showed cross resistance to halofantrine. Gene amplification and overexpression of vacuole-associated ATP transporter (*pfmdr1*) have been demonstrated in mefloquine resistant clones selected *in vitro* . However, a direct link between *pfmdr1* function and mefloquine resistance remains to be demonstrated (Wilson *et al.*, 1989).

2.3.2. Quinine resistance

Quinine, the first antimalarial, is a quinoline containing drug derived from the chinchona tree. The use of this drug is presently limited to the treatment of cerebral malarial due to its severe side effects. Moreover, there are some reports of resistance to quinine from several parts of the world (Cowman and Foote, review, 1990). During the past decade the minimal inhibitory concentration (MIC) of quinine for *Plasmodium* to be effective has risen. In this situation and in severe malaria, high doses are required with the inconvenient that quinine is toxic at that high doses producing many side effects (Looareesuwan, 1992).

2.3.3. Halofantrine resistance

Halofantrine is a quinine like alcohol such as mefloquine. Initial trials with halofantrine demonstrated that its effective against multidrug resistant *P. falciparum* in both Thailand and

Africa (Looareesuwan *et al.*, 1992). However, a later study in Thailand have shown halofantrine to cure only 30 to 70 % (Bunnag *et al.*, 1990) even though it had not been extensively used. Although poor drug absorption has been cited as a reason for a number of treatment failures (Bryson *et al.*, 1992), the possibility of resistance or decreased parasite susceptibility can not be ruled out.

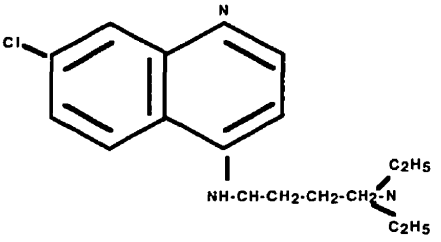
2.4. Cross-resistance

In a reaction to the appearance and spread of resistance, novel quinine analogs of chloroquine such as mefloquine, halofantrine and amodiaquine have been synthesized but resistance to these drugs have been reported suggesting that cross-resistance between antimalarials is emerging and it may be due to similarities in structure (fig.2) and/or mode of action. Nevertheless, acquisition of chloroquine resistance by a parasite does not necessarily imply resistance to other antimalarials but it may predispose the parasite to become resistant to them. Furthermore, there are several problems to be considered in assessing the question of cross-resistance in malaria: a) many studies are done on fresh isolates from patients, therefore, it is difficult to determine whether there is only one population of parasites present or several with different drug sensitivities and b) as there are few places where only one drug is used, it is possible that parasites have successively acquired resistance to many drugs. However, it seems that there is evidence of some cross resistance between antimalarials .

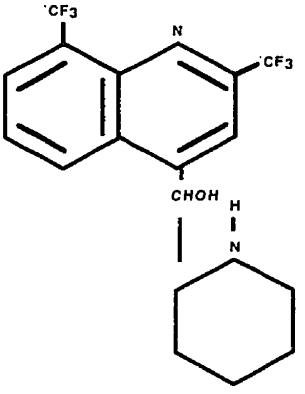
For example, in Cameroon, 240 isolates of *P. falciparum* from different areas were surveyed and a correlation was found between chloroquine and quinine resistance. At the same time, adjacent regions without chloroquine resistance also lacked quinine resistant parasites (Brasseur, 1986). This study confirms previous observations in which resistance to quinine is often associated with resistance to chloroquine (Cowman and Foote, review, 1990).

Mefloquine resistance has been found in regions where mefloquine has never been used, suggesting that such resistance is due to cross-resistance (Brasseur, 1986). Webster *et al.* (1985) working in different areas of Thailand studied sensitivity to mefloquine, quinine, halofantrine and chloroquine. In that study, no correlation between chloroquine and the other chemiotoxics was

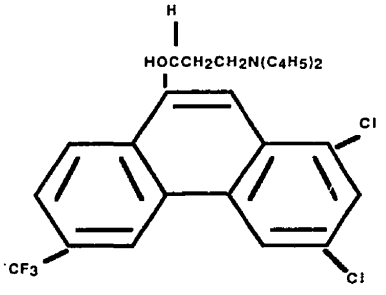
Chloroquine



Mefloquine



Halofantrine



Amodiaquine

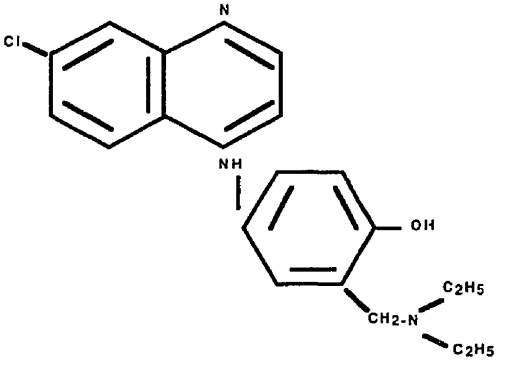


Fig. 2. Chemical structures of some of the quinoline containing antimalarials.

found. However, a correlation between mefloquine, quinine and halofantrine was seen .

Draper *et al.* (1988) tested parasites for sensitivity to chloroquine, amodiaquine, quinine and mefloquine in a small community in East Africa in which chloroquine was the only drug used to treat malaria. They found a rise in the average of the MIC of chloroquine as expected but surprisingly they also found a gradual increase in the MIC for both quinine and amodiaquine and some sporadic cases of mefloquine resistance.

3. MULTIDRUG RESISTANCE

3.1. General aspects

Multidrug resistance (MDR) describes a complex phenotype that is exemplified by the expression of resistance to a broad range of structurally and functionally dissimilar drugs. This phenotype has been observed in tumour cells exposed to sublethal concentrations of cytotoxic drugs. Some of the characteristics of the above tumour cell lines are listed in table 1.

Table 1. Biochemical changes in multidrug resistant cells

a. Increase in resistance to unrelated cytotoxic drugs.
b. Decreased cellular accumulation of drugs.
c. Enhanced drug efflux.
d. Collateral sensitivity to membrane active agents.
e. Overexpression of a 170 to 180 kDa membrane glycoprotein (P-glycoprotein).
f. Reversal by chemosensitizers.

The development of resistance to multiple chemotherapeutic drugs following the exposure to a single cytotoxic agent (Bench Hansen *et al.*, 1986), is one of the most interesting characteristics of drug resistant cells and also a major obstacle in the clinical treatment.

Paradoxically, MDR can display increased sensitivity to certain compounds (e.g. steroids, detergents).

Although many cellular changes have been described in MDR cells, the above characteristics are more consistently identified in cells that overexpress P-glycoprotein (P-gp). Thus the hallmarks of P-gp mediated MDR are: a) Enhanced drug efflux or reduced drug accumulation ; and b) the reversal of the drug resistance phenotype with a group of compounds referred as chemosensitizers or reversing agents. These chemosensitizers include non-cytotoxic drugs analogs, Ca^{++} channel blockers, calmodulin inhibitors, lysosomotropic agents and cyclosporin A (Georges *et al.*, review, 1990).

3.2. P-glycoprotein as a mediator of MDR in tumour cells

The overexpression of 170 kDa membrane glycoprotein was shown to be the most consistent biochemical alteration identified in MDR cells. P-glycoprotein was first described in colchicine resistant Chinese hamster ovary (CHO) cells and in actinomycin D resistant Syrian hamster cells (Juliano and Ling, 1976). This protein is normally expressed at barely detectable levels in drug sensitive cells but has been shown to be overexpressed in numerous independently derived MDR cell lines (Nielsen *et al.*, 1992). It is now believed that P-gp present in the cell membrane, allows the efflux of cytotoxic drugs in an energy-dependent manner allowing MDR cells to survive high concentrations of cytotoxic drugs (Bradley *et al.*, 1988). (Fig. 3)

Sequence analysis of P-gp revealed a tandemly duplicated molecule. Each half is divided in two domains, consisting of a hydrophobic and a hydrophilic region (Gerlach *et al.*, 1986; Gros *et al.*, 1986). The hydrophobic domain encodes six putative transmembrane sequences while the hydrophilic domain encodes a large cytoplasmic domain containing one ATP binding site.

P-gp is a member of the ATP-binding cassette (ABC) family of membrane transporters (Higgins *et al.*, 1986). Members of this family include the bacteria periplasmic transporter protein hemolysin B (Gerlach et al., 1986), the cystic fibrosis membrane regulator CFTR protein (Hyde *et al.*, 1990; Riordan *et al.*, 1989) and the MHC I proteins (Monaco, 1992).

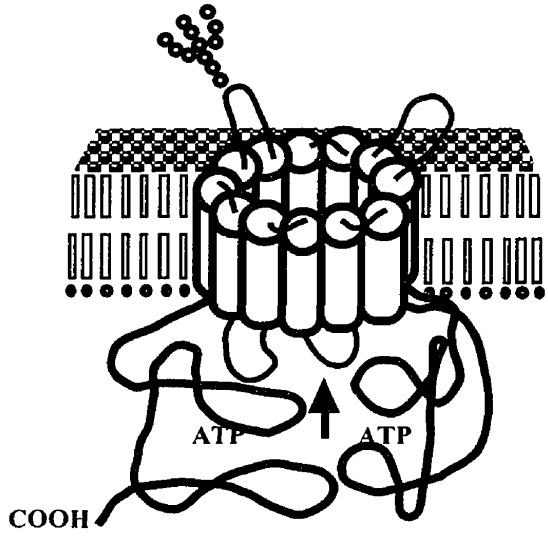


Fig. 3. Model of P-glycoprotein as a channel-forming energy dependent drug efflux pump. The 12 transmembrane domains (here drawn as cylinders in the lipid bilayer) form a pore. The nucleotide binding domains (ATP) have been localized to he cytoplasmic side using monoclonal antibodies.

The normal function of P-gp is presently not known. However, in some tissues and organs, P-gp is thought to mediate the transport of metabolites and xenobiotics (Childs and Ling, review, 1994). These functions of P-gp are consistent with some results. In one study both alleles of P-gp were deleted from the mouse genome. After analysis of tissues and organs that express high levels of P-gp from knockout mice, was found that these mice accumulated more drugs than normal mice (Schinkel *et al.*, 1994). More recently, P-gp 1 has been shown to mediate the transport of various short chain lipids (van Helvoort *et al.*, 1996). Taken together, although the normal function of P-gp remains to be confirmed, its function in tumour cells as drug efflux pump is well accepted.

P-gp homologous have been identified in procaryotes and parasites. For example, P-gp homologous have been cloned from emetine resistant *Entamoeba hystolitica*, vinblastine resistant *Leishmania tarantulae, Leishmania donovani,* and chloroquine resistant *P. falciparum*

(Dallagiovanna *et al.*, 1994). However, the role of the latter P-gp homologous in drug resistance is not well understood. Table 2 shows a list of P-gps homologous and other related putative transporters in parasites.

Table 2. P-glycoproteins and other traffic ATPases in parasites

Parasite genus	gene	comments
Entamoeba	*Ehpgp1-6*	Multigene family with two pseudogenes. One gene overexpressed in an emtine-resistant clone.
	Ehabc1	
Leishmania	*PgpA*	Involved in resistance to arsenite and antiamonials.
	PgpB-E	Closely related to pgpA; more related to MRP than to MDR1.
	mdr1	Involved in resistance to drugs. Part of mammalian MDR spectrum.
Plasmodium	*Pfmdr1*	Controversial role in resistance
	Pfmdr2	Related to yeast HMT1; involved in cadmiun resistance.
Schistosoma	*Smdr1*	
	Smdr2	

3.3. Similarities between multidrug resistance in tumour cells and in malaria

Chloroquine resistant *P. falciparum* shares some similarities with multidrug resistant tumour cells. For example, chloroquine resistance in *P. falciparum* was reversed by verapamil that reverses the MDR phenotype of tumour cells (Martin *et al.*, 1987; Bitonti *et al.*, 1988). Moreover, methoxyverapamil, diltiazen and other Ca^{++} channel blockers have been shown to potentiate the effect of chloroquine against resistant *P. falciparum* (Kyle *et al.*,1990).

Another characteristic shared by P-glycoprotein-mediated MDR in tumour cells and chloroquine resistant malaria is the rapid efflux of cytotoxic drugs. Several studies (Krogstad *et*

al., 1992; Slater *et al.*, 1993) have shown that chloroquine resistant strains of *P. falciparum* accumulate less drug than sensitive ones. Although the rate of uptake of chloroquine into the parasite has been demonstrated to be similar, the efflux of chloroquine in resistant strains was shown to be 40-80 times greater than in sensitive strains.

As indicated earlier, the mammalian MDR phenotype is usually accompanied by amplification of the MDR gene with increased transcription leading to higher P-gp expression. The identification of P-gp homologous (*pfmdr1* and *pfmdr2*) in *P. falciparum* using PCR primers that hibridize to conserved sequences in ATP binding domains led to the speculation that the over expression of *pfmdr* genes may mediate chloroquine drug resistance in *P. falciparum* (Wilson *et al.*, 1989; Foote *et al.*, 1989). .

