The Cellular and Molecular Basis of Co-artemether (Artemether-Lumefantrine) Action.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

by

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Declaration:

I declare that the work presented in this thesis is all my work and that it has not been submitted for any other degree.

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Michael. Musakiriza. Makanga. (2002)

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ABSTRACT

Co-artemether is a novel oral fixed combination of two mutually complementary antimalarial agents, artemether (ARM) and lumefantrine (LMF) in a ratio of 1:6. Combination therapies that include an artemisinin derivative are believed to have a major role in the control of both malaria and the spread of drug resistance. The ARM/LMF fixed combination has proved to be highly efficacious against *Plasmodium falciparum* (*P.f*) even against multi-drug resistant *P.f* infections. Artemisinin derivatives, such ARM are the most active antimalarial compounds available for human use. They are now widely used in the tropical world (South East Asia and in sub-saharan Africa for treatment of both uncomplicated and severe malaria. Hitherto, albeit ARM is widely used, there is paucity of information elucidating the mechanisms associated with the toxicity of ARM and the novel LMF to the malaria parasites.

The aim of this study was to characterise the cellular and molecular mechanisms of the processes associated with co-artemether (ARM and LMF) drug induced toxicity to malaria parasite at therapeutically relevant drug concentrations. Explicitly, the study endeavored to:

(i) Define the drug exposure-parasite death relationship.
(ii) Assess morphological changes associated with drug effect, using electron microscopic ultra-structural studies to investigate both dose dependent and time dependent drug induced cellular alterations following ARM and LMF treatment *in-vitro.*
(iii) Determine relevant drug targets: probe the role of intracellular iron, and of the haemoglobin degradation pathway in the mechanism of action of ARM and LMF.
(iv) Apply comparative proteomics to study differences in protein expression profiles between drug treated and untreated *P.falciparum* parasite populations, to explore the global parasite response of ARM and LMF drug pressure at the proteome level.

Baseline growth inhibition sensitivity studies were performed to determine the required inhibitory concentrations for subsequent studies. The drug exposure death relationship was then investigated to define the duration of time required for irreversible drug toxicity with ARM and LMF *in–vitro.* Ultrastructural changes induced by the exposure of human erythrocytes infected with the K1 isolate of *P.falciparum* (CQ resistant), to ARM and LMF at three inhibitory concentrations (IC_{20}, IC_{50} & IC_{90}) were studied over a 24 hr period. The results of both the time dependent growth inhibitory sensitivity studies and electron microscopy showed rapid onset of action of ARM observed after one hour of exposure and irreversible toxicity effects after 5-8 hours. This contrasted with the effects of slower acting LMF, observed after 3 hours and irreversible toxicity effects after 8-12 hours. Prominent ultra-structural changes in both ARM and LMF treated parasites involved the digestive vacuole and were occurring at different rates and with different morphological features in the two groups.

The importance of intracellular iron in ARM and LMF mechanisms of action was investigated using three iron chelators with different lipid solubility properties (CP94.HCl, desferrioxamine, and deferoxamine-hydroxyethyl starch) as probes. The results evidently showed that ARM and its active metabolite DHA's antiparasitic effects were grossly antagonized by highly lipid soluble iron chelator CP94.HCl The interaction of CP94.HCl with LMF however was additive in contrast to CQ (positive control) which displayed antagonism. The source of this chelatable iron necessary for the bio-activation of ARM was however not clear. The role of haeme and the haemoglobin degradation pathway in general was therefore further investigated using different proteinase inhibitors: Ro40-4388, ALLN and E64 against CQ-resistant isolate K1 and CQ sensitive isolate HB3. Isobologram analysis of the fractional inhibitory concentrations (FICs) of proteinase inhibitors with the FICs of ARM, and LMF, in the main showed antagonism. These results suggest that the mechanisms of action of both ARM and LMF are dependent on efficient haemoglobin degradation which is evocative of the electron microscopic findings.

A global investigation of the differences in protein expression profiles between drug treated and untreated *P.falciparum* parasite populations was done using a proteomic approach. The differentially extracted *Plasmodial* proteins were separated using two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), the 2-D gels were then digitized with a densitometer and analysed with gel imaging software PDQuest^{TM}, to identify qualitative and semi-quantitative alterations in protein expression patterns followed mass spectrometry, to formally characterize the protein repertoire involved in the processes associated with drug toxicity to *Plasmodium falciparum* following ARM and LMF treatment. An attempt was thereafter done to formally characterize the differentially expressed *Plasmodial* proteins and these were clustered according to relatedness within specific biochemical processes in different biochemical path ways.

Abbreviations

[^{3}H]	Tritiated
µM	micro-moles
2-D PAGE	Two Dimensional Polyacrilamide Gel Electrophoresis
ACTH	Adenocorticotrophic hormone
ALLM	N-acetyl-L-leucyl-L-leucyl-methional
ALLN	N-acetyl-L-leucyl-L-leucyl-norleucinal
APS	Ammonium persulphate (Ammonium peroxydisulfate)
AQ	Amodiaquine
ARM	Artemether
ARTS	Artesunate
ATP	Adenosine triphosphate
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CQ	Chloroquine
DDT	Dichlorodiphenyl-trichloroethane
DHA	Dihydro-artemisinin
DHFR	Dihydrofolate reductase enzyme
DHPS	Dihydropteroate synthetase enzyme
DMSO	Dimethyl sulphoxide
DNase	Deoxy-ribonuclease
DTT	Dithiothreitol
DV	Digestive vacuole
E64	l-transepoxy-succinyl-leucylamido-(4- guanidino) butane
EDTA	Ethylenediamine-tetraacetic acid
EIR	Entomological Inoculation Rate
ES-MS	Electro Spray Mass Spectrometry
Fe(III)PPIX	Ferri III Protoporphyrin IX (Haem)
FIC	Fractional Inhibitory Concentration
HAP	Histo-aspartic protease
HEPES	N-[2-hydroxyethylpiperazine-N`-[2-ethanesulfonic acid] buffer
HF	Halofantrine
HPLC-ECD	High performance liquid chromatography with electrochemical detection
Hr	Hour
IC	Inhibitory Concentration
IEF	Isoelectric focusing
IPG	Immobilised Polyacrylamide Gel
kDa	Kilo Dalton
L	Linear
LMF	Lumefantrine
MALDI-MS	Matrix Assisted Laser Desorption Mass Spectrometry
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
Min	Minutes
mM	milli-molar
MQ	Mefloquine
MS	Mass Spectrometry
NL	Non-Linear
nM	nano molar

°C	Degree Celsius
PBS	Phosphate buffered saline
PHC	Primary Health Care
PMF	Peptide Mass Fingerprint
PMI	Plasmepsin I
PMII	Plasmepsin II
PMSF	Phenylmethylsulfonyl fluoride
PSD	Sulfadoxine/Pyrimethamine (Fansidar)
PTM	Post Translational Modifications
QHS	Artemisinin
QN	Quinine
r	Correlation coefficient
RBM	Roll Back Malaria
RNase	Ribonuclease
Ro	Roche compounds such as Ro40-4388, Ro61-7835, Ro61-9379
RT	Room temperature
S	Second
SB	Sulfabutaine
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
TBP	Tributyl phosphine
TCTP	Translationally Controlled Tumor Protein
TEMED	*N,N,N',N'*-tetramethyl-ethylenediamine
TFA	Trifluoro acetic acid
U.K.	United Kingdom
U.S.A	United States of America
UV	Ultra violet
V/V	Volume by volume
W/V	Weight by volume
WHO	World Health Organisation

TABLE OF CONTENTS

TABLES:

FIGURES

CHAPTER 1

1. INTRODUCTION

1.1. MALARIA

Malaria remains the most important protozoan parasitic disease of man and one of the main causes of morbidity and mortality in tropical countries of the world [1]. In this regard we are principally concerned with infection with *Plasmodium falciparum* which is responsible for almost all the mortality compared with *P.vivax, ovale* and *malariae.* Despite considerable efforts towards malaria control over the past decade, malaria remains a serious disease, a life-threatening disease with serious social and economic impact. Malaria, unlike most other infectious diseases, but, regrettably as with HIV, has a global mortality that is rising and not falling [2]. A large percentage of the fatalities occur in Africa, south of the Sahara upon children; however, malaria is endemic throughout most of Southeast Asia, the Indian subcontinent, the South Pacific region and Latin America. Malaria, either alone or in combination with other diseases, is estimated to kill between 1.1 and 2.7 million people worldwide each year, and over 2400 million, almost half of the world's population, remain at risk [3]. The high-risk groups include children below the age of five years, travelers, migrants from non-malarial regions moving into malarial regions, and populations with repressed immune system, including pregnant women (especially first pregnancy) and individuals suffering from HIV [4-6]. Over time, the human immune system

adjusts to the malaria parasite and adult mortality in endemic areas is fairly low due to acquired immunity.

Of particular concern are the acknowledged facts that drug resistant *Plasmodium falciparum* **(refer to figure 1.1)** field isolates are increasing at a rate that exceeds new drug development [7-16]. Furthermore, changes in global ecosystems and weather patterns have been implicated in a resurgence of malaria in South America and Africa [17, 18]. Not surprisingly, the *Plasmodium falciparum* disease patterns display enormous geographical variations and as more and more studies are carried out, greater variability is uncovered.

Hackett LW (1937) prudently observed:

"Everything about malaria is so moulded and altered by local conditions that it becomes a thousand different diseases and epidemiological puzzles. Like chess, it is played with few pieces, but is capable of an infinite variety of situations."

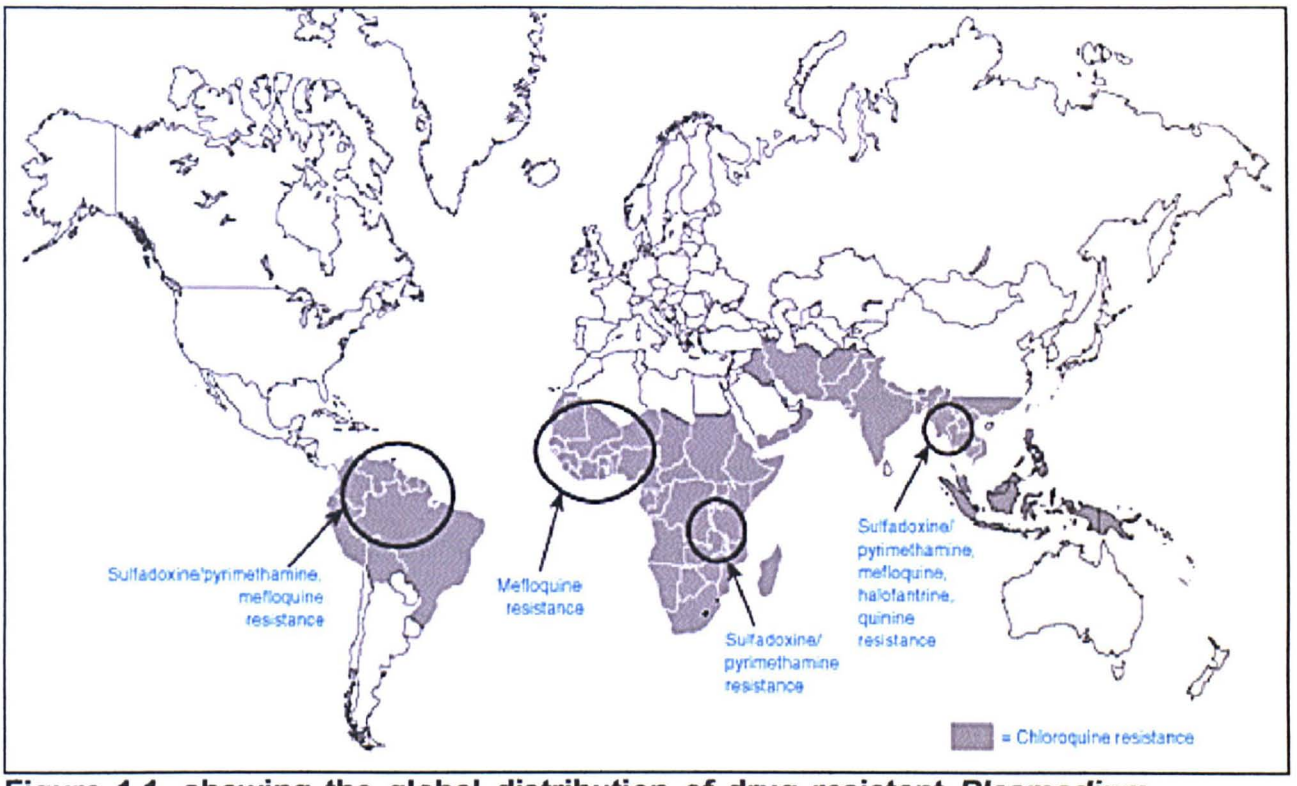


Figure 1.1. showing the global distribution of drug resistant *Plasmodium falciparum* malaria.

1.1.1. Historical background

Malaria is a health and social problem and few diseases (with the possible exceptions of acquired immune deficiency syndrome (AIDS) and tuberculosis) have had a greater impact on human social and economic development than malaria. Malaria has been with man for thousands of years. Fossil mosquitoes have been found in geologic strata 30 million years old and cadavers of mothers dating back 3000 years have been found with splenomegaly subsequently diagnosed to have been associated with with *P. falciparum* infection [19]. The nomenclature of fevers often reflected their supposed origin. "Malaria," named by the Italians in the 18th century derived from the notion that 'foul air' caused febrile disease. The earliest references to periodic fevers can found in Chinese *Nei Ching*, (Canon of Medicine) of 2700 BC, however the Greek physician

Hippocrates (460-377 BC) is attributed with the first detailed description of the clinical manifestation and some of the complications of malaria [20]. Malaria is also documented in the medical literature of ancient India where it is referred to as the dreaded affliction, "the King of Diseases" [19]

Descriptions of the patho-physiological features of malaria appeared in the 18th century. In 1716, Lancini documented the pigmented appearance of the spleen and brain of patients who had died following malaria, but it was not until 1847 that Meckel reported black granules in the blood of a patient dying from malaria. In 1878, Charles Louis Alphonse Laveran, a French army surgeon stationed in Algeria (North Africa), spent time examining post-mortem tissue from patients that had died of malaria. He also found the "pigmented bodies" earlier found and described by others, but he was the first to grasp their significance. On 6th November 1880 Laveran first described exflagellating gametocytes of *P. falciparum* in a fresh blood film from a patient with malaria and this marked first major breakthrough in understanding the aetiology of this long recognised disease syndrome.[20, 21]. In 1907 he received a Nobel Prize as "initiator and pioneer of the pathology of protozoa"[19].

In 1884 Gerhardt reported a key element of Koch's postulates, following the successful inoculation of healthy people with blood from patients with malaria. In 1885, Marchiafava and Celli gave the parasite its generic name *plasmodium* after describing development of malaria parasites within red blood cells developing at the expense of their substance, converting their haemoglobin into melanin pigment (now haematin), and that multiplying by fission. Golgi observed that the

febrile paroxysms were coincident with division and multiplication of parasites and by 1889 had determined that the different types of malaria were caused by distinct species of parasites. In 1891 Romanowsky developed the polychrome staining which permitted more detailed morphologic studies.

In 1894 Manson suggested the mosquitoes' possible involvement in malaria transmission. After four years of assiduous microscopic examination of dissected mosquitoes Sir Ronald Ross discovered the life cycle of the malaria parasite in 1897. During the period 1898-1899, Grassi and colleagues demonstrated that the entire extracorporeal phase of the human malaria parasite's life cycle occurred in the anopheles mosquitoes. 30 years later James and Tate documented that sporozoites disappear from the blood within 30 minutes of infection. Shortt Garnham and colleagues completed the elucidation of the corporeal life cycle of malaria parasites in 1948-49 by demonstrating the development of pre-erythrocytic forms of both *Plasmodium falciparum* and *Plasmodium vivax* in human liver. At this point the basic understanding of the parasites life-cycle was complete.

1.1.2. The Malaria parasite and its life cycle

Malaria is an acute and chronic disease caused by obligate intracellular protozoa of the genus *Plasmodium.* Four species of *Plasmodium* cause disease in humans: *P. malariae* (Laveran, 1881), *P. vivax* (Grassi and Feletti, 1890), *P. falciparum* (Welch, 1897) and *P. ovale* (Stephens, 1922) but *P. falciparum* causes most problems as a result of its prevalence, virulence, pathophysiological

consequences and drug resistance patterns. The pathophysiological disturbances and symptoms of malaria are attributed solely to the asexual erythrocytic forms and the pathogenicity of *P.falciparum* is attributed to its rapid rate of asexual reproduction and its ability to sequester in the microvasculature of vital organs, notably the brain, where they cause microvascular obstruction.

The life cycle of malaria is complex and this has rendered identification of suitable drug targets particularly arduous due to difficulties in developing appropriate experimental systems for investigation. All human *Plasmodial* species undergo sexual and asexual stages during their life cycle [22]. The sporozoites are the infective stage of the parasite life cycle and are injected along with saliva into the subcutaneous capillaries of the human host as the female anopheline mosquito prepares to take a blood meal. Male mosquitoes do not transmit the disease as they feed only on plant juices.

The inoculated sporozoites are viable in the peripheral blood for a short duration, typically less than 30 minutes. Some of the sporozoites are destroyed by the host reticuloendothelial system but many enter hepatocyte cells within the liver where they undergo asexual multiplication (exoerythrocytic schizogony/merogony). Upon maturation, the pre-erythrocytic tissue schizonts rupture and release more than 10,000 merozoites. This is the first asexual stage of the parasites development. The released merozoites find their way into the blood stream of the host where they infect healthy erythrocytes. Once inside the erythrocyte the parasite undergoes a 48 h life cycle. This is the second asexual stage of the parasite's development, termed erythrocytic schizogony, and it is this phase of

the parasite's life cycle that results in the clinical symptoms of the disease. During this stage the parasite matures within the red cell as a trophozoite. These trophozoites undergo nuclear division forming schizonts. Fully developed schizonts rupture releasing large numbers of merozoites, which are able to invade further healthy erythrocytes, therefore initiating a new cycle of erythrocytic schizogony. It is the rupture of the infected red cells and release of debris and toxic waste products from the parasite's development, which stimulates a host response manifesting itself as the clinical symptoms of malaria i.e., fever, chills and agues etc. During maturation within the red cell the parasite remodels the host erythrocyte membrane introducing parasite derived proteins which form electron dense protrusions termed knobs. These knob structures included the highly polymorphic, antigenically variable family of molecules (*Plasmodium falciparum* erythrocyte membrane protein 1, PfEMP1) that is implicated in the development of immunity [23, 24] and involved in the cytoadherence phenomenon [25-27]. Other molecules such as the human erythrocyte anion transporter structurally related to the band 3 protein [28] and the Duffy Binding Like proteins [29] have also been associated with cytoadherence.

The sexual stage of the parasite's life cycle begins when some merozoites, on entering a red blood cell, develop into male and female gametes. The anopheline mosquito takes up these gametocytes when it blood feeds on a malaria-infected host, thus initiating the sporogonic phase of development. The male gametocyte exflagellates, releasing eight flagellated male gametes which can fuse with the female gametocyte forming a zygote. The zygote matures into a mobile ookinete,

which penetrates the mid gut wall of the mosquito forming an oocyst. The oocyst under-goes a process of asexual sporogony releasing sporozoites which make their way through the haemocoel of the mosquito and subsequently sequester in the mosquito salivary glands, from where they can be transmitted back to the host when the mosquito takes another feed. **Figure 1.2.** Illustrates the life cycle of the *Plasmodium spp.*

One significant difference in the life cycles of the four species of malaria is that the a proportion of the sporozoites produced by the benign tertian malarias, namely *P.vivax* and *P.ovale* which invade the liver cells, have the capacity to transform into dormant stages called hypnozoites. These do not cause clinical symptoms but are able to produce a relapse of the disease, months to years after the initial infection. However, the mechanism explaining this development process and the subsequent initiation of disease relapse is ill understood. The *P. falciparum* and *P. malariae* species of human malaria do not have equivalent stages in their life cycles.

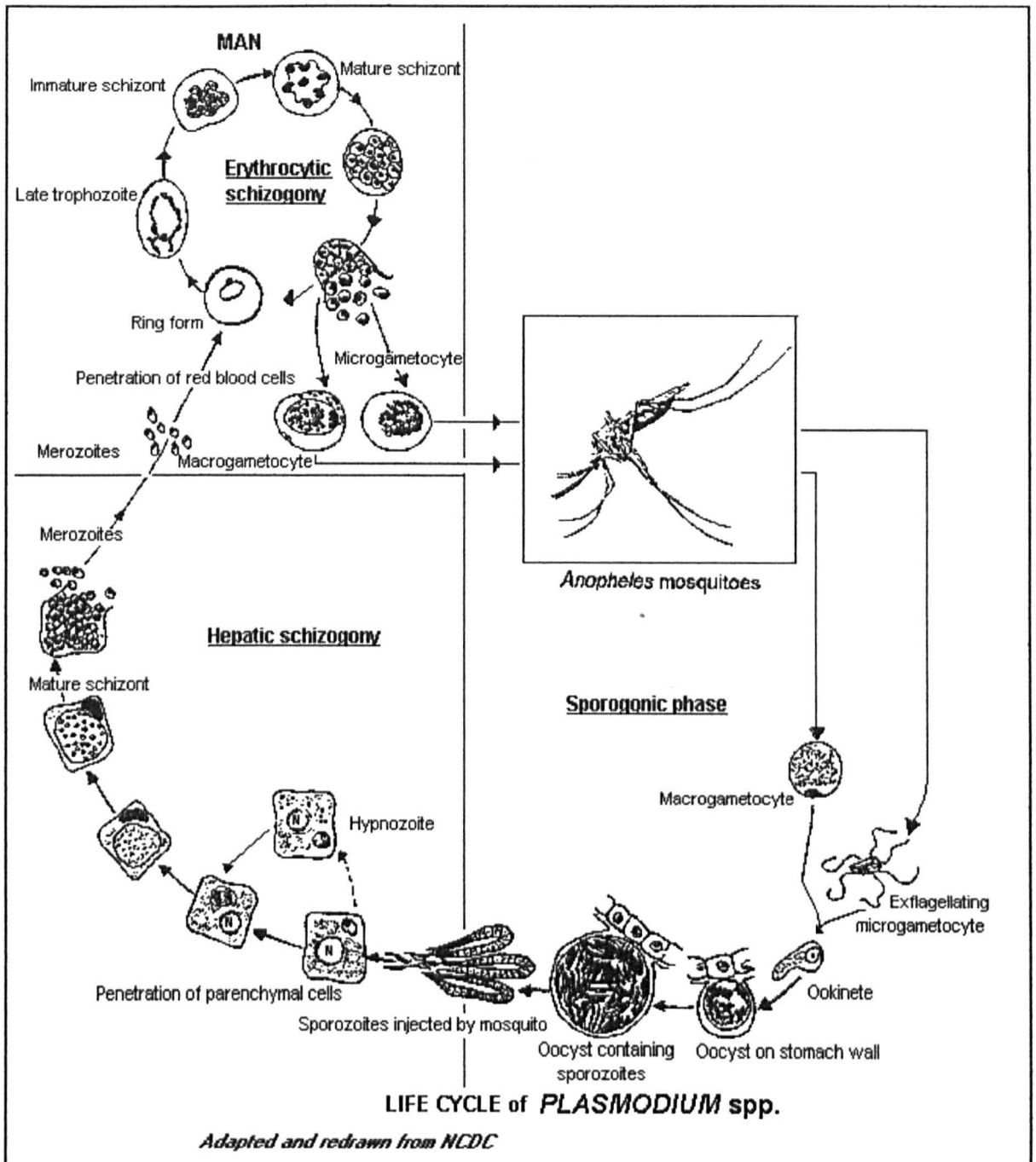


Figure 1.2. Illustrating the life cycle of the *Plasmodium spp.*

1.1.3. Drug resistance of malaria parasites

The phenomenon of drug resistance in malaria parasites has been defined as 'the ability of a parasite strain to multiply or survive in the presence of concentrations of a drug that normally destroy parasites of the same species or prevent their multiplication' [30]. The drug resistance may be relative, ie. yielding to increased doses of the drug tolerated by the host, or complete resistance, i.e. withstanding maximum doses tolerated by the host [31]. Drug resistance has been reported for almost all antimalarial agents in current clinical use with the exception of the artemisinin derivatives.

The WHO has been monitoring multi-drug resistance and recent data indicate the following patterns [3, 32] **(refer to figure 1.1)**.

1 Chloroquine-resistant strains of *P. falciparum* are spread throughout Southeast Asia (including Indonesia, Malaysia and Phillipines), the Indian sub-continent, South America and Africa. Despite the intensification of resistance during the last 2 decades, chloroquine still remains the first-line treatment for malaria in most of these countries [33]. Chloroquine resistance has not been reported in Egypt and Central America.

2 Chloroquine-resistant strains of *P. vivax* were first documented in 1989 and are now found in India, Indonesia, Papua New Guinea, Myanmar Vanuatu and Brazil [34-40]

3 Antifolate (pyrimethamine, sulphadoxine) resistance is wide spread in Southeast Asia and South Africa and also is found in East and Central Africa and other focal areas [41-50].

4 Mefloquine resistance is a significant problem in Thailand, Cambodia, Myanmar and South America. Reduced susceptibility to mefloquine has also been reported in Africa but clinical response rates remain high [51-62].

5 Resistance to quinine is increasing in Southeast Asia, South America and in a few areas in Africa [56, 57, 61, 63-73]. In regions where failure to respond to quinine has been demonstrated treatment with artemisinin is now advocated and may offer the only prospect of cure [63, 64]. Quinine is still considered to be highly effective in treatment of falciparum malaria in Africa and is the drug of choice for severe disease in most countries.

Several strategies have been proposed to attenuate further development resistance based on our understanding of the factors resulting in the selection of drug resistant and multidrug resistant falciparum malaria.

1. Rational drug use is essential. The inappropriate use of the limited and shrinking pharmacopoeia of affordable and effective antimalarial drugs should not be allowed to continue. Mass drug administration, including wide-spread chemoprophylaxis in endemic areas, as part of global eradication programmes, should be avoided or carefully controlled. The use of drugs with

exceptionally long elimination half-lives, in patients living in endemic areas, needs to be minimized [74]. Well-controlled combination chemotherapeutic regimens may offer a highly effective malaria treatment and may reduce the chances of resistant parasites being selected.[75-77]. The development of co-artemether is part of this initiative and in order to ensure patient compliance, the individual components have been ideally formulated together into a single tablet.

2. More research: New therapeutic strategies and a better understanding of the mechanisms of action and mechanisms of drug resistance to current drugs needs to be addressed.
3. New drugs, such as the artemisinin derivatives, halofantrine, atovaquone, pyronaridine and lumefantrine should be restricted and their use carefully controlled and monitored.
4. Simple treatment regimens are preferred, to increase compliance and minimize costs. And single-dose treatments should be monitored.
5. Post-therapy follow-up is essential, with, with a particular focus on differentiating between recrudescence and re-infection. R1 resistance may go unnoticed if follow-up is inadequate, but this is the first indication of potential problems with existing regimens or strategies of malaria control.

6. Drugs with high levels of resistance should be withdrawn from use with subsequent retesting in the clinical setting to establish if resistance patterns change. Re-introduction may eventually be appropriate.
7. Vector control is an essential part of all global strategies. Personal protection with bed-nets and appropriate use of insecticides is a highly regarded prophylactic strategy.
8. National and region-specific strategies should be implemented, depending on resources available. Issues like implementation of new initiatives to facilitate compliance and to encourage dispensers and retailers to educate their patients on the need to complete a full course of treatment [78] and more effective surveillance should be encouraged, both to monitor efficacy and to document adverse reactions.

1.1.3.1. WHO classification of clinical response or resistance to antimalarial drugs.

Resistance to antimalarial drugs may be demonstrated by *in vitro* tests while the clinical efficacy remains essentially unaffected due to the combination of modest effects on the parasite or host erythrocyte and host defence mechanisms, principally the reticuloendothelial system. In recognition of this anomaly, the WHO developed an *in vivo* classification of response (or resistance) to chloroquine in the 1970s **(Table 1)** [79, 80]. This classification has been

advocated for the assessment of response to artemisinins and other drug treatment regimens [79].

Table 1. Classification of response to antimalarial drug treatment [79, 80].

Classification	Response	Criteria
S	Sensitive	Marked reduction in the asexual parasitaemia to less than 25% of the admission parasite count within 48 h commencing drug treatment. Clearance of asexual parasitaemia within seven days and no recrudescence (radical cure).
RI	Low-grade resistance	Marked reduction in asexual parasitaemia to less than 25% of the admission parasite count within 48 h of commencing drug treatment. Clearance of asexual parasitaemia within seven days. Recrudescence within 28 days of commencing drug treatment.[a,b]
RII	High-grade resistance	Marked reduction in asexual parasitaemia to less than 25% of the admission parasite count within 48 h of commencing drug treatment. Parasitaemia is not cleared although parasites may be undetectable for a short period of time. Early recrudescence.[a]
RIII	High-grade resistance	No significant reduction in asexual parasitaemia within 48 h of commencing drug treatment. Parasitaemia is not cleared and may rise despite drug treatment

[a]. Recrudescence may be difficult to differentiate from reinfection. For RI and RII classifications to be considered valid, patients must remain in a non-endemic area where the risk of reinfection is low.

[b]. The length of time for recrudescence may be substantially longer for drugs with exceptionally long elimination half-lives.

The above classification, however, is difficult to apply to areas with intense transmission. Therefore in 1996 WHO introduced a modified protocol based on clinical outcome targeted at a practical assessment of theraeutic response in areas with intense transmission (Assessment of Therapeutic Efficacy of Antimalarial Drugs for Uncomplicated Falciparum Malaria in Areas with Intense Transmission). Therapeutic response may be graded according to WHO criteria into adequate clinical response, early and late treatment failure. The test procedure is based on a 14 day follow up with clinical, parasitological, haematocrit and fever assessment on days 0, 3, 7 (days 3 and 7 without haematocrit) and 14 (WHO, 1996).

1.1.4. Prevention and Treatment of Malaria

The control of human malaria is based on three principle approaches with a fourth strategy, vaccines, actively being sought:

1. Vector control
2. Health education and Primary Health Care (PHC)
3. Vaccine development
4. Chemotherapy

1.1.4.1. Vector control

An integrated malaria transmission control strategy including measures like: bed net use, indoor spraying or other domestic adult vector control measures and larval control, is likely to have a significant impact on the epidemiology of malaria

[17, 81-84]. The epidemiology of malaria is conventionally defined in terms of entomological inoculation rate (EIR) [2]. That is the number of times individuals are bitten by a mosquito carrying malaria parasites per unit time--in this case, a year. As the entomological-inoculation rate falls, the intensity of transmission falls or becomes more seasonal [2].

An early programme of vector control was initiated based upon the prevention of mosquito breeding. This method of disease control was known as source control, species sanitation, and involved methods such as altering habitats in a way that would encourage the vector to avoid certain areas. However, this method of disease control failed to achieve widespread implementation and was later phased out in the 1950's when insecticides were introduced [1, 85].

In 1955, the WHO adopted a policy to eradicate the malaria vector, the anopheline mosquito, worldwide [1] following the development of effective insecticides. The basic tenet was that as the *Plasmodium* parasites need the mosquito to develop sexually, if the mosquitos were killed the transmission of the parasites would be blocked. Houses were sprayed using the then widely accepted insecticide dichlorodiphenyl-trichloroethane (DDT) to kill the vector. However, inexplicably, Sub-Saharan Africa where the most of the world's malaria occurred was excluded. Although the strategy achieved much success, producing tremendous benefit of wellbeing to a billion people (WHO, 1989), it became apparent that it would be impossible to eradicate the disease. The programme was abandoned in 1969. It was decided to resolve the problem by "control" strategies. It was argued that the previous policy was unrealistically

simplistic: insecticide resistance by mosquitoes developed in areas of high transmission [1, 86]. However, it was also thought that there was need to further understand the biology of the disease rather than over-spending on eradication. However, some reports claim that it was abandoned because of DDT's toxicity, "donor fatigue", logistics and economic problems facing the west in the early 1970's [87].

Global eradication of the malaria vector is still presumably off the agenda of the WHO as an achievable objective, although safer and more environmentally friendly pesticides than DDT have since been made available e.g., the pyrethroids. The WHO emphasis of the past based on malaria eradication by indoor spraying is shifting and a wider multidisplinary approach is being adopted. This is also reflected in the flexibility of the Roll Back Malaria (RBM) policy, which advocates an open strategy of critical evaluation, and malaria control programmes geared to diverse and changing local situations. The organisation encourages the use of a combination of insecticides along-side larvicides plus the original method of environmental management to reduce vector numbers [88-90]. Vector control is now used solely as a means of reducing the rate of malaria transmission. It has been suggested that the benefits of effective control programmes will be only transitory in areas of high malaria endemicity and that deaths and disease will not be prevented but postponed [91]. Furthermore, it has also been argued that a reduction in the incidence of infection, due to reduction in transmission by efficient vector control, will prevent the natural development of immunity to infection by the host [91]. It is not easy to define the relationship

between the rate of transmission, the incidence / severity of infection, or death nor is it known if this relationship is in any way linear. Therefore, although the number of incidents of infection may be reduced per annum by efficient vector control, some argue that these infectious incidents would be of a more serious nature when they occurred. It is clear that vector control alone cannot completely eradicate the disease and must be used only as a first line of defence against the disease in conjunction with the use of chemotherapeutic agents [1]. Additionally it is important that effective control programmes involve the endemic community at large, the existing general health services and policy makers [88, 89].

1.1.4.2. Health education and Primary Health Care (PHC).

Malaria control has to be integrated into the basic health system of each country and there is a need to improve health education and development of primary health care. The integration of antimalaria activities within the primary health care system is a useful approach that has been succesfully tested in some places [1, 90, 92-95]. Patterns of such integration depend on different epidemiological, socioeconomic, cultural, and other factors. Malaria control within the framework of primary health care demands full commitment by the government concerned, constant support of the community and a close cooperation with all other sectors of the health system. Training of national professional and auxiliary staff and health education of the community are equally important.

The availability of primary health care facilities in malaria-endemic areas goes a long way in disease management through early diagnosis and prompt treatment.

Such clinics are also used to educate the local community in control through personal protection measures. Health education helps affected populations seek medical attention, which is fundamental to malaria control. It promotes the use of insecticide-impregnated bed nets, biological control methods involving the use of toxic agents against the mosquito vector in order to reduce the incidence of infection [3, 96, 97]. These methods are usually quite cheap and can provide a good first line of defence against the disease vector. In some parts of the world, the disease has been contained quite effectively through personal (individual or house-hold) protection measures such as protective clothing, repellants, bednets, or community/population protection measures e.g., use of insecticides or environmental management to control transmission. In areas of high endemicity, such as those found in tropical Africa, vector control, initially by household spraying [89, 98, 99] has been reported successful in reducing mortality and morbidity from malaria. Additionally the use of bed nets (impregnated with pyrethroids) and curtains [100-112] has helped to reduce mortality and morbidity due to malaria, and should be promoted.

1.1.4.3. The Malaria Vaccine

The complexity of the *Plasmodium* malaria life cycle, the transmission dynamics in different endemic settings and the spread of resistance to various drugs by the parasite and to insecticides by the vector render control strategies very difficult. An effective vaccine against malaria would represent a major strengthening of control. Research efforts to identify and select antigens for vaccine development

have been substantial, particularly in the past 20 years. Vaccines are being developed at a global level; clinical trials are ongoing in USA, Columbia, Switzerland, Australia, Papua New Guinea, The Gambia and Tanzania [113-125]. These include field trials with SPf66, the Colombian malaria vaccine and several *Plasmodium falciparum* candidate vaccines under Phase I testing, including NYVAC-7, a multi-antigen, attenuated recombinant vaccinia virus.

A cost effective vaccine must be capable of being incorporated into appropriate health delivery programmes and must provide a sufficient duration of immunity. Unfortunately, it is difficult to predict when one such effective vaccine will be made available. There are five main strategies being followed in the development of a malaria vaccine:

1. Anti-sporozoite vaccines, designed to block the sporozoite from invading or developing within the hepatocytes (anti-infection vaccine);
2. Blocking merozoite invasion of red blood cells and inhibiting development of schizonts (anti-disease or asexual stage).
3. A newer approach is development of antidisease vaccines which aim to alleviate morbidity by suppressing immunopathology in the host. Antidisease vaccines are based on neutralizing parasite components that induce host pathology, leaving the parasite itself directly unaffected. Blocking the adverse pathology-inducing effects of cytokines and parasite sequestration (disease modifying vaccine).
4. Transmission blocking vaccines, designed to arrest the development of the mosquito, thereby reducing or eliminating transmission of the disease and

5. Combinations of the above (multi-stage, multi-antigen).

Much attention has been focussed on SPf66, a synthetic polypeptide based on pre- erythrocytic and asexual blood-stage proteins of *Plasmodium falciparum.* In 1988, Patarroyo et al., [126] reported that this vaccine induced significant protection resulting in a delay or suppression of development of *Plasmodium falciparum* parasitaemia in immunized human volunteers. Following this initial observation extensive field trials have been performed [119, 127-130]. Although the early clinical trials in Colombia showed the safety and protective efficacy of this vaccine in areas of low to moderate transmission [131], the subsequent trials undertaken in areas of high transmission e.g., Latin America, Africa and Thailand failed to confirm this efficacy [128, 130, 132-135].

Despite the challenges in vaccine development, the task still continues. The individual immune mechanisms that are responsible for the acquired immunity remain uncertain, but classical transfer experiments with polyvalent gamma globulin from immune donors to non-immune individuals showed that antibodies play an important role. Potential targets for malarial vaccines include antigens on the surface of the sporozoites and the merozoites. Several protein antigens from *P. falciparum* have been characterized at the molecular level, and most of the characterized antigens have the common characteristic that they are recognized by immune sera from individuals living in malaria endemic areas [136].

One major challenge in vaccine development is that the parasite's life cycle is complex, the developmental stages in humans are morphologically and antigenically distinct, the immunity is stage specific [137]. Another important factor is the capacity of the parasite to evade the immune response as a consequence of antigenic diversity. The existence of antigenic polymorphisms allows for the switching of the expression of these variant antigenic molecules during the parasite's life cycle. Immune responses generated to one allelic form may not recognise another form. There is a large family of genes in *P.falciparum* called the *var* genes that encode highly variant antigens e.g., PfEMP1; which function as erythrocyte receptors for endothelial cells. Differential expression of these genes helps the parasite to escape the host immune response. We are hopeful that the potential role of post genomics in vaccine development will usher in knowledge to circumvent the current problems. The development of a successful malaria vaccine still has a long way to go. Therefore, in the meantime the emphasis still lies with the use of chemotherapeutic agents, to control the disease.

1.2. POTENTIAL CHEMOTHERAPEUTIC TARGETS IN *P.FALCIPARUM*

The malaria parasite must offer a wide selection of putative chemotherapeutic targets, though very few have been validated it is anticipated that at least some of these will generate effective and safe compounds of the future. Some of the targets of the current antimalarial drug armoury can broadly be put into three categories [138]:

(a) Targets implicated in processes occurring in the *Plasmodium* digestive vacuole: these include haemoglobin catabolism and haem detoxification; redox processes, and free radical reactions (these will include antioxidant defence mechanisms which also occur in the parasite cytoplasm and the host cell). The digestive vacuole membrane is also thought to be involved in drug accumulation and extrusion and may therefore lend itself to chemotherapeutic attack.

(b) Enzymes involved in macromolecular and metabolite synthesis: these include nucleic acid metabolism, phospholipid metabolism, glycolysis, and tubulin assembly.

(c) Proteins responsible for membrane dependent processes (including trafficking and drug transport) and signaling.

Pathway	Enzyme/Process	Notes
Haemoglobin metabolism	Plasmepsin I,II (aspartic proteinases; Cathepsin D-like)	Genes cloned and expressed; structure of plasmepsin II known; available for screening.
	Falcipain (cystein proteinase; cathepsin L-like	Cloned and expressed, but with low yield
	Haem polymerization, depolymerisation	Chemical reaction? Assay available
	Haem synthesis	Parasite has unique ALA synthase
Folate metabolism	DHFR-TS	Gene highly expressed, human enzyme and mutants also available for screening
	PPPK-DHPS	Gene expressed, but inefficiently; mutants resistant to sulfa drugs available
Pyrimidine synthesis,	Carbamoyl-phosphate synthase II	Parasite growth suppressed by ribozymes targeted to the gene
electron	DHODase	Enzyme purified. Target for atovaquone?
transport	Cytochrome oxidase	Target for atovaquone?
Purine salvage	HGPRT	Expressed, but very low yield
RNA/DNA	RNA polymerase (35 kb)	Inhibited by thiostrepton, rifampin?
	Ribonucleotide reductase	Presumed target for iron deprivation
	Topoisomerase I and II	Genes for both enzymes cloned. Topo II target for quinolones?
Glycolysis	LDH	Gene cloned and expressed. HTS underway. Structure solved (unique cleft near active site)
Phospholipid metabolism	PC/phosphatidylserine synthesis	Inhibitors of choline transport; potential antimalarials
Artemisinin receptors	Iron haem as activator of protein alkylation	Haem-binding model developed, but not clear if compound activity could be predicted
Oxidant damage	Potentiation of oxidant drugs	"Xanthone hypothesis": xanthone formed from oxidant process, active antimalarial
Oxidant defence	Glutathione reductase	Flavoenzymes, copurifies with glutamate dehydrogenase
	SOD	Cu/Zn-SOD in the FV; Mn-SOD in the RBC Respective roles unclear, none available for screening

Table 2. Summary of principal Chemotherapeutic Targets in *Plasmodium* [138]

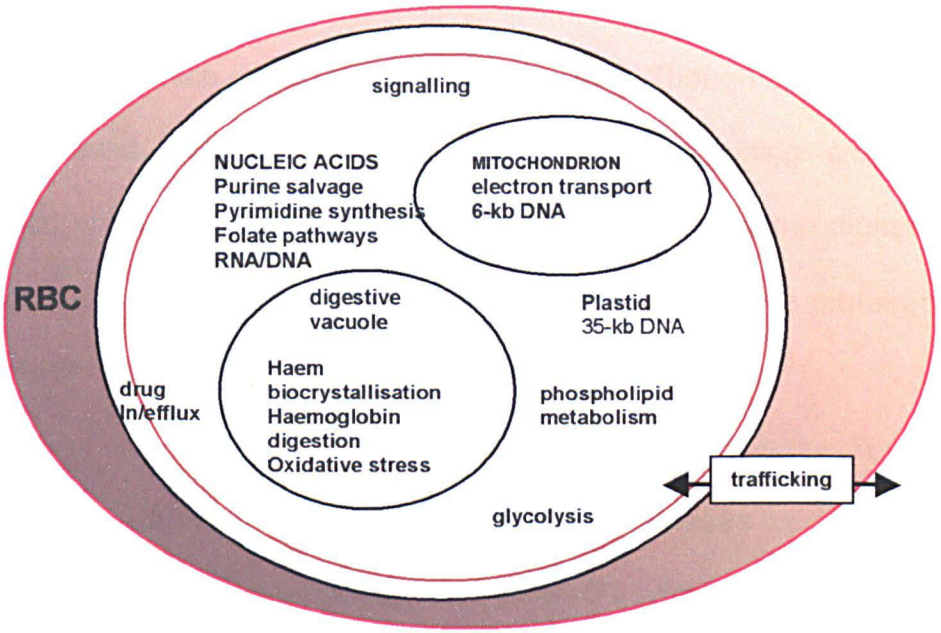


Fig. 1.3. Schematic showing the principal chemotherapeutic targets in *Plasmodia* [347].

1.2.1. Processes in the digestive Vacuole

Intraerythrocytic *Plasmodium* utilizes host cell haemoglobin presumably as a principal source of amino acids for parasite protein. The processes of haemoglobin degradation and haem detoxification occur in the *plasmodial* digestive vacuole and are essential for parasite survival. In contrast to other metabolic pathways the parasite lacks an alternative process for haemoglobin utilization and haem detoxification. These processes therefore, represent unique chemotherapeutic targets [139, 140], and may explain the long period of effective use that quinoline antimalarials have served in contrast to the other drugs such as the antifols.[138] Quinine and 4 aminoquinoline antimalarials are thought to act by binding to haem ([141, 142]. It is postulated that the drug and haem binding results in formation of drug-haematin complex or accumulation of free haematin (unpolymerised haem) as consequence of inhibition of haem

crystallisation which produces toxicity. [143, 144]. Though still subject to debate, it is believed that resistance to the quinoline-containing antimalarials has emerged via drug accumulation/extrusion mechanisms at the digestive vacuole membrane level rather than at the binding site of a putative protein drug target [145, 146].

1.2.1.1. Haemoglobin catabolism and proteinases

Plasmodium falciparum degrades nearly 25-75% of its host cell haemoglobin as a source of amino acids to supplement its limited capacity for de novo amino acid synthesis. A greater percentage of the haemoglobin degradation occurs during the 6-12 h period of the late trophozoite stage of its life cycle [147]. Proteases of *Plasmodium* play a key role in the processing of parasite encoded proteins, schizont rupture, erythrocyte invasion and haemoglobin degradation [148]. Haemoglobin hydrolysis appears to be mediated by acid cysteine, aspartic, and metalloproteases, and then a neutral aminopeptidase. Four aspartic proteases are currently known to be involved in the haemoglobinolytic process: Plasmepsins (PMs) I, II, and IV and histo-aspartic protease [149-154],. Aspartic proteases account for 60-80%, and cysteine protease account for 20-40% of the globin-degrading activity in purified digestive vacuoles [155-157]

The first aspartic proteinase (plasmepsin I) makes the initial cleavage of the native haemoglobin tetramer at the Phe33-Leu34 bond, unraveling the molecule for further proteolysis. A second aspartic proteinase (plasmepsin II) is believed to have preferential activity on acid-denatured globin. The two aspartic proteinases

are present in the digestive vacuole at different times in the parasite cycle (plasmepsin I at the ring stage, plasmepsin II in the trophozoite and young schizonts) [138], however the relevance of this observation is unclear. The initial aspartic proteinase cleavage exposes globin to further proteolytic digestion [149, 156-158] and leads to the release of FPIX (Fe (II) protohaematoporphyrin). After the tetramer is open, the cysteine protease (falcipain) recognizes the globin (apoprotein) but not native haemoglobin. Falcipain cleaves globin readily into small peptides and amino acids for parasite growth and maturation [140]. A recent study however, suggested the involvement of cysteine proteinase in an early step of haemoglobin degradation [159]. Recently, a novel metallopeptidase activity, falcilysin, was purified from food vacuoles and characterized. Falcilysin appears to function downstream of the aspartic proteases plasmepsins I and II and the cysteine protease falcipain in the hemoglobin proteolytic pathway [151]. There is ample evidence supporting vacuolar proteinases as valid chemotherapeutic targets: inhibitors of these enzymes have been shown to block haemoglobin degradation and kill the parasites in culture [152], although results of *in-vivo* experiments have been less successful [160].

1.2.1.2 Haem polymerisation / Bio-crystallisation

The haem detoxification pathway of the malaria parasite *Plasmodium falciparum* is a potential biochemical target for drug [161-163]. Haem, (Fe (III) protohaematoporphyrin) is toxic, and intra-erythrocytic stage malaria detoxify it in the digestive vacuole. Malaria parasites sequester the haem, which is potentially

toxic to biological; membranes and parasite enzymes [156], into an insoluble innocuous crystalline form [164], haemozoin (or malaria pigment) [157]. Haemozoin consists of polymerized Fe (III) protohaematoporphyrin subunits linked together through an iron carboxylate bond between the central iron of the molecule and the propionate side chain of the next haem molecule [165, 166]. Ridley, 1996 [167] reviews the various mechanisms of the polymerization process: earlier research implicated the involvement of haem polymerase [141, 165] or histidine-rich proteins (*Plasmodium falciparum* histidine- rich protein-2 (Pfhrp-2) [162, 168] in the initiation of the process in the parasite. However, other researchers now believe that rather than being enzyme-mediated, haem polymerization is actually a chemical process, dependent only on the presence of haem-derived material associated with haemozoin and not on protein [169].

1.2.1.3. Oxidative stress

Oxidative stress is an important mechanism in the destruction of malaria and other parasites [158, 170-175]. Intra-erythrocytic and hepatic-stage malaria parasites are confronted with oxidative effects resulting from reactive oxygen species (ROS) derived from the parasites themselves, the erythrocyte, or the host immune cells. This necessitates that the parasites build up antioxidative defense against these ROS. These include three oxidant defense enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, in varying amounts during its growth cycle [172, 176-181]. The erythrocyte itself has potent antioxidant defenses that counteract the constant production of ROS by

oxidation of haemoglobin (Fe^{2+}) to methaemoglobin (Fe^{3+}). Although the parasite has its own hexose monophoshate shunt activity ([175] including glucose-6-phosphate dehydrogenase (G6PD), it also utilizes the host reducing powers as well. This is supported by the natural selection for G6PD deficiency in malaria-endemic areas.

Endoperoxides are believed to act via inducing oxidative stress. Evidence for this includes their synergism with other oxidant drugs and oxygen and a reduction in activity produced by agents that lower oxidative stress [182]. The specific nature of this oxidative stress is not fully elucidated although a role for iron de-sequestration is probably involved since the drugs are strongly antagonized in vitro by iron chelators [183, 184]. These drugs bind tightly with haem and there is a correlation between antimalarial activity and strength of haem binding of various artemisinin derivatives [185]. It has been hypothesized that haem binding results in drug activation to free radical intermediates that alkylate essential parasite proteins, leading to loss of function [184, 186, 187]. The chemical mechanism of activation of the artemisinins and other trioxanes, is proposed to occur with the help of ferrous ion, initially giving rise to an oxygen-centred and then a carbon-centred radical, followed by an Fe(IV)=O species, and finally an epoxide, which is an highly active alkylating agent [188].

1.2.2. Glycolysis

P.falciparum lacks a functional tricarboxylic acid cycle and is totally dependent on glycolysis for energy generation. [189, 190]. A compound that selectively inhibits

the parasite's ATP- generating machinery would therefore be a potential antimalarial agent. The parasites increase glucose consumption 50-100 fold as compared to uninfected erythrocytes. Contrary to mammalian cells and most aerobic organisms, pyruvate does not enter the citric acid cycle in *Plasmodia*, most of the glucose is metabolized to lactic acid, the end product of the glycolytic pathway. The parasite contains the complete set of glycolytic enzymes of the Embden-Meyerhoff pathway [189]. Some enzymes such a hexokinase, enolase and pyruvate kinase are vastly increased over corresponding levels in uninfected red cells.[189] Glycolytic enzymes are believed to associate with membrane components facilitating channeling of substrate during triosephosphate metabolism. Potential chemotherapeutic targets in the glycolytic pathway may involve: interactions causing inhibition of the activity of an enzyme, or perturbation of the micro-organization of consecutive enzymes in the metabolic pathway. Below are some of the glycolytic enzymes from the malaria parasite *P. falciparum* that may be of potential use as drug targets.

Hexokinase:

This is the first enzyme in the pathway which converts glucose to glucose -6-phosphate. It bears 25% identity with that of the human enzyme and there is a 25 fold increase of hexokinase activity following infection of the erythrocyte by the malaria parasite [191]. *P.falciparum* activity has a lower affinity for glucose as compared to that of the human enzyme [189]. In addition to supplying glucose-6-phosphate for glycolysis, hexokinase also plays a role in reduction of glutathione

by the hexose monophosphate shunt. The hexose monophosphate shunt (HMS) produces NADPH for reductive antioxidant protection and for metabolic regulation, as well as ribose- 5-phosphate needed for the synthesis of nucleic acids. The parasite hexokinase is structurally different from the human enzyme, with a large stretch of hydrophobic residues at the C-terminus missing. This is an important feature given the fact that glycolytic enzymes of parasites are known to be membrane bound [191].

Aldolase:

This enzyme converts fructose-1-6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate and is the third enzyme in the glycolytic pathway. It is potentially important for drug design as well as for vaccine development [191]. It has been reported to elicit an immune response in monkeys and the antibody to the enzyme offers partial protection [192]. Despite this the enzyme shows high sequence identity with that of the human enzyme [193-195] with an exception at the C-terminus where there is low sequence identity this may be a potentially useful feature for drug design strategies [196, 197].

Lactate dehydrogenase (LDH):

LDH the terminal enzyme of anerobic Embden-Meyerhoff glycolysis, plays an important role in the carbohydrate metabolism of human malaria parasites; LDH catalyzes the reduction of pyruvate by NADH to lactate. Gylcolysis is an essential

source of energy for *Plasmodium*, and inhibition of LDH halts NAD regeneration. Consequently, the parasite has evolved to avoid inhibition by high levels of pyruvate or lactate and so, unlike the human enzyme, there is no substrate inhibition by pyruvate. LDH has been expressed and has been used for the detection of the malaria parasite [198]. *Plasmodial* LDH differs from its human counterpart by the presence of a 5 amino acid insertion at the pyruvate binding site. The crystal structure of *P.falciparum* LDH shows a significant displacement in the position of the NADH cofactor compared with other LDH structures [199] and may explain the lack of inhibition by pyruvate of the malaria enzyme. A distinct cleft has been reported adjacent to the active site that could be a good target for bulky inhibitors like gossypol derivatives.

***Triosephoshate isomerase* (TPI):**

This dimeric glycolytic enzyme catalyzes the isomerisation of D-glyceraldehyde-3-phosphate to dihydroxyacetone phosphate. *P.falciparum*. TPI has highly conserved sequences and active site residues making it hard to design inhibitors specific for the parasite enzyme. This is unlike TIM from the parasite *Schistosoma mansoni* which was shown to be a surface antigen and has been considered for vaccine development [199].

ARM has also been reported to induce glycogen reduction in schistosomes related to an inhibition of glycolysis rather than an interference with glucose uptake [338], the effect on *Plasmodium* is yet to be investigated.

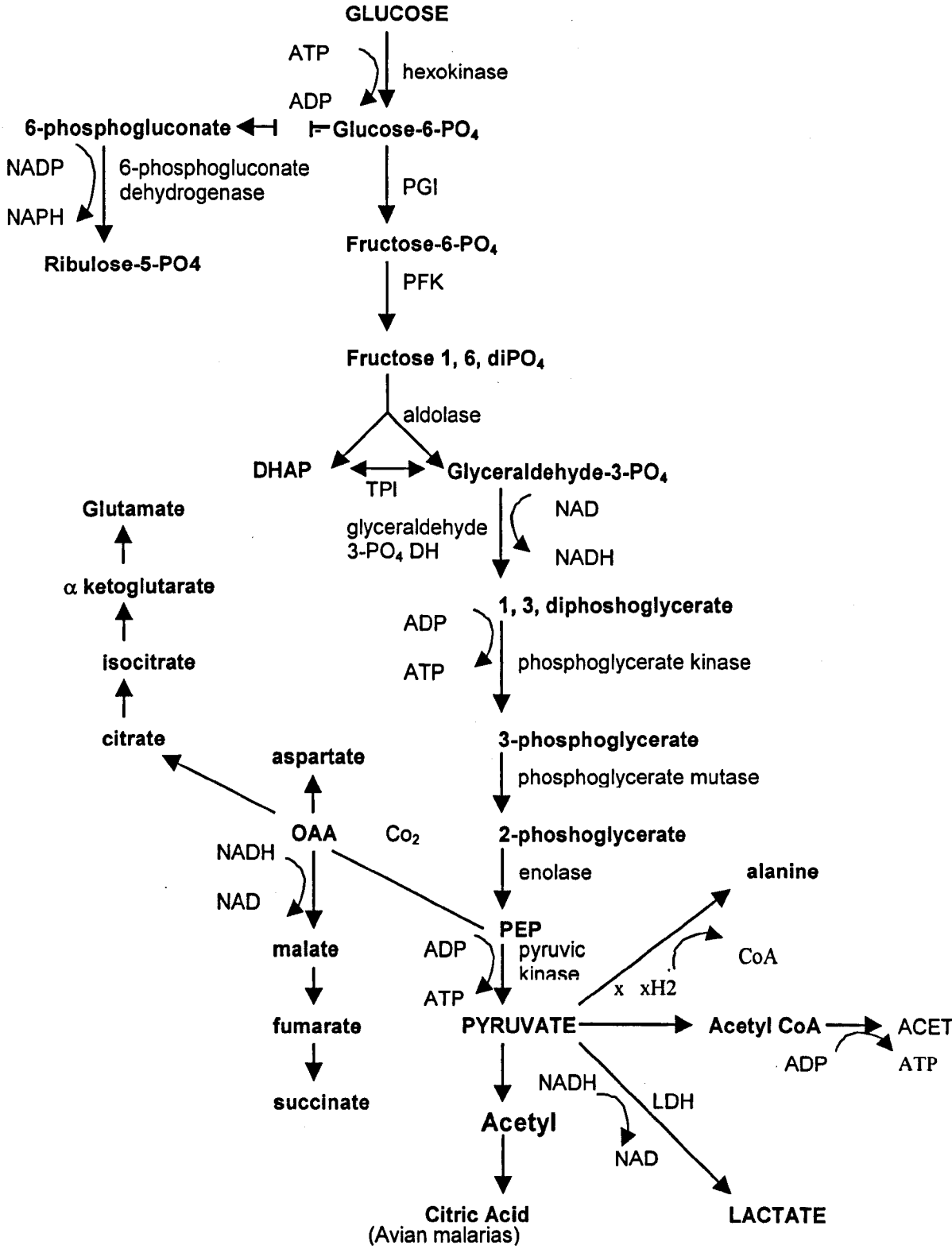


Figure.1.4. ***Proposed pathway of carbohydrate metabolism in Plasmodium. ADP, adenosine diphosphate, PGI, glucose phosphate isomerase; PFK, phosphofructokinase; DHAH, dihydroxyacetone phosphate; TPI, triose phosphate isomerase; DH, dehydrogenase, PEP, phosphoenolpyruvate; LDH, lactic dehydrogenase; OAA, oxaloacetate*** **[190].**

1.4.3. Nucleic Acid Metabolism

The synthesis of nucleic acids by malaria parasites necessitates the availability of purines and pyrimidines (the constituents of the nucleotides) as well as an adequate supply of ribose sugars and phosphate. The biosynthesis of purines, pyrimidines, and folates and mitochondrial transport are intimately linked. **Table 1.3** below summarizes the major differences that exist between *Plasmodial* and the corresponding human pathways.