Immmunofluorescence and immunoelectrone microscopy using antibodies against the *pfmdr1* gene product (Pgh1) localized the protein at a relevant site, the proposed target of chloroquine: the food vacuole (Cowman *et al.*, 1991). However, based on the localization of the *pfmdr1* gene product (Pgh1) in the food vacuole, higher levels of *pfmdr1* or Pgh1 would result in increased rather than decreased drug accumulation in resistant isolates. Further quantitative immunoblotting analysis has shown that equal levels of Pgh1 are expressed in chloroquine resistant (7G8, K1, V1, CSL2) and chloroquine sensitive isolates (HB3, 3D7, FC27). Therefore, differences in Pgh1 expression are not likely to be responsible for chloroquine resistance. High levels of Pgh1 has been demonstrated in a chloroquine resistant line FAC8 (Cowman *et al.*, 1991). This increased in Pgh1 was shown to be due to gene amplification. Nevertheless, the selection of FAC8 mutants with higher levels of resistance led to the deamplification of the *pfmdr1* gene. Thus, in the latter clone, resistance to chloroquine appears to be inversely related to *pfmdr1* gene amplification. Interestingly, the decrease in *pfmdr1* levels correlates with increased sensitivity to mefloquine (Barnes *et al.*, 1992).

Moreover, transfection of *pfmdr1* cDNA was shown to increase the sensitivity of CHO transfectant mutant to chloroquine. However, transfection of a mutant *pfmdr1* cDNA carrying 2 point mutations (Ser ^{1034} to Cyst and Asn ^{1042} to Asp) did not result in increased sensitivity toward chloroquine (van Es *et al.*, 1994). More recently, it has been shown that the *pfmdr1* gene

expressed in a heterologous yeast system functions as a transport molecule (Volkman, 1985).

Ruetz and coworkers (1996) introduced wild type and mutant variants of *pfmdr1* in the yeast *Saccharomyces cerevisiae* to analyzed the effect of *pfmdr1* expression on resistance to quinoline containing antimalarial drugs. These results showed that the expression of wild type *pfmdr1* is responsible for resistance to quinine, quinacrine, mefloquine and halofantrine in yeast cells. Interestingly, a mutated *pfmdr* carrying point mutations in transmembrane 11, did not confer resistance. However, it could not be demonstrated that *pfmdr1* is localized in yeast plasma membrane, likely due to the short half life of this protein (Ruetz *et al.*, 1996).

3.4. Differences between multidrug resistance in tumour cells and in malaria

Chloroquine resistance in *P. falciparum* has been linked to a form of multidrug resistance found in tumour cell lines. However, there are some differences between them. On one hand, in mammalian MDR tumour cells, P-gp expression is sufficient to confer resistance to multiple drugs (Guild *et al.*, 1988) and the level of P-gp correlates with the level of drug resistance (Endicott and Ling, 1988). By contrast, a correlation between Pgh1 overexpression and chloroquine resistance has not been demonstrated. In the parasite quantitative immunoblot analysis showed that the protein is expressed at equal levels in chloroquine resistant and sensitive isolates. In addition, photoaffinity labeling of cells extracts from chloroquine sensitive and chloroquine resistant parasites with a photoactive analogue of chloroquine did not result in photoaffinity of Pgh1 (Foley *et al.*, 1994). Thus, unlike P-gp in tumour cells, Pgh1 does not interact directly with chloroquine.

The presence of the *pfmdr1* gene in some chloroquine resistant isolates in equal amount to that in chloroquine sensitive led Foote et al. to suggest that the *pfmdr1* gene in chloroquine resistance may be mutated. Sequence analysis of *pfmdr1* gene from two chloroquine sensitive and five chloroquine resistant isolates revealed the presence of single amino acid mutations in *pfmdr1* sequence in chloroquine resistant isolates. Nevertheless, given the slow occurrence of chloroquine resistance in the field which is in contrast with the situation seen with pyremethamine; it has been suggested that a *pfmdr1* allele competent for chloroquine resistance and a mutation in a second

gene is required (Foote *et al.*, 1990). The point mutation in *pfmdr* was suggested to inhibit chloroquine transport by Pgh1 and reduce accumulation of drugs in the digestive vacuole in chloroquine resistant parasites. Isolates of *P. falciparum* from 3 areas of Sudan were studied to determine their sensitivity *in vitro*. Chloroquine resistance was detected in diverse isolates from different places indicating that resistance is spreading. However, studies using diverse methods did not detect mutations in the *pfmdr1* gene (Awad el Kariem *et al.*,1992). These results are consistent with earlier genetic cross by Wellems et al. (1990) which failed to show a correlation between the inheritance of *pfmdr1* and chloroquine drug resistance.

Another difference between multidrug resistance in tumour cells and in malaria is that anticancer drugs permeate into cells relatively slowly thus allowing for the efficient efflux of drugs (Fojo *et al.*, 1985). In comparison, the influx of drugs in malaria infected RBCs is faster and thus more difficult for a pump to deal with such rapid influx (Geary *et al.*, 1986). A pump that would be able to work with this flux rates should be present in a higher copy number or have high turnover number.

In addition, it has been shown that some antagonists that reverse drug resistance in MDR cancer cells do not potentiate the effect of chloroquine in drug resistant malaria (e.g.. calmodulin antagonists) (Kartz *et al.*, review, 1991). Furthermore, Bray and co-workers (1993) showed that the ability of verapamil to increase steady state chloroquine accumulation was found to be insufficient to explain the increase in chloroquine sensitivity caused by the drug. On the other hand, when chloroquine accumulation was increased by raising the pH gradient, the shift in sensitivity to chloroquine could be predicted (Bray *et al.*, 1993). Moreover, it has been found that in MDR cancer cells is possible to restore cellular drug concentrations to levels found in the drug sensitive parent using reversing drugs (Georges *et al.*, review, 1990). Reversal of chloroquine drug efflux in resistant parasites remains higher than that of sensitive parasites even in the presence of MDR-reversing agents (Krogstad *et al.*, 1987).

MDR cancer cells display cross resistance (Endicott and Ling, 1989) but in malaria parasites multidrug resistance is not the same. For example, the chloroquine resistant clone W2 is sensitive to mefloquine, while the chloroquine sensitive clone D6 is resistant to mefloquine (Oduola *et al.*, 1988).

Taken together, the role of *pfmdr1* in chloroquine resistance remains unclear. However, the fact that chloroquine resistance is associated with enhanced drug efflux and decreased accumulation of drug, suggests the presence of another chloroquine efflux mechanism.

4. PROTEASOMES

4.1. Introduction

There are two different proteolytic pathways in mammalian cells involved in protein degradation: Lysosomal and non lysosomal pathways. Lysosomal degradation occurs when proteins enter the cell from the extracellular millieu. This lysosomal degradation takes place mainly under stress conditions. However, most cellular proteins are degraded by a non-lysosomal pathway via large protein complexes denominated proteasomes (Ciechanover, 1994).

Proteasomes act on proteins that have been specifically marked for destruction by the covalent attachment of a small protein called ubiquitin. Degradation of a protein via the ubiquitin pathway involves two steps: 1. Signalling of the protein by attachment of multiple ubiquitin molecules forming a multi-ubiquitin chain that is recognized by the proteasome and 2. Degradation of the targeted protein with the release of free and reutilizable ubiquitin. Conjugation of ubiquitin is an ATP requiring process in which the carboxy terminus of ubiquitin becomes attached by an isopeptide bond to lysine residues on the protein substrate or on other ubiquitins (fig.4) (Rechteiner *et al.*,
review, 1993).

The temporary activation of a latent proteasome by substrates such as ubiquitin conjugates and linkage to ATP hydrolysis may help to explain how these structures can be present in the cytosol or in the nucleus without causing extended damaged to cellular proteins. Unlike lysosomes, proteasomes are not segregated in a membrane enclosed space. Therefore, their activity has to be controlled.

This non-lysosomal protein degradation pathway was discovered about 20 years ago where by it was shown to mediate the rapid elimination of highly abnormal proteins (Goldberg,

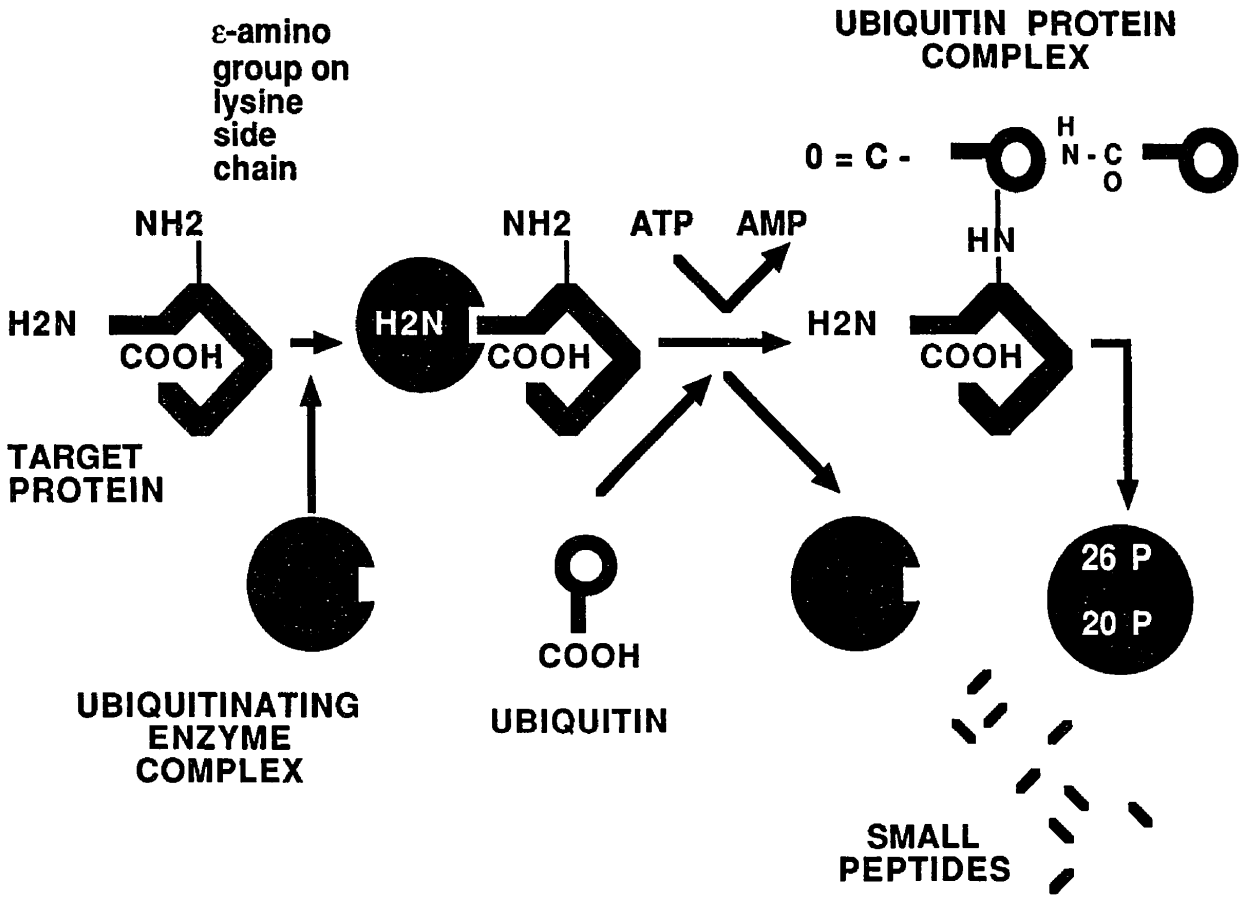


Fig. 2. Ubiquitin dependent protein degradation. A target protein is recognized by the ubiquitinanting enzyme complex. A repeated series of biochemical ractions joins ubiquitin molecules together to produce a multiubiquitin chain. Finally, the proteasome cuts the target protein into a series of small fragments.

Now it is known that this pathway is involved in the degradation of many critical regulatory proteins that must be rapidly eliminated for normal cellular growth and metabolism. The ubiquitin pathway has been been implicated in the degradation of mitotic cyclins, oncoproteins, the tumour suppressor protein P53, several cell surface receptors, transcriptional regulators and mutated and damaged proteins (Ciechanover, 1994). Recent studies have shown that the proteasome has an important role in antigen presentation, proteolytic proccesing of precursor proteins and also in the degradation of proteins in an ubiquitin independent manner (Coux *et al.*, review, 1996). In addition, and of interest to the topic of this thesis, the ubiquitin-proteasome degradation pathway has been implicated in drug resistance in yeast (Jungmann *et al.*, 1993) and plants (Leyser *et al.*, 1993). In one study in yeast, the expression of the ubiquitin dependent proteolysis pathway was shown to be activated in response to cadmium exposure and that mutants deficient in specific ubiquitin conjugating enzymes are hypersensitive to cadmium. Moreover, mutants in the proteasome are hypersensitive to cadmium suggesting that cadmium resistance is mediated by degradation of abnormal proteins. This indicates that cadmium toxicity may be due to cadmium-induced formation of abnormal proteins.