Intraerythrocytic-stage *Plasmodia* are incapable of *de novo* purine synthesis unlike mammalian cells [190], and therefore depend on preformed host purine precursors. On the other hand, they are unable to salvage either pyrimidine bases or nucleosides and have to synthesize pyrimidine nucleotides *de novo* [190], whilst mammalian cells can either salvage or synthesize *de novo* pyrimidine nucleotides. The parasite depends on *de novo* synthesis of folate cofactors, although exogenous folates can be salvaged [138, 199]. Differences in the *Plasmodial* purine pathway, pyrimidine metabolism and electron transport, together with folate pathway in contrast with the analogous processes in the host, provide exploitable potential targets for antimalarial chemotherapeutic attack **(refer to figure 1.5)**.

Pathway	Plasmodium	Mammal
Pyrimidines	Synthesizes pyrimidines *de novo*; cannot salvage bases/ nucleotides	Can either synthesize or salvage pyrimidine nucleotides
Purines	No *de novo* synthesis; depends on host-derived hypoxanthine as source of purine precursors	Can either synthesize or salvage purine nucleotides-hypoxanthine waste product
Folate cofactors	Can either synthesize or salvage folate precursors	No *de novo* synthesis; rely on external sources

Table 3. Metabolism of nucleic Acids in Humans and *Plasmodia* [138].

The reduction of ribonucleotides to deoxyribonucleotides, the precursors of DNA, is catalyzed by ribonucleotide reductase (RNRase) and this is a rate-limiting reaction in DNA replication and repair. RNRase is a cell-cycle-regulated, iron requiring allosteric heterodimeric enzyme. It is likely to be a target of iron chelation therapy [200, 201]: deprivation of iron from the enzyme by iron chelators may result into inhibition of DNA synthesis and parasite death. It has been shown that antisense oligonucleotide directed against RNRase displayed selective inhibition of *P.falciparum* [202], and this supports the potential of this enzyme as a target for antimalarials.

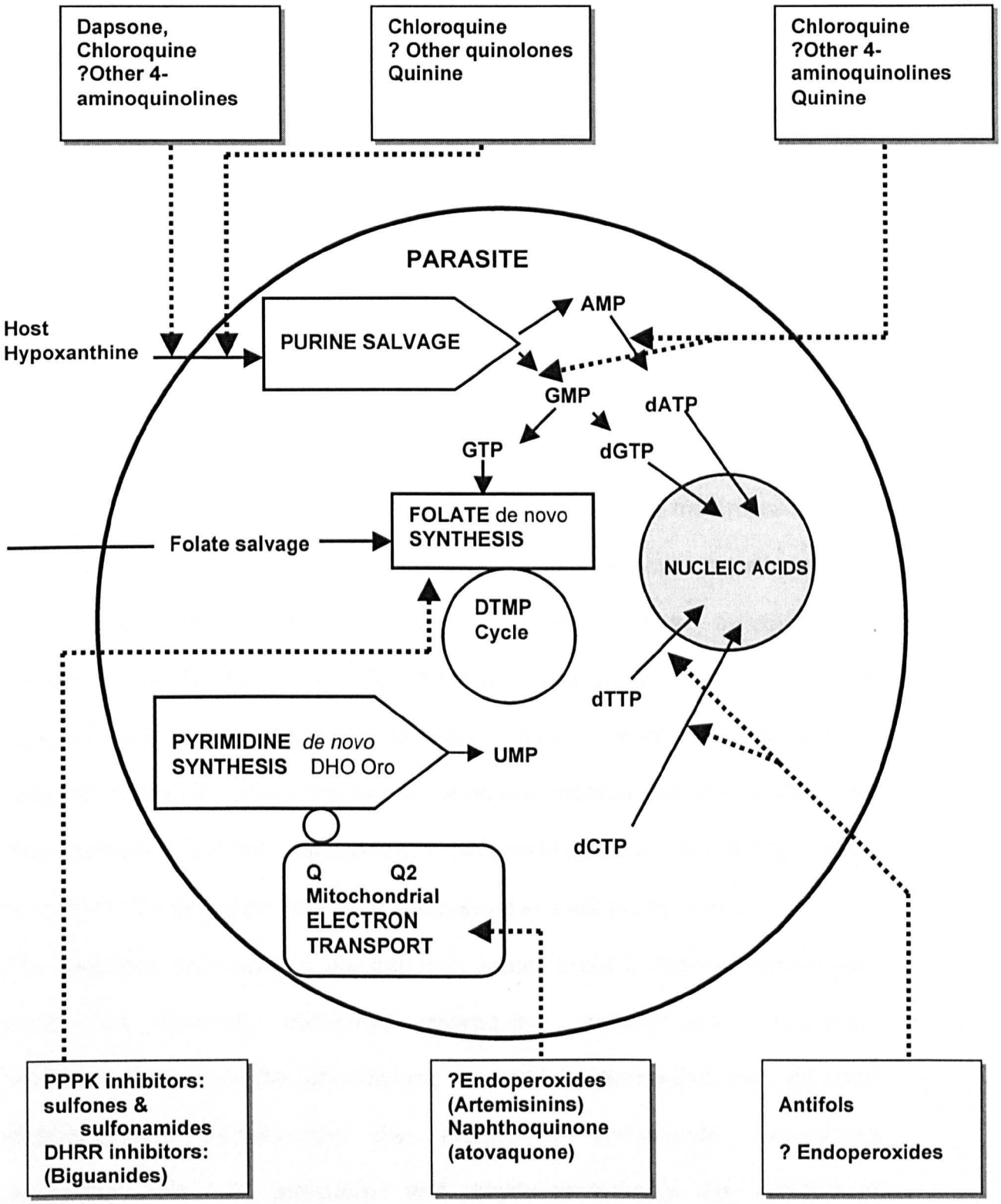


Fig. 1.5 ***Possible points of attack of antimalarials drugs on Plasmodial synthesis of nucleic acids.***

Mammalian cells have been reported to sustain reversible inhibition with iron chelators, cells recovering after removal of drug pressure, whereas parasites appear to have limited capacity to recover from iron chelator-induced iron depletion [200]. Proteins responsible for membrane processes, including trafficking, drug transport and signaling are potentially important and require careful investigations to rule out their involvement in endoperoxide drug action.

1.3. CHEMOTHERAPY FOR *FALCIPARUM* MALARIA

The available non-chemotherapeutic approaches currently in operation in some of the endemic areas can only achieve a reduction in disease transmission rather than eradication of malaria. Since presently there is no effective malaria vaccine, malaria chemotherapy remains the mainstay of malaria control by clearing the parasites from the host, depending on drug sensitivity of the area involved. Fundamentally the treatment of *falciparum malaria* involves killing asexual parasites in the circulation (the sexual forms are important for the spread of the drug resistance, but not pathogenic), while providing supportive therapy to the host [203]. The killing of liver forms is important in malaria prevention.

The available antimalarials fall into five broad groups: the quinoline-related compounds (quinine, quinidine, chloroquine, amodiaquine, mefloquine, halofantrine, lumefantrine, primaquine); the antifols (pyrimethamine, proguanil, chlorproguanil, trimethoprim; the artemisinin compounds (artemisinin, artemether, arteether, artesunate and dihydroartemisinin); the tetracyclines (doxycylcine and tetracycline); the napthoquinone (atovaquone). Of the available

antimalarial agents, the artemisinin drugs have the broadest time window of action on the asexual malarial parasites, from medium sized rings to early schizonts, and they also produce the most rapid therapeutic responses [147, 204].

Several antibacterial drugs including sulphonamides and sulphones, the tetracyclines and macrolides, also have antiplasmodial activity, although in general their action is slow and they are used in combination with other antimalarial drugs. The tetracyclines are particularly useful in that they retain activity against multidrug resistant parasites [205, 206].

1.3.1. Quinoline antimalarials

1.3.1.1. Cinchona Alkaloids

Quinine

The introduction of cinchona into Europe, ranks not only as one of the greatest events in the history of medicine but as one of the great factors in the civilisation of the world. (Sir William Osler, regius professor at Oxford, 1892) [19]

The first quinoline antimalarial drugs were alkaloids extracted from the cinchona tree, which was introduced from South America into Europe by the Jesuits. Recognition of the therapeutic value of the 'fever bark tree', cinchona dates back to the 17th century well before the parasitic nature of malaria was understood. The active components contained in the bark include quinine, its diastereomer

quinidine, and the desmethoxy diastereomers cinchonidine and cinchonine [207, 208].

Quinine (QN) and quinidine continue to be important in the therapy of malaria either alone or in combination with antibiotic antimalarials [209-213]. Quinidine is a more potent antimalarial than QN, but has higher cardiotoxicity (concentration-dependent changes in the ECG, systemic blood pressure, and heart rate) [213, 214].

The mode of action of QN is not fully elucidated. QN is however, known to accumulate in the acid food vacuoles of malarial parasites and may inhibit the parasite haem polymerisation process [161] Structure-activity studies suggest that the conformation around the atoms C-8 and C-9 of the cinchona alkaloids, particularly the direction of aliphatic N-H and (9C) O-H bonds relative to each other, are crucial to antimalarial activity [207]. QN has relatively low potency and a narrow therapeutic range. It is however the drug of choice for severe malaria because of its reliability (in the setting of chloroquine resistance) rather than its potency. Indeed, there appears to be an inverse relationship between chloroquine resistance and QN and mefloquine resistance in *Plasmodium falciparum* [215].

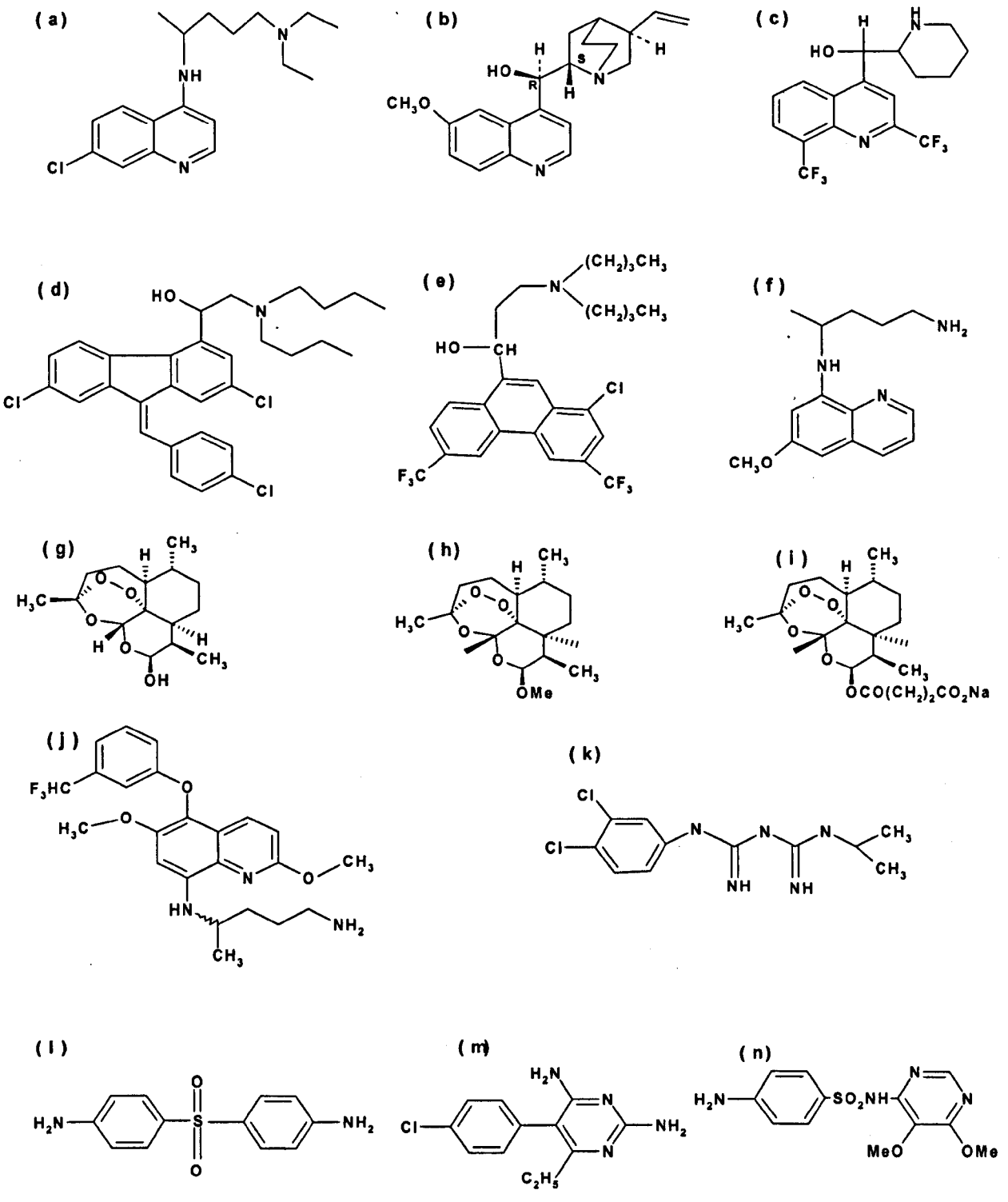


Figure.1.6. The chemical structures of drugs in widespread use for *falciparum* malaria are shown here: chloroquine (a); quinine (b); mefloquine (c); lumefantrine (d), halofantrine (e); premaquine (f); dihydroartemisinin (g); artemether (h);artesunate (i); terfenoquine (j); chlorproguanil (k); dapsone (l); pyrimethamine (m); sulphadoxine (n).

In addition, the lag between introduction of the drug and onset of effects is longer for QN than for chloroquine. Clinically, in comparison with artemisinin derivatives (such as artemether), the effect of QN upon the rate of parasite clearance is slow [216], while rates of clearance achieved by pyrimethamine-sulphadoxine (Fansidar) are comparable to those achieved with QN [213].

In vitro, the effects of QN on nutrient importation by *Plasmodium falciparum* are confined to the late ring and trophozoite stages, comparable with chloroquine *[204]*. Quinine does not prevent sequestration or further development of formed meronts and does not kill the preerythrocytic or sexual stages of *P.falciparum*. QN is gametocytocidal against *P.vivax*, *P. ovale*, and *P. malariae* [217-220]. QN's pharmacokinetic and adverse effect profiles make it a poor choice for prophylaxis.

1.3.1.2. 8-Aminoquinolines

The 8-aminoquinolines were the first drugs to be synthesised for use against malaria. Currently, the 8-aminoquinoline primaquine [221] introduced in 1950 as an antimalarial, is the only available drug for treatment of exoerythrocytic stages of *Plasmodium vivax* and *P.ovale* although a congener, tafenoquine, is being developed. Primaquine [221] is normally not used in treatment of malaria but may be used for terminal prophylaxis after leaving a malarious area (only for selected group of travellers) and as a gametocytocidal drug in *P.falciparum* infections.

1.3.1.3. 4-Aminoquinolines

The 4 Aminoquinoline chloroquine (CQ) has been in clinical use since 1943 [222] and has been the main-stay of *falciparum malaria* chemotherapy; with the advantages of being cheap, safe and practicable for outpatient use [203, 223]. CQ is effective against the erythrocytic stages of all four *Plasmodium* species which cause human malaria with the exception of mature *Plasmodium falciparum* gametocytes. Regrettably, since its heyday in the 1950s and 1960s, the efficacy of CQ has been declining and over the last 3 decades CQ resistance has reached all regions of the world where CQ is used [33, 224-227]. The once universally sensitive and relatively benign parasite, P. vivax, has also emerged as resistant to CQ in some areas [34-36, 38, 39, 99, 228-231]. CQ may no longer be the most important antimalarial in the world [232]. CQ acts mainly on the large ring-form and mature trophozoite stages of the parasite. It is more rapidly acting than quinine. CQ is also indicated in the treatment of hepatic amoebiasis, and for some collagen vascular diseases like rheumatoid arthritis.

Amodiaquine is another clinically useful 4-aminoquinoline with a similar mode of action to CQ. A systematic review of amodiaquine treatment in uncomplicated malaria [233] collated convincing evidence that amodiaquine is superior to chloroquine especially in areas with considerable chloroquine resistance has been undertaken. Nevertheless, its clinical utility may be curtailed by partial amodiaquine cross resistance with chloroquine. Amodiaquine treatment is reported to be safer than amodiaquine prophylaxis. Although amodiaquine is more toxic than chloroquine, it has also suggested to be a less toxic alternative to

pyrimethamine/sulphadoxine (PSD) in HIV infected patients in sub-Saharan Africa [233, 234].

1.3.1.4. Synthetic quinoline methanols.

The quinoline methanols, which are structural analogs of quinine [235, 236], were the most promising group of compounds following the Walter reed screening programme in 1963. Although the first compounds examined displayed a strong photosensitising action, mefloquine (a prototype of this group) developed later has no appreciable photosensitivity action. Mefloquine has proven to be effective in antimalarial chemotherapy in the field over the last approximately 20 yrs, especially against chloroquine-resistant strains of malaria parasites [58, 237]. Mefloquine has efficacy against the late trophozoite stage of all species of human malaria but, akin to most antimalarial drugs other than the artemisinins, has little activity against circulating ('ring' forms) of *Plasmodium falciparum.*

The mode of action of mefloquine is unknown, but may be similar to quinine due to the structural similarity. Mefloquine also interacts specifically with erythrocyte membrane band 7.2b protein (stomatin) and (in parasitized erythrocytes) with two further high-affinity proteins of molecular mass 22 and 36 KDa [238]. The role of these specific mefloquine-binding proteins in mefloquine's antimalarial action is still unclear.

Mefloquine is only available as an oral preparation and therefore only used for treatment of uncomplicated malaria especially in areas with multidrug resistance like Thailand. Unfortunately, in recent years, resistance to mefloquine in parts of

Southeast Asia (particularly the Thai borders with Burma and Cambodia) [239] has led to its combination with artemisinin derivatives; treatment failure is less likely after combination treatment than with mefloquine monotherapy [79, 240-245]. Mefloquine has a very long plasma half-life (15-33 days and a mean of 21.4 days) and this makes it a practicable drug for chemoprophylaxis [61, 246-254]. Unfortunately, mefloquine is a non-competitive inhibitor of acetylcholinesterase and butyrylcholinesterase, which is thought to account for some of the gastro-intestinal (e.g. nausea and vomiting) and central nervous system toxicities (e.g. hallucinations and disorientation) seen at relatively high drug concentrations. Mefloquine can also cause serious idiosyncratic adverse reactions [255-258] but these are rare.

1.3.1.5. Phenanthrene methanols

This is another important class of compounds that resulted from the Walter Reed screening programme in which the quinoline portion of the 4-quinoline methanols was replaced by a different aromatic ring system to form the aryl amino alcohols e.g. halofantrine and novel lumefantrine (formerly benflumetol).

Halofantrine (HF) is effective against the erythrocytic stages of all four human *Plasmo*dial species. Both *in-vitro* and *in-vivo* studies suggest that it has activity against chloroquine-resistant parasites and has similar antimalarial potency to mefloquine [259, 260], the principal metabolite of HF, also exhibits equivalent antimalarial activity [261, 262]. The mechanism of action HF is not yet well understood. Its actions are stage specific showing greatest activity against

mature trophozoite and schizont stages. Like quinine and MQ, HF evokes similar ultra-structural changes in mouse erythrocytes infected with *P.berghei*, although HF also induces mitochondrial damage [263].

HF like MQ is an expensive oral antimalarial without a parenteral formulation, for uncomplicated malaria. Pharmacokinetic studies have revealed extensive intra- and inter-subject variability in drug absorption. Drug bio-availability is improved if taken after a fatty meal. HF resistance is worsening in parts of Thailand requiring higher doses to achieve a cure. Further more, HF and MQ cross resistance has been documented [51, 264-267]. HF is not recommended for chemo-prophylaxis, but has been used for self presumptive treatment; the risk of cardio-toxicity [QT prolongation and arrhythmias], especially associated with (+) -isomer has led to a reappraisal of its therapeutic role [243, 268-270].

Lumefantrine

Lumefantrine (LMF) is a synthetic racemic fluorene derivative with the chemical name 2 dibutylamino-1-[2,7-dichloro-9-(4-chlorobenzylidene)-9H-fluoren-4-yl]-ethanol ($C_{30}H_{32}Cl_3NO$) (refer to fig.1.3). LMF conforms structurally, physico-chemically and in terms of antimalarial activity with the class II aryl amino alcohol blood schizontocides including quinine, mefloquine, and halofantrine [271]. It is highly lipophilic and is almost insoluble in water and aqueous acids. The *dextro-* and *laevo-* rotatory enantiomers resulting from the dibutylamino-1-ethanol substitution at position 4, and the racemate of LMF display very similar

antimalarial activity *in-vitro*, and therefore no specific enantiomer is selected for clinical use [272].

Recent *in-vitro* work has shown that desbutyl-LMF a putative metabolite of LMF, has highly significant potency and is also being evaluated as an antimalarial [273]. LMF is highly lipophilic. The pharmacokinetic properties of LMF are reminiscent of those of halofantrine, with variable bioavailability in malaria patients [274]. LMF oral bioavailability is very dependent on food (considerably improved when administered after a fatty meal) and is consequently poor in acute malaria but improves markedly with recovery.[274-276]. ARM and LMF are synergistic *in vitro* against *P.falciparum* [277], and the two drugs have complementary pharmacokinetic and pharmacodynamic properties (the main rationale for developing fixed dose combination of co-artemether – see below) [278].

The detailed mechanism of action of LMF is not known, but like the 4-aminoquinolines and the quinoline methanols the drug accumulates within in the parasite possibly within the parasites digestive vacuole.[141, 168, 169, 279-281].

1.3.1.6. A unifying hypothesis of quinoline action:

The quinoline antimalarials namely: the 4 aminoquinolines chloroquine (CQ) and amodiaquine (AQ), the quinoline methanols quinine (QN) and mefloquine (MQ), and the phenanthrene methanol halofantrine (HF) and possibly lumefantrine (LMF) put forth selective toxicity towards the erythrocytic stages of malaria parasites and their development was based on the knowledge of quinine

structure and activity.[282]. There is compelling evidence supporting the central role of a haem dependent mechanism in the action of the 4-aminoquinolines and the quinoline and phenanthrene methanol antimalarials [283].

Studies in our research group [283-287] have shown that the selective antimalarial activity of CQ and related compounds stems from the extensive saturable uptake of these drugs into malaria parasites. It has been demonstrated that CQ uptake in *Plasmodium falciparum* is determined by binding to ferriprotoporphyrin IX (FPIX) [285-287]. Specific proteinase inhibitors that block the degradation of hemoglobin and have been shown to stop the generation of FPIX also inhibit CQ uptake. CQ resistance is characterized by a reduced affinity that is reversible by verapamil. The affinity of CQ binding to ferriprotoporphyrin IX (CQ-FPIX binding) had earlier been shown to be reduced in resistant parasites. The mechanism responsible for reduced binding affinity was overcome by verapamil and various lysosomotropic agents, and was thought to be the basis of CQ resistance [288].

Recent in-vitro and in-vivo studies have associated CQ resistance with point mutations in the gene *pfcrt* [16, 229, 289-294]. This transmembrane protein contains 10 putative transmembrane domains and localizes to the parasite digestive vacuole (DV), the site of CQ action, where increased compartment acidification is reported to associate with PfCRT point mutations. Mutations in PfCRT may result in altered CQ flux or reduced drug binding to hematin through an effect on DV pH [289]. Point mutations in PfCRT have also been associated

with increased susceptibility to quinine, mefloquine, and artemisinin and its metabolite DHA.

Polymorphisms in *Pfmdr1*, which encodes the P-glycoprotein homolog Pgh-1 of Plasmodium falciparum that localises to the digestive vacuole [295, 296] have been linked to CQ resistance. Pgh1 has also been implicated in resistance to mefloquine and halofantrine [297].

1.3.2. Antifols

These include pyrimethamine and biguanides inhibitors of dihydrofolate reductase enzyme (DHFR). Pyrimethamine is used most widely in synergistic combination with inhibitors of dihydropteroate synthetase (DHPS) (sulfonamides and sulfones), an 'earlier' enzyme in the parasites folate pathway.

The biguanides; proguanil and chloroproguanil are considered the safest of all antimalarials. These are prodrugs requiring CYP P450 dependent cyclisation to their active metabolites cycloguanil and chlorcycloguanil respectively.

The DHFR inhibitors inhibit development of mature trophozoite/schizont stage of the asexual parasite, in addition to having pre-erythrocytic and sporonticidal activities. Pyrimethamine is also used as a chemotherapeutic agent for toxoplasmosis.

The conversion of 2′-deoxyribosyluracil monophosphate to 2′-deoxyribosylthymine monophosphate is essential for normal DNA synthesis in mammals and protozoa, and this is achieved by donation of a methyl group by methylene-tetrahydrofolate (mTHF) and is catalysed by thymidylate synthase.

Dihydrofolate, the oxidized product, must be reduced back to replenish the 'pool' of mTHF: the rate-limiting step of this process is catalysed by DHFR. The antifols compete with dihydrofolate for DHFR by binding to the active site with higher affinity. Several researchers have reported spontaneous mutations of *Plasmodium falciparum* dhfr gene occurring [45, 298-305] and proliferation of the mutants if they have an advantage [306], usually under drug pressure. Resistance to pyrimethamine has been associated with point mutations in dhfr at positions 108, 51 and 59 in Africa the incidence is on the increase), whereas parasites from Southeast Asia often also have mutation at position 164. Pyrimethamine-sulphadoxine (PSD) is usually deployed as a successor to chloroquine [307]. PSD is also cheap and practicable in an out patient setting (only a single dose is required because of slow elimination from the body) and is highly efficacious in much of Africa and South America [308]. Unfortunately, pyrimethamine combinations are prone to rapid emergence of resistance when introduced for mass use. Resistance to sulphadoxine has also been demonstrated and this is attributed to point mutations of dhps gene [309]. Within the DHPS, polymorphisms in the codons 436, 437, 581, 540 and 613 have been reported to cause sulphadoxine resistance, but no single polymorphism has been associated with resistance to sulphadoxine/pyrimethamine and only limited data are available on the global distribution of these polymorphisms [12, 302, 310-312]., which are associated with *in vivo* resistance.

1.3.3. Artemisinin and its derivatives.

"The artemisinins are a fascinating and unique family of compounds discovered, or rediscovered...by the Chinese approximately 30 years ago, a sesquiterpinelactone peroxide with the business end of the molecule being the peroxide bridge and substitutions giving us the different derivatives'.

Professor Nick White, 2002 [2].

1.3.3.1. Historical review of the artemisinins.

The Chinese medicinal plant Quinghao (Artemisia annua L, annual worm-wood, sweet worm-wood) has been in traditional Chinese medicine for over 2000 years [313]. The earliest reference to the use of Quinghao goes back to "52 prescriptions or recipes for 52 Kinds of diseases", found in the Mawangudi Tomb in an era dating back to 206 BC-AD 23. The first description of Quinghao as an antimalarial agent is in *Zhouhou Bei Ji Fang* (Handbook of Prescriptions for Emergency Treatments) by Ge Hong (AD 281-340) [313, 314]. As part of a collaborative program of screening traditional herbal remedies, the active moiety of qinghaosu (extract of quinghao), was isolated from the leaves and flowering tops of the herb in the early 1970s [315]. Pharmacological studies showed that it possessed good *in vitro* antimalarial activity and subsequent studies in animal models proved encouraging, but recrudescence was observed in primates [314]. Early clinical trials, conducted in China in the 1970s, confirmed qinghaosu (QHS) to be an exceptional antimalarial agent with low toxicity and high efficacy against human malaria parasites, including those resistant to conventional malaria treatment [316], and the WHO has supported research into qinghaosu since

1979. In 1982 *The China Cooperative Research group* published an important series of reports on qinghaosu and its derivatives as antimalarials detailing a range of studies on the chemistry & synthesis, efficacy, metabolism and toxicity of QHS and several semi-synthetic derivatives [317-322]. The semi-synthetic derivatives of QHS (**refer to figure 1.7**) were developed in an attempt to produce compounds with greater potency than the parent drug. Initial studies proved that the peroxide group was essential for antimalarial activity [320, 321]. The first stage of the synthetic process was reduction of QHS with sodium borohydride to produce dihydroartemisinin (DHA), which was found to be 4-8 times more potent than QHS [320]. Derivatives that were synthesized and introduced into clinical practice included artesunate (ARTS), a hemisuccinate ester of DHA that was regarded as potentially useful because the water soluble sodium salt could be given by the intravenous route, and artemether (ARM), a lipid-soluble compound found to be 2-5 times more potent than DHA [321].

The artemisinin derivatives represent an exciting breakthrough in the treatment of malaria. They are being used extensively in South- East Asia and increasingly in Africa. Currently more than two million people have received antimalarial treatment with artemisinin, artesunate, or artemether [323].

1.3.3.2. Structure, chemical characteristics and formulations.

Artemisinin compounds are structurally distinct from all other classes of antimalarials, consisting of a sesquiterpene lactone ring with a unique endoperoxide bridge essential for their antimalarial action **(1.7)** [188, 324].

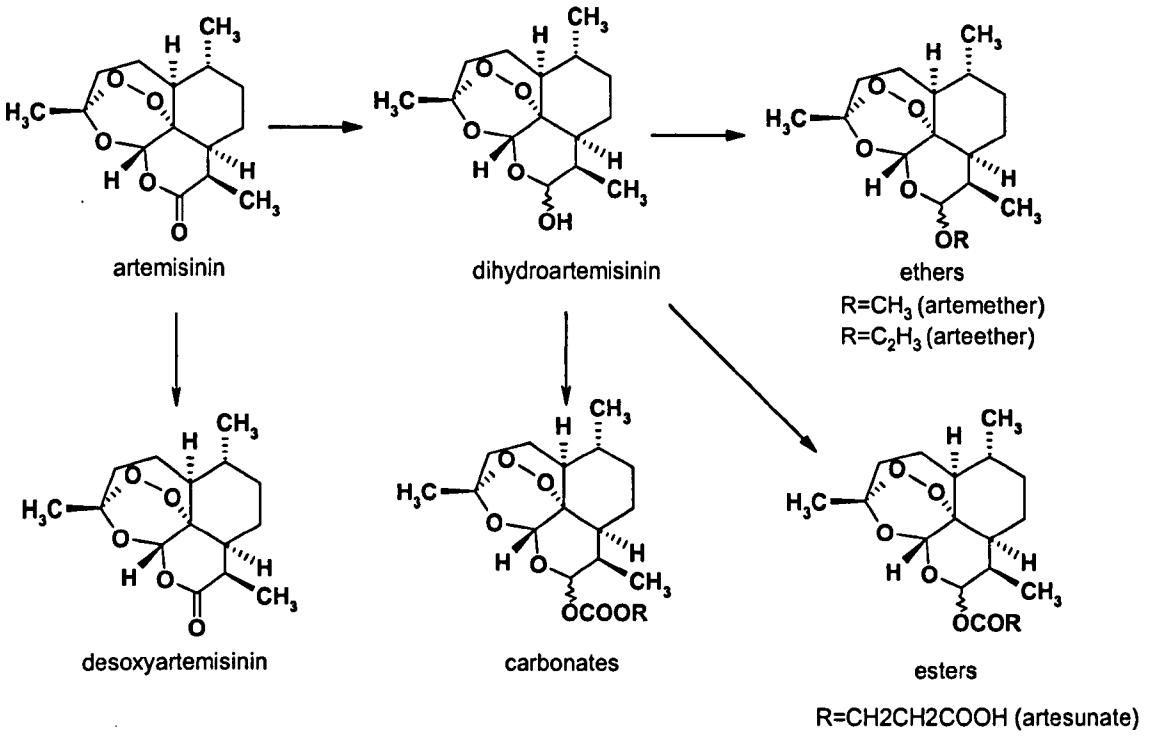


Figure 1.7. artemisinin derivatives.

ARM is a semi-synthetic methyl ether derivative of artemisinin, a natural 1,2,4-trioxane. It occurs as an odourless, white crystalline powder, with molecular weight of 298.38, solubility in water of 128mg/L and in ethanol (96%) about 10% at 20^{o} C, and it is chemical stable below 25^{o}C for more than 2 years. ARM is available in ampoules for intra-muscular injection in groundnut oil or as capsules and tablets for oral administration [316]. The other artemisinins, artesunate (water soluble hemisuccinate) is available in oral, parenteral and suppository formulations; and arteether (lipophilic ester like ARM) is available as an im formulation. Both the lipophilic and hydrophilic derivatives are converted to dihydroartemisinin (DHA), the active metabolite. DHA per se is available in an oral preparation.

1.3.3.3. Proposed mode of action

ARM is a semi-synthetic sesquiterpene lactone, methyl ether derivative of QHS. Like other artemisinins, its structure includes a unique endoperoxide bridge (c-o-o-c), the essential pharmacophore for its parasiticidal effect [187, 325, 326]. The precise mechanism(s) of action of this class of antimalarials is still not completely understood, although different theories have been proposed.

ARM exhibits selective toxicity [327, 328] by the enhanced uptake of the drug into the parasite. ARM like other artemisinin drugs has a high lipid solubility, and readily crosses both erythrocyte and parasite membranes [327-331]. *Plasmodium falciparum* infected erythrocytes have been shown to take up and concentrate [^{3}H] dihydroartemisinin better than the uninfected erythrocytes [332]. The uptake is concentration dependent, being greater than 300-fold at 3 μg/L and less than 25-fold at 300 μg/L. Furthermore, the incorporation of QHS is 5-fold higher in intact parasites than in isolated haemozoin [188]. Artemisinin compounds bind selectively to malaria-infected erythrocytes to as yet largely unidentified targets. There is strong evidence that the artemisinin drugs gain access to and are concentrated in the parasites digestive vacuole and possibly the mitochondria of the malaria parasite [330, 333-336]. The accumulation mechanisms are not well characterized, but may involve an inherent capacity of the drug to cross both cell and parasite membranes.[337, 338].

An alternative explanation is based on an interaction with intravacuolar haem [146, 281, 286, 287, 339], leading to irreversible accumulation of the drugs in the parasite vacuole. Once inside the digestive vacuole, an interaction of the trioxane

pharmacophore of artemisinins with the intra-parasitic ferrous haem (ferroprotoporphyrin IX, Fe(II)PPIX), or exogenous, or non-haem iron(II) serving as the transition metal ion [340]. This interaction results in the reductive scission/cleavage of the endoperoxide bridge invoving the transfer of an electron from the transition metal ion and the formation of a Fe[IV]=O species [341]. The endoperoxide bridge is cleaved first; this is followed by intramolecular electronic rearrangements which results in generation of highly reactive carbon-centred radicals [342]. This is followed by the second step of alkylation, which involves the formation of covalent adducts between the drug and both parasite and erythrocyte proteins (biomolecules) [183, 184, 187, 343-345]. The specific target proteins that are alkylated and result in death of parasite have yet to be identified. The C-centred radicals can alkylate haem itself although the biological significance of this is debatable. There is however a correlation between antimalarial activity against *Plasmodium falciparum in-vitro,* of a number of artemisinin derivatives with their affinity of binding with ferroprotoporphyrin IX [346]. The correlation points to the biological significance of the interaction of these derivatives with ferroprotoporphyrin IX as a potential target for this class of antimalarials. The antimalarial activity and presumably the interaction between ferroprotoporphyrin IX and artemisinin is inhibited by antioxidants (free-radical scavengers) such as α-tocopherol, catalase, dithiothreitol, ascorbate and reduced glutathione [182-184, 187], while free-radical generating compounds such as doxorubicin, miconazole, canestin and artemitin, enhance the activity *in-*

vitro [184]. This evidence supports the argument that the killing of the parasites is mediated by free radicals.

It has been observed that a trioxane able to generate a C-centred radical, but without alkylating ability toward a haem model, was devoid of of toxicity against *Plasmodium* [347]. This may imply that either haem alkylation is biologicaly important or the ability to generate C-centred radicals is not essential for antimalarial activity [342]. Another possibility is that radicals might alkylate other target proteins such as transitionally controlled tumour protein (TCTP) [348], or the histidine-rich protein (HRP) [349]. Proteinases involved in the haemoglobin degradation pathway may also be potential targets of alkylation [342]. Dihydro-artemisinin, an analogue of artemisinin has also been reported to bind haemoglobin-H resulting in the ineffectiveness of the drug in malaria parasites residing in α-thalassemic erthrocytes [350, 351].

There are controversial reports over the ability of artemisinin and its derivatives to inhibit dimerization of ferric haem to produce haemozoin [164] consequently exerting their effect in an indirect manner by enhancing the build-up of toxic monomeric (ferrous) haem [352-354]. Pandey and collaegues [352] also argue that endoperoxides can also initate the breakdown of haemozoin already present in the parasite food vacuole, further aggravating the build-up of toxic monomeric haem, which may not be detoxified by the already blocked haem polymerisation pathway.

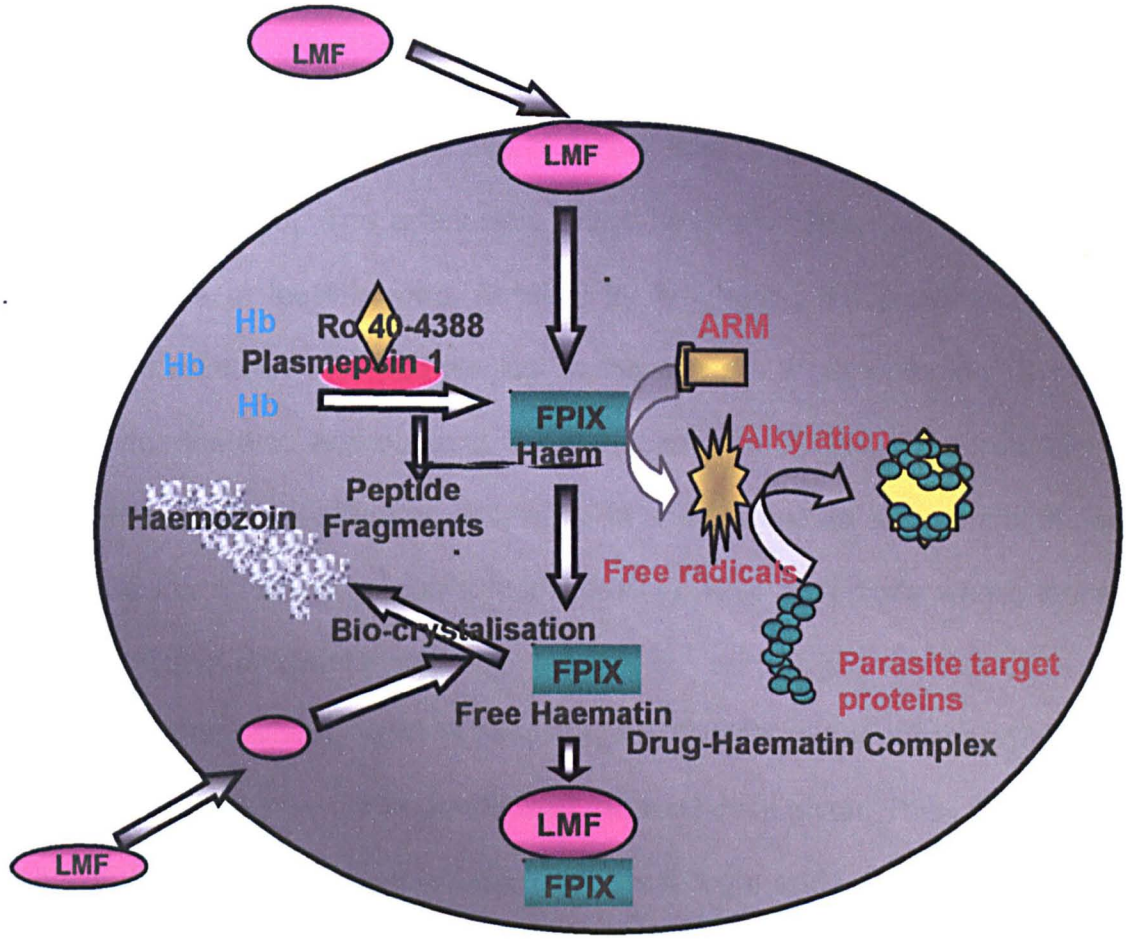


Figure.1.8. Schematic of the parasite digestive vacule showing the proposed mechanisms of action of artemether (ARM) and Lumefantrine (LMF)

1.3.3.4 Antimalarial activity and pharmacodynamics.

Artemether (ARM) and artesunate (ARTS) are the two artemisinin derivatives which are in greater clinical use and both are synthesized from the reduction product dihydroartemisinin [313, 355-357]. As described previously the artemisinin derivatives have the broadest time window of action on the asexual malarial parasites, from medium sized rings to late schizonts [204, 358], and they produce a more rapid clinical and parasitological response compared to other antimalarial drugs [359]. The activity against the later stages of the parasite

development prevents the occurrence of merogony and this eliminates or attenuates the occasional sharp rise in parasitaemia that may occur immediately after treatment [360]. The artemisinin drugs have also been shown to prevent cytoadherence at least in vitro, possibly by preventing the progression of the young and relatively less pathological to the mature, more pathological stages which cytoadhere to and obstruct the microcirculation [361]. Data from clinical trials however, demonstrate equality of ARM and quinine for severe malaria and indicate a trend toward greater effectiveness of ARM in regions where there is recognized quinine resistance [362].

Like other artemisinins, ARM is associated with high recrudescence [363-368] that varies with duration of treatment and the total dose given. This may partly be explained by the low bio-availability via the oral route and very short elimination half-lives of these compounds. However, studies with ARM combined with a long acting schizontocide like mefloquine and LMF indicate better curative effects [240, 274, 275, 360, 369-373]. There is little or no cross-resistance with other antimalarial drugs [374] and resistance to the endoperoxides has not yet developed despite the wide spread clinical use [323, 375]. Various studies have shown cross sensitivity between artemisinins and quinolines such as mefloquine and quinine, although this may not be of clinical relevance. Sidhu et al [294] recently reported that pfcrt mutations (one of the candidate genes for chloroquine resistance) are associated with increased susceptibility to artemisinin and quinine and minimally affected amodiaquine activity. Polymorphisms in pfmdr1, the gene encoding the P-glycoprotein homologue 1 (Pgh1) protein of *Plasmodium*

falciparum, have been linked to chloroquine sensitivity. Mutations in Pgh1 are also implicated in confering resistance to mefloquine, quinine and halofantrine. The same mutations have been reported to influence parasite resistance towards chloroquine in a strain-specific manner and the level of sensitivity to the structurally unrelated compound, artemisinin.

The current situation of minimal or no resistance to artemisinins is reassuring and is no cause for complacency; artemisinins ought to be used rationally. The artemisinin drugs should be used in combination with a long acting antimalarial to protect both drugs against the emergence of resistance [76, 240, 274, 376-378]. The combination of artemisinin drugs with mefloquine, and the fixed combination ARM-LMF have been studied widely, and no significant drug interactions have been found [240, 274, 379]. *In-vitro* studies involving combination of artemisinin drugs with standard antimalarials, showed that both mefloquine and tetracycline showed marked synergism with artemisinin, whilst primaquine showed potentiation. Conversely, combinations of artemisinin with pyrimethamine and with chloroquine were antagonistic against the NF54 and K1 strains of *P. falciparum* [380].

1.3.3.5. Pharmacokinetics

The artemisinin drugs currently in clinical use have proved remarkably difficult to assay in body fluids, consequently there is insufficient reliable pharmacokinetic data published [381]. The current method of choice for measuring the artemisinin drugs is high performance liquid chromatography with electrochemical detection

(HPLC-ECD). This is a difficult and time-consuming procedure although well validated [378, 382]. ARM has been the subject of two large clinical studies [383, 384] and several smaller studies, but pharmacokinetic data from patients are from two reports comprising of 25 adults with *P.falciparum* malaria [385, 386], another 26 children [387] and other studies in healthy volunteers [379, 388, 389].

Following oral administration, ARM is rapidly, but not completely absorbed, with considerable inter-individual variability in plasma concentration profiles [360]. There is extensive first pass metabolism and rapid biotransformation involving demethylation of ARM to DHA, the major active metabolite [390]. ARM has an elimination $t_{1/2}$ in the order of 2-7h, with clearance exceeding hepatic blood flow in all except a small group of patients with acute renal failure [385]. Peak plasma concentrations are variable, ranging from 668 μg/L following a 10 mg/kg i.m. dose in one group of healthy volunteers to 1,754 μg/L following a 5 mg/kg i.m. dose in another group [391]. After oral administration, the peak concentrations of ARM is achieved after 3 h and the drug is rapidly cleared from the body, elimination half life being 4-5 h [386, 391] and that of DHA (its active metabolite) is 1.9 h [391].

ARM binds highly to human plasma proteins; (33%) acid glycoprotein, 17% to albumin, 12% to high density lipoproteins (HDL), 9.3% to low density lipoproteins (LDL) and 12% to very low density lipoproteins (VLDL) [385, 392] Plasma protein binding of other artemisinin compounds range from 43% for artenimol to 81.5% for artelinic acid [378].

Most of the clinical pharmacokinetic studies have shown moderate plasma concentrations of DHA, indicating that this is the principal metabolite. The active metabolite, DHA, appears to be eliminated more slowly than the parent compound and contributes to nearly all the in vivo antimalarial activity [391].

Multiple dosing with oral artemisinin is associated with reduced plasma concentrations on day 6 compared with day 1, suggesting autoinduction of hepatic metabolism [393]. Metabolic pathways for the biotransformation of artesunate, artemether, arteether and DHA are different to those for the parent compound artemisinin. The hydrolysis of artesunate is very rapid (considered a prodrug). Artemether and arteether are metabolized predominantly by the hepatic cytochrome CYP3A. DHA is cleared by biotransformation to biologically inactive glucuronides. The induction of artemisinin metabolism, however involves principally CYP2B6, which plays a minor role in the clearance of other compounds [394].

1.3.3.6. Toxicity.

The artemisinin compounds seem remarkably free from toxic adverse effects, although neurotoxicity seen in animal studies with the lipid soluble derivatives gives some cause for concern. *In-vitro* toxicological studies have indicated that artemisinin derivatives have specific neurotoxic effects in high doses [335, 395-399]. The general toxicity profile in experimental animals however has been good, but in all mammal species tested to date, high parenteral doses of certain artemisinin derivatives (arteether and artemether) can produce a limited and

unique selective pattern of damage to certain brain stem nuclei, particularly those involved in auditory processing. The toxicity of artemisinin derivatives to neuronal cells appears to be iron dependent, since it is potentiated by haem in neuroblastoma cells *in-vitro* [344, 345, 396], and appears to involve protein alkylation [400] the proposed mechanism in the antimalarial action of artemisinin derivatives. Despite the extensive use of the peroxide antimalarials, there have been very few reports of clinically significant toxicity in humans [243, 368, 401-403].

1.4. COMBINATION THERAPY FOR MALARIA.

1.4.1. The rationale of combination therapy

The philosophy behind the combination of drugs is based on the understanding that if the parasite is attacked on several fronts by drugs with independent modes of action then it will be less likely to develop resistance to each of the constituent drugs. The principle has successfully been employed in antituberculous, anticancer, anti AIDS & early HIV-1 infection chemotherapy, and is currently being promoted for malaria chemotherapy [75-77, 404]

Resistance arises from mutations in specific gene sequences. The probability of resistance developing simultaneously to two independent mechanisms of action is extremely low, of the order of once in 10^{12} treatments depending on the drug. This frequency is the product of the probabilities of the acquisition of a resistant mutation to each drug. The incidence of a resistant parasite emerging is then the

a function of this probability and the number of parasites in a typical infection [307].

Combinations are likely to impede the development of resistance substantially as compared to sequential use of single agents. This is supported by the results of a number of studies that have examined the efficacy of drug combinations in malaria chemotherapy [76, 241, 405-411].

The investigations involving combinations of antimalarial drugs that will impede the selection of *Plasmodium falciparum* drug resistance, is currently focused on the use of a member of the artemisinin family in association with a relatively long-acting blood schizonticide such as mefloquine, pyronaridine, or lumefantrine. Artemisinins achieve a massive reduction of the parasite biomass but have a short half-life, whilst the long acting partner eliminates any survivors [376, 407, 412-415]. This is clearly demonstrated with co-artemether (ARM + LMF) fixed dose combination.

1.4.2. Data review of co-artemether (ARM / LMF)

Co-artemether (CGP 56697) is an oral fixed combination of ARM-LMF (formerly benflumetol). The two components of the combination were developed in the People's Republic of China, and the combination is currently being co-developed by Novatis, formerly Ciba-Geigy (since 1996; Ciba-Geigy, patent WO9202217) for international registration and marketing in a joint venture with the Chinese partners.

In-vitro work has demonstrated that LMF and ARM have synergistic action against *Plasmodium falciparum* [277]. The two drugs also have complementary pharmacokinetic and pharmacodynamic properties [274, 276, 278]. More importantly, the ARM component has the advantages of having a fast onset of action and very potent intrinsic antimalarial activity (substantially reducing parasite biomass), however it has a short half-life ($t_{1/2}$ = 1 – 2 h) which minimizes the period of parasite exposure to subtherapeutic blood levels. In contrast LMF which is slowly absorbed and eliminated ($t_{1/2}$ = 40 –105 h), the short half life and rapid parasite clearance time of ARM means that fewer parasites are exposed to LMF alone after elimination of ARM. Furthermore, parasite exposure occurs at maximum blood levels of the drug [415]. An added advantage of artemisinin combinations is the 90% reduction in gametocyte level in treated patients. These features minimize the probability that a resistant mutant will survive therapy and may also reduce overall malaria transmission rates [76, 244, 416-422].

1.4.3. Formulation and Dosage

ARM and LMF fixed combination tablet comprises of ARM 20 mg and LMF 120 mg (a ratio of 1:6). This formulation is known as CGP-56697, or co-artemether (Riamet®, Novartis Pharma, Basel, Switzerland). Coartemether is indicated for treatment of malaria patients (adults and children) in countries with endemic falciparum malaria. It is not for use as a prophylactic antimalarial. No liquid formulation for paediatric use or parenteral formulation of the combination is available.

Currently there are two regimens for coartemether: the 4-dose and 6-dose regimens.

Adult dosage schedule

For adults (35 kg or more)

In multi-drug resistant areas: A 6-dose regimen is recommended (24 tablets, 480 mg ARM and 2880 mg LMF, given over 3 days). Therapy is delivered over 3 days as one dose (4 tablets) is given at the time of initial diagnosis, after 8 hours, and subsequently twice daily (morning and afternoon) for 2 more days.

In semi-immune patients in areas where resistance to other antimalarials has developed, use of the 4-dose regimen (16 tablets, 320 mg ARM and 1920 mg LMF, given over 2 days) is recommended. Therapy is delivered over 2 days: one dose (4 tablets) is given at the time of initial diagnosis and then again 8, 24, and 48 hours thereafter.

Dosage schedule for small children and low-weight malaria patients

In these patients, the timing of administration of coartemether remains the same, but both the 4-dose and the 6-dose regimen require adjustment for body weight:

- 5 to <15 kg 1 tablet per dose
- 15 to <25 kg 2 tablets per dose
- 25 to <35 kg 3 tablets per dose
- 35 kgs and above, adult dose (4 tablets per dose)

The minimum body weight limit is 5 kgs for the 4-dose regimen and 10 kgs for the 6-dose regimen. The WHO in collaboration with Norvatis Pharma. is currently conducting clinical trials in Africa (Kenya, Tanzania and Nigeria) assessing the

safety and efficacy of the 6-dose regimen in children and infants down to 5 kg body weight.

1.4.4. Clinical trials

ARM-LMF combination was first registered for oral treatment of malaria in China in 1987. Since 1992 clinical trials have been conducted in China and in several other countries: Two studies were conducted in Africa (The Gambia and, Tanzania), one in Asia (India), one in Europe (travelers from the Tropics returning to France and the Netherlands), and four in South East Asia (Thailand). Two studies included children aged one to five years [408, 423], two included both adults and children [271, 424], and four studies included participants over 13 years of age [370, 371, 406, 425], and currently there an going WHO supported multi-centre study including children (5kg-25kg) in Kenya, Tanzania and Nigeria. 3265 patients were recruited in the concluded studies: 2200 adults, 424 children and 641 infants and small children, and in total 2024 patients were treated with co-artemether. Pregnant women were not recruited in the clinical trials. Fear of potential toxicity has limited the use of the artemisinin derivatives during gestation period in humans since animal studies have documented teratogenicity when the drugs are administered at high doses [249].

Good antimalarial efficacy and safety of the ARM-LMF combination have been confirmed in these studies. The six-dose regimens of ARM-LMF provides a highly effective and very well tolerated treatment for multidrug-resistant falciparum malaria [426]. The combination was well tolerated by all age groups

and was also better tolerated than mefloquine-containing drug regimens, with a significantly lower incidence of nausea, vomiting and dizziness [271]

1.4.5. Pharmacokinetics of coartemether

There is limited pharmacokinetic data for both co-artemether and ARM in animal models, although some ADME studies were done with ^{3}H-lumefantrine in mice (unpublished data). These studies showed slow absorption and uptake into erythrocytes, high protein binding, rapid tissue distribution, and slow excretion as unchanged compound via bile and faeces.

In humans there is still limited pharmacokinetic data. ARM and its active metabolite DHA are analysed by an HPLC method with electrochemical detection [382] and LMF is analyzed by an HPLC method with UV-detection [427]. Recently some clinical pharmacokinetic studies have been conducted [240, 274-276, 379, 425, 428, 429]

Absorption and bioavailability

A Thai clinical trial [278] showed that the pharmacokinetic properties of LMF and ARM differ markedly. Oral LMF is absorbed slowly and its oral bioavailability is influenced considerably by concomitant intake of food, particularly fats.

Under fasted conditions, oral ARM is rapidly absorbed though not completely [430], reaching peak plasma concentrations about 2 hours after dosing, whilst LMF – a highly lipophilic molecule- is absorbed after a lag period of up to 2 hours, with peak plasma concentrations at 6 to 8 hours post-dose [276, 278,

428]. Pharmacokinetic-pharmacodynamic analysis have shown that the determinant of the efficiency of the combination is the area under the LMF plasma concentration-time curve [276, 278].

Under fasted conditions, the oral bioavailability of both ARM and LMF is unpredictable and low. However, high-fat meal has been shown to increase the bioavailability of LMF 16 fold and that of ARM more than 2-fold [431]

In a clinical situation, food has been shown to increase the absorption of LMF in patients [276]. During acute malaria, there is marked intra- and inter-patient variability with regard to LMF absorption, possibly because of differences in food in-take. Acutely ill patients are disinclined to eat and tend to avoid high fat foods. Therefore, to improve bioavailability, patients need to be encouraged to take coartemether with a normal diet as soon as food can be tolerated.

Distribution

Colussi and colleagues [392] have also reported that both ARM and LMF are highly bound to human serum proteins in vitro (95.4% and 99.9%, respectively). Under physiological protein concentrations, the distribution in blood showed that 33% of ARM was bound to alpha(1)-acid glycoprotein, 17% to albumin, 12% to high density lipoproteins (HDL), 9.3% to low density lipoproteins (LDL) and 12% to very low density lipoproteins (VLDL), with binding capacities (nKa) of 3.2 x 10(5), 6.2 x 10(3), 2.1 x 10(5), 1.7 x 10(6) and 2.0 x 10(7) lmol(-1), respectively. 77% of LMF was bound to HDL, 7.3% to LDL and 6.6% to VLDL, with binding capacities of 2.7 x 10(7), 2. 6 x 10(7) and 2.4 x 10(8) lmol(-1), respectively. A

negligible fraction of LMF was bound to albumin and α-(1)-acid glycoprotein. The fraction in erythrocytes was around 10% for both ARM and LMF. The high protein binding may contribute to the longer half life of LMF.

Metabolism

An additional source of significant differences in the pharmacokinetic properties of ARM and LMF, are differences in the hepatic metabolic enzyme profiles, in particular inter-individual variability in the activity of CYP3A. ARM is rapidly and extensively metabolized by human hepatic microsomes (mostly through the enzyme CYP3A4/5) in vitro and in vivo, with substantial first pass metabolism [432]. DHA is the active metabolite. Studies with inhibitors of intestinal CYP3A4 (grapefruit juice) doubled the plasma concentrations of ARM. Mefloquine, another antimalarial is described as a substrate (and possible inhibitor) of CYP3A4, however there no clinically relevant risks due to pharmacokinetic drug-drug interaction expected at the enzymatic level following co-administration of co-artemether with CYP3A4 substrates with similar affinity to that of mefloquine [275].

LMF is also metabolized predominantly by the hepatic microsomal enzyme CYP3A4 and desbutyl LMF is the metabolite. At therapeutic plasma concentrations, LMF significantly inhibits the enzyme CYP2D6 in vitro [432]. Therefore, co-administration of compounds with low therapeutic indixes and significant metabolism by CYP2D6 (e.g neuroleptic agents and tricyclic antidepressants) might cause clinically relevant drug interactions.

Elimination and Excretion

ARM is rapidly cleared from plasma with an elimination half life of about 2-3 hours. Conversely, LMF is cleared more slowly, with an elimination half life of 2-3 days in healthy volunteers, and 5-10 days in patients with *falciparum* malaria [275]

Currently there is no urinary excretion data available for humans. Animal studies showed no evidence of ARM in faeces or urine of rats and dogs. Various unidentified metabolites were detectable in both faeces and urine. LMF was eliminated through the liver and bile and primarily excreted in the faeces in rats and dogs, with relatively low qualitative and quantitative recovery of metabolites [429].

1.4.6. Toxicity

Both artemether and co-artemether appear to show a slight degree of acute (single dose) toxicity following oral and intraperitoneal drug administration to mice but not in rats and dogs, based on available data [433]. The toxic effect of co-artemether was more evident in male mice after oral administration while no sex difference was shown after intraperitoneal administration, with a ratio of oral to parenteral toxicity of about 1:3. LMF however shows practically no acute toxicity in mice, rats, and dogs.

Embryogenicity has been noted with co-artemether in rats at 100mg/kg p.o. but is well tolerated at 30 mg/kg without teratogenic effects. In rabbits, maternal effects and embryotoxicity occurred at all doses $\geq$ 210 mg/kg. ARM showed embryotoxic effects at about 20mg/kg i.m. in mice, 11mg/kg i.m. or 20mg/kg p.o

in rats, and at 3mg/kg i.m or 30 mg/kg p.o. in rabbits, respectively. LMF displayed impairment of foetal testogenesis after ≥ 500 mg/kg and interference with the formation of spermatoblasts of the paternal rats at 1000 mg/kg with sterilising effect has been noted. No evidence of teratogenic effects up 1000 mg/kg has been noted in rabbits and rats (unpublished data).