In another study, in arabidopsis, it was shown that resistance to auxin (a plant hormone required in many aspects of growth and development) is mediated by a protein with significant sequence similarity to the ubiquitin activating enzyme E1. After screening for mutants of arabidopsis resistant to exogenous auxin, one gene was identified, AXR1 (Leyser *et al.*, 1993). This gene is defined by recessive mutations that confer auxin resistance to the roots.This similarity suggests that the ubiquitin pathway may have an important role in plant hormone action. Studies in vertebrate cells indicate that several membrane receptors are ubiquitinated in response to ligand binding. By analogy the function of the auxin receptor might be altered by ubiquitination. Another possibility is that the concentration of proteins that mediate auxin response are regulated by ubiquitin mediated proteolysis.

4.2. Proteasomes: Structure and function

The proteasome has been highly conserved during evolution and simpler forms are found in archaebacteria and eubacteria. Although the form of the proteasome most isolated and studied is the 20S particle, a distinction must be made between this form and the 26S particle which is a larger structure. Some reports on eukaryotic proteases appeared during 1970's but it was not until 1980 that descriptions of the multi-catalytic protease (MCP) complex were presented by Wilk and Orlowski (1983) and Hase *et al.* (1980). Both groups isolated the 20S proteasome, a 700 kDa protease composed of several subunits. Although the 20S proteasomes were first described playing an important role in intracellular protein degradation, sometime later it was discovered that these particles by themselves do not degrade ubiquitinated proteins but can assemble with other protein complexes. One of the macromolecular assemblies formed, the 26S proteasome complex, functions as an ATP dependent protease in the ubiquitin pathway (Coux *et al.*, review, 1996).

4.2.1. The 20S proteasome

The 20S proteasome is a 700 kDa particle with an essential role in the ubiquitin pathway. Electron microscopy studies have revealed that this complex contains multiple subunits ranging in size between 20 and 35 kDa. These subunits are stacked into a barrel shaped complex with a clear central hole which may be a site of entry for protein subunits (Coux *et al.*, review, 1996).

The molecular organisation of the 20S proteasome was clarified after the discovery of the highly related protein complex of simpler composition present in archaebacter of the genus thermoplasma. Thermoplasma 20S proteasome is composed only by two different types of subunits, α and β. In thermoplasma, the two outer rings of the 20S proteasome are composed of seven α subunits, while each inner ring contains seven β subunits. A similar type of organisation has been proposed for eukaryotic 20S proteasomes . However, the subunit composition although related to α and β in thermoplasma, is different (Peters, 1994).

The 20S proteasome has a highly organized and evolutionary conserved structure (Rivett,

1993). However, different particles containing variable subunits can exist in certain cells. This has been demonstrated in the case of two 20S proteasome subunits that are encoded in the major histocompatibility II locus of mammalian cells by the LMP2 and LMP7 genes. These genes can be expressed after stimulation with γ -b interferon leading to the formation of particles in which two constitutive subunits are replaced by the MHC- encoded polypeptides.

Based on this, it has been thought that 20S proteasomes are involved in antigen presentation.

4.2.2. The 26S proteasome complex

Although the 20S proteasome is essential in the ubiquitin pathway, by itself this particle can not digest ubiquitin conjugates and requires additional components. Previous studies have shown that the 20S particle forms the proteolytic core of a larger protein complex responsible for protein degradation in the ubiquitin pathway. This proteolytic complex has been named the 26S proteasome.

The 26S protease was first isolated from rabbit reticulocytes (Houg *et al.*, 1987) composed of more than 25 polypeptides subunits. The complex can not be detected in lysates of ATP depleted reticulocytes but can be reconstituted in the presence of ATP from 3 conjugates factors CF1, CF2 and CF3.

Furthermore, the 26S proteasome has been found in the cytoplasm and in the nucleus suggesting that the lifespan of both cytosolic and nuclear proteins is controlled by this system.

After imunodepletion experiments in which the 20S particle was removed from cellular extracts, it was shown that the 20S proteasome is required for ubiquitin dependent protein degradation (Matthews, W. *et al.*, 1989). Other evidence for the contribution of the 20S subcomplex to the structure and function of the 26S proteasome is that some yeast mutants of the 20S proteasome accumulate ubiquitin conjugates and do not degrade substrates of the ubiquitin system (Peters, 1994). Moreover, it has been found that highly purified 26S proteasomes contain all polypeptides of the 20S particle (fig. 5).Electron microscopy and digital image analysis showed that the 26S proteasome contains two rounded complexes on either of the barrel shaped 20S

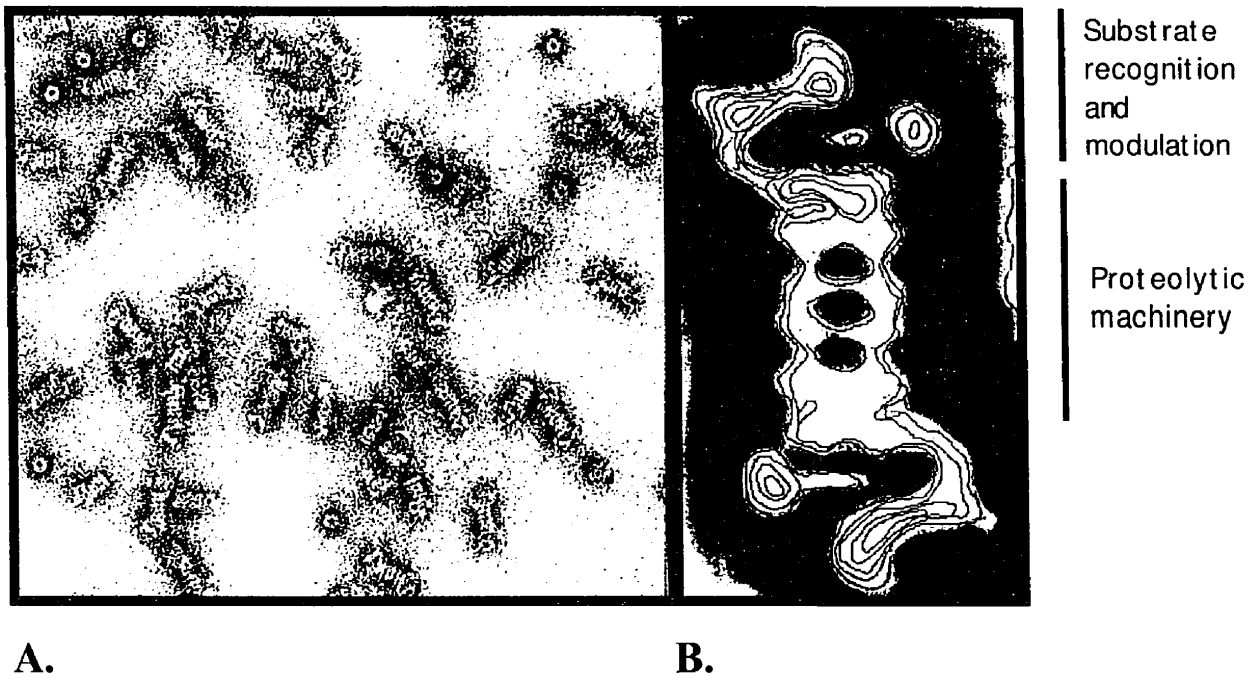


Fig. 5. A Proteasome. A. A large model of negatively stained particles. **B.** A 3-D model of a single complete proteasome complex derived by computer based image processing of such images.

particle. These caps correspond to the PA700 activator or 19S cap complex and contain an invagination that may be the site for substrate entry, presumably with regulatory functions (Coux *et al.*, review, 1996)

4.2.3. Subunit 4 of the 26 S complex

The recent cloning of several of the subunits of the 26S proteasome has revealed that some of these subunits belong to a new family of ATP binding proteins. The hallmark of these ATPases is the presence of one or two copies of a highly conserved module of about 230 amino acids (Coux*et al.*, review, 1996).

Subunit 4 of the 26S proteasome is one of the ATPases identified as a member of this newly recognised ATPase family (Dubiel *et al*, 1992). Six other proteins with high sequence identity to S4 have been identified and they include: human TBP1 (Nelbock *et al.*, 1990), pig valosine containing protein (Koller *et al.*, 1987), *Xenopus laevis* p97 (Peters *et al.*, 1990), *Saccharomyces cerevisiae* CDC48p (Frohlich *et al.*, 1991), Chinese hamster N-ethylmalemide fusion protein (Wilson, D. W. *et al.*, 1989) and *S. cerevisiae* PAS1p (Erdmann *et al.*, 1991). Although these proteins encode a highly conserved domain, their NH_2-terminal 150 residues are more divergent, hence may provide for substrate specificity. The ATPase module and the COOH terminal regions posses higher homology including several stretches in which 60-90 % of the residues are identical.

Surprisingly, some of these proteins were initially identified as regulators of gene transcription. For example, TBP1 was identified as a protein that binds the HIV tat protein, and its over expression led to tat down regulation (Nelbock *et al.*, 1990). On the other hand, overexpression of MSS1 increased tat mediated transactivation and SUG1 restored induction by a truncated form of the GAL 4 transcription factor. These observations suggest that these proteins are transcription factors (Shibuya *et al.*, 1992 and Swaffield *et al.*, 1992). However, it has been proposed that like S4, these three proteins can be subunits of the 26S protease.

The degradation of ubiquitinated proteins must involve several steps: a) substrate binding, b) removal and breakdown of the polyubiquitin chain, c) unfolding of the substrate and its

injection into the 20S particle and d) release of oligopeptides products. Possible roles have been proposed for the ATPases in 26S function.

It has been suggested that ATP hydrolysis serves to reverse binding between S4 and its substrate, promoting presentation of the latter to the active sites of the 26 Protease complex. ATP was also shown to be essential for the assembly of the 26S complex (Reichtener *et al.*, 1993). It is possible that ATPases promote the unfolding of ubiquitinated target proteins and the entry of a polypeptide into the 20S particle.

RATIONALE AND OBJECTIVES

1. RATIONALE

The role of *pfmdr1* in chloroquine resistance in *P. falciparum* is not clear. However, given the increased efflux of chloroquine in resistant *P. falciparum* isolates and its reversal with verapamil, it has been suggested that a P-gp homolog, other than *pfmdr*, could mediate chloroquine drug resistance. To investigate this possibility, several clones of *P. falciparum* resistant to actinomycin D were previously isolated *in vitro* in our laboratory starting with a drug sensitive clone (3D7 chloroquine sensitive). The rationale for using actinomycin D to select drug resistant parasites that are likely to express a P-gp drug efflux pump similar to that in MDR tumour cells is based on several observations: a) Actinomycin D has been shown to induce the overexpression of P-gp in several tumour cell lines (Choi *et al.*, 1988). b) The mechanism of actinomycin D cytotoxicity is well understood, actinomycin D, a DNA intercalator agent, binds to GC nucleotides and inhibits DNA transcription (Sobell,1980). c) This hydrophobic drug can diffuse across the lipid bilayer and concentrate in the parasite given that red blood cells are void of DNA. d) Chloroquine resistant isolates of *P. falciparum* showed decreased sensitivity to actinomycin D (unpublished results and Wirth D., personal communication). Given the mode of action of actinomycin D, we reasoned that to escape the effect of this drug the parasite would have to mutate large portions of its genomic GC DNA sequence or decrease the intracellular accumulation of the drug by overexpression of an enhanced drug efflux, decrease influx or drug sequestering mechanisms.

Taken together, we predict that the resistance to actinomycin D is likely due to the overexpress of an energy dependent drug efflux pump similar to P-gp. However, other mechanisms of drug resistance were investigated. For example, the expression of *pfmdr1* gene in actinomycin D drug resistant clones.

2. OBJECTIVES

Drug treatment of malaria has become difficult and if unchecked could result in failure to control the disease due to resistance to different antimalarials. However, the cause of drug resistant remains undetermined. The ultimate goal of this study is to understand the mechanism of multidrug resistance in *P. falciparum*. The specific aims are:

1. Determine the expression of *pfmdr1* in drug resistant *P. falciparum* selected *in vitro* with actinomycin D (3D7R/actD) or chloroquine(FAC8/CQ). This was done by Southern and Northern blots of DNA and RNA isolated from the various drug resistant clones.

2. Determine the presence of P-gp homolog, other than *pfmdr1*, using RT-PCR and primers to conserved sequences in ABC-transporters.

3. Clone and sequence some of the PCR fragments.

(References for the **Introduction**, **Literature Review** and **Rationale and Objectives** fol low the **General Discussion**)

MANUSCRIPT I

Multidrug Resistant *Plasmodium Falciparum* : Actinomycin D Selected Parasites Show Cross-Resistance To Antimalarial Drugs And Rhodamine 123.

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Running head: Selection and characterization of Actinomycin D-resistant *Plasmodium falciparum*

Keywords: Malaria; *P. falciparum; pfmdr1*; P-glycoprotein; Actinomycin D; Chloroquine resistance; Rhodamine 123; Multidrug resistance.