CHAPTER 2

GENERAL EXPERIMENTAL PROTOCOLS

2.1. CULTURE SYSTEMS FOR PARASITE MAINTENANCE

In-vitro cultures of parasite isolates of *Plasmodium falciparum* were used throughout this study, and were cultured by an adaptation of the methods of Trager and Jensen [434]and [435]. All culture work was carried out using standard aseptic techniques in an Envair class II laminar flow safety cabinet. Consumables, such as tissue culture flasks, centrifuge tubes, universal bottles and bijous were of pre-sterilised disposable plastic. All of the glassware used for sterile culture work was autoclaved at 120°C, 15 atmospheres for 15 min and cooled prior to usage. Solutions were sterilised either by filtration through a 0.2 μm acrylic filter (Gelman Sciences Inc. UK) or by autoclaving. Protective surgical gloves were used, and regularly rinsed with 70 % ethanol: water, when working in the laminar flow safety cabinet to reduce chances of contamination. The basic culture techniques used are outlined below:

2.1.1. Parasite isolates

Different parasite isolates of *Plasmodium falciparum* were used in the preliminary stages of this work, namely; K1, TM6, HB3 & 3D7 isolates. The K1 isolate was however selected for the remaining experiments in the thesis, on the basis of its robustness in continuous culture. HB3 and 3D7 isolates were kindly provided by

Professor D. Walliker, Department of Genetics, University of Edinburgh, UK. The K1 isolate (cloned in house) was obtained from Professor D.C. Warhurst, London School of Hygiene and Tropical Medicine (London, UK). Dr P. Tan-areya, Department of Microbiology, Mahidol University, Bangkok, Thailand provided the TM6 isolate.

The original source and CQ sensitivity status of these isolates is summarized below (**Table 4**). Isolates with a CQ IC50 of less than 80 nM are defined as susceptible and isolates with IC50 more than 80 nM are defined as resistant.

ISOLATE	SOURCE	CQ SENSITIVITY
K1	Thailand	Resistant
TM6	Thailand	Resistant
HB3	Honduras	Sensitive
3D7	Unknown	Sensitive

Table 4. Origin and CQ sensitivity of the isolates of *P. falciparum* used in this thesis.

2.1.2. Culture medium

For experiments in the first half of this thesis, lyophilised RPMI 1640 supplied as 103.9 g/10 L containing L-glutamine (Sigma Chemical Co, UK) was used. Culture media was prepared in bulk following the manufacturer's instructions. Briefly, 10.43 g lyophilized RPMI 1640 was added to 9 litres (90 % of the final volume) of distilled water. To the suspension, 20 g of sodium bicarbonate (Sigma Chemical

Co, UK) was added and the whole suspension was allowed to dissolve for 1 to 2 h with continuous mixing using a magnetic stirrer. To facilitate dissolution, the pH was usually adjusted to 4.0 with 1 M hydrochloric acid. The pH of the medium was thereafter re-adjusted to 7.2 using a 1 M solution of sodium hydroxide, after complete dissolution. The volume was then made up to a final volume of 10 litres with distilled water. Finally, the medium was aseptically filtered into 500 ml pre-autoclaved bottles through a 0.2 µm acrylic filter using a Millipore (UK) peristaltic pump. The stock medium, red/orange in colour was stored between 2 - 8°C for up to 2 weeks. An aliquot of the filtered media was kept at 37°C for 24 h before use to monitor for contamination. Contamination was characterised by increased turbidity and or colour change from red/orange to yellow as a consequence of increased acidity due to lactic acid production by the contaminating microorganisms. Prior to use, the medium was supplemented with serum and HEPES buffer as described later.

For the remaining part of the study, 500 ml aliquots of factory reconstituted RPMI-1640 (Sigma Chemical Co, UK) were used. It is sterile filtered, endotoxin tested, with L-glutamine and sodium bicarbonate present and therefore ready to be supplemented to make complete medium. Complete medium was prepared by addition of 12.5 ml of 1M HEPES (N-[2-hydroxyethylpiperazine-N`-[2-ethanesulfonic acid]) buffer (Sigma Chemical Co, UK), 1 ml of 10 mg/ml gentamycin solution (Sigma Chemical Co, UK) and 50 ml of pooled human AB+ serum (see section 2.1.3 below) to the 500 ml aliquot of RPMI-1640. The

complete medium was then incubated for 24 h prior to use in order to check for contamination. Contamination was characterised as described previously. The medium was used for up to one week. Any unused medium was discarded.

2.1.3. Serum

The North West Regional Blood Transfusion Centre, Liverpool, kindly supplied human AB+ serum in 100-250 ml bags produced from a single unit of whole blood. To minimize fluctuations in serum performance, approximately 10 - 15 bags were pooled together into a pre-autoclaved glass flask. The pooled serum was aliquoted into 50 ml tubes and stored at –20°C until used. Prior to use, the serum was aseptically thawed out in a 37°C water bath for 10 - 15 minutes.

2.1.4. Uninfected erythrocytes

Human blood of group O Rhesus positive that had been tested negative for anti-HIV (human immunodeficiency virus) and anti-hepatitis B antibodies, was also kindly supplied by the Regional Blood Transfusion Centre, Liverpool. This blood, obtained no longer than 48 h after collection, was unsuitable for transfusion because of irregular bag-volume. It was supplied in citrate-phosphate-dextrose bags and kept at 4°C. Upon receipt, the fresh blood was transferred aseptically into sterile 50 ml centrifuge tubes and stored at 4°C for up to three weeks.

Prior to usage, the blood was washed with RPMI 1640 without additional supplements followed by centrifugation at 500 g for 5 min at room temperature.

The serum and buffy coat layer were carefully removed using a 10 or 20 ml syringe fitted with a sterile Kwill. Washing was repeated 2 or 3 times until no buffy coat remained and the supernatant assumed the colour of the wash medium. The washed packed erythrocytes were stored as packed cells at 4^0C for up to 1 week. Unused cells discarded after one week.

2.1.5. Gas phase

It is well documented that prolonged parasite growth requires an environment with lower O_2 concentration and a higher CO_2 concentration than atmospheric air [436]. The gas phase used in all experiments was composed of 93 % N_2, 3 % O_2 and 4 % CO_2 (prepared and supplied by British Oxygen Special Gases, UK). Tissue culture flasks were gassed aseptically, in a class II Envair laminar flow cabinet as follows: The gas from the cylinder was delivered to the laminar flow cabinet via a length of silicone rubber tubing. The gas passed through a 25 mm micro-flow disc filter assembly of 0.2 µm pore size (Gelman Sciences Inc., UK) into another length of silicone rubber tubing. The terminal end of this rubber tubing was connected to a 2.5 ml syringe barrel, connected to an acrylic filter of 0.2 µm pore size. Culture flasks were gassed via individual kwills fitted to the terminal 0.2 µm acrylic filter. Fresh sterile kwills were used to gas each individual flask. The duration of gassing was 30 s for 50 ml and 60 s for 200 ml capacity culture flasks.

2.1.6. Parasite cultivation method

A modification of the methods of Trager and Jensen [434] and [435] was used to maintain parasite in continuous culture in pre-sterilised plastic tissue culture flasks (Nunc, UK) of 50 or 200 ml capacity. The haematocrit or cell density in the flasks varied between 1 % and 4 % but was commonly 2%. Cultures were initiated by seeding a red cell/complete medium suspension with parasitised red cells from either another culture or with parasitised cells retrieved from cryopreserved stocks (see section 2.1.9). Cultures were usually initiated at about 0.1 % parasitaemia and 2 % haematocrit. When parasites were required in large volumes for protein extraction experiments, higher parasitaemias were often employed and the haematocrit increased to 4 % for the last developmental cycle. This would however require the culture medium to be changed atleast twice in a period of 24 hrs.

When parasitaemias were low (less than 1.5 %) culture medium was changed every 48 h. At higher parasitaemias, the medium was changed every 24 h. The procedure for this was as follows: spent medium was carefully and aseptically decanted from above the static cell layer. Pre-warmed complete medium was then added to make up the volume to 15 ml in 50 ml capacity flasks or 50 ml in 200 ml flasks. The flasks were then gassed as described in section 2.1.5. Once gassed, the culture flasks were placed in the incubator at 37°C. The parasites were subcultured (see section 2.1.7) when the target parasitaemia had been reached (usually at 10 to 15 % parasitaemia).

2.1.7. Sub-culturing of parasites

The process of sub-culturing was as follows: the parasitised cell suspension was centrifuged at 300 g for 5 min at room temperature and the supernatant was discarded. An appropriate volume of parasitised cell pellet was added to a fresh sterile culture flask. Fresh uninfected red cells and medium were added to create the required haematocrit and parasitaemia depending on size of flask used. The flask was then labeled, gassed and incubated as described. The remainder of the parasitised cells were used either in an experiment, cryopreserved (see section 2.1.8) or discarded.

2.1.8. Cryopreservation (freezing) and retrieval of parasite cultures

A modification of the method of Rowe *et al.*, 1968 was used as described in detail below.

2.1.8.1. Preparation of cryoprotectant solution

Physiological saline (0.95 %; w/v) was prepared by dissolving 1.9 g of sodium chloride (Sigma Chemical Co, UK) in 200 ml of distilled water. This prepared saline solution was then used to dissolve 8.4 g of sorbitol (Sigma Chemical Co, UK). 70 ml of glycerol (Sigma Chemical Co, UK) was thereafter added to the above solution and sterile-filtered using a 0.2 µm acrylic filter before use.

2.1.8.1. Procedure of cryopreservation (freezing)

Cultures of high parasitaemia (greater than 5 %), predominantly ring infected cells, were transferred into sterile 15 ml or 50 ml centrifuge tubes and centrifuged at 500 g for 5 min at room temperature. The supernatant was discarded and a volume of cryoprotectant equal to the volume of packed cells was added. The suspension was allowed to equilibrate for 5 min at room temperature. Aliquots of 500µl - 1000µl of the suspension were placed into screw-capped cryotubes (Nunc, UK), labeled appropriately and then transferred into liquid nitrogen for storage.

2.1.9. Retrieval of parasite cultures

The cryopreserved cultures were removed from the liquid nitrogen storage tank and the vials quickly thawed at 37°C. Prior to decanting the thawed out cultures into a 15 ml centrifuge tube, 70 % ethanol: water was used to wipe the rim of the vial to minimize contamination of the cultures. An equal volume of 3.5 % sodium chloride (w/v; Sigma Chemical Co, UK) was then added and centrifuged at 500 g for 5 min. The supernatant was discarded and a second volume (half the initial volume) of the 3.5 % sodium chloride was added, mixed and centrifuged as before. The supernatant was discarded and replaced with 0.5 ml of complete RPMI-1640 medium, and centrifuged as before. Then by adding 5 ml of complete RPMI-1640 medium each time, the pellet was washed twice more. Finally, the pellet was suspended in a volume of complete RPMI-1640 medium and made up to the required haematocrit with freshly washed uninfected red blood cells. The

contents of the tube were then prepared for culture after placing them in sterile 50ml culture flasks. Cultures were then gassed and placed in the incubator at 37^0C.

2.2. ROUTINE MONITORING OF PARASITAEMIA

In order to monitor the parasitaemia of the cultures, Giemsa-stained thin blood films were prepared each morning. These were prepared from each culture flask by spreading a drop of cultured parasitised cells onto a fresh, clean glass microscopic slide, and air drying. Films were methanol-fixed for approximately 30 sec before being stained in 10 % Giemsa stain (BDH, UK) in buffered distilled water, pH 7.2 for 20 min. The slides were then removed, rinsed carefully and thoroughly under running tap water, dried and examined with a light microscope (Zeiss, Germany) observed with immersion oil and on objective at X100 magnification. The parasitaemia was calculated from the number of infected cells expressed as percentage of the total number of cells counted in 5-10 fields of the blood film as shown below:

Percentage parasitaemia = number of infected cells/total number of cells x 100 %

2.2.1. Decontamination of cultures

As a rare occurrence, bacterial contamination would affect culture growth, and decontamination of precious cultures was attempted using penicillin-streptomycin solution. Cultures were first washed in complete medium by centrifugation at 500 g for 5 min. The medium was discarded and replaced with medium containing

1000 units and 200 µg/ml penicillin-streptomycin (Gibco, UK) solution respectively. Cultures were treated until the blood films were devoid of visible bacteria on daily blood films for a total of three days. However, if contamination persisted after 3 days treatment, cultures were discarded and fresh ones retrieved from liquid nitrogen.

2.2.2. Synchronisation of parasite cultures

Highly synchronous cultures were used throughout the studies described in this thesis Parasite synchrony was maintained by 5 % sorbitol treatment of parasitised cells [437]. This technique selectively lyses the mature forms of the parasite, which are more permeable to sorbitol. Due to osmotic effect this causes them to swell, eventually lyse and die after washing with normal medium, leaving only the young ring forms unaffected.

Cultures with a high proportion of ring stage parasites were transferred into sterile 15 or 50 ml centrifuge tubes and centrifuged 500 g for 5 min at room temperature. The supernatant was discarded and the parasitised cell pellet was resuspended in 5 volumes of the sterile 5 % sorbitol. The suspension was left to stand at room temperature in the laminar flow cabinet for 10 - 15 min and then centrifuged at 500 g for 5 min at room temperature. The parasitised cell pellet was then washed 2–3 times in 10 volumes of complete medium. Finally, the remaining parasites were re-introduced into culture for a minimum of 48 h prior to use in the different experiments. Occasionally it was necessary to re-synchronise

the cultures to keep them tightly synchronous, this was performed after 48 h in culture.

2.2.3. Stage-specific parasite isolation

When trophozoites were required from a culture of parasites of mixed stages, these were separated (concentrated or enriched) from the schizonts, ring or uninfected erythrocytes by a method using a percoll density gradient coupled with differential centrifugation as developed by Kramer et al [438]. An isotonic solution of 90 % Percoll (Sigma Chemical Co, UK) was prepared by diluting 9 parts of percoll to 1 part sterile complete RPMI-1640 medium. The solution was further diluted to 65 % percoll solution using complete RPMI-1640 medium. Then 20 ml of the 90 % Percoll solution followed by 20 ml of the 65 % solution were carefully dispensed aseptically into a sterile 50 ml centrifuge tube. The parasitised cell pellet, adjusted to 50 % haematocrit with complete medium, and was then carefully layered onto the gradient. The gradient was then centrifuged at 7000 g for 30 min. Three distinct bands were commonly seen after centrifugation. These bands were collected separately using a sterile glass Pasteur pipette. They were then washed 2 to 3 times at 500 g for 5 min at RT to remove percoll. Giemsa-stained thin blood films were prepared and the parasitaemia determined. The top band usually contained pigment and cell debris and late schizonts while the second band contained concentrated trophozoites. The third or bottom band contained usually uninfected erythrocytes and ring infected erythrocytes.

This technique was however not used for parasites used in the proteomic studies because it was thought to be quite "stressful" to the parasites in its own right.

2.2.4. In vitro parasite drug sensitivity assay

The activity of ARM, LMF, DHA, CQ and various other compounds used in these studies were tested against the CQ sensitive (HB3 & 3D7) and CQ resistant (K1 & TM6) isolates of *P. falciparum*. For the *in vitro* assay, an adaptation of the method of Desjardins et al [439] was used to assess parasite viability after drug treatment with the various compounds. The method relies on the incorporation of [^{3}H]hypoxanthine into nucleic acids by live parasites, and thus inhibition of uptake of the radiolabeled nucleic acid precursor by the parasites serves as the indicator of antimalarial activity.

2.2.5. Preparation of drug dilutions

Drugs used in these studies were dissolved / diluted in the following solvents: water, ethanol, methanol or dimethyl sulphoxide (DMSO) or a combination of these solvents. The concentration of the stock drug solution was 10^{-2}M in most instances. The stock solutions were then serially diluted in double distilled water or complete medium (without [^{3}H]hypoxanthine) to achieve the required range of drug concentrations for each assay. The drug concentrations were made up at double the targeted final concentration required in the assay plate, making provision for a 50% dilution following addition of an equal volume of inoculum. The final concentration of organic solvent in the assay plates was always less

than 0.1 %, a concentration previously shown to have no effect on parasite growth.

Drug dilutions were prepared such that when 100 µl of drug plus inoculum was added to the wells, an inoculum size of 1 was achieved. All of the dilutions were set up in plastic bijous prior to plating them out into the 96-well microtitre plate. The controls were set up in the same way, without drug.

2.2.6. Parasite preparation

Parasites were synchronised at the ring stage (see **section 2.2.2**), 48 h prior to use. Parasitaemia was assessed as described in **section 2.2**. The cell suspension was centrifuged at 500 for 5 min at RT and the supernatant discarded. The packed cells were diluted with washed fresh uninfected erythrocytes to give a final parasitaemia of 1 % and a haematocrit of 10 % using complete medium (without [^{3}H]hypoxanthine). The inoculum size of the parasitised cell suspension is calculated by using the relationship:

$$\text{Inoculum} = \text{parasitaemia} \times \text{haematocrit}$$

A 100-µl of this parasitised cell suspension was added to each tube containing 900 µl of each drug concentrations thereby achieving a final volume of 1 ml.

2.2.7. Preparation of 96-well micro-titre plates

Sterile and individually wrapped 96-well microtitre plastic plates (Nunc, UK) were used in this study. The wells arranged in 8 columns (A through to H), with each column containing 12 rows (sequentially numbered 1 through to 12). For the assay, the outer wells (columns A & H and rows 1 & 12) were not used as previous work has shown that these wells do not support good parasite growth [440]. Under sterile conditions and very carefully, each assay was performed in triplicate, using three adjacent wells in a row in the plate (for example B, C and D), leaving room for a second assay on the same plate. Using an automated pipette (Gilson Pipetteman, Gilson, UK) the control wells representing rows 6 and 7 received 100 µl of inoculum without drug. Then 100 µl of culture inoculum plus drug as described above was added to the wells in rows 2 - 5 and 8 – 11 (2 bearing the lowest and 11 the highest drug concentration). After setting up the assay, the plates were covered with their own sterile lids labeled appropriately and placed in a modular incubation chamber (Flow, UK), gassed for 5 min and then incubated for 24 h at 37°C for 24 h. At the end of 24 h incubation period, the plates were removed from the chamber and [^{3}H]hypoxanthine was added to each well (see **section 2.2.8**).

2.2.8. Preparation and addition of [3H]hypoxanthine

[^{3}H]Hypoxanthine (NEN, USA), supplied in 5-ml volumes of 5 mCi/ml sterile aqueous solution was used. The specific activity of each batch was approximately 50 Ci mmol^{-1}. Aliquots of the solution, 1mCi/ml were diluted 20-fold

with hypoxanthine free complete RPMI-1640 producing a 50 µCi/ml working solution.

At the end of the initial 24 h incubation period, 5 µl (0.25 µCi) of the [^{3}H]hypoxanthine working solution was added to each well of the assay plate using an automatic pipette (Gilson Pipetteman, UK) sterile tip. The plates were then shaken gently to ensure that the contents of each well were thoroughly mixed before being placed in the modular chamber for gassing. The chamber was then returned to the incubator for a further 24 h incubation period.

2.2.9. Harvesting of the assays

After the second 24 h incubation period was complete, the plates were removed from the incubation chamber and harvested. The plates were shaken gently to achieve thorough mixing of the contents of each well, and then harvested using a Tomtec semiautomatic cell harvester 96® (Wallac, UK). Printed glass fibre filter mats of type 1450-421 (Wallac, UK) specific for the 1450 MicroBeta™ liquid scintillation and luminescence counter which bind DNA and thus ideal for measuring the [^{3}H]hypoxanthine, were used. The filter mat was carefully placed in the appropriate section of the cell harvester and the culture plate was loaded as per the manufacturers' instructions. The culture plate contents were then harvested onto the filtermat until the wells were clear of culture material and cells. These filter mats were then partially dried under reduced pressure, by a

stream of air, and there after transferred from the harvester and allowed to dry fully in an oven set at 60°C prior to scintillation counting.

2.3. SCINTILLATION COUNTING OF THE HARVESTED ASSAY PLATES

Melt-on scintillation sheets (Wallac, Finland) were then placed on top of the dried filtermats and inserted into a sample bag (Wallac, Finland). The perimeter of the bags were sealed using a 1295-012 heat sealer (Wallac, Finland), before transferring them into the 1495-021 micro sealer (Wallac, Finland). The edges around the sealed perimeter of the mat were then trimmed to fit in the defined area of scintillation counter loading cassettes. Each sample was then placed in a cassette ready for counting using the 1450 MicroBeta Trilux liquid scintillation and luminescence counter (Wallac, Finland).

2.3.1. Data analysis

To assess the effect of a drug on parasite growth [^{3}H]hypoxanthine incorporation in the presence of drug was compared with that of controls without drug. The amount of radiolabelled hypoxanthine incorporated was measured as counts per minute (cpm). Using an Excel-based programme devised in our laboratory, triplicate mean cpm were automatically calculated for controls and drug-treated groups and the mean cpm of drug-treated groups expressed as a percentage of the control mean cpm (which represented 100% growth).

Data were presented graphically in the form of a log-dose response curve using the Grafit computer programme package (Erithacus Software, Staines, UK). The graph was produced by plotting drug concentration on the abscissa (x axis) versus the percentage of control parasite growth on the ordinate (y axis). The Grafit computer programme package uses an interactive four-parameter logistic method to fit the data to a dose-response curve. This allows the calculation of IC_{50} values. These IC_{50} values were used as measure of antimalarial drug potency.

2.3.2. Drug combination assay

A modification of the antimalarial potency of drugs in combination was assessed using a modification of the method of Berenbaum [441, 442]. Initially the sensitivity of each drug to be tested in combination was tested individually against a particular parasite isolate as described in **section 2.2.4**. Using the obtained IC_{50} values for each drug, working drug solutions with drug concentration set at 16 times the IC_{50} values of the individual drugs were prepared (this allows the IC_{50} concentration of each drug to be in the range of the fourth serial dilution). These two working drug solutions (x16 IC_{50}) were then titrated against each other in fixed ratios as shown in **Table 5** below.

DRUG A	DRUG B
10	0
9	1
7	3
5	5
3	7
1	9
0	10

Table 5. Showing the fixed ratio combination used to assess the in vitro interaction of drugs used in the drug combination studies.

Each combination was then serially diluted 8 times. These dilutions (corresponding to micro-titre plate well rows 2-11) were then processed as for the standard sensitivity assay described in **section 2.2.4.**

The fractional inhibitory concentration (FIC) (which denotes the IC_{50} of the drug in the combination / IC_{50} of the drug when tested alone) of each drug is calculated and plotted on an isobologram as described by Berenbaum [441, 442] using the relationship:

FIC of drug A = IC_{50} of drug A in combination/IC_{50} of drug A alone

FIC of drug B = IC_{50} of drug B in combination/IC_{50} of B alone.

Drug interactions when represented graphically on an isobologram (see **figure 2.1**), may conform to one of the three recognized patterns: Additivity (zero interaction), here the observed effect of drugs in combination is the summation of

the expected dose-response of the individual agents; Synergy (potentiation, augmentation, enhancement) where the effect is greater than expected; and Antagonism, where the effect is less than expected.

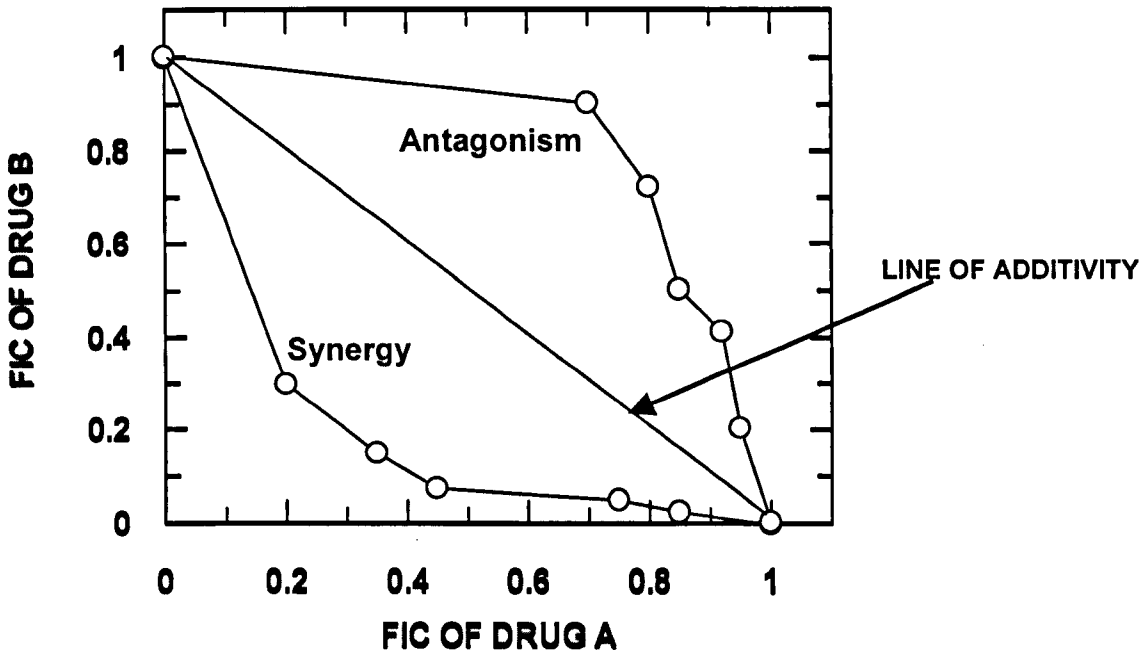


Figure 2.1. A hypothetical isobologram

From drugcombinations where there is zero interaction, a linear relationship exists between the two FICs (when the IC_{50} values are converted into the fractional inhibitory concentrations the data describes a straight line and links the points x=1, y=0, to x=0, y=1). If the results obtained produce an upward (convex) curve from this line then this is indicative of an antagonistic relationship. In contrast, if it is a downward (concave) curve below this line then this indicates a synergistic relationship. These relationships can be explained with regard to the fractional concentrations observed in each combination; for zero interaction the sum of the component FIC values are equal to1; for synergy the sum of the FIC

values are less than 1; for antagonism the sum of the FIC values are greater than 1.

2.4. CHARACTERISATION OF THE TIME REQUIRED ACHIEVING IRREVERSIBLE DRUG TOXICITY AGAINST *PLASMODIUM FALCIPARUM* PARASITES FOLLOWING ARM & LMF TREATMENT.

Five culture flasks were set up for either of the drugs tested (ARM and LMF). Cultures of *Plasmodium falciparum* K1 isolates were used, maintained at a haematocrit of 2 % and parasitaemia of 2 %, in complete medium. In four of the flasks in either group, the parasites were exposed to a final drug concentrations corresponding to their IC_{10}, IC_{20}, IC_{50}, and IC_{90} as calculated from the dose response curves using the Grafit computer programme package. The fifth flask in either group was the drug free control maintained under the same conditions as the treated parasite cultures. A total culture volume of 5000 µl per flask was used initially and 400µl samples removed at time intervals; 0, 1, 3, 5, 8, 12 & 24 hours post drug exposure. Culture was carefully removed and aseptically washed x 3 with complete medium at 2000 g for 1 min, and then re-suspended in complete medium. Aliquots of 100 µl of these samples (in triplicate) were then put into 96 micro-well micro-titre plates. [^{3}H] hypoxanthine (5 µl of 50 µCi/ml) were added to the samples after 24 hr incubation period, and then re-incubated for a further 24 hrs. All flasks and micro-wells were incubated at 37^{0}C and gassed for 30 seconds prior to each incubation period. Parasite viability at each drug concentration and drug exposure times was measured by assessment of [^{3}H]

hypoxanthine incorporation into parasite nucleic acids as described by Desjardins [439]. Samples were processed for scintillation counting as described in **section 2.3**. Parasite viability after drug exposure was expressed as a percentage of the control growth (without drug).

2.5. *PLASMODIUM FALCIPARUM* ULTRA-STRUCTURAL STUDIES: ELECTRON MICROSCOPY.

A modified protocol of that described by Langreth et al [443] was adapted for these investigations. Infected erythrocytes at a parasitaemia between 10 - 15 % in culture medium were added slowly to a fixative consisting of 2 % (v/v) glutaraldehyde, 0.12 M sucrose, 0.2 μM $CaCl_2$, 0.1 M Na cacodylate-HCl buffer at pH 7.4. Fixation was performed over 1 hr at room temperature. The fixed cells were then centrifuged at 500 rpm for 10 minutes and rinsed x 4 in cold 0.1 M Na cacodylate-HCl buffer, pH 7.4 containing 0.12 M sucrose followed by an overnight buffer rinse. The samples were then post-fixed in 1.5 % (w/v) osmium tetroxide, 0.12 M sucrose, 0.1 M Na cacodylate-HCl buffer, pH 7.4 at 4^0C for 1-2 hrs. After rinses in buffer and water, the cells were stained in 0.5 % (w/v) aqueous uranyl acetate for 1-2 hrs at room temperature, and then rinsed in water. Dehydration and embedding were performed in graded solutions of ethanol: 70 % ethanol for 1hr; 90 % ethanol for 1hr with 2 changes; 100 % ethanol for 1hr with three changes. The samples were transferred into propylene oxide for 30 minutes; into 1:1 propylene oxide: Epon 812 resin for 2 hrs; then into 1:2 propylene oxide: Epon 812 resin overnight and finally in Epon 812 resin all

day with 2 changes. The specimens were then polymerized for 48 hrs at 80^0C in beam capsules topped with resin. Thin sections of 110 nm thickness were cut with a Reichert ultracute ultramicrotome and mounted onto bare 200 mesh hexagonal copper grids. Staining was then performed with 2 % uranyl acetate for 20 minutes at room temperature. Sections were later washed in distilled water and then immersed in Reynold's lead acetate for 5 minutes at room temperature. Finally, the samples were allowed to dry and observed at 80 kV with a Phillip CM 10 transmission microscope. The negatives were recorded onto Kodak 4489 film.

2.6. PROTEOME ANALYSIS OF *PLASMODIUM FALCIPARUM*

2.6.1. Background to proteome analysis of *Plasmodium falciparum* (2-D gel protocol development).

2.6.1.1. Introduction

These types of investigation apply comparative proteomics to study differences in protein expression profiles between antimalarial drug treated and untreated *P.falciparum* parasite populations. This is an attempt to understand the molecular basis of of artemether (ARM) and lumefantrine (LMF) drug action, by capturing and analyzing the dynamic protein changes induced by perturbations of *P.falciparum* parasites following treatment with these antimalarial agents. We have employed high-resolution two dimensional gel electrophoresis [444] using immobilized pH gradients (IPG-Dalt) [445] to resolve and array *plasmodial* proteins, sequentially extracted by differential solubilization [446]. Two

dimensional electrophoresis using IPGs is the core separation technology adopted for protein display prior to post-separation analysis [447]. Subsequent analysis of the visualised protein expression patterns was performed using image analysis software, PDquest™. Protein spot identification was then achieved with biological mass spectrometry (MALDI) coupled with database comparisons.

The proteome (PROTEins expressed by a genOME or tissue) [448] is the collective term for all of the proteins produced from the instructions encoded by the genetic material in the cell. Proteomes are dynamic and reflect the state of a biological system [449]. Proteomics is the global analysis of proteins of a cell, organism or biological fluid, a process which requires stringently controlled steps of sample preparation, 2-D electrophoresis, image detection and analysis, identification and characterization of proteins by mass spectrometry and genomic database search, and bioinformatics for protein identification and database searching [450-452]. Proteomics offers information that is unique and valuable, independent from, but complementary to, genomic data.

As the acting macromolecules in cells, proteins are potential targets for most therapeutic agents. Proteins are therefore expected to be the most relevant markers (compared to mRNA profiling) for monitoring biological perturbations such as disease and drug treatment effects. 2-DE protein profiling can reveal virtually all proteins present in a cell or tissue at any given time and can provide information about (a) post- and co-translational modifications critical to our understanding of proper physiological protein function, because proteins are dynamically modified and processed, (b) subcellular localization, and (c) protein

translocation [453] for proteins that may undergo physiological or biochemical transport across membranes within an organism. There is also increasing evidence showing poor correlation between mRNA and protein abundance [449, 454, 455] and therefore genomic information alone cannot be extrapolated to represent the functional status of a biological system.

2-D gel electrophoresis is a biochemical separating technique for proteome analysis, based upon two independent but complementary and efficient separating principles. It involves separation of proteins according to charge (pI) by isoelectric focusing (IEF) in a polyacrylamide gel with a pH gradient and high urea concentration in the first dimension, and separation according to size (Mr) by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. The resolving capacity of the 2-DE gels is dependent on the separation length in both dimensions and is usually considered to be proportional to the total gel area available for separation.

High-resolution 2-D electrophoresis is the only technique that can separate thousands of gene products with diverse properties simultaneously, and is compatible with semi-quantitative analysis of these products. Each spot on the resulting two-dimensional array may correspond to a single protein species in the sample, but quite often may be a mixture of proteins. This method is characterized by a number of important features; (1) extremely high resolving power, (2) tolerance to crude protein mixtures, and in principle it can be used for all proteins (detergent compatibility), (3) tolerance to relatively high sample loads,

(4) 2-D gels are very efficient fraction collectors, and (5) the separated proteins are conserved in the gel matrix for further analysis at any desired time [456].

2-D electrophoresis using IPGs was used instead of the classic 2-D electrophoresis, which uses carrier ampholytes [444]. The former bears the advantages of higher resolution, improved reproducibility for inter-laboratory comparisons, increased loading capacity for micro-preparative 2-D electrophoresis with subsequent spot identification by mass spectrometry and Edman sequencing, and an extended basic pH limit for 2-D electrophoresis [445].

In brief, 2-D electrophoresis using IPGs involves: (1) sample preparation, (2) IPG strip rehydration, (3) IEF, (4) IPG strip equilibration, (5) SDS-PAGE, (6,7) Visualization & analysis of resultant 2-D array of protein spots.

2.6.1.2. Sample preparation

Sample preparation is a vital factor for the overall performance of the 2-DE technique. The goal of sample preparation is to maximize solubilisation of all proteins (including hydrophobic species), protein disaggregation and removal of interfering components (such as nucleic acids, and lipids), and to minimize, proteolytic degradation, artifactual oxidation, carbamylation, and conformational alteration of proteins (co-analytical modifications) [454, 457].

The solubilisation process involves breaking molecular interactions, including disulphide bridges, the main forces holding proteins together; non-covalent interactions like ionic bonds, hydrogen bonds, and hydrophobic interactions (between proteins and other compounds); and covalent bonds found mainly

between proteins and some coenzymes. Urea, a non-ionic reagent is the chaotrope of choice for 2-D electrophoresis, a common constituent of sample preparations, used as the denaturant in the first dimension of 2 DE. The use of thiourea in addition to urea has recently been shown to increase solubilisation, particularly for intractable proteins such as membrane proteins [446, 458, 459]. Urea and thiourea disrupt hydrogen bonds and are used when hydrogen bonding causes unwanted aggregation or formation of secondary structures that affect protein mobility.

To ensure complete sample solubilisation of the hydrophobic residues exposed as a result of chaotropic denaturation, and prevention of aggregation through hydrophobic interactions, a non-ionic or zwitterionic detergent (surfactant) is always included in the sample solution. In the recent years the sulphobetaine CHAPS, a zwitterionic detergent, has become the surfactant of choice and is generally used at between 2-5% in 8 M urea [460]. It increases the solubility of hydrophobic proteins. Traditionally, nonionic polyol mixtures such as the Triton X-100 and Nonidet P-40 have been used [444, 461], but are of less purity compared to CHAPS. New sulfobetaine surfactants such as N-decyl-N,N-dimethyl-3-ammonio-1-propane sulfonate (SB 3-10), when used in combination with thiourea/urea mixture, provide a wide range of powerful sample solutions for 2-DE. However, they suffer from poor solubility in high concentrations (> 5M) of urea [462, 463]. 2-mercaptoethanol is discouraged because of its buffering effect above pH 8 (ref). SDS an ionic detergent, is poorly compatible with IEF, though low amounts may be used in the initial sample solubilisation prior to IEF,

provided that high urea concentrations and nonionic or zwitterrionic detergents are present to ensure complete removal of the SDS from the proteins during IEF. The final concentration of SDS should be 0.25% or lower, and the ratio of the excess detergent to SDS should be at least 8:1 [464, 465]

Inclusion of reducing agents in the sample solution helps to break any disulphide bonds present and to maintain all proteins in their completely reduced state. Addition of an excess of a sulphydryl (free thiol containing) reductant such as dithiothreitol (DTT) at concentrations ranging from 20 to 100 mM is commonly used. Dithierythritol structurally akin to DTT is an alternative. Non-thiol reductant Trialkyl phosphines such as tributyl phosphine (TBP), have recently been reported to increase the solubility of proteins during the IEF and to increase transfer of proteins to the to the second dimension [466]

Addition of carrier ampholytes or IPG Buffer (0.5-2% v/v) can be beneficial, enhancing protein solubility by minimizing protein aggregation due to charge-to charge interactions and scavenging cyanate ions [451]. They do not disturb the IEF very much because they migrate to their pIs, where they become uncharged.

In addition to chemical solubilisation techniques, mechanical procedures of cell disruption like trituration (cells lysed by shear forces resulting from forcing cell sample through a small orifice under high pressure) and sonication (in short bursts to avoid heating), facilitate disaggregation of protein molecules. Maintenance of protein integrity by blocking degradation due to proteolysis is critical. This may be achieved by maintaining low temperature (near 4 ^{0}C) since proteases are less active at low temperatures. Use of protease inhibitors such as

PMSF, EDTA, EGTA, and peptide protease inhibitors (such as aprotinin, leupeptide, pepstatin, bestatin) are recommended in combination and this offers sufficient protection against proteolysis. In addition, most tissue proteases are inactive above pH 9, consequently proteolysis can be inhibited by preparing the sample in the presence of tris base, sodium carbonate, or basic carrier ampholytes [452].

Presence of nucleic acids, especially DNA may variously affect the separation of proteins by IEF. DNA complexes are dissociated during denaturing conditions as occurs in sample preparation. This inhibits protein entry and slows migration in the IPG gel, and furthermore DNA binds to proteins in the sample and causes artifactual migration and streaking [457]. Nucleic acids are efficiently broken down by sonication. Nucleic acids may also be degraded by addition of a suitable pure (i.e. protease-free) DNase/RNase endonuclease mixture to the sample solubilisation solution. An alternative method is to utilize the ability of synthetic carrier ampholytes to form complexes with nucleic acids and then remove the complexes by ultracentrifugation [467].

Samples containing urea must not be heated because this can introduce significant charge heterogeneity due to carbamylation of the proteins by isocyanate formed in decomposition of urea.

2.6.1.3. First dimension isoelectric focusing

The first dimension of 2-D electrophoresis is isoelectric focusing (IEF), which involves separation of proteins in a pH gradient until they reach a stationary

position where their net charge is zero (isoelectric point (pI)). Proteins are amphoteric molecules and thus have a zwitterionic character and, depending upon the pH of their surroundings, can bear a positive, negative or zero net charge. The net charge of a protein is the sum of all the negative and positive charges of its amino acid side chains and amino- and carboxyl-termini [452]. Proteins are positively charged at pH values less than their pI and negatively charged at pH values greater than their pI. The presence of a pH gradient is critical to the IEF technique. Proteins in a pH gradient, migrate when subjected to an electric field, those with a positive net charge migrating toward the cathode while those with a negative net charge migrating toward the anode, until they reach their isoelectric points. If because of thermal diffusion a protein moves from the pH region of its isoelectric charge, it gains a charge and migrates back electrophoretically to its "correct" position, which corresponds to the isoelectric point. Thus isoelectric focusing is a concentration end point method, which forms sharp and highly concentrated protein bands. The resolution magnitude is dependent on the slope of the pH gradient and the electric field strength. This explains the high voltages characteristic of IEF, typically in excess of 1,000 V (**refer to table.6**). When isoelectric points are reached in the pH gradient, there is very little ionic movement in the system, resulting in a very low final current (typically below 1 mA). The optimum focusing time required achieving the best quality and reproducibility is the time needed for IEF separation pattern to achieve the steady state [468, 469]. The optimum focusing time must be

established empirically for each combination of protein sample, protein loading and particular pH range and length of IPG gel strip used.

The first-dimension separation procedure involves IPG gel rehydration, sample application, and isoelectric focusing. The technique using IPG strips for the first dimension of 2-D electrophoresis, has been introduced and further developed by Gorg et al [447]. Immobilised pH gradients are based on the principle that the pH gradient is co-polymerised, and thus insolubilised within the fibres of the polyacrylamide matrix [470, 471]. Covalent binding of the IPG eliminates "cathodal drift" or, "plateau phenomenon", a major set back associated with carrier ampholyte IEF. Use of IPG strips for first dimension gives superior 2-D maps in terms of resolution and reproducibility. The features of the immobilized pH gradients that have caused a shift from the traditional technique to the IPG strip method include:

(1) Immobilized pH gradients are very reproducible because the covalently fixed gradients cannot drift, and are not modified by sample composition [447].

(2) The film-supported gels are easy to handle. They can be picked up at either end with forceps or gloved fingers.

(3) The plastic support minimizes stretching and breaking of the gels.

(4) IPG technology offers a wide and stable pH range; more very acidic and basic proteins can be separated [469, 472, 473].

(5) IEF separation distance can be greatly increased by use of very narrow pH intervals in long gel strips, which allow high special resolution and high

protein loading [474]. This allows poorly expressed proteins to be visualized directly [475].

(6) Different sample application methods are feasible; commonly the sample can be introduced into the dried IPG strips during rehydration [476].

(7) Various additives, like detergents and reductants, can be added to the rehydration solution

(8) Availability of pre-cast dry strip gels minimizes the handling of toxic acrylamide monomers, reduces the preparation time and effort, and the reproducibility of pH gradient is improved.

2.6.1.4. Sample application

Sample application may be achieved passively by either including it in the rehydration solution or by direct application to the rehydrated IPG strip using sample cups or sample wells. In-gel sample rehydration or rehydration-loading of the IPG is the preferred method of sample application, where the protein sample is included in the rehydration solution (refer appendix), using the entire IPG gel for sample application, as suggested by [476]. This has been shown to eliminate the formation of precipitates at the application point often associated with loading with sample cups, because there is no discrete application point. This results in improved resolution throughout the pH range of the gel, and allows precise control of protein amounts and sample volumes loaded into the IPG gels. The method is also technically simpler, avoiding problems of leakage that can occur

when using sample cups. Regardless of where proteins start in the pH gradient, they migrate in the electric field to their corresponding isoelectric points (pIs).

IPG strip rehydration solution

IPG strips are rehydrated prior to IEF. This is performed in the Immobiline Drystrip Reswelling Tray if the Multiphor II system is used for IEF or in IPGphor strip holders, if the IPGphor is used. The specific protein solubility requirements of the sample determine the choice of the optimal rehydration solution. Generally, a typical rehydration solution consists of (1) a chaotropic agent, urea 8 M commonly used (solubulises and denatures proteins, unfolding them to expose internal ionisable amino acids). Addition of thiourea to urea, has been reported to improve membrane protein solubilisation [446, 458, 459]; (2) a non-ionic and zwitterionic detergents like CHAPS, Triton X-100, or NP-40 usually in concentrations of 0.5 to 4% (to solubilise hydrophobic proteins and minimize protein aggregation); (3) a reducing agent (cleaves disulphide bonds to allow proteins to un fold completely), like DTT or DTE used commonly in concentrations of 20-100 mM. Use of non-thiol reductant tributyl phosphine has also been documented [466]; (4) Carrier ampholyte mixture or IPG buffer of desired pH range (enhances sample solubility and produces more-uniform conductivity across the pH gradient during IEF without affecting the shape of the gradient); (5) tracking dye (bromophenol blue) (for observing IEF progress at the beginning of the protocol)

2.6.1.5. Second–dimension

SDS PAGE (SDS-polyacrylamide gel electrophoresis) is an electrophoretic method for separating polypeptides according to their molecular weight (MW). SDS-PAGE consists of four steps: (1) second-dimension gel preparation, (2) equilibration of the IPG strip(s) in SDS buffer, (3) transfer of the equilibrated IPG strip on to the SDS gel, and (4) electrophoresis.

The technique requires that all the proteins in the mixture have the same net charge per gram, for the movement through the gel to be solely based on the molecular mass of the proteins. This is achieved by performing the separation in polyacrylamide gels containing sodium dodecyl sulphate (SDS). The charge modifier SDS is an anionic detergent (surfactant) that denatures proteins by binding to them. SDS inundates the intrinsic charge of proteins, thus disrupting hydrogen bonds and hydrophobic interactions and preventing protein aggregation, such that they all have the same charge density and free solution electrophoretic mobility. Addition of a reducing agent to cleave disulphide bonds, totally unfolds the protein molecules. When proteins are treated with both SDS and a reducing agent such as DTT, separations exclusively by molecular weight are possible. There is a log-linear relationship between the relative distance of migration of the SDS-polypeptide micelle and the molecular weight, for a certain molecular weight range depending on the polyacrylamide percentage used [452]. The polyacrylamide gels are made by an aqueous free radical polymerization of the monomer acrylamide and the cross-linker *N,N'*-methylenebisacrylamide (Bis), using a redox initiator system such as ammonium persulphate and *N,N,N',N'*-

tetramethylethylenediamine (TEMED) [477]. Commonly SDS-PAGE in a Tris-chloride / Tris-glycine buffer according to Laemmli [478] is employed. A stacking gel is not needed in 2-D protein separation, because the proteins are pre-separated by IEF and migrate from a gel into another gel. Vertical and horizontal flatbed systems can be used with similar results [479].

NB: Polyacrylamide gel composition is indicated by two different percentages:

$$\%T = \text{Total acrylamide} = \frac{g\,(\text{acryl} + \text{bis})}{100\ ml} \times 100$$

$$\%C = \text{Crosslinker} = \frac{g\,(\text{bis})}{g\,(\text{acryl} + \text{bis})} \times 100$$

The total percentage of acrylamide determines the pore size. Single percentage (homogenous) gels containing 12.5 % total acrylamide were used in this study.

2.6.1.6. IPG strip equilibration

Prior to transfer of IPG strip onto the SDS gel, the strip(s) have to be equilibrated in SDS buffer. The equilibration step saturates the IPG with the SDS buffer system required for the second dimension separation. Special precautions have been taken to minimize protein losses during this stage, while maximizing transfer of proteins on to the second dimension, and these are reflected in the modified composition of the equilibration buffer [479]. The equilibration solution contains: (1) Equilibration buffer (50 mM Tris-HCl, pH 8.8) maintains IPG strip pH in a range suitable for electrophoresis.

(2) Urea (6M), in combination with glycerol minimizes the effects of electro-

endosmosis by increasing the viscosity of the buffer [480]. The carboxylic groups on the IPG gels become ionized in the electric field, and because they are fixed in the gel matrix, they cannot migrate. This result in a counter-flow of hydrogen (H_3O^+) ions towards the cathode: electro-endosmosis. This effect may cause partial losses of proteins, which are carried towards the cathode with the electro-osmotic flow, thus causing interference with protein transfer from the IPG strip to the second dimension.

(3) Glycerol (30%), in combination with urea, minimizes electro-endosmosis water transport and enhances transfer of protein from the first to the second dimension [480]

(4) Reductant DTT preserves the fully reduced state of the denatured, unalkylated proteins.

(5) Sodium dodecyl sulphate (SDS) denatures proteins and forms negatively charged proteins-SDS complexes. The amount of SDS bound to a protein, and the resultant negative charge, is directly proportional to the mass of the protein.[452] Consequently, the electrophoretic migration of the SDS coated proteins in a sieving polyacrylamide gel separates proteins on the basis of their molecular mass.

(6) Iodoacetamide (IAA) alkylates thiol groups on proteins, preventing their re-oxidation during electrophoresis. IAA also alkylates any free DTT that may otherwise migrate through the second dimension SDS-PAGE gel resulting in point streaking observed after silver staining, and eliminates other silver-staining artifacts [481]. Equilibration with iodoacetamide is also used to minimize

unwanted reactions of cysteine residues (i.e., when mass spectroscopy is performed on the separated proteins) [452].

(7) Tracking dye (bromophenol blue) facilitates monitoring of electrophoresis.

The optimum equilibration period of 15 minutes in DTT followed by another 15 minutes in iodoacetamide, is seemingly long but necessary. The charged carboxylic groups of the immobilized pH gradients act like a weak ion exchanger and prevent the diffusion of proteins out of the strip. On the other hand, SDS being negatively charged slows down its diffusion into the strip [482]. An alternative procedure of equilibration of IPGs using tributyl phosphine (TBP) has been documented [466]. TBP is reported to have advantages of being uncharged and thiol free, and therefore unable to migrate during SDS-PAGE, and requiring a single step of equilibration. However, we were unable to demonstrate the superiority of TBP over DTT in our investigations.

2.6.1.7. Detection techniques

Most detection methods used for SDS gels can be applied to second dimension gels. Features that characterize an ideal detection technique include; a wide linear dynamic range, high sensitivity, ability to be quantitative, compatibility with further analysis using mass spectrometry, fastness, non toxicity, affordability (price) and non-dependence on living cells for labeling. None of the available techniques combines all these features. Autoradiography and fluorography are

the most sensitive detection methods, although silver stains, Coomassie Brilliant blue staining are the most frequently used methods.

The most sensitive non-radioactive and widely used technique to detect protein spots on two-dimensional gels is silver staining, picking up protein amounts down to 0.2 ng. It is however much less stoichiometric, with the intensity being linear over 40-50 fold range in concentration from 0.04 ng/mm^2 to 2 ng /mm^2. There are two main types of silver stains employed for 2-D gels: silver nitrate and silver diamidine procedures. The latter shows better sensitivity for basic proteins, but is incompatible with tricine buffer in the gel, and silver mirror development on the gel surface is a common feature. In the silver nitrate method (used in this study) the silver is more weakly bound to the proteins (silver binds to the amino acid side chains, primarily the sulphydryl and carboxyl groups of proteins), it can be modified for mass spectrometry compatibility [483, 484]. Silver staining is a complex, multi-step process, and many variables can influence the outcome. High purity reagents (including water used for preparing the staining reagents) and precise timing are necessary for reproducible, high-quality results.

The Coomassie Brilliant blue staining, although 50-fold less sensitive than silver staining, is relatively simpler with good quantitative linearity than silver, with a 20-fold range (10-200ng) of linearity [485]. It is compatible with mass spectrometry.

Negative staining with imidazole zinc is very sensitive, picking protein amounts down to 0.2 ng. This method stains only the background, and not the proteins and this gives a very good recovery yield for further analysis with mass spectrometry [486]. The down side of it is that it cannot be used for quantification.

Fluorescence staining methods are less sensitive than silver staining detecting protein amounts down to 2-8 ng. They however have broad linear dynamic ranges of about 10^4, and they are compatible with subsequent mass spectrometry analysis with enhanced recovery of peptides from in- gel digests [487]. Currently available fluorescent dye stains include SYPRO® Ruby (most sensitive), SYPRO® Red and Orange. Unfortunately all these dyes are relatively expensive, and a fluorescence scanner or a CCD camera is required. Fluorescence labeling with Bimane or Cydyes prior to isoelectric focusing gives similar sensitivities and dynamic ranges to fluorescence staining.

Currently, blotting of 2-D gels is mainly used for immuno-detection. The transfer efficiency of electroblotting is not sufficient for the general detection of all the proteins.

2.6.1.8. Image analysis of 2-DE gels using PDQUEST™

The work flow of Pdquest™ comprises of the following steps following image acquisition by scanning.

(a) 2-DE gel image optimisation and editing which involves smoothing, contrast enhancement, edge detection and background subtraction. Background subtraction helps to eliminate meaningless changes of the background.

(b) Protein spot detection: is a multiple step procedure in which the gel image is processed and spot centres are marked according parameters one selects. The spots are then fitted to a gaussian

model. Gaussian fitted spots are more rounded and well defined compared to the original gel spots. Having a Gaussian model minimises having areas of overlapping and streaking allowing for more accurate quantitation, and gaussian fitted spots are the ones used for higher level analysis and data basing in pdquest™.

(c) Gel matching (comparison of identical spots in serial gels enabling comparative analysis of alterations in protein spot expression, under various experimental conditions): this is done by creating Pdquest™ "matchsets". Matchsets, are groups of gels with spots to be compared and the they comprise of the following elements:

(i) *Matchset members*, which are gel spots images containing gaussian images of gels created from the original scans. These gaussian spots are used for matching and quantitation.

(ii) *Member images,* these are copies of the original gel scans and they contain the original spot data as well as the processing associated with spot data. One can toggle between the member with gaussian spots and the member images of the original spots. The original scan files however, remain unchanged and are not included in the matchset.

(iii) *Matchset standard,* this is the synthetic gel image from the spots of the various members of a matchset generated by Pdquest™, that represents all spot data in the matchset. It is

based on a template chosen from one of the matchset members. And the criteria for selecting the ideal template gel as the standard include: selecting a gel with a large number of spots, one that is representative of the experiment as a whole, a control gel and in which the spots are well resolved and are of good quality. All matching and higher level calculations are performed using the standard.

(d) Data handling by generating replicate groups: Because of the complexity and inherent imprecision of the process of loading and running 2-D gels, different gels loaded with the same samples may result in spots with differing amounts of proteins. To circumvent this hurdle, the same sample is run on multiple gels and spot data from these duplicate gels are combined for more accurate quantitation using Pdquest™ replicate groups function. In this case the software requires that the duplicate groups are included in the same matchset in order to combine them in a replicate group. Using Pdquest™ one is then able to show the average quantity of spots in each replicate group (replication group quantitation mode), or provide histograms showing the quantity the spot in each member (quantitation mode).

(e) Normalization of gels: Is a process of compensating for non-expression related variation in protein spot intensity, important for

accurate quantitation (Pdquest™ uses house keeping proteins for this).

(f) Gel data analysis using analysis sets: This involves grouping of proteins spots generated using Pdquest™, based on various statistical criteria (eg using student's T test, Boolean analysis or higher level analysis based on two other analysis sets) or a manual selection of ones own design. Analysis sets are used to create and study categories of proteins that are scientifically meaningful, through determination of qualitative and or quantitative changes in protein expression. Analysis sets are created and displayed in the match set standards.

(g) Data presentation and interpretation (assigning detected spots molecular mass, M_r and isoelectric point, pI; comparison of spot quantitation and reflection of alteration trends of spots easily illustrated with graphs, scattergrams and bar charts)

(h) The analysis can be maintained in databases that link this information to the original gel images using Pdquest™, and 2-DE databases can be created.

2.6.1.9. Protein spot identification

Mass spectrometry (MS) has become the technique of choice for the identification of proteins separated by gels [488-492]. The technique is applicable to all proteins and combines the advantages of greater sensitivity and high

throughput. Traditionally, protein electroblotting and Edman sequencing have been employed as tools for *de novo* sequencing and protein identification (details of these are not discussed in this thesis).

2.6.1.10. Mass spectrometry

Mass spectrometry (MS) is an analytical technique that accurately determines molecular mass measurements of any particular biologically interesting molecule (e.g., proteins, peptides and DNA), or the component molecules of a mixture.

Basically, mass spectrometers used for the analysis of proteins or peptides comprise of two main parts: an ion source which introduces sample into the machine, and a device to measure the mass of the introduced ions. The molecules are ionized in the gas phase, accelerated by an electric field in a vacuum and enter a mass analyzer, which allows the measurement of their mass to charge ratio (*m/z*).

Basically, a mass spectrometric analysis can be considered to comprise of the following steps: (1) Sample Introduction, (2) Ionisation, (3) Mass Analysis, and (4) Ion Detection / Data Analysis.

The samples for mass spectrometric analysis may be introduced in gas, liquid or solid states. The available different ionisation techniques include (a) Electron Impact and Chemical Ionisation, (b) Plasma Desorption Mass Spectroscopy (PDMS), (c) Fast Atom Bombardment (FAB), (d) Thermospray and Particle Beam Ionisation, (e) Electrospray Ionisation (ESI), and (f) Matrix-Assisted Laser

Desorption Ionisation (MALDI). Of these, the last two are the most popular, and the two main instruments used currently: Matrix Assisted Laser Desorption Ionisation (MALDI), a development of Karas and Hillen-Kamp (1988), and Electrospray (ESI) invented by Yamashita and Fenn (1984).

2.6.1.11. Matrix Assisted Laser Desorption Time of Flight-Mass spectrometer. (MALDI-TOF)

The MALDI-TOF MS generates ions from solid-phase samples and measures their mass in a flight tube.

The protein and peptide mixture generated by in-gel trypsin digestion (or chemical cleavage) from individual selected gel spots, are mixed and co-crystallized with a matrix molecule (low molecular weight compounds with an absorption maximum at the wavelength of the laser), such as α cyano 4 hydroxy cinnamic acid, on to a stainless steel or gold-plated target. When the matrix-embedded sample is irradiated with a pulsing laser, the small matrix molecules absorb the energy. The transfer of laser energy from the matrix to the peptides causes their desorption into the vacuum where they are accelerated through an electric field facilitated by a high voltage grid. All ions (peptides) with their different masses bear the same kinetic energy ($1/_2mv^2$, where m is the mass and v is the velocity), light ions arriving at the detector sooner than heavy ions. The recorded time of flight (TOF) measurements are used to calculate the masses of ions in m/z (mass per charge) with an accuracy of better than ± 0.5 Da. This accuracy can be improved further at the expense of sensitivity, by extending the

time between ionization and acceleration (delayed extraction), and by positioning an ion mirror (reflectron) to extend the flight path and focus the ions [493]. MALDI-MS instruments equipped with a reflectron can utilize the decomposition of metastable ions (post-source decay (PSD) capability) to obtain peptide sequence information, and partial protein sequence information by monitoring fast metastable ion fragmentation (in-source decay) can be obtained [494]. High molecular weight peptides and proteins are only detected in the *linear mode*, and thus most TOF instruments can be used in both modes.

MALDI-MS characteristically generates low charge (almost exclusively singly charged ions) states (m/z) leading to less complex spectra when mixtures of proteins and peptides are analysed. MALDI-MS also tolerates moderate buffer and salt concentrations in the analyte mixture (allows peptides taken from in-gel or membrane digests to be analysed directly), and these advantages make MALDI the preferred ionization technique for peptide mass fingerprint (PMF) analysis. The MALDI-MS generated PMF can be compared to "virtual" fingerprints obtained by theoretical cleavage of protein sequences available in the avalanche of accessible protein and DNA databases. The top-scoring proteins are retrieved as possible candidate proteins. MALDI-MS is now considered to be the technique of choice for high throughput protein and peptide identification. By this method more than 90% of the analysed proteins can be identified directly with certainty and with high sequence coverage against mature databases. MALDI-TOF data however, do not provide a complete coverage of peptides. Therefore, for *de novo* sequencing and intensive posttranslation

modification (PTM) analysis electrospray ionization (ESI) tandem mass spectrometers are required.