(submitted for publication)

ABSTRACT

The development and rapid spread of drug-resistant *Plasmodium falciparum* is a major health concern in areas where malaria is endemic. Resistant malaria falciparum to chloroquine, halofantrine and mefloquine are now common in many regions in Thailand and Africa. Moreover, recent reports of multidrug resistant malaria falciparum in Thailand points to the urgency for greater understanding of the mechanism(s) of drug resistance in malaria. In this study, we have selected actinomycin D-resistant *P. falciparum* in vitro from a parental drug sensitive 3D7 clone. Interestingly, the actinomycin D resistant *P. falciparum* are also less sensitive to antimalarial drugs (chloroquine and mefloquine) and Rhodamine 123. Comparison between parental (3D7) and actinomycin D-resistant (3D7R/actD2) *P. falciparum* did not show differences in the copy number or the level of expression of *pfmdr1* previously implicated in chloroquine or mefloquine drug resistance. Taken together, the results of this study describe the isolation of a multidrug resistant *P. falciparum* selected with a single drug. Furthermore, we show that *pfmdr1* is not involved in this resistance phenotype.

INTRODUCTION

Chloroquine (CQ) has been very effective in the prophylaxis and treatment of malaria. However, the development and spread of chloroquine-resistant *Plasmodium falciparum* has limited the use of CQ in regions where malaria is endemic (1). Resistance to other antimalarial drugs (mefloquine, halofantrine, and quinine) has also been reported and therefore threatens future use of these antimalarials (2, 3, 4). This recent reports indicate in consequence that multidrug resistant parasites are a major concern given the number of annual malaria infections (200-300 million) and the lack of new antimalarial drugs (5).

The mechanism of chloroquine resistance in *P. falciparum* is not well understood; however, it has been known some time that chloroquine-resistant parasites accumulate less drug than sensitive ones (6, 7). The demonstration that the reduced drug accumulation in resistant parasites is due to enhanced efflux (8, 9) and that verapamil (classical MDR reversing agent; 10) can reverse the resistance phenotype (11) linked CQ resistance to P-glycoprotein drug efflux mechanism in tumor cells. This link was extended further by the demonstration that both CQ and quinine reverse P-glycoprotein-mediated MDR and are substrates for P-gp efflux pump (12).

A P-glycoprotein homolog Pgh1, encoded by *Pfmdr1*, was shown to be overexpressed in some CQ-resistant parasites (13); however, Pgh1 is localized to the digestive vacuole membrane hence its action would result in concentrating CQ in the digestive vacuole (14). In agreement with these results, transfection of wild-type and mutant *pfmdr1* in heterologous systems showed that only the former increased the sensitivity of CHO cells to CQ (15). In a recent study by Ruetz et al. (1996), transfection of *Pfmdr1* in a heterologous system in yeast was shown to confer resistant to mefloquine, halofantrine and quinine (16).

Thus whilst these results link the function of Pgh1 to CQ-resistance, there are some studies suggesting that there are not related. For example, a genetic cross between CQS and CQR clones of *P. falciparum* showed no correlation between the inheritance of *pfmdr1* gene and CQ-resistance in the recombinant progeny (17). Furthermore, photoaffinity labeling experiments using a photoactive analogue of CQ or MQ did not demonstrate a direct binding to Pgh1 (18, 19). In

addition, no correlation was found between CQ resistance and mutations in *pmdr1* gene (20). More recently, selection of CQR or MQR *P. falciparum in vitro* did not show a change in the copy number or the levels of *pfmdr1* expression in resistant isolates (21, 22). Taken together, the role of *pmdr1* in CQ and MQ-resistance in *P. falciparum* remains unclear.

P-glycoprotein homologs have been identified in other parasites; however only few have been linked to drug resistance (for review see, 23). Furthermore, among the many P-gp homologs that have been isolated in *Entamoeba* and *Leishmania*, only *Ehpgp1* and *ldmdr1* have been shown to mediate a multidrug resistance phenotype to hydrophobic drugs (24, 25). Interestingly, in both parasites, resistant clones were selected with hydrophobic drugs that are known substrates for P-gp (26). Thus while P-gp can confer resistance to a broad spectrum of structurally and functionally dissimilar drugs, some drugs may be more effective in inducing its expression (27).

To understand further the mechanism of multidrug resistance in *P. falciparum* and to determine if a P-gp homolog other than Pgh1 is responsible for resistance to hydrophobic drugs, it was of interest to select *P. falciparum* clones for resistance to hydrophobic drugs that are well known substrates for P-gp. ActinomycinD, a DNA intercalating agent (28), was chosen since it has been previously shown to be tightly associated with the overexpression of P-gp in MDR tumor cells (29). In this report, we describe the selection and characterization of an actinomycin D-resistant clones of *P. falciparum* . Our results show that actinomycin D resistant parasites are cross-resistant to chloroquine and mefloquine in addition to other structurally dissimilar drugs (eg. Rhodamine 123).

MATERIALS AND METHODS

Parasite culture - The parasites were cultured *in vitro* according to the method of Trager and Jensen (30). Parasites were maintained on 5% suspension of type O erythrocytes in RPMI 1640 media supplemented with 28 mM NaHCO3, 32 mM hypoxanthine, 2 mM glutamine, 25 mM HEPES, pH 7.4, and 10% human plasma. Ten ml of the above parasite suspension is added to each of T25 flasks and flushed with 3% O2, 4% CO2, and 93% N2. Flasks were incubated at 37 ^{0} C with constant shaking. The Plasmodium falciparum clones 3D7 (CQS) and FAC8 15 (CQR; a kind gift from Dr. A.F. Cowman at Walter and Eliza Hall Institute in Australia) were grown continuously without and with 15 ng/ml of chloroquine, respectively. The 3D7R/actD2 was cultured as above but in the presence of 2 ng/ml of actD.

Selection and cloning of drug resistant parasite - The CQS isolate (3D7) was cultured in the presence of increasing concentrations of actD (figure 1). Drug was added to 5% culture and allowed to incubate at 37 ^{0} C for 48 hours at which point the parasitemia was checked and the growth of the parasite was scored. The drug pressure was removed for 48 hours when the parasitemia fell below 0.2%. This cycle of drug selection was continued until a parasite culture that was able to withstand 2 ng/ml of actD (3D7R/actD2) was obtained. As indicated in figure 1, several actD resistant isolates were obtained over a period of 21 months and cloned by limited dilution in 96-well plates. Briefly, 200 ml of 0.05% parasitized red blood cells (RBCs) was aliquoted into each well of a 96-well plate and incubated at 37 ^{0} C. The media was changed daily and fresh RBCs were added on day 14. On day 21, smears of each well were made and positive clones isolated and expanded. This cloning procedure was repeated two times and three clones that grew continuously in 2 ng/ml of actD were isolated (3D7R/actD2.1, 2.2, and 2.3). All three clones showed similar resistance profile. One of the actD-resistant clones, the 3D7R/actD2.1 was used throughout this study (referred to in the text from here on as 3D7R/actD2). Throughout the selection process, the haematocrite was kept constant at 5%, as changes in the haematocrite affect the bioavailability of the drug. The actD-resistant clones have been in culture for at least one year

and were shown to be stably resistant to the drug even when the drug was withdrawn for several months.

Cytotoxicity assay - Malaria cultures are washed three times with 5 ml of RPMI 1640 (Gibco.Brl) and synchronized twice with 15% sorbitol in RPMI 1640 for 5 minutes at 37 ^{0} C (31). After synchronization, parasitized RBCs were resuspended in equal volume of 10% sera in RPMI and washed two times by centrifugation at 200 x g for 5 minutes. For cytotoxicity assays, the cultures were diluted to 0.1% parasitemia in 2% Haematocrite. One hundred microliter volumes of synchronized parasitized RBCs were aliquoted into 96-well tissue culture plates containing 100 µl of cytotoxic drugs (CQ, actD, MQ or Rh123) or chemosensitizers (VRP). Plates were incubated in a gassed chamber at 37 ^{0} C for three days. Following the third day, 100 µl of the top media was carefully removed and replaced with fresh media without or with drugs. The latter step was repeated for days 5 and 6 of the cytotoxicity assay. On the seventh day, 50 µl (0.1 mci/ml) [3H] Hypoxanthine monohydrochloride (NEN Dupont, Inc) was added to each well of the 96-well plates and allowed to incubate for an additional 17 hours at 37 ^{0} C. Parasitized RBCs were harvested on a Millipore glass fiber sheets (Skatron,Cat # 11731) pre-saturated with 3% Bovine Serum Albumin in phosphate buffer saline (PBS) using a multi-well cell-harvester (Skatron, Inc.). The Millipore membranes were allowed to dry and placed in tubes containing scintillation fluid (NEN Dupont, Inc.) The amount of incorporated [3H] Hypoxanthine was determined by flourometry using liquid scintillation counter (LBK Wallac, 1219 Rack Beta, Inc.).

DNA and RNA extraction - Parasites were collected by centrifugation and resuspended in 0.1% saponin in PBS. The parasite cell pellet was washed in 0.9% NaCl. For DNA extraction the resultant pellet was incubated overnight at 55 ^{0}C in a lysis buffer containing 50 mM Tris HCl, pH 7.5, 0.1 M NaCl, 1mM disodium ethylenediaminetetra acetate, 0.5% sodium dodecyl sulfate and 100 mg/ml proteinase K. The lysate was repeatedly extracted with phenol-chloroform. The aqueous phase containing DNA was precipitated with ethanol in the presence of 3M sodium acetate. For RNA extraction, synchronized cultures of parasitized RBCs (5%

parasitemia) were mixed with equal volume of Trizol and incubated at room temperature. Cell lysates were extracted with chloroform and the aqueous phase was precipitated with isopropanolol following a short incubation at room temperature. Following centrifugation at 4 ^{0} C, the RNA pellets were washed with ethanol and kept under ethanol at -70 ^{0} C if not immediately used.

Southern and Northern blots - Southern and Northern blot analysis were performed using standard procedures (32). For Southern blot, 10 μg sample of DNA from sensitive and resistant clones was digested with EcoRI and electrophoresed through a 0.7% agarose gel. Digested DNA was transferred to a Hybond-N nylon membrane (Amersham) and the membrane was pre-hybridized in a solution containing 6 x SSC, 5 x Denhardt's reagent, 0.5 % SDS, 100 mg/ml salmon sperm DNA, and 50% formamide at 42 ^{0} C. ^{32}P nick translated probes coding for parts of pfmdr1 gene (3331-3744 bp (13)) or the α-tubulin gene (690 - 950bp (33)) were added separately and hybridization was allowed for 18 hr. The membrane was washed with 2 x SSC with 0.5 % SDS at room temperature for 10 min and 1 x SSC with 0.1% SDS at 65 ^{0} C. For Northern blot analysis, 10 μg of total RNA from sensitive and resistant parasites previously synchronized in sorbitol was fractionated with glyoxal / DMSO and electrophoresed on a gel containing 1% agarose, transferred to a nylon membrane and probed with *pfmdr1*-specific probe at 42 ^{0}C. The membrane was washed for 30 minutes at room temperature in 0.5 x SSC. The signal was developed by exposing the membrane to an X-ray film with an intensifying screen.

PCR and DNA sequencing - PCR was done to check for mutations in *pfmdr1* gene sequence in a CQR-associated region (15) between 3D7R/actD2 and 3D7. The reaction was made using 10X PCR buffer (100 mM Tris-HCl (pH 9), 500 mM KCl, 15 mM MgCl2), 200 mM of each dNTP, 100 pmol of each primer, 200 ng template DNA (3D7 and 3D7R/actD2), and 5 units of Taq DNA Polymerase in a 50 μl reaction for 1 cycle of 94 ^{0} C x 1min., 40 cycles of 94 ^{0} C x 1min (denaturation), 45 ^{0} C x 2min (annealing) and 72 ^{0} C x 3 min. (extension), 1 cycle of 72 ^{0}C x 7 min. (34). The PCR fragment was purified from a gel slice and cloned into a TA-cloning

vector, PCRII, according to the manufacturers protocol (Invitrogen, Inc.). Plasmid DNA was prepared and used for double stranded DNA sequencing (dideoxy method) (35).

RESULTS AND DISCUSSION

Selection of *P. falciparum* clones resistant to actinomycinD - The selection of drug resistant clones was done by stepwise drug pressure with increasing concentrations of actD (figure 1). Red blood cells infected with 3D7, the chloroquine sensitive clone of *P. falciparum,* were grown in the presence of sublethal concentrations of actD for 48 hours. Following a given drug pressure, the surviving parasites were transferred to fresh media and allowed to recover for 48 hours in the absence of drug. The drug pressure was applied multiple times until no decrease in parasite level was observed at a given drug concentration. Using this protocol (figure 1), few cultures that were able to grow in 2 ng/ml of actD were isolated and cloned two times by limiting dilution. One of the above clones was designated as 3D7R/actD2. It should be mentioned that actD at relatively high concentrations killed the parasite within two to three days, while lower concentrations required much longer incubation times to show toxicity.