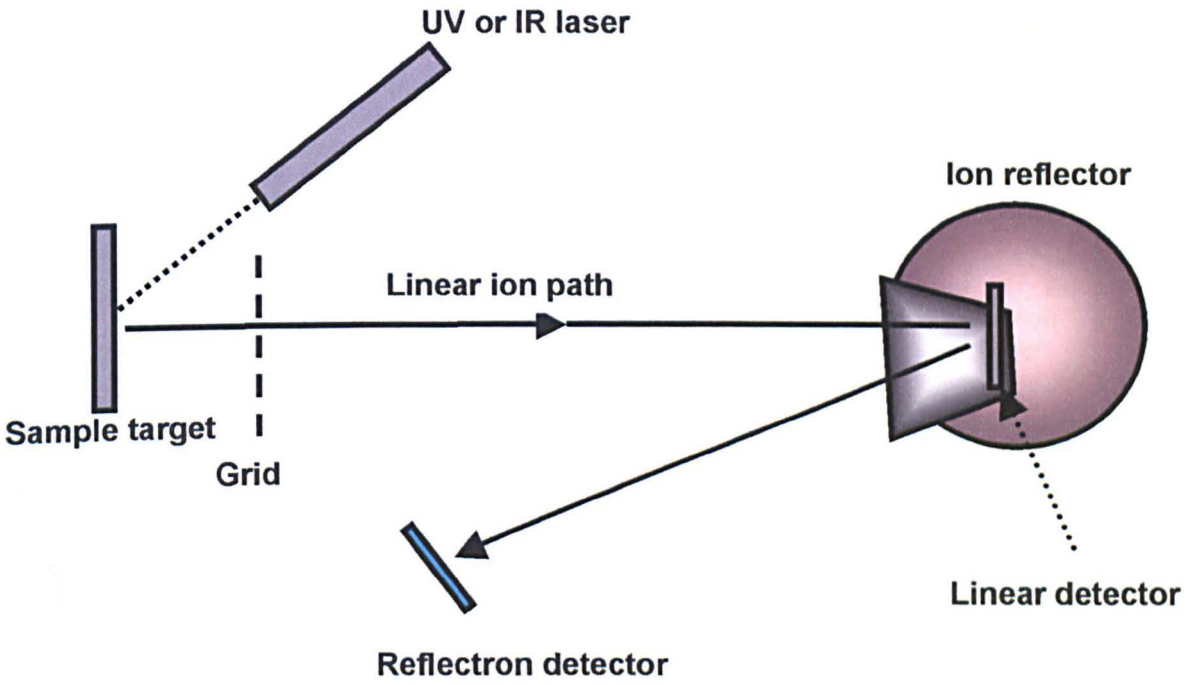


Figure 2.2. Schematic showing MALDI-TOF MS instrument.

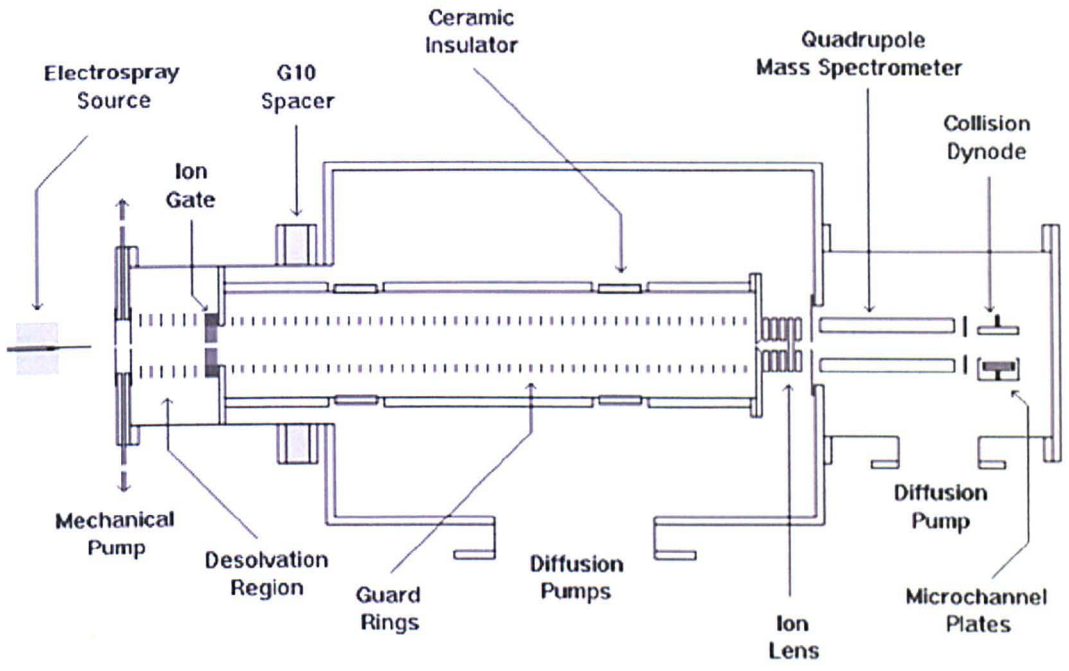


Figure 2.3. Schematic showing electrospray mass spectrometer.

2.6.1.12. Electrospray Ionisation-Mass Spectrometry (ESI-MS)

This generates ions samples in liquid, and measures their mass in a quadrupole or in a time of flight detector device (**refer to figure 2.3**). And generally, ESI-MS offers the opportunity to obtain at least partial structure (sequence) information from the fragmentation of individual peptides [495].

The analyte in solution (eluted peptides derived from a single spot dissolved in a volatile solvent) in micro-litre volumes is delivered to the end of the high-potential capillary, either under pressure from a pump, or by electrostatic forces alone via a nanospray capillary [484]. This results in a spray of fine highly charged droplets

containing protein, generated at atmospheric pressure in an electrostatic field. These droplets are directed towards the inlet of the mass spectrometer, which is held at lower potential. With the help of dry gas and heat (hot nitrogen gas), the solvent evaporates releasing a constant stream of peptide ions into the gas phase (Coulombic explosion). These are introduced through a capillary or a tiny orifice to the high vacuum environment of the mass analyser. Multiple protonation occurs, and ESI yields a series of multiply charged ions from each peptide. The resulting spectrum is complex and requires deconvolution to produce a theoretical mass spectrum of ions as though they were all singly charged.

ESI is commonly used with a triple mass analyzer, in which the first quadrupole is used for the selection of a peptide ion. These selected ions are directed to a second quadrupole where they are fragmented by collision with argon gas (inert gas). The masses of the resulting fragments are then analysed in the third quadrupole. The quadrupole mass analyzer is a mass filter which under preset physical conditions only allows ions with a totally defined mass /charge ratio to pass through, and the rest of ions that cannot pass through are lost. Continuous change of the potential at the quadrupole, allows ions of different masses to pass sequentially (scanning), and the intensity of the ion flow is recorded according to the *m/z* ratio

Nanoelectrospray, "nanospray" [484] is currently the method of choice for high sensitivity MS and MS/MS experiments [494]. In this approach, highly concentrated solutions are sprayed at very low flow rates of $nLmin^{-1}$ (nanospray ESI). This provides the advantages of increased sensitivity, and very long

measurement times are made available with samples of few microlitre volumes. Electrospray mass spectrometers can be coupled on-line to capillary electrophoresis and liquid chromatography systems [456].

2.6.1.13. Mass Analysis.

There are different equipments available on mass spectrometers for mass analysis and these include:

(a). Sector Instruments:

These use fixed magnetic and electric fields to separate ions of different mass and energy. To improve the focusing, i.e., the resolving power of these mass spectrometers, a device termed an energizer (an electrostatic analyzer (ESA)) is placed in the ion optic pathway, often before the magnet. Such an instrument is capable of a mass resolving power exceeding 100,000. The necessary geometry and size of high resolution sector instruments depend on the mass range, sensitivity and resolving power that the analyst wishes to achieve. Some of the unique advantages are their relatively high mass range, sensitivity and resolving power, together with compatibility with a wide range of ionisation techniques. The disadvantages are their size and cost compared to most mass spectrometers.

(b). Quadrupole Mass Filter and Quadrupole Ion Trap Instruments:

The quadrupole mass filter has now become one of the most widely used types of mass spectrometers because of its ease of use, small size and relatively low cost. Mass separation in a quadrupole mass filter is based on achieving a stable trajectory for ions of specific m/z values in a hyperbolic electrostatic field.

The resolution will increase with increasing mass, because ions of higher mass have lower velocity. However, the transmission efficiency will decrease, due to the longer time ions of higher masses spend in the quadrupole.

One of the advantages of a quadrupole mass filter over a sector instrument is the low voltage applied to the ion source, i.e., the kinetic energy of the ions is in the order of 5-10 eV, compared with several keV for a sector instrument. This eliminates high voltage problems and makes interfacing to GC and LC easier. Other advantages are its good transmission efficiency, high scan speed, and wide acceptance angle to give high sensitivity

(c). The quadrupole ion trap:

Is based on the same principle as the quadrupole mass filter, except that the quadrupole field is generated within a three-dimensional trap. As is the case with quadrupole mass filters, the quadrupole field is closest to the theoretical ideal in the center of the trap. For this reason, a moderator gas like helium is often introduced into the trap in addition to the sample, to dampen the oscillations of the ions and hence concentrate them in the center of the trap. As the name suggests, the ion trap can store ions over a long period of time making it possible

to study gas phase reactions. In particular, the ion trap has excellent MS/MS capabilities. The mass range of commercial instruments is 650 Da, scanned at over 5000 Da/sec. By reducing the scan speed to 0.015 Da/sec, a resolving power of $1.2x10^7$ (full width at half maximum, FWHM) can be achieved. Also, under special conditions, i.e., resonance with external field to cause ejection, a mass range of up to 45,000 Da and sensitivities in the attomole range have been obtained. The very high sensitivity of the ion trap is a consequence of the fact that all ions formed can in theory be detected. However, space charge effects (ion-ion coulombic interactions) reduce the accuracy of mass assignment for an ion trap. Even though ion/molecule reactions take place within the trap, EI spectra generally compare well to EI spectra acquired on quadrupole mass filters. The applications potential of the ion trap is very great. Size, speed, sensitivity, MS/MS capabilities and compatibility with most ionization techniques favor further development of this mass analyzer.

(d). Time-of-flight (TOF) mass spectrometry:

The principal of mass analysis in a TOF analyzer is based on the principle that ions of different m/z values have the same energy, but different velocities, after acceleration out of the ion source. Thus, the time required for each to traverse the flight tube is different: high mass ions take longer to reach the detector than low mass ions. Several ionization techniques are suitable for TOF mass analyzers by which ions are generated or ejected from the ion source over very short periods of time. Today's linear TOF MS instruments are capable of attaining

a resolution of 1 part per 1000. Reflectron TOF instruments are capable of a resolving power of over 10,000.

TOF analyzers have some special advantages. In contrast to sector instruments and quadrupoles, all ions accelerated out of the ion source of the TOF instrument will reach the detector, giving it a relatively high sensitivity. Of course, the ions must be pulsed into the analyzer, usually at rates of 10-10,000 Hertz, depending on the particular instrument arrangement, and so, some dead time exists when ions are not being analyzed. Also, the mass range of TOF analyzers is virtually unlimited and any practical upper limit is dictated by the ionization process and by detector efficiency. The combinations of time-of-flight mass spectrometry with MALD, PD and ES have produced effective tools, due to their relatively low cost, high sensitivity, speed and ease of operation.

(e). Ion Cyclotron Resonance Mass Spectrometry (Fourier transform techniques (FT-MS)

Like the ion trap, FT-MS is capable of storing ions within a cell. It consists of three pairs of parallel plates arranged as a cube, used for trapping, excitation or detection, respectively. The cell lies within a strong magnetic field which is perpendicular to the trapping plates.

An outstanding feature of FT-MS is the extremely high resolving power that can be attained; for example, using electrospray ionization, a resolution of over $2x10^6$ has been achieved. Another important feature of FT-MS is its MS/MS capability,

which makes it an important tool for basic research in gas phase chemical research.

2.6.1.14 Protein Identification using Peptide Mass Fingerprinting (PMF) and Peptide Fragmentation (PF).

2.6.1.14.1. Peptide Mass Fingerprinting (PMF)

This is the most widespread technique for protein identification [494, 496]. In gel digestion of proteins separated by 2-D GE, using a proteolytic enzyme such as trypsin, generates a mixture of peptides specifically cleaved at certain amino acids (e.g. trypsin specifically hydrolyses peptide bonds at the carboxylic sites of lysine and arginine residues). The masses of the peptides resulting from this cleavage are measured with great precision using mass spectrometry (MALDI-TOF MS or ESI-MS). The results of all peptide masses are then compared with the theoretical (mathematically derived) peptide masses for proteins in databases. Theoretical peptides are generated using the knowledge of the preferred cleavage motifs of the experimentally used enzyme. The matching output is a list of proteins ranked by number of peptides shared with the unknown protein, where the correct identification for an unknown protein is likely to be that with the largest number of peptide "hits". Reliable protein identification is dependent on several parameters: the accuracy of fragment mass determination, the number of masses submitted for query, the mass distribution of the query mass, the number of masses matching between sample and database protein, the size of the sequence database, gel spot and database information applying to

the same species (little confidence can be ascribed to cross-species matches, with the likely exception of highly conserved proteins), and the number of modifications considered [494].

Different approaches have been suggested to generate additional data in situations where the acquired MALDI mass fingerprint data are inadequate for reliable identification of an unknown proteins: (a) re-measuring the spectrum using different parameters to improve the spectrum quality, (b) generation of additional fingerprints with a protease of different specificity, and (c) use of peptide fragmentation techniques such as PSD and MS/MS to derive valuable sequence information such as a larger part of amino acid sequence or post-translational modification.

2.6.1.14.2. Peptide Fragmentation.

Peptide fragmentation to generate partial sequence of peptides is a useful tool for unambiguous protein identification, and a necessary complement to peptide mass fingerprinting. Sequential fragmentation of peptides can be achieved enzymatically or chemically, removing amino acids one by one from the N- or C-terminus of a peptide to generate a ladder of peptides. Alternatively, mass spectrometric techniques using post-source decay (PSD) or collision-induced dissociation (CID) in MALDI-MS and ESI-MS respectively can be used to fragment peptides. The sole objective of the technique is to produce spectra containing a series of peaks differing by the mass of amino acid residues, and therefore enabling stretches of peptide sequence to be logically concluded.

The analysis of peptide post-source decay (PSD) fragments in refrectron MALDI mass spectrometers yields some sequence information [488]. MALDI-PSD however, often produces incomplete fragmentation, making the *de novo* interpretation of sequence difficult or impossible, and therefore it is not extensively used for peptide sequencing. The method of choice for peptide fragmentation and sequencing is currently collision-induced dissociation (CID), which is done by triple quadrupole ESI-MS or recently introduced MALDI-ToF instruments coupled with a collision cell. The manual interpretation of spectra from CID is still difficult however computer programs that facilitate automatic interpretation are now available. Complete interpretation of the CID spectra is not necessary for purposes of protein identification. The "peptide sequence tag" (a few residues of sequence obtained from a CID spectrum) [495], together with the peptide parent ion mass and the distance in mass units to the N- and C-termini of the peptide, often suffice for specific protein identification. Use of "peptide sequence tags" however, requires expert operators, and involves manual manipulations and data analysis (interpretation of data still requires considerable effort).

The combination of peptide mass fingerprints with sequence tags both identifies the protein in the database and also pinpoints protein modifications [497, 498].

2.6.1.15. Analysis of Posttranslational Modifications

Posttranslational modifications (PMs) exert significant effect on the functions and properties of proteins and therefore are an important area of consideration in

proteome analysis. There are several different types of co-and post translational modifications, all of which can influence a protein's charge, hydrophobicity, conformation and/or stability [499-504]. Characterisation of PMs however, still remains a problem area in proteomics.

Separated proteins, which show an indication of PMs, require detailed investigation. Information from mass spectrometric analysis such as a deviation of the observed isoelectric point of a protein from that calculated from the DNA sequence is a good indication of a modification. Many of the modifications cannot be reliably predicted from DNA sequence, so analytical methods are required to both identify the modification and also to localize it on the protein. The precise determination of the type and the position of the posttranslational modification, can be done using special mass spectrometric techniques such as precursor scan or neutral loss scan and the mass spectrometric sequencing methods MALDI-PSD and nanospray ESI-MS/MS. Edman sequence analysis, though tedious is also a useful identification tool. Partial modifications at several sites of a protein commonly associated with phosphorylations and glycosylations are particularly difficult to analyse. These may require separation of the mixture by nano-HPLC with on line mass spectrometric analysis to be performed.

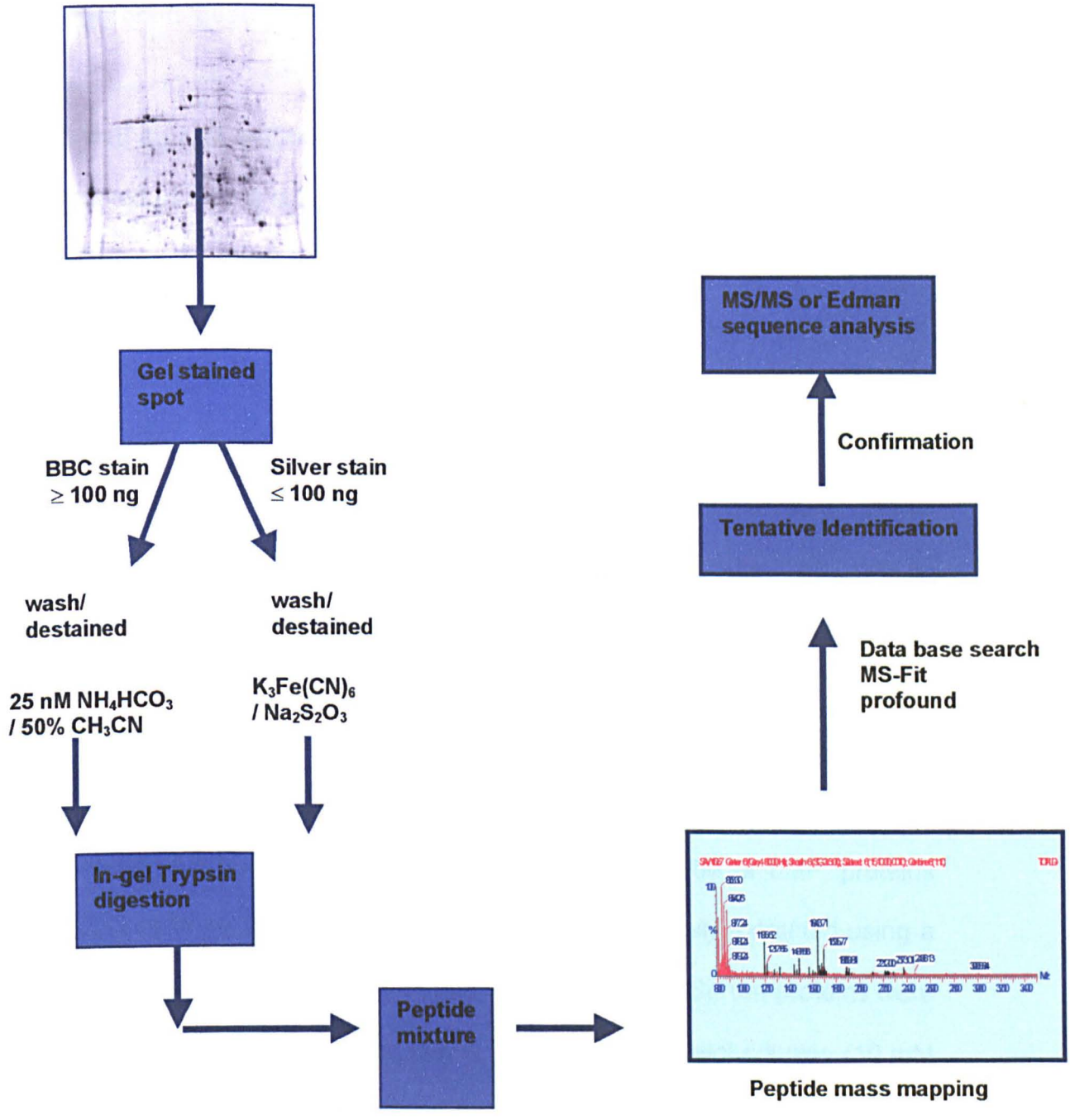


Figure 2.4. Schematic showing protein identification with peptide mass fingerprinting.

2.6.2 Protocols used in the proteome analysis of *P.falciprum.*

2.6.2.1. Cell culture and treatment

Synchronized parasites cultures (**refer to section 2.2.2**) at the trophozoite stage of the parasite life cycle (22-26 hours post-invasion, 10-15% parasitaemia and 4% haematocrit) were usedm in these studies . Parasite cultures were exposed to 10 nM of ARM, or 200 nM of LMF (drug concentrations corresponding to their respective IC_{90} values), for 5 and 6 hours respectively (exposure times associated with irreversible parasite toxicity). An equal amount of culture material without drug (control) was maintained under the identical culture conditions as the drug treated. Both sets of cultures were then processed for parasite protein extraction.

2.6.2.2. Sample preparation

After 5 hours or 6 hours of parasite exposure to either ARM or LMF, proteins from both drug treated and control cultures, were sequentially extracted using a modification of the protocol described by Molley et al [446]. Serum proteins were removed by repetitive washing with sterile Tris-buffered sorbitol solution (10 mM Tris / 250 mM sorbitol pH 7.0) followed by centrifugation at 2000 g at 21^0C for 5 minutes and removal of the buffer drug (this was repeated X3). The parasites were then freed from infected erythrocytes by saponin lysis (0.15% in Tris-buffered sorbitol) for 10 minutes at 21^0C, and then washed three times with ice-cold Tris-buffered sorbitol followed by centrifugation at 5000 g for 15 minutes to

sediment parasites from red cell membranes (debris). The crude parasite extract was then re-suspended in Tris extraction buffer (50 mM Tris-HCl pH 7.4; 0.1x volume of 1mg/ml DNAse, 0.25mg/ml RNAse A in 50 mM $Mgcl_2$; 1 mM PMSF, 30 μg aprotinin) at 0^0C. This was followed by trituration through a 27G needle and followed by ultrasonication at 0^0C using an ultrasonic processor (Vibra Cell™). The Tris-soluble fraction was then separated at (0^0C, 10,000 g, 15 min) and the supernatant stored at $^-80^0$C for later use. The resultant pellet was then washed twice with 50mM Tris-base and then subjected to lysis solubilising solution (8 M urea, 4% w/v CHAPS, 100 mM DTT, 50 mM Tris-base (pH 7.4), 10% SDS, 0.5% Pharmalytes 3-10), vortexed, ultrasonicated (in short bursts to avoid heating & foaming) and centrifuged at 10,000 g, 0^0C for 15minutes. The supernatant was collected and stored at $^-80^0$C for later use. The pellet was again washed twice with 50mM Tris-HCl (pH 7.4). The resulting pellet was then solubilised in enhanced extraction solution (5M urea, 2M thiourea, 2% CHAPS, 2% SB 3-10, 40 mM Tris-base, 0.5% CA), vortexed, ultrasonicated and centrifuged as before and the supernatant recovered, aliquoted and stored at $^-80^0$C until used. The protein concentration of each parasite extract was determined using a modification of the Bradford dye-binding assay (Bio-Rad) [505] with bovine serum albumin as the standard. Protein quantification in solution in the presence of urea and detergent works only with the modification according to [506], where the sample is acidified with 0.1 mol/L HCl prior to assay.

2.6.2.3. Modified Bradford Assay

1. Bradford dye reagent (Biorad protein assay dye) was diluted with deionised water in a ratio of 1:3. The diluted solution was then filtered through fast filter paper (401) under gravity.
2. A calibration series comprising of a blank, 5, 10, 20, and 40 μg BSA was made up with a BSA stock solution of 1 mg/ml, (BSA used here as the protein standard).
3. All test samples and standards (calibration samples) were made up to 40μl with deionised water.
4. 5 μl of lysis buffer was added to each sample, including the standards but excluding samples extracted with lysis buffer.
5. 10 μl of 0.1 M HCl solution were then added [506], followed by the addition of 2 ml diluted dye solution to all samples.
6. The optical density of the samples was then measured spectrophotometrically at 590 nm wave-length, using the zero BSA sample as the blank.
7. Using the BSA standard curve, the protein concentration of the test samples was then determined using Grafit computer soft ware.

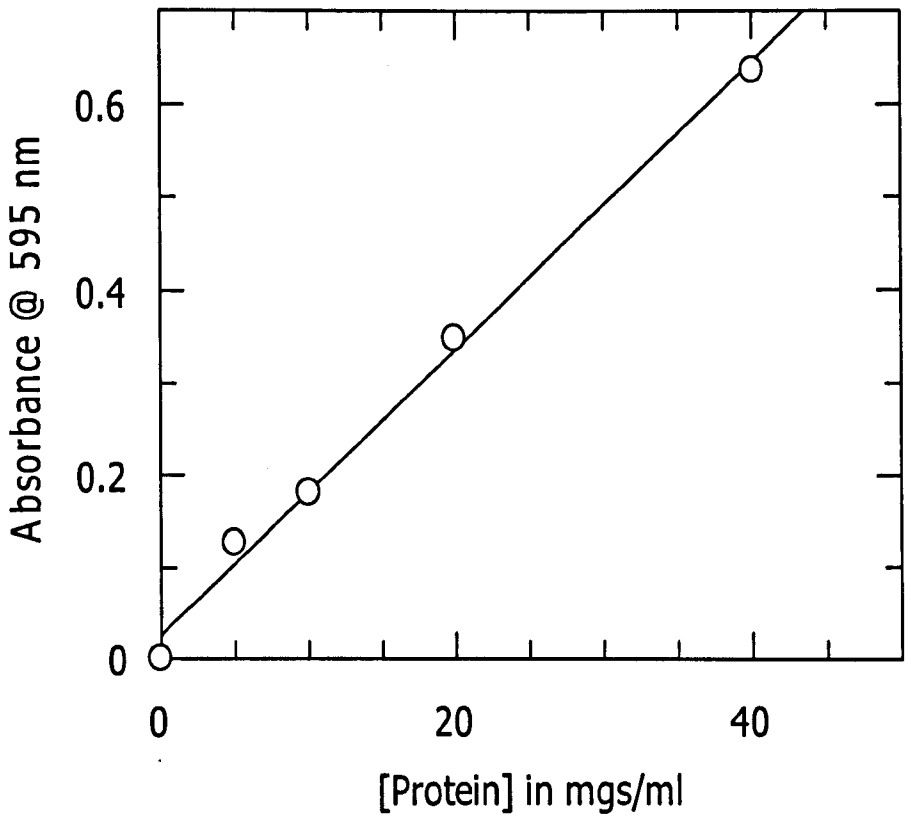


Fig.2.3. Calibration curve of BSA protein standards in the modified Bradford assay. Each point represents the mean of three protein determinations.

2.6.2.4. First dimension isoelectric focusing

Isoelectric focusing was performed using Multiphor® II flat bed electrophoresis system (Pharmacia Biotech, Uppsala, Sweden). Ready-made IPG strips, (Immobiline Drystrip gels (T=4%, C=3%) with a pH gradient 3-10 L (linear) 130 mm length) from Amersham pharmacia biotech were used. The protein samples (100 μg for analytical gels and 300 μg for micro-preparative gels) were mixed with a rehydration solution (8M Urea, 3% CHAPS, 0.5% Triton X-100, 0.5% IPG buffer 3-10, 10 mM DTT, and a trace amount of bromophenol blue) to obtain a

final volume of 250 µL per gel. The mixture was in-gel applied for reswelling of the dry immobilised pH gradient (IPG) strips passively overnight (a minimum of 10 hrs) at room temperature. The IPG strips were over laid with 2 mls of IPG Cover Fluid (mineral oil) to minimize evaporation and urea crystallization. The rehydrated IPG strips were removed from the Reswelling Tray with a pair of forceps, rinsed with deionised water to remove excess rehydration solution (in order to prevent formation of urea crystals on the gel surface during IEF), and excess moisture drained off with dump filter paper. The IPG strips were then positioned in the DryStrip aligner with the gel side facing upwards in the grooves, with the acidic end near the anode. 5mm wide, 11 cm long electrode paper strips, moistened with distilled water were then placed across the cathodic and anodic ends of the aligned IPG strips. The electrodes were then carefully inserted to contact the electrode strips, and about 50 ml of mineral oil poured onto the strips. IEF was initiated using and the following running conditions from 0 to 300 V in 1 min, then 300V for 1h, 1000V for 1h, 2500V for 1h and 3000 V for 21h (Biorad power pac 3000). The power supply was programmed in gradient mode with the check the current option turned off. To establish cooling, the temperature on the Multitemp II Thermostatic Circulator was set at 20^0C. The bromophenol blue tracking dye front migrates toward the anode as isoelectric focusing proceeds, but leaves the IPG strip before focusing is complete, therefore clearing of the dye doesn't imply that the sample is focused.