Characterization of the actinomycin D-resistant clones - To determine the effective concentration of actD that can inhibit the growth of the parental and actD-resistant *P. falciparum* by 50 % (IC_{50}), parasitized RBCs were grown in the absence and presence of increasing concentrations of actD in 96-well plates and the relative growth of parasites was determined from the incorporation of [3H] Hypoxanthine (see Materials and Methods). The results in figure 2 show that the IC_{50} for 3D7 and 3D7R/actD2 to be 0.6 ng/ml and 2 ng/ml, respectively. These results indicate that the IC_{50} of 3D7R/actD2 is 3-fold higher than the parental 3D7 *P. falciparum*. It should be mentioned here that parasites in the above cytotoxicity assay were exposed for a total of seven days to drugs rather than the three day assay usually used. This was found to give more reproducible results and was more accurate when measuring the cytotoxic effect of actD on *P. falciparum*. However, the increase in parasitemia, as determined from the incorporation of

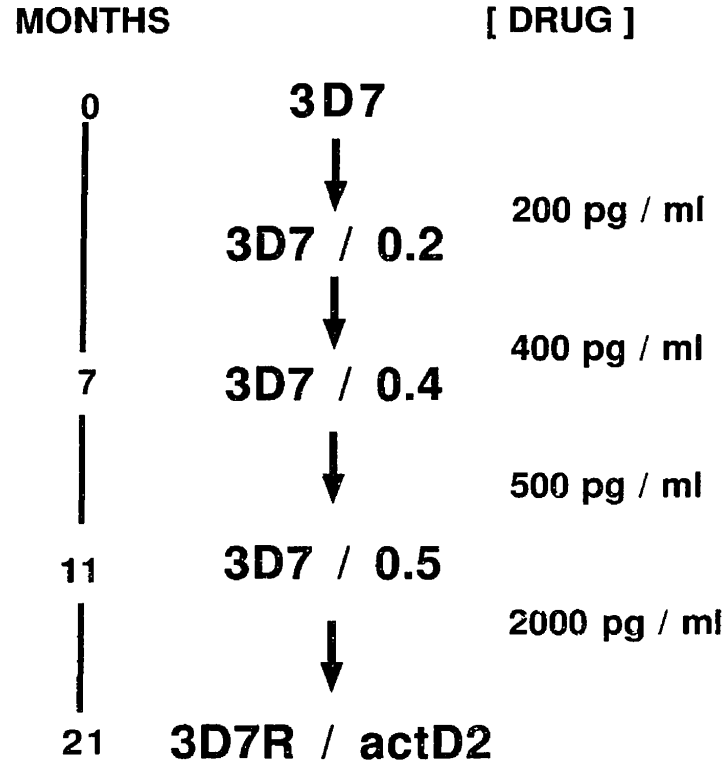

Fig. 1. Selection of *P. falciparum* clones resistant to actinomycin D.

[3H] hypoxanthine, was consistently seen at the lowest non-toxic concentration of actD (figure 2).

Previous studies (10, 36), using actD resistant tumor cells lines that overexpress P-glycoprotein, have shown that verapamil (VRP) and other chemosensitizing agents can reverse the multidrug resistance phenotype of drug resistant cells at concentrations that are not toxic to the parental drug sensitive cells. To determine if VRP had a similar effect on the actD resistance phenotype in *P. falciparum*, parasitized RBCs with 3D7 or 3D7R/actD2 were grown in the presence of actD with or without 2 mM VRP. Our results (data not shown) did not reveal any change in the IC_{50} of 3D7 or 3D7R/actD2 in the presence of 2 mM VRP. Thus, VRP does not reverse actD resistance in 3D7/actD2 cells. Furthermore, we have found that higher concentrations of VRP to be very toxic to 3D7 and 3D7R/actD2 using a seven day incubation cytotoxicity assay. The reversal mechanism of MDR by VRP and other unrelated compounds is not entirely clear. It has been shown that VRP competes with cytotoxic drugs for P-gp drug binding and is effluxed from resistant cells (37, 35). Based on these observations it was proposed by some that reversal of MDR of tumor cells with VRP is indicative of a multidrug efflux mechanism. However, the identification of another energy-dependent multidrug efflux mechanism in tumor cells that is mediated by multidrug resistance-associated protein (MRP; (38)) and is not reversed with VRP (39) is inconsistent with the latter suggestion. The reversal of CQ resistance by VRP reported earlier (11) is unclear since in that study different strains of *P. falciparum* (CQS and CQR parasites) were compared. Hence, the observed reversal effect of VRP may have been due to differences in sensitivity between different strains of *P. falciparum* . Furthermore, VRP did not increase the sensitivity of MQ resistant *P. falciparum* (22) nor did it reverse the MDR phenotype of other parasites that overexpress a P-glycoprotein-homolog (e.g. *Leishmania donovani*) (40, 25).

Actinomycin D-resistant *P.falciparum* are less sensitive to antimalarial drugs and to Rh123. To further characterize the actD-resistant *P. falciparum*, it was of interest to examine the cross-resistance profile of 3D7R/actD2 *falciparum* to other cytotoxic drugs such as the anti-malarial drugs (CQ and MQ) and to Rh123. The latter drug (Rh123) is a cationic fluorescent dye that accumulates specifically in negatively charged cellular compartments, such as the mitochondria. It was shown earlier (36) that Rh123 accumulates in the mitochondria in *P.* .

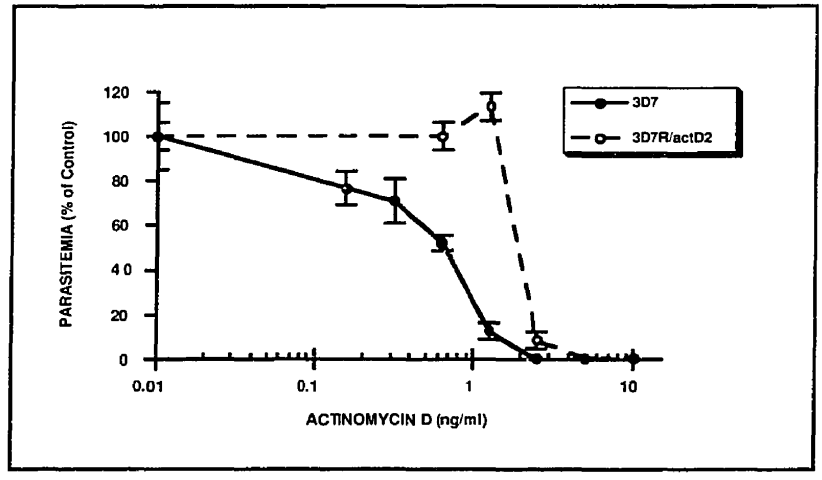

Fig. 2. Dose-response curves of drug-sensitive (3D7) and actD-resistant (3D7R/actD2) *P. falciparum.* 3D7 and 3D7R/actD2 parasites at 0.1% parasitemia were cultured in 2% hematocrine in the absence and in the presence of increasing concentrations of actD. The effect of actD on the growth of 3D7 and 3D7R/actD2 was determined from the incorporation of [3H) hypoxanthine following a seven day incubation without or with drug. The effect of actD on the parasite growth is expressed as percent of control (without drug). Each point is expressed as mean ± standard error of a representative of three experiments carried out in triplicate.

Figure 2. Certad, et al.

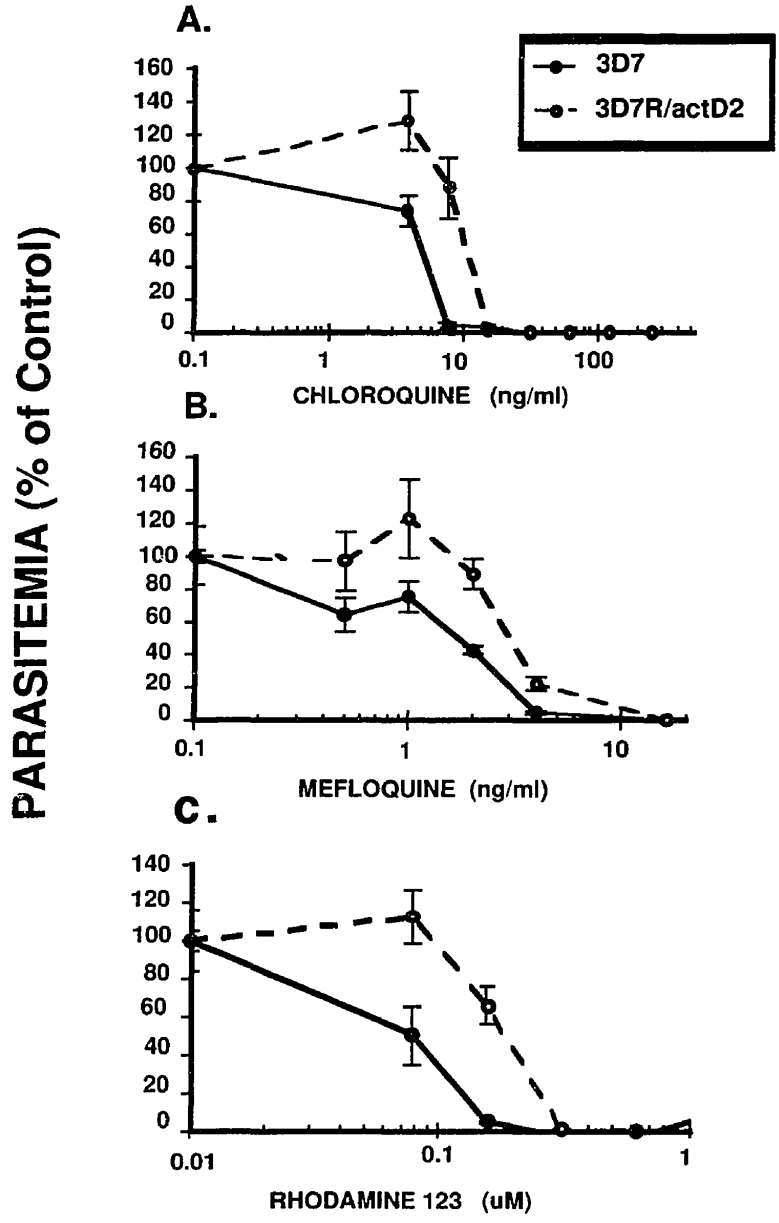

Fig. 3. Cross-resistance of 3D7R/actD2 clone to chloroquine and Rhodamine123. Dose-response curves for 3D7 and 3D7R/actD2 in the presence of increasing concentrations of chloroquine (A), mefloquine (B) or Rhodamine123 (C). The effects of drugs on the growth of the parasites were determined as in figure 3.

Table 1

Clone	IC_{50}*			
	Actinomycin D (ng/ml)	Chloroquine (ng/ml)	Rhodamine (μm)	Mefloquine (ng/ml)
3D7	0.65 ± 0.18	5.00 ± 0.75	0.08 ± 0.02	2.00 ± 0.35
3D7R/actD2	1.90 ± 0.20	10.50 ± 2.25	0.20 ± 0.04	3.30 ± 0.29

* Fifty percent inhibitory concentration (IC_{50}) determinations for actinomycin D, chloroquine, rhodamine 123 and mefloquine with *Plasmodium falciparum* 3D7 and 3DR/actD2. Values are means ± SD of at least three separate determinations.

falciparum and is toxic to the parasite. Rh123 has been shown to interact directly with P-glycoprotein from tumor cells (42) and is a model substrate for P-glycoprotein drug efflux pump.

Furthermore, *Leishmania donovani* selected for resistance to vinblastine (which induces P-glycoprotein in tumor cells) was shown to overexpress a P-glycoprotein homolog and to mediate the transport of Rh123 (25). The results in figure 3 show the effect of increasing concentration of CQ, MQ or Rh123 on the growth of 3D7 (CQS) and 3D7R/actD2 (actD-resistant) parasites. The actinomycin D- resistant clone (3D7R/actD2) was less sensitive to CQ, MQ and Rh123 than the parental drug-sensitive 3D7 *falciparum* (figure 3). Table I shows the IC_{50} values for 3D7 and 3D7R/actD2 in the presence of CQ, MQ, or Rh123. It should be mentioned that similar cross-resistance profile was observed with other actD resistant clones (data not shown). The observed decrease in sensitivity of 3D7R/actD2 toward CQ, MQ and Rh123 is consistent with results from previous studies where the levels of drug resistance are higher towards the selecting agent but vary towards other structurally dissimilar drugs (For review, 42). Moreover, the observed decrease in sensitivity to CQ in 3D7R/actD2 is consistent with results from another studies where in vitro selected *P. falciparum* with CQ. In that study (2), the IC_{50} of sensitive and resistant *P. falciparum* were less than two fold different.

Pfmdr1* expression in actinomycin D resistant *P. falciparum - There is now growing evidence that the expression of *pfmdr1* gene (a P-glycoprotein homolog) correlates with resistance to MQ, halofantrine, and quinine in *P. falciparum* (44, 22, 43). Moreover, *pfmdr1* has been implicated in CQ drug resistance in certain isolates of *P. falciparum*. However, the role of *pfmdr1* in CQ drug-resistance remains unclear . To determine if *pfmdr1* is involved in the resistance to actD, *pfmdr1* gene copy number and level of expression were compared between 3D7R/actD2 and the parental 3D7 *falciparum*. The CQR *P. falciparum* FAC8 15 was used as a positive control, since it was shown previously to contain three copies of *pfmdr1* gene and to express high levels of *pfmdr1* mRNA (14, 22). Genomic DNA from 3D7 (CQS isolate), FAC815 (CQR isolate) and 3D7R/actD2 were digested with EcoRI and separated by agarose gel electrophoresis, transferred, and probed with a 413 bp cDNA fragment that encodes for sequences

between transmembrane 11 and 12 (3331-3744bp) (13) of *pfmdr1*. Figure 4a shows a representative Southern blot where 9 Kb EcoRI fragment that hybridized to *pfmdr1* probe is found in all three cell lines. FAC8 15 showed an extra EcoRI fragment of approximately 6 Kb, not present in either 3D7 nor in 3D7R/actD2 (figure 4a). This fragment corresponds to the internal junction of the *pfmdr*1 amplicon (44). Although some differences in the intensity of the 9 Kb fragment in 3D7 and 3D7R/actD2 can be seen they were not reproducible. Further, similar results were also obtained using another cDNA probe that encodes for sequences in the ATP binding domain of pfmdr1 (from 1732bp to 2280; data not shown). Equal DNA loading was confirmed by subsequent reprobing of the same Southern blot with an α-tubulin-specific probe from *P. falciparum* (33). The results in figure 4 show that equal amounts of DNA were loaded in each lane.

To compare the levels of *pfmdr1* expression between the above three cell lines of P. falciparum, Northern blot analysis was performed using 10 μg of total RNA of 3D7, FAC8 15 and 3D7R/actD2 trophozoites isolated from synchronized cultures. Figure 4b shows a Northern blot probed with *pfmdr1* specific probe used earlier (transmembrane 11 to 12). These results show high levels of *pfmdr1* expression in FAC8 15 but not in 3D7 or 3D7R/actD2. Figure 4b shows an Ethidium bromide stained blot with the ribosomal RNA indicating equal loading of RNA.

To address the possibility that mutations in *pfmdr1* sequence (13) have occurred following our selection of 3D7 *P. falciparum* with actD and maybe responsible for resistance to actD, the region encoding these amino acid changes were amplified from 3D7 and 3D7R/actD2 by PCR and compared. The results (data not shown) show identical sequence for 3D7 and 3D7R/actD2. Thus, no changes to *pfmdr1* gene have occurred during the selection of actD resistant clones.

In conclusion, we have selected actD resistant *P. falciparum* that show decreased sensitivity to CQ, MQ and Rh123. Given the differences in the structure and function of these drugs, our results suggest that 3D7R/actD2 *falciparum* are multidrug resistant. Moreover, the resistance phenotype of 3D7R/actD2 does not involve changes in *pfmdr1* copy number, mutations or increased expression. Work is in progress to identify the molecular changes that confer the multidrug resistance phenotype onto 3D7R/actD2.

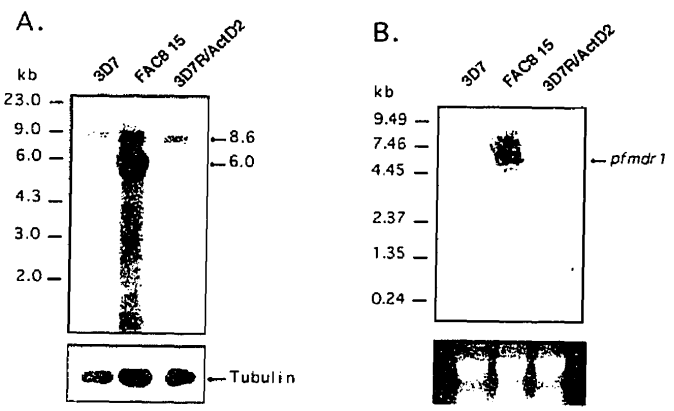

Fig. 4. Amplification or expression of *pfmdr1* in 3D7, 3D7R/actD2 and FAC8 15 clones of *P. falciparum*. For Southern blot analysis, ten microgram DNA from 3D7 (CQ-sensitive), FAC8 15 (CQ-resistant), or 3D7R/actD2 (actD-resistant) *P. falciparum* was digested with EcoR I, transferred to N-nylon membrane and probed separately with a *pfmdr1*-specific probe (A) or an a-tubulin-specific probe. For Northern blot analysis, ten microgram total RNA from synchronized 3D7 (CQ-sensitive), FAC8 15 (CQ-resistant) and 3D7R/actD2 (actD-resistant) *P. falciparum* was fractionated, electrophoresed and transferred to a membrane followed by hybridization with a *pfmdr1* probe (B). The ethidum bromide stained gel is shown as a control for loading. The migration of the DNA or RNA marker bands is indicated to the left of figures 4A and 4B.

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CONNECTING STATEMENT

In the first manuscript, we have selected a cell line, 3D7R/actD2 that is resistant to actinomycin D and that shows cross resistance to other drugs different in function and in structure (chloroquine, mefloquine and rhodamine 123). Furthermore, we showed that *pfmdr1* previously implicated in drug resistance in malaria is not involved in this resistance. To investigate the possibility that a P-glycoprotein homolog, other than *pfmdr1*, can mediate the transport of drugs and confer resistance to chloroquine and other antimalarials, we have set out to do PCR using oligonucleotide primers which hibridize to highly conserved sequences in the nucleotide binding folds of ABC transporters and we identified a gene homolog to the S4 subunit of the 26S proteasome complex. This subunit 4 belongs to a family of newly recognized ATPases.

MANUSCRIPT II

Cloning And Characterization Of Proteasome Subunit 4 ATPase From *Plasmodium falciparum*

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Keywords: Malaria; P. falciparum; Subunit 4 ATPase; 26S Proteasome.

(in preparation)

ABSTRACT

In eukaryotic cells, intracellular protein degradation occurs via the non-lysosomal pathway. The ATP-ubiquitin-proteasome pathway has been shown to regulate cell growth and differentiation by modulating the levels of several regulatory proteins and transcription factors. In this study, we describe the cloning and characterization of the proteasome subunit 4 ATPase from *Plasmodium falciparum* . The open reading of the *P. falciparum* S4 ATPase (PFS4) shows high level of sequence identity to that of yeast, mouse, drosophila and human S4 proteins. Southern blot analysis shows a single gene copy of PFS4 *P. falciparum.* Analysis of PFS4 expression during the parasite intraerythrocytic life cycle shows higher levels of PFS4 expression in ring stage than in trophozoites. Interestingly, northern blot analysis shows a marked decrease in PFS4 mRNA level in a multidrug resistant clone of *Plasmodium falciparum* selected with actinomycin D compared to the parental drug sensitive parasite.

INTRODUCTION

Plasmodium falciparum is the most lethal form of human malaria and is responsible for more than 200 million cases annually and over 1 million deaths. One of the most important forms of control has involved the use of antimalarial drugs. However, the emergence of resistance at the same time in different areas of the world where malaria is endemic, has compromised the control of this disease.

In an attempt to develop new means to combat drug resistance in malaria, we have begun a molecular analysis of this parasite. Specifically, we are interested in the role of intracellular proteases in the growth and life-cycle of *P. falciparum* . In fact, much is known about parasite secreted proteases and lysosomal protein degradation (for review, Mckerrow *et al.*, 1993). For example, the degradation of hemoglobin which occurs largely via lysosomal proteases in the food vacuole has been a major site for antimalarial drugs (for review, Cowman and Foote, 1990) . However, little is known about the non-lysosomal protein degradation pathway which now had been shown to be evolutionary conserved and is found in prokaryotic and eukaryotic cells (Rivett, 1993). By contrast to the lysosomal degradation of intracellular proteins that occurs primarily under stressed conditions; the non-lysosomal protein degradation is more selective and mediates the turnover of cellular proteins in addition to the removal of abnormal proteins generated under stressed conditions (Ciechanover, 1994).

In eucaryotes, proteins degraded by the latter pathway are generally tagged by the covalent attachment of a short polypeptide known as ubiquitin (Ciechanover, 1994). The ubiquitin conjugation and the degradation of ubiquinated proteins are ATP dependent processes found in the cytosol and the nucleus (Rechteiner *et al.*, 1993). The ATP-dependent degradation of ubiquitinated proteins occurs within a large barrel shaped cytosolic complex (2000 kDa) known as the proteasome or 26S complex (Houg *et al.*, 1987). The ubiquitin-proteasome pathway regulates normal cell growth and differentiation by mediating the degradation of mitotic cyclins, oncoproteins, the tumor suppressor protein P53, and other regulatory proteins (Ciechanover, 1994). Furthermore, this pathway is involved in antigen presentation and in a proteolytic

processing of precursor proteins (Coux *et al.*, 1996). More recently, the ubiquitin-dependent proteolysis has been shown to mediate cadmium- or arsenite-resistance in yeast (Jungmann *et al.*, 1993 and Klemperer *et al.* 1989) and auxin-resistance in plants (Leyser *et al.*, 1993).

The mechanism by which the 26S complex mediates the hydrolysis of ubiquitin-conjugated proteins is not understood. However, the multisubunit 26S proteasome consists of 20S core complex (the catalytic core) and the 19S cap complex (the regulatory cap). The crystal structure of the 20S proteasome from yeast was resolved to a resolution of 2.4 Å (Groll, M. *et al.* 1997; Nature). The latter study suggests a complex mechanism of protein hydrolysis involving the beta subunits in the 20S proteasome with the 19S cap mediating the ATP-dependent unfolding of ubiquinated proteins (Reichtener *et al.*, 1993). Furthermore, it has been suggested that the ATPase promotes the entry of polypeptides into the 20S particle. The 19S complex consists of several subunits and contains five ATPases.

The Subunit 4, found in the regulatory cap complex, is a member of a novel eukaryotic ATPase family (Dubiel, K. *et al,* 1992) that include: human TBP1 (Nelbock *et al.*, 1990), pig valosine containing protein (Koller et al., 1987), *Xenopus laevis* p97 (Peters *et al.*, 1992), *Saccharomyces cerevisiae* CDC48p (Frohlich *et al.*, 1991), Chinese hamster N-ethylmaleimide fusion protein (Wilson, D. W. *et al.*, 1989) and *S. cerevisiae* PAS1p (Erdmann *et al.*, 1991). Members of this ATPase family share highly conserved sequences surrounding the ATP binding site. However, their N-terminal 150 residues are divergent and therefore are thought to mediate their substrate specificities.

In this study, we report cloning and characterization of the S4 homolog (PFS4) in *P. falciparum.* The open reading frame predicts 385 amino acids residues and shows high sequence identity to that of yeast, mouse, drosophila and human S4 proteins. Analysis of PFS4 expression during the parasite intraerythrocytic life cycle showed higher levels of PFS4 expression in the ring stage than that of the trophozoite. We also show that PFS4 is expressed at lower levels in a multidrug resistant clone of *Plasmodium falciparum* selected with actinomycin D.

MATERIALS AND METHODS

Parasite growth- Parasites were cultivated *in vitro* according to the method of Trager and Jensen (Trager, W. and Jensen, J. B., 1978). Parasites were maintained on 5% suspension of type O erythrocytes in RPMI 1640 media supplemented with 32 mM hypoxanthine, 25 mM HEPES buffer, 10 % human plasma and 1 gr. of glucose . Parasite cultures (10 ml in T25 tissue culture flasks) were flushed with 3% O_2, 4% CO_2 and 93% N_2 and incubated at 37 ^{0} C with constant shaking. The drug sensitive clones (3D7) were a gift from Dr. A.F. Cowman at Walter and Eliza Hall Institute in Australia). The 3D7R/actD2 was cultured as above but in the presence of 2 ng/ml of actD.

PCR and DNA sequencing- PCR was done using primers with conserved sequences in ABC transporters. The sequences of the sense and antisense primers are 5` GG(A/T) AT(A/T)G T(A/T)G G(A/T)(A/ C) G(A/T)A C(A/T)G G(A/T)(A/T) C(A/T)G G (A/T)A AT 3` and 5` ATC (A/T)AC (A/T)GC (A/T) GC (A/T)GT (A/T)GC (C/T)TC AT 3` respectively. PCR reaction was made using 10X PCR buffer (100 mM Tris-HCl (pH 9), 500 mM KCl, 15 mM MgCl2), 200 mM of each dNTP, 100-200 pmol of each primer , template DNA 200 ng., Taq DNA Polymerase (5 U /(l), in 50 (l reaction for 1 cycle of 94 ^{0} C x 1 min., 40 cycles of 94 ^{0} C x 1 min., 40 ^{0} C or 37 ^{0} C x 2 min. and 72 ^{0} C x 3 min., 1 cycle of 72 ^{0} C x 7 min. (Innis *et al.*, 1990). The PCR fragment was purified from a gel slice and cloned into a TA cloning vector (TA-PCR II) according to the manufacturers protocol (Invitrogen, Inc.). Plasmid DNA was prepared and used in double stranded DNA sequencing (dideoxy method) (Brown, T.A. 1994).

RACE PCR- Total RNA was prepared from FAC8 15 *falciparum* as previously described (Certad et al. Chapter 3) using Trizol. The RNA pellet was washed with ethanol and was kept in ethanol at -70 ^{o} C if not immediately used. One ug of total RNA was used for RACE PCR (Gibco.Brl BRL). For 5` RACE, an antisense gene specific primer (5` CTG ATA CTA ATG

GAT CCA CTT C 3`) was used for the synthesis of cDNA by reverse transcriptase. Prior to PCR, a tdt-tailing step attached an adapter sequence to the unknown 5` sequences of the cDNA. Specific DNA was amplified by PCR using a gene specific primer (5` TAA AAC TGA ACA ACC AGG TTC 3`) and adapter primers that targeted the 5` terminus. For 3' RACE, a pol-T was used with reverse transcriptase first and then followed by PCR using a gene specific primer (5`ACC TAA CCC AGA TAC CAA AAC3`) that annealed to a region of known sequences close to the 3` end and an adapter primer that targeted the poly(a) tail region .

Expression of PFS4 in *P. falciparum*- The level of PFS4 expression in the two stages (ring and trophozoite) of the malaria erythrocytic cycle was determined by quantitative PCR (Foster, 1994). Briefly, parasitized RBC cultures were synchronized twice with 15% sorbitol and the parasites replaced in T25 flasks (10 ml) at 5% parasitemia and 5% hematocrite in fresh erythrocytes. RNA was prepared from parasitized RBCs following a second cycle of synchronization , from the ring stage and 24 hours later from the mature trophozoite stage. Equal amounts of RNA from both stages were used for RT-PCR. cDNA was synthesized by reverse transcriptase and amplified by PCR using specific primers (5` GGT ACT TTG GAA GAA ATT ATT GAT G 3` and 5` TAT ATC AGC ACC TGA CAA AAA TC 3`) in the presence of a 486 bp competitor fragment that was constructed. The PCR products were electrophoresed in a 1.5% agarose gel containing Ethidium bromide. The results were scanned and analyzed with NIH-image program .

Southern and Northern Blot Analysis- Southern and Northern blots were performed using standard procedures (Sambrook et al, 1989) For Southern blot, 10 µg of DNA from 3D7 *P. falciparum* was treated with BamH1, Cla1 and EcoR1 overnight at 37 ^{0} C. The DNA was electrophoresed through a 0.7% agarose gel and transferred to a Hybond-N nylon membrane (Amersham). Following pre-hybridization of the membrane (in 6 xSSC, 5 x Denhardt`s reagent, 0.5 % SDS, 100 µg/ml salmon sperm DNA, 50% formamide at 42 ^{0} C), the membrane was hybri

dized overnight with ^{32}Pi ATP labelled probe of 856 bp coding PFS4 sequences. The membrane was washed in 2 x SSC with 0.5 % SDS at room temperature for 10 min. and in 1 x SSC with 0.1% SDS at 65 ^{0} C.

For Northern blot analysis, 15 μg of total RNA from asynchronized FAC815 or synchronized drug-sensitive (3D7) or drug-resistant (3D7R/actD2) *P. falciparum* was fractionated with glyoxal /DMSO and electrophoresed on a gel containing 1% agarose. Total fractionated RNA was transferred to a nitrocellulose membrane and hybridized at 42 ^{0} C. The nitrocellulose membrane was incubated with ^{32}Pi labelled PFS4 probe as described above. The membrane was washed for 20 min. at room temperature in 1 x SSC with 0.1% SDS, followed by one wash of 20 min in 1 x SSC.

RESULTS AND DISCUSSION

The cloning of the PFS4 was accomplished by a combination of PCR cloning and 5` and 3` RACE PCR. Using degenerate sense and antisense primers (see methods) that encode for the consensus sequences of the A and B Walker motif of the nucleotide binding domain (Higgins *et al.*, 1986), a 200 bp fragment was amplified. An open reading of ~60 amino acids was used to search the protein data bank (GenBank library). The results of the latter sequence homology search showed high sequence identity to a family of new ATPases that included the S4 subunit of the 26S proteasome. Using poly-T and other specific primers, two other fragments were isolated by 3' and 5' RACE PCR, respectively (Figure 1). Following the cloning and sequencing of the latter fragments, a 1157 bp sequence which encodes for most of the S4 ATPase subunit was isolated. The high AT base composition is characteristic of malaria genome (Levitt, 1993).

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aaaatgtcgattaaaattattaaaaattagaaaggataaaagattatttattattagaagaagaat
atataacaaatcaagagcagattaaaagtagtgatgataagaattatgtgaaattgaaaatagatg
atttaagaggatcacctgtgaatgtaggtactttggaagaaattattgatgaaaatcatggtatta
tagctacatctgttggtccagaatattatgtaaatatattatcatttgttgataaagatttattag
aacctggttgttcagttttattaaataataaaacaaatagtgtagtaggaatattattagatgaag
tggatccattagtatcagttatgaaagtagaaaaagctccattagaatcttatgcagatattggtg
ggttagaatcacaaatacaagaaataaaagaagctgttgaattacctctaacacatccagaacttt
atgaagatattggtatcaaaccacctaagggtgttatcttatatggtccaccaggtactggtaaaa
cattattagctaaagctgtagctaatgaaacctctgctacatttttaagagttgtgggttctgaat
taatacaaaaatatttaggtgatggtccaaaattagttagagaaatgtttaaagttgcagaagaac
atgcaccttccattgtttttattgatgaaattgatgccgtaggtaccaaaagatatgaagctacaa
gtggaggagaaagagaaattcaaagaactatgttagaactattaaaccaattagatggtttcgatt
ctagaggtgatgttaaagttattatggctactaatagaatcgattcattagatcctgcactaatcc
gaccaggtagaattgatagaaaaatacaattacctaacccagataccaaaacaaaaagaagaatct
ttcaaatacatactagtaaaatgactatgtctcctgatgtagatttagaagaatttgttatgtcaa
aagatgatttgtcaggtgctgatataaaagctatatgtacagaagctggactcctagctcttagag
aaagacgaatgaaaataacacaagcagatttacgtaaggcacgagataaggcattattccaaaaga
aaggaaacataccagaaggtttatacttataaaaa
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Fig. 1. The nucleotide sequence of the *Plasmodium falciparum* S4 subunit.

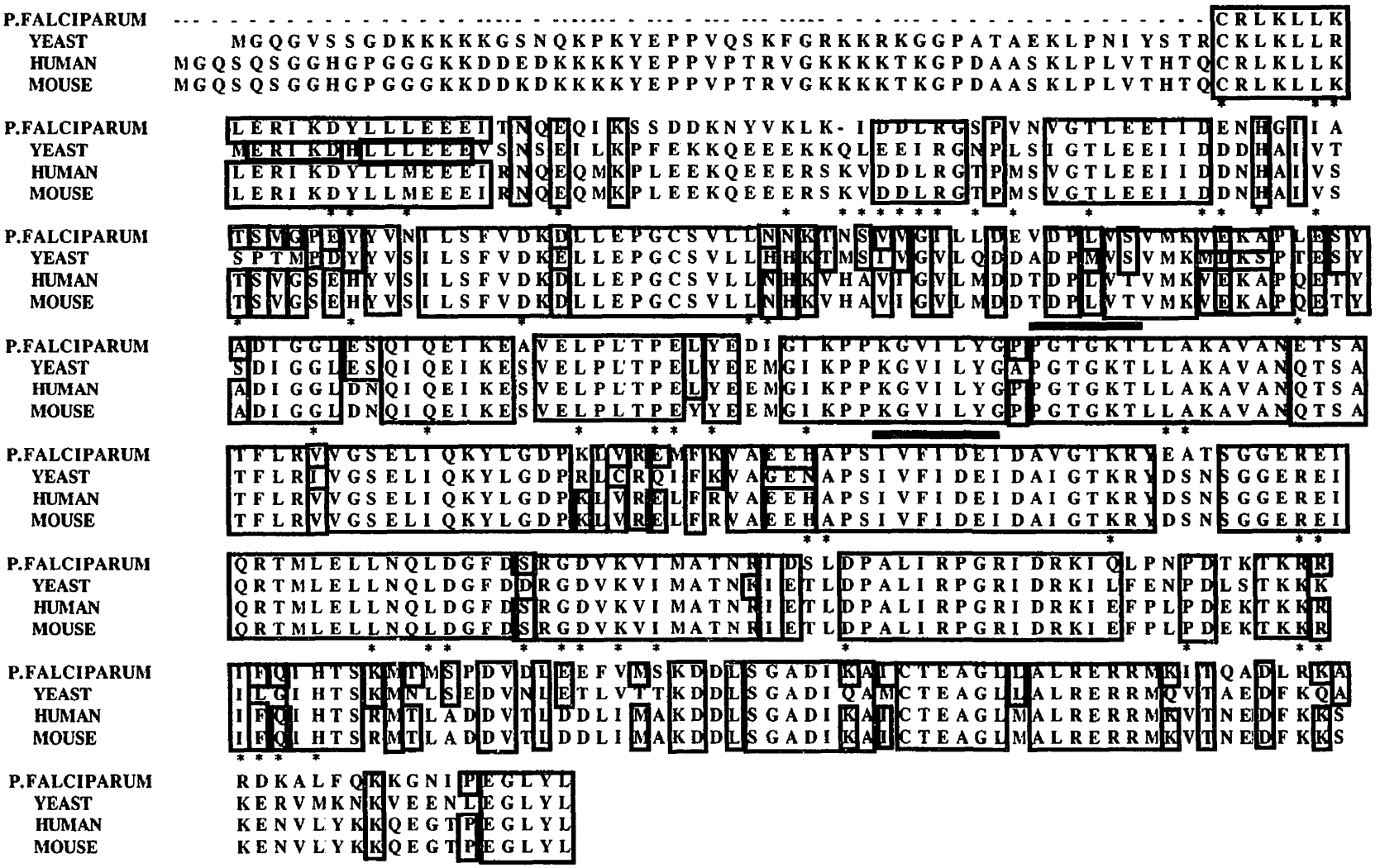

Fig. 2. Alignment of the deduced amino acid sequence of the P. falciparum S26PF gene with homologous sequences. The sequence shown correspond to S4 subunit of yeast, human and mouse. The boxes indicate identical residues and asterisks (*) beneath the alingment represent conservative replacements. Horizontal lines above the alignment show the ATP binding domains.

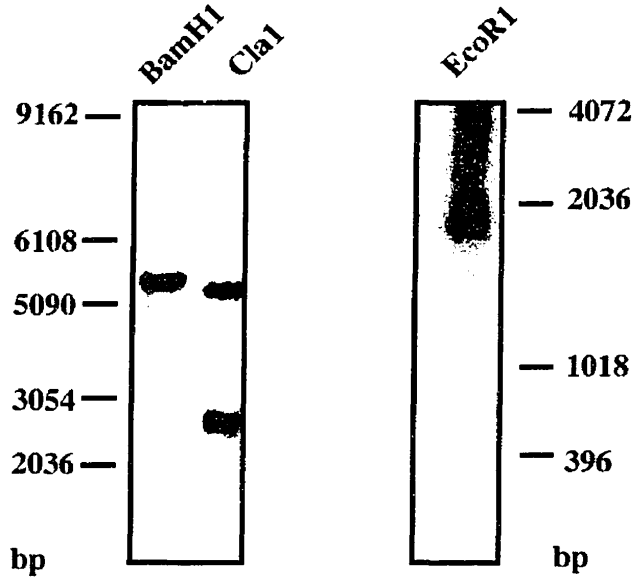

Fig.3 . Southern blot analysis of DNA from FAC8 15. Different samples of FAC8 15 containing ten micrograms of DNA were digested with BamH1, Cla1 and EcoR1 respectively, transferred to N-nylon membrane and probed with a 241bp fragment of PFS4.

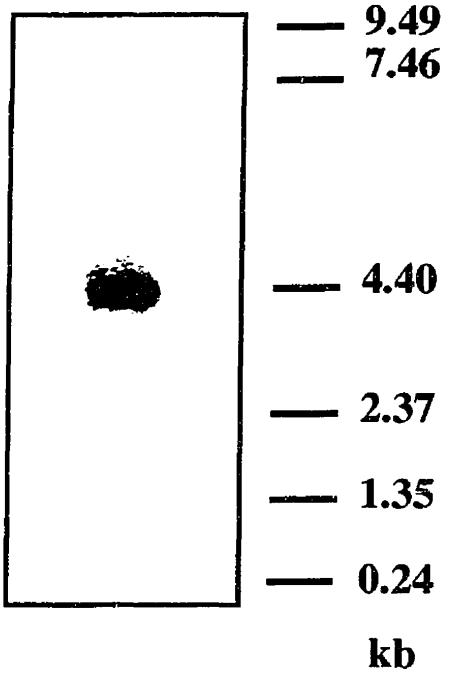

Fig. 4 . Northern blot analysis of the PFS4 transcript in *P. falciparum*. The size of the message is aproximately 4.4 kb. Sizes of RNA markers are shown to the rigth.

SPECIE	IDENTITY	HOMOLOGY
drosophila	79 %	92 %
human	75 %	89 %
mouse	75 %	89 %
yeast	72 %	88 %

Table. 1.Comparison of S4 sequences in differences species

Although the first ~50 amino acids are missing from the PFS4 gene, sequence homology searches shows PFS4 to share the highest sequence identity with that of yeast, mouse, drosophila and human S4 ATPases (table 1). The sequence identity is higher in the middle of the protein which contains the ATP binding domain. The sequence identity between PFS4 and the other S4 ATPases is less in the C- and the N-domains (figure 2). Although the function of the S4 ATPase is still not clear, it has been suggested that it may play a role reversing binding between S4 and its substrate and in the unfolding or transport of ubiquinated proteins for proteolysis within the 19S core complex (Reichtener *et al.*, 1993).

To determine if the PFS4 gene is a single or multigene family in *P. falciparum,* DNA from FAC8 15 strain was digested with different enzymes and hybridized with a 856 bp fragment of PFS4. The results in figure 3 show 5.3 kb and 1.9 kbp fragments for BamH1 and EcoR1 digestions, respectively. Cla1 digest shows two fragments of 2.5 kb and 5 kb (figure 3). The presence of the second fragment in Cla1 digest is due to the presence of an internal Cla1 site in PFS4 sequence. Given the size of the coding sequence of the PFS4 gene (~ 1.3 kbp), we predict a single copy gene for PFS4 in 3D7.

Analysis of total RNA from asynchronous parasites demonstrated the presence of a single 4.4 kb band (Figure 4). The differences between the coding sequence of 1.3 kb and the size of the message suggest the presence of a large 5' and/or 3' untranslated regions. Earlier studies have demonstrated that malarial mRNA have the longest 5' untranslated sequences of 400 - 1000 nucleotides (Levitt, A. 1993). Moreover, polyadenylation of malarial mRNA have been shown to occur about 300 bp past the TAA stop codon (Levitt, A. 1993). Thus although differences between the expected and found sizes of PFS4 mRNA may be due to the long 5' and 3' untranslated sequences, further sequencing of these two regions should shed more light on this difference.

Given the role of the ubiquitin-proteasome in the control of the cell cycle in eukaryotic cells, it was of interest to determine levels of PFS4 mRNA expression during the different stages of the parasite erythrocytic life cycle. Parasites cultures were synchronized twice by incubation with 15% sorbitol and total RNA was prepared for RT-PCR from ring and trophozoite stages of the parasite. Figure 5 shows the results of an RT-PCR using two PFS4-specific primers (see Methods). The results in figure 5 show higher levels of a 856 bp fragment in lanes 2 (ring stage) versus that in 1 (trophozoite stage). These results suggest higher metabolic activity in ring stage versus trophozoite stage.Thus, confirming the role of the ubiquitin-proteasome degradation pathway in normal cell metabolism (Ciechanover, 1994).

Earlier studies have shown that changes in the ubiquitin-proteasome pathway can confer cadmium- or arsenite-resistance in yeast (Jungmann, *et al.* 1993; and Klemperer *et al.* 1989). Although the precise mechanism by which the ubiquitin-proteasome pathway confers resistance to these drugs is not clear, it may be speculated that changes in the half-life of regulatory proteins that are substrates for the proteasome degradation could lead to some form of drug resistance. In mammalian cells, modulation of the levels of p53 tumor suppressor or the transcription factor NF-kB can lead to drug resistance by preventing drug induced apoptosis (For review, Shimamura *et al.*, 1996 Beg *et al.*, 1996).Taken together, it was of interest to compare the levels of PFS4 mRNA expression between a drug sensitive and resistant *P. falciparum* selected for resistance to actinomycin D. The selection and characterization of the actinomycin D resistant *P. falciparum* has been described elsewhere (Certad, et al. 1997, see chapter 2). Figure 6 shows a Northern blot analysis of total RNA from asynchronous parasite cultures of sensitive and resistant *P. falciparum*. Interestingly, the results in figure 6 show an increase in the levels of PFS4 mRNA in the sensitive relative to that in actinomycin D resistant parasites.This result suggests that one possible mechanism of actinomycin D resistance may involve a decrease in the metabolic rate of the parasite given the mode of action of the 26S proteasome responsible for normal growth and metabolism inside the cell (Reichtener *et al.*, 1993) .

Further work is required to determine if a similar decrease in PFS4 at protein level is

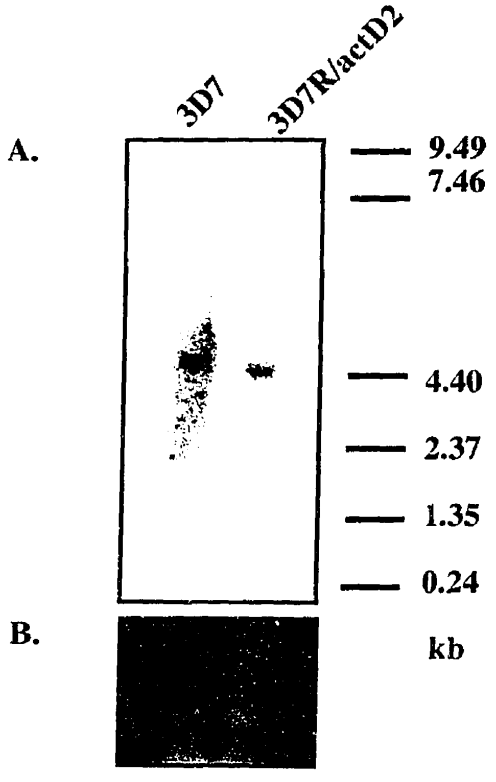

Fig.6. Expression of PFS4 in 3D7 and 3D7R/actD2 clones of *P. falciparum*. A. Northern blot analysis was done using 15 micrograms of total RNA from 3D7 and 3D7R/actD2. The total RNA was fractionated, electrophoresed and transferred to a membrane followed by hybridization with a PFS4 probe. **B.** The ethidium bromide stained gel is shown as a control for loading.

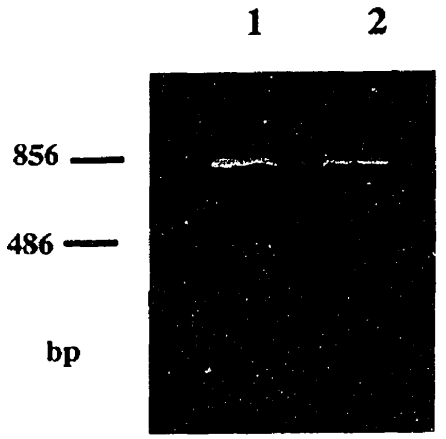

Fig.5. RT-PCR using a competitor to determine the expression of PFS4 at different stages of the life cycle of the parasite. Lane 1 corresponds to trophozoite stage. Lane 2 corresponds to ring stage. Two fragments were obtained: The upper one corresponds to the 856 fragment expected from the cDNA template and the lower one corresponds to the 486 bp fragment expected from the competitor constructed by deletion of one portion of the 856 fragment. The higher ratio between the two fragments (upper and lower) in lane 2 is indicating that there is higher expression of this gene in the ring stage of the parasite.

found between drug sensitive and actinomycin D resistant *P. falciparum*. If the latter difference is confirmed at the protein level, it would be of interest to examine other drug resistant *P. falciparum* for changes in PFS4 levels.

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GENERAL DISCUSSION

Malaria is a major disease of tropics. Infection with drug resistant strains of P. falciparum is a public health problem in almost all these endemic areas (Peters et al, 1985). The appearance of high level of resistance to chloroquine and other quinines (previously very effective) together with the shortage of new antimalarial of major health concern.

Studies of the mechanism of chloroquine resistance have revealed similarities with the multidrug resistant phenotype observed in tumour cells (Endicott and Ling, 1989) where neoplastic cells develop resistance to a wide range of drugs that share functional and structural properties. This MDR phenotype is believed to be mediated by rapid efflux and decreased concentration of drugs inside the cells. In tumor cells, this rapid drug efflux is mediated by increased expression of an energy dependent transmembrane transporter (or P-glycoprotein). P glycoprotein is a member of the ABC family of membrane transporters (Higgins et al, 1986).

The suggestion that chloroquine resistant *P. falciparum* was due to rapid efflux of the drug in chloroquine resistant strains (Krogstad *et al*, 1987) and that this efflux can be reversed by verapamil and other compounds (Slater *et al*, 1992) raised the possibility that this drug efflux can be mediated by an energy dependent drug efflux pump and led to a search for P-gp homologues.

At present, two genes, *pfmdr1* and *pfmdr2*, have been isolated. However, *pfmdr2* (Zalis *et al.*, 1992) encodes a protein with a different structure which is not typical of the P-gps encoded by mammalian MDR genes and there is no evidence to suggest that *pfmdr2* is involved in drug resistant phenotype. *Pfmdr1* was shown to share sequence and structural homology with the human P-gp in MDR cells (Wilson *et al*, 1989) but the role of *pfmdr1* in drug resistance remains controversial. Therefore, to investigate further that a P-gp associated mechanism of drug resistance in *P. falciparum* can mediate an MDR phenotype that includes resistance to chloroquine, we hypothesized that actinomycin D can induce the expression of P-gp homologues in resistant strains of *P. falciparum* and we selected *in vitro* ActD-resistant clones. The principal findings of this research are summarized bellow.

In this thesis, we describe the selection of a *P. falciparum* clone (3D7R/actD2) that is

resistant to 2 ng/ml of ActD. Limited characterization of these cells showed that the IC_{50} of 3D7R/actD2 is 3 folds higher than the IC_{50} of the parental cell line 3D7. More importantly, we show that 3D7/ActD2 *falciparum* are also resistant to other antimalarial drugs (eg., chloroquine and mefloquine) and to Rhodamine 123. Rh 123 has been shown to interact directly with P-gp from tumour cells (Nare et al., 1994) and to be a model substrate or P-gp. Also it has been found that Rh 123 accumulates in the mitochondria of the parasite and is very toxic. *Leishmania donovani* selected for resistance to vinblastine was shown to overexpress a P-gp homologue and was also shown to transport Rh 123 (Chow *et al.*, 1993). Taken together, our results in chapter 2 demonstrate the isolation of a multidrug resistant parasites following the selection with a single drug. On going studies will establish the full cross-resistance spectrum of 3D7R/actD2 clone.

Previous studies (Georges *et al.*, 1990), Divo *et al.*, 1985) using ActD resistant tumour cell lines that over express P-gp have shown that VRP and other compounds can reverse the MDR phenotype of drug resistant cells. However, verapamil does not reverse the resistance phenotype of 3D7R/actD2. Although it is not clear to us at this point why VRP does not reverse the actD resistance, it likely that actD resistance in 3D7R/actD2 is mediated by a multidrug resistance mechanism other than P-glycoprotein. For example, another energy dependent multidrug efflux mechanism in tumour cells is mediated by the Multidrug Resistance Associated protein MRP (Coles *et al.*, 1992) that is not reversed by VRP (Cole *et al.*, 1994). It is interesting to note that unlike the multidrug resistant phenotype of 3D7R/actD *falciparum*, CQ-resistant *falciparum* do not show a multidrug resistant phenotype. Moreover, we also show that *pfmdr1* does not mediate a multidrug resistant phenotype seen in 3D7R/actD2.

Manuscript II focuses on studies involving the use of oligonucleotide primers which hybridize to highly conserved sequences in the nucleotide binding domains of ABC transporters and as a result we have identified a gene with a very high homology to the S4 subunit of the 26S proteasome member of a family of ATPases (Dubiel *et al.*, 1992), that seems to be very highly conserved. Its presence is required throughout the complete erythrocytic cycle of the parasite. but the level of expression is higher at ring stage level than at trophozoite stage level. Surprisingly, when we compared the expression of this gene in 3D7R/actD2 and 3D7, we found that there is a

decrease in the expression in the resistant strain in comparison with the sensitive one, suggesting that this gene is involved in drug resistance may be as a part of other different changes taking place inside the parasite. Given the mode of action of the 26S proteasome, involved in normal growth and metabolism inside the cell (Reichtener et al., 1993), we speculated that a possible mechanism of action of PFS4 responsible for drug resistance could involved a decrease in the metabolic rate of the *Plasmodium.*

There are several possibilities for future research in this area. For example, transport studies are required to determine differences in drug accumulation or drug efflux in these cell lines. Furthermore, *P. falciparum* with higher levels of resistance towards ActD can be isolated and the cross resistance profile to CQ, MQ, Rh 123 and other lipophilic drugs can be determined.

It is possible that the PFS4 gene is involved in resistance alone or as a part of other different molecular changes conferring a multidrug resistant phenotype. Further investigation could be helpful to find these changes and to elucidate better the role of PFS4 gene and its involvement in drug resistance. For example, some transfection studies could be done. On the other hand, the finding of this gene could be useful if it is considered as a target for designing new antimalarial drugs.

In the control of malaria, non invasive approach as vector control, improvement in housing, anopheles biting prevention and comprehensive antimalarial programs are significant measures to prevent the appearance of the disease but once the infection has taken place, chemotherapy is required. As the experience of the past decades demonstrates, resistance to available drugs will continue to occur and a big effort is still needed in the developing of new antimalarials.

Multidrug resistance in malaria remains a great challenge and still many aspects have to be examined but it is hoped that the findings presented here can be useful in the understanding of the mechanism of drug resistance and use in the developing of new therapeutic strategies for *P. falciparum* infection.

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IMAGE EVALUATION TEST TARGET (QA-3)

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