IPGs Length/pH	Phase	Voltage (V)	Current (mA)	Power (W)	Duration (h:min)	Vh (recommended)
13 cm/pH 4-7L	1	300	2	5	0:01	1
	2	3500	2	5	1:30	2900
	3	3500	2	5	3:45-4:20	6100-9100
	Total				**5:15-5:50**	**9000-12000**
13cm/pH3-10L/NL	1	300	2	5	0:01	1
	2	3500	2	5	1:30	2900
	3	3500	2	5	3:10-4:00	11100-14100
	Total				**4:40-5:30**	**14000-17000-**
18cm/pH4-7L	1	500	2	5	0:01	1
	2	3500	2	5	1:30	3000
	3	3500	2	5	5:40-7:40	2000-27000
	Total				**7:10-9:10**	**23000-30000**
18cm/pH3-10L/NL	1	500	2	5	0:0.1	1
	2	3500	2	5	1:30	3000
	3	3500	2	5	4:50-6:20	17000-22000
	Total				**6:20-7:50**	**20000-25000**

Table 6. Showing Immobiline Drystrip IEF guidelines for Multiphor II used in this study

2.6.2.5. Second– dimension SDS-PAGE

After the IEF run, the IPG gel strips were incubated at room temperature for 2 x 15 min with gentle shaking in 10 mL of equilibration solution containing Tris-HCl buffer (50 mM, pH 8.8), 6 M urea, 30% w/v glycerol, 2% w/v SDS, and a trace of bromophenol blue. DTT (1% w/v) was added during the first and iodoacetamide (4% w/v) during the second equilibration step [447]. Both solutions were freshly made prior to IPG strip equilibration. After equilibration, the IPG strips were aligned on filter paper moistened with deionised water Excess equilibration solution was drained before they were applied to vertical SDS gels [445, 452]. The second dimension separation was performed with home made 12.5%

homogenous vertical SDS-polyacrylamide gel slabs (gel plate size 180 x 160 mm, and gel thickness 1.5 mm) employing the tris-glycine system of Laemmli [478]. The gel sandwich was prepared as per the Hoefer SE 600 series user manual The gels (refer to table 2.3) were poured up to 0.5 cm from the top of the plates and overlaid with a thin layer (500 μL) of water-saturated n-butanol immediately after pouring to minimize gel exposure to oxygen and to create a flat gel surface. The focused and equilibrated IPG strips were then dipped in SDS electrophoresis buffer for lubrication [452] and then transferred onto the upper edge of the SDS gel and sealed with agarose solution (Tris base 25 mM, glycine 192 mM, SDS 0.1% w/v, agarose 0.5% w/v). Electrophoresis was performed at 20^0C and a constant current of 25 mA per gel using SE 600 series vertical slab gel electrophoresis units. A constant current setting is traditionally used with a discontinuous buffer system so that the rate of electrophoresis migration remains unchanged throughout the run. Under these conditions, voltage increases as the run proceeds.

Components (12.5 %) gel.	70 ml (2 x Gels) (ml)	140 ml (4 x Gels) (ml)	280 ml (8 x Gels) (ml)
Acryl/Bis Solution (30%) 37.5:1	33	66	132
Deionised water	20	40	80
1.5M Tris (pH 8.8)	17	34	68
TEMED	30 µl	60 µl	120 µl
Ammonium Persulphate (100 mg/ml)	350 µl	700 µl	1400 µl

Table 7. Showing components of 12.5 % SDS PAGE gel used as second dimension.

2.6.2.6. Internal standards for 2 D

Internal standards are extremely important for the determination of the pH and molecular weight ranges of a specific 2-D gel system and for constructing calibration curves for calculating the relative pI and M_r of unknown proteins [507]. In these investigations, BIO-RAD's 2-D SDS PAGE standards were used, which were mixed with experimental samples prior to 2 D gel electrophoresis. To eliminate the possibility of the standards interfering with the migration and identification of unknown proteins, standards were also electrophoresed in parallel with the biological samples on a separate reference gel.

2.6.2.7. Detection techniques

Most detection methods used for SDS gels can be applied to second-dimension gels. Using staining procedures, proteins can be visualized as spots with varying properties or features, such as size, darkness/brightness and location on the gel. Silver staining and Coomassie staining were used in these investigations and the protocols used are described below.

2.6.2.7.1. Silver staining of analytical 2-D gels

This protocol is based on the protocol of Blum et al. [508], with modifications [509, 510].

1. In order to fix the proteins in the gels and remove any interfering compounds in the 2-D gel (glycine, Tris, SDS, and carrier ampholytes), gels were fixed overnight in fix solution (5% acetic acid, 40% methanol)
2. Gels were then washed in deionized water for 0.5 hr.
3. Sensitized with (0.3 g Sodium dithionate / L) for 1-2 minutes, with an aim of increasing the subsequent image formation.
4. The gels were washed gels x2 with deionised water, for less than 2 minutes.
5. Silver impregnation was then performed, by soaking the gels in silver nitrate solution for 40 minutes (2g Silver nitrate plus 250 μL formaldehyde made up to 1 L).
6. The gels were then rapidly washed in deionised water for about 20 seconds.

7. Gels were then developed in image developer solution (30g Sodium carbonate, 250 μL formaldehyde, 10 mg Sodium thiosulphate, made up to 1L) for up to 10 minutes. The thiosulphate reduces the background allowing for thorough development of the image [508].
8. Development was stopped by dipping the gels in stop solution (50g/L Tris, acetic acid 20 ml / L).
9. Final stabilization of the image was then achieved by thorough rinsing of the gels in deionised water for about 30 minutes.

Note. Both Developer and sensitizing solutions should be made up fresh prior to use, and all solutions prepared in clean glassware with deionised, distilled water.

2.6.2.7.2. Coomassie staining of preparative 2-D gels

Staining reagents

Coomassie blue G-250 (CBB G-250) stain was used and the following staining reagents were prepared:

2. Gel fixative solution: 7% glacial acetic acid in 40% (v/v) methanol.
3. Stock solution CBB G-250 stain: 10% w/v ammonium sulphate in 2 % w/v phosphoric acid (2 g of 85 % phosphoric acid in 100 ml distilled water), and 0.125% w/v CBB G. The stock staining solution should not be filtered because the colloidal dye particles formed get retained on the filter. The

stock staining solution is stable for prolonged periods but requires thorough agitation prior to use.

Staining with CBB G-250

1. The gels were placed in the fixative solution for 20 minutes or more (Fixation is done immediately after electrophoresis and can be continued over night but is not a critical step for this stain).
2. The fixative solution was decanted.
3. Immediately prior to staining, 4 parts of stock solution were combined with 1 part of methanol and mixed for 30 seconds.
4. The gels were then placed in the staining suspension and incubated overnight for 18-24 hrs.
5. The gels were de-stained with 10% acetic acid in 25% (v/v) methanol for 60% seconds with gentle shaking using the orbital shaker.
6. Using 25% methanol, the gels were repeatedly rinsed until desired contrast was achieved.
7. Finally, the gels were then subjected to densitometric scanning, and then wrapped in plastic wrap and stored at 4^0C (could alternatively be stored for several weeks in 25% (v/v) ammonium sulphate at room temperature).

2.6.2.8. Scanning of gel image (data acquisition)

Data was acquired from the 2-DE gels by scanning with a GS-710 Imaging Densitometer (BioRad). This is a laser scanner characterized by high

densitometric and geometric (spatial) resolution, which can be used to scan both wet and dried gels stained with silver or Coomassie blue. The scanning process converts the 'analog' gel image into a digital representation for further computer-based processing.

2.6.2.9. Image analysis of 2-DE gels

Computerized gel analysis using PDQUEST soft ware, was used in order to handle the large amount of data acquired from the 2-DE gels coupled with the need for an objective, reproducible, qualitative and quantitative analysis of the data. Use of PDQUEST enabled the following to be performed:

(a) 2-DE gel image processing (smoothing, contrast enhancement, edge detection and background subtraction),

(b) protein spot detection and quantitation,

(c) gel matching (comparison of identical spots in serial gels enabling comparative analysis of alterations in protein spot expression under various experimental conditions),

(d) normalization of gels (A process of compensating for non-expression related variation in protein spot intensity, important for accurate quantitation),

(e) data analysis by generating analysis sets used to create and study groups of proteins that are scientifically meaningful through determination of qualitative and or quantitative changes in protein expression,

(f) data presentation and interpretation (assigning detected spots molecular mass, M_r and isoelectric point, pI ; comparison of spot quantitation and reflection of alteration trends of spots easily illustrated with graphs, scattergrams and bar charts)

(g) The analysis could be maintained in databases that link this information to the original gel images.

The identified protein spots of interest following PDQUEST computer analysis, were then processed for characterization using biological mass spectrometry as described below.

2.6.2.8. Characterisation of protein spots by MALDI-MS

(This is a modification of the protocol adapted from Courchesne and Patterson [511].)

The work area, microfuge tubes, and all utensils used were cleaned with 50% v/v MeOH/0.1% v/v TFA solution, and allowed to dry. The protein spots of interest identified from preparative gels were carefully excised and sliced into $1mm^2$ pieces before tryptic digestion [483]. Concisely, the chopped gel pieces were washed in 50% acetonitrile/25mM ammonium bicarbonate, pH 7.8, and dried in a vacuum concentrator. To the dried gel pieces, 4-10 μl digestion buffer (10 μg/ml modified sequencing grade trypsin (Promega) in 25 mM NH_4HCO_3 was added and incubated overnight at 37^0C. Resulting peptides were extracted by addition of 4 μl water followed by 7 μl 30% acetonitrile/0.1% triflouro-acetic acid (TFA), vortexing and brief centrifugation. This step could be repeated depending on the

volume of gel pieces. The extracts were concentrated in a vacuum concentrator to approximately 5 μl. The concentrated sample extracts were then mixed 1:1 with matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid (HCCA; Aldrich) in 50% acetonitrile/50% ethanol/0.001% TFA) containing internal standard, adrenocorticotrophic hormone (ACTH, 50 fmol/μl), and 1μl of mixture was loaded unto a 96-position target. Using a MALDI mass spectrometer (M@LDI, Micromass), peptide mass fingerprints were obtained semi-automatically and resultant mass lists searched against non-redundant protein database (Swiss-Prot/Trembl) using ProteinLynx 3.4 (Micromass) software.

CHAPTER 3

THE CELLULAR EFFECTS ON *PLASMODIUM FALCIPARUM* FOLLOWING TREATMENT WITH ARTEMETHER OR LUMEFANTRINE *IN-VITRO.*

3.0. INTRODUCTION.

The principal problem in managing the chemotherapy of malaria is that there are few defined drug targets in the cell. It is apparent that our understanding of the fundamental biology of malaria parasites and the mechanisms of action of antimalarial existing drugs must be broadened. Currently the parasite feeding process, implicating the food vacuole, is the major target which is the proposed site of action of quinoline drugs (such as chloroquine, mefloquine and amodiaquine) [140, 143, 146, 222, 284, 512-521] the main antimalarials used to control *P.falciparum.* However there is increasing resistance to these drugs. Nucleic acid synthesis is another putative target to which antifolates (such as dihydrofolate reductase inhibitors and sulpha drugs) are directed, but resistance has emerged with these too [9, 45, 145, 522-525]. There is therefore urgent need to validate existing drug targets and to develop and characterize new drug targets in order to evolve effective chemotherapeutic strategies against malaria in the future.

Co-artemether, a novel oral fixed combination of two mutually complementary antimalarial agents, artemether and lumefantrine (benflumetol), has proved to be

highly efficacious with an excellent profile of safety and is better tolerated than regimens that contain mefloquine [271]. Until now, albeit artemether is widely used, there is paucity of information explaining artemether (ARM) and lumefantrine (LMF) drug action. This study seeks to characterize the cellular processes associated with drug induced parasite death following treatment of infected erythrocytes with therapeutic concentrations of ARM or LMF and appropriate exposure time.

In this study parasite viability was determined based on the incorporation of [^{3}H] hypoxanthine into the nucleoprotein of the parasites (human erythrocytes infected with *P.falciparum* in continuous culture *in-vitro*) following exposure to a range of therapeutically relevant drug concentrations [439]. This enabled baseline assessment of the sensitivity of different chloroquine sensitive and resistant isolates to LMF, ARM and its active metabolite dihydroartemisinin (DHA) to be established. Subsequently, the drug exposure death relationship was then investigated to define the duration of drug exposure required for irreversible drug toxicity with ARM & LMF *in–vitro*. Such *in-vitro* micro-tests are useful for the monitoring of drug sensitivity of malaria parasites because they are quantitative and largely independent of host factors such as immunity and the bioavailability and disposition of drugs [526].

Having established the relationship between drug exposure and parasite death the morphological consequences of this process were investigated by electron microscopy. The effect of drug exposure on parasite morphology is drug specific [331, 527-531]. It is assumed that the site of earliest ultra-structural changes can

be considered to be the initial site of action of these drugs [147, 330, 519, 529, 530, 532-538].

3.1. METHODS

3.1.1. *In vitro* parasite drug sensitivity assay.

Baseline *in-vitro* drug response assays were carried out with antimalarials: ARM, LMF, DHA, and CQ tested against the CQ sensitive (HB3 & 3D7) and CQ resistant (K1 & TM6) isolates of *P. falciparum*. An adaptation of the method of Trager and Jensen [439] was used to assess parasite viability after treatment with the various drugs used in these *in-vitro* assays. The method relies on the incorporation of [^{3}H]hypoxanthine into nucleic acids by live parasites, and thus inhibition of uptake of the radio-labeled nucleic acid precursor by the parasites serves as the indicator of antimalarial activity.

Estimates of drug concentrations that inhibit 10 %, 20 %, 50 % and 90 % of the uptake of radio labeled hypoxanthine (IC_{10}, IC_{20}, IC_{50}, and IC_{90}, respectively) were derived from non-linear regression analysis. **Table 8,** shows the IC_{50} of each drug for the individual isolates. The geometric mean (IC_{10}, IC_{20}, IC_{50}, IC_{90}) values of ARM and LMF in nano-Molar concentrations are summarized in **table 9**. These values were then used in the subsequent time dependent assays for determination of time and minimum drug concentrations required to achieve irreversible drug toxicity on *P.falciparum*. The drug concentrations that were used in these assays were as indicated in **table 9**.

3.1.2. Characterisation of the time required to achieve irreversible drug toxicity against *Plasmodium falciparum* parasites following ARM & LMF treatment.

Five culture flasks were set up for each of the drugs tested (ARM and LMF). Cultures of *Plasmodium falciparum* K1 isolates were used as the target parasite, maintained at a haematocrit of 2 % and parasitaemia of 2 %, in complete medium. In four of the flasks from each group, the parasites were exposed to a final drug concentration corresponding to their IC_{10}, IC_{20}, IC_{50}, or IC_{90} (**refer to table 9**) as calculated from the dose response curves using the Grafit computer programme package. The fifth flask in each group was the drug free control, maintained under the same conditions as the treated parasite cultures. A total culture volume of 5000 µl per flask was used initially and 400µl samples were removed at time intervals; 0, 1, 3, 5, 8, 12 & 24 hours post drug exposure. Culture was carefully removed and aseptically washed x 3 with complete medium at 2000 g for 1 min, and then re-suspended in complete medium. Aliquots of 100 µl of these samples (in triplicate) were then put into 96 micro-well micro-titre plates. 5µl (0.25 µCi/ml) of the reconstituted [^{3}H] hypoxanthine solution was added to the samples in each well after 24 hr incubation period and then re-incubated for a further 24 hrs. All flasks and micro-wells were incubated at 37^{0}C and gassed for 30 seconds prior to each incubation period. Parasite viability at the various drug concentrations and drug exposure times was measured by assessment of [^{3}H] hypoxanthine incorporation into parasite nucleic acids as described by Desjardins et al [439]. Samples were processed for scintillation

counting as described in **section 2.3.** Parasite viability after drug exposure was expressed as a percentage of the control growth (drug free culture).

3.1.3. *Plasmodium falciparum* ultra-structural studies: Electron microscopy

A modified protocol of the one described by Langreth et al [443], was adapted for these investigations. The infected erythrocytes were processed for electron microscopy as detailed in section 2.5.

3.2. RESULTS

3.2.1. *In-vitro* sensitivity of different *P. falciparum* isolates to DHA, ARM, CQ and LMF antimalarial drugs.

Table 8 Shows the sensitivity of CQ sensitive isolates (HB3 and 3D7), and CQ resistant isolates (K1 and TM6) to different antimalarial drugs (CQ, ARM, DHA and LMF) *in-vitro*. The results show that DHA and its methyl derivative, artemether (ARM) are highly potent against both CQ sensitive and CQ resistant isolates, with mean IC_{50} values less than 10 nM against all isolates tested. LMF also is a potent antimalarial, effective at nM concentrations though less potent than DHA and ARM. The results also show an inverse relationship between the CQ sensitivity pattern and that of LMF. This type of relationship has been seen with other phenanthrene and quinoline methanol structures [539]. The CQ resistant isolates show greater sensitivity than the CQ sensitive isolates to LMF.

DRUG	HB3 ISOLATE	3D7 ISOLATE	K1 ISOLATE	TM6 ISOLATE
CQ	13.6 ± 4	27.0 ± 3.35	140.22 ± 13.4	99.17 ± 8.22
ARM	2.8 ± 0.1	4.73 ± 0.17	5.7 ± 0.32	5.6 ± 0.31
DHA	1.12 ± 0.04	1.53 ± 0.14	2.01 ± 0.02	1.69 ± 0.1
LMF	279.23 ± 9.71	208.81 ± 3.03	123.41 ± 11.77	37.14 ± 2.05

Table 8. Shows in-vitro sensitivities of HB3 & 3D7 (CQ sensitive strains) and K1 & TM6 (CQ resistant strains) of Plasmodium falciparum parasites to CQ, ARM, DHA and LMF. The data represents mean ± sd of IC50 values (nM) derived from at least 3 different assays.

Antimalarial drug	IC_{10} (nM)	IC_{20} (nM)	IC_{50} (nM)	IC_{90} (nM)
Artemether	4.33 ± 0.54	4.8 ± 0.35	5.7 ± 0.32	7.7 ± 0.38
Lumefantrine	72.39 ± 12.31	87.98 ± 11.82	123.41 ± 11.77	212.73 ± 12.45
Chloroquine	39.65 ± 5.62	66.01 ± 8.62	140.22 ± 13.4	432.19 ± 14.23

Table 9. Showing geometric mean IC10, IC20, IC50 and IC90 of ARM, LMF and CQ derived from linear regression analysis in the K1 parasite.

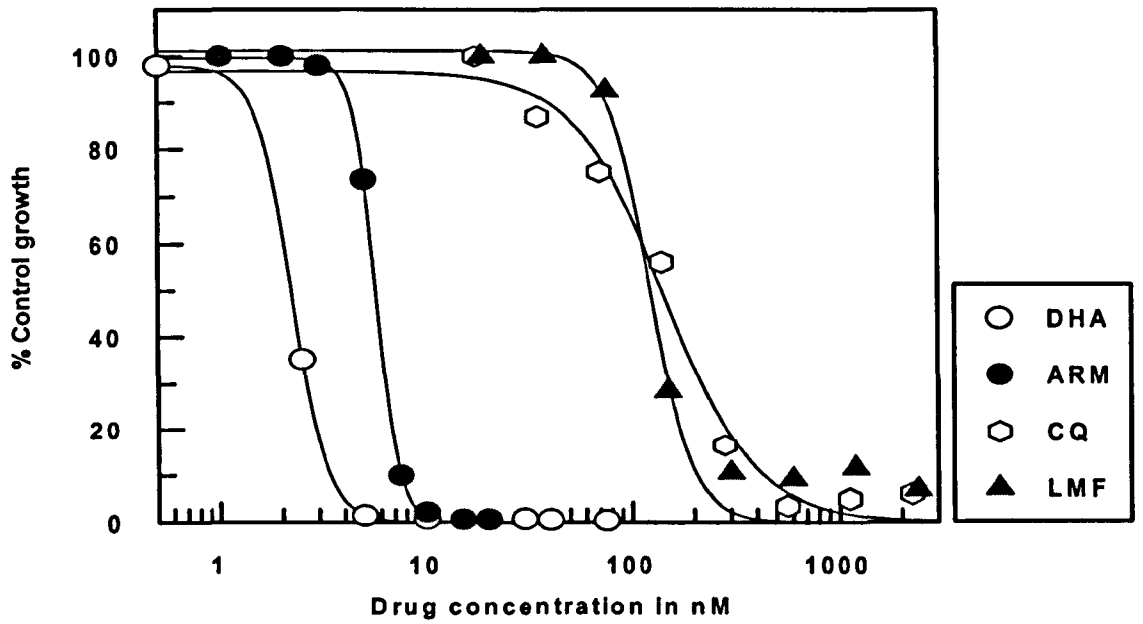


Figure 3.1. Showing the relationship between the sensitivity K1 (CQ resistant isolate) to DHA, ARM, CQ, & LMF.

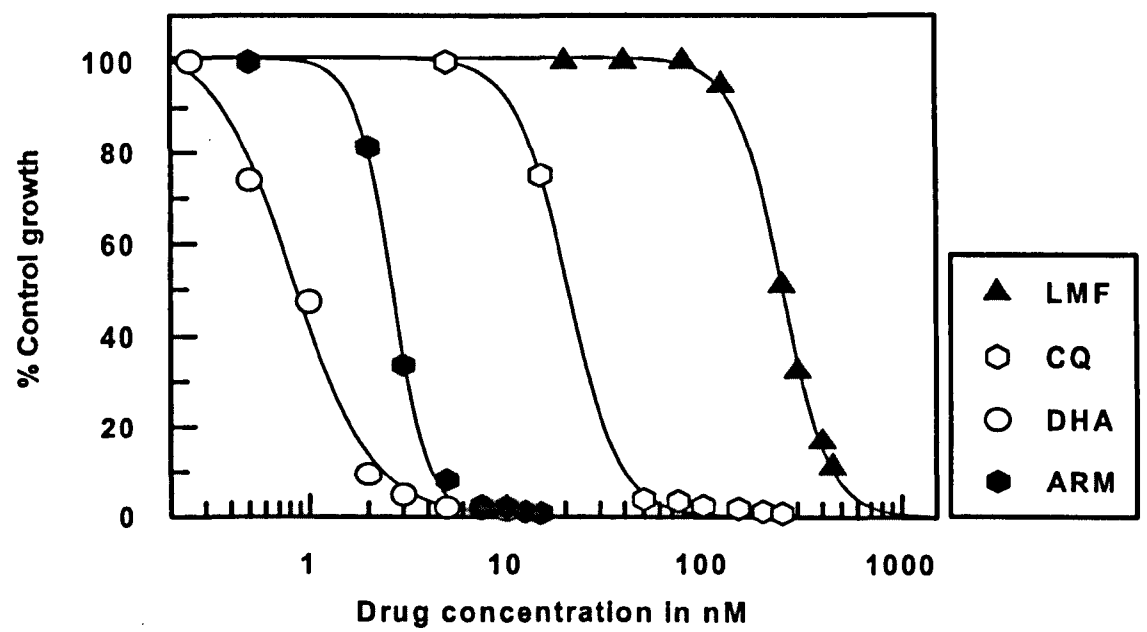


Figure 3.2. Showing the relationship between the sensitivity of HB3 (CQ sensitive isolate) to DHA, ARM, CQ and LMF.

3.2.2. Characterisation of the time required to achieve irreversible toxicity of ARM and LMF against K1 isolates of *P.falciparum*.

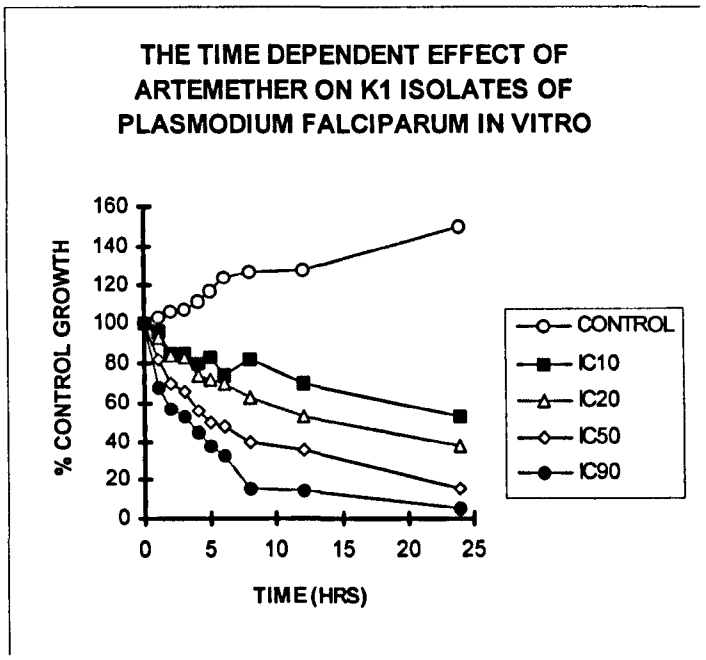


Figure 3.3

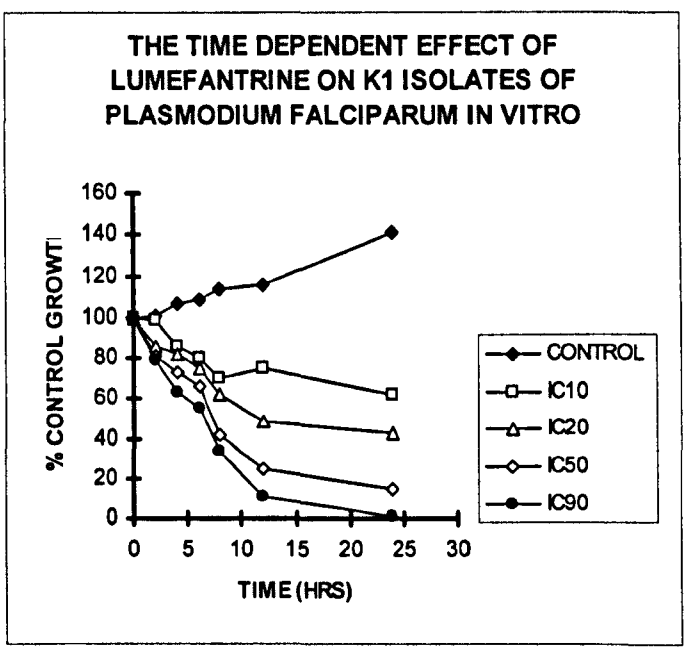


Figure 3.4

Figures 3.3 & 3.4, show the time dependent inhibitory effects of ARM and LMF used in varied concentrations, on the growth of *P. falciparum* K1 isolate following exposure to drug at IC_{10}, IC_{20}, IC_{50}, or IC_{90} concentrations.. The untreated control cultures produced continuous growth. The results of the treated cultures indicate that ARM is a fast acting antimalarial (effective at less than 10 nM concentrations) with remarkable effects in the first hour of exposure and that after 5 hours of exposure, the parasites are irreversibly damaged with ARM drug concentrations ≥ 5 nM. There is however significant parasite survival (about 40-60% of control growth) at IC_{10} and IC_{20} **(refer to table. 9.)** even after 24 hrs of exposure to ARM. LMF too displays potent inhibitory effects but at a higher drug concentration. In contrast to ARM, the inhibitory effect of LMF occurs more slowly with noticeable effects seen after 3 hours and the parasites are irreversibly inhibited after 8-12 hours of exposure to the drug. There is significant residual parasite growth at sub-therapeutic concentrations (IC_{10} & IC_{20}) comparable to that observed in the ARM treated group.

3.2.3. *P.falciparum* ultra-structural changes following ARM & LMF treatment using Electron Microscopy.

3.2.3.1. Control electron micrographs of *P. falciparum* (K1 isolate).

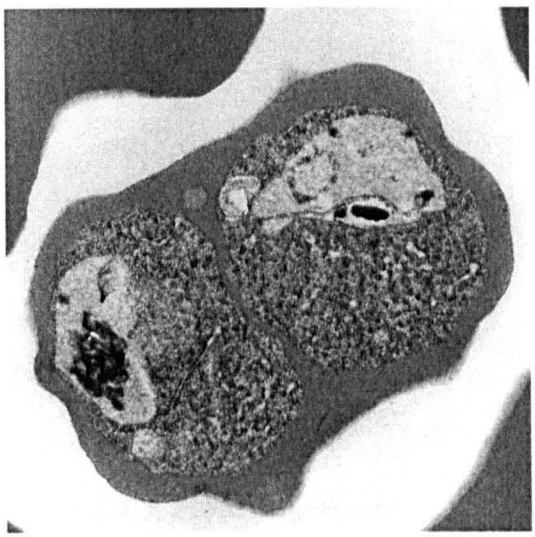


Figure 3.5. X 15500 magnification.

Indicates an erythrocyte with double infection 24 hrs post invasion

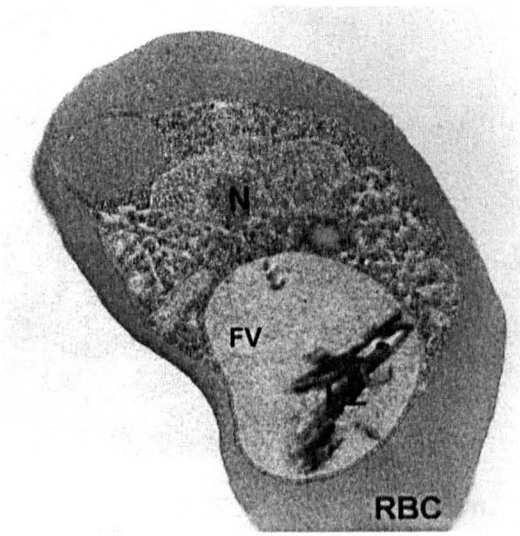


Figure 3.6. X 15500 magnification.

27 hrs post invasion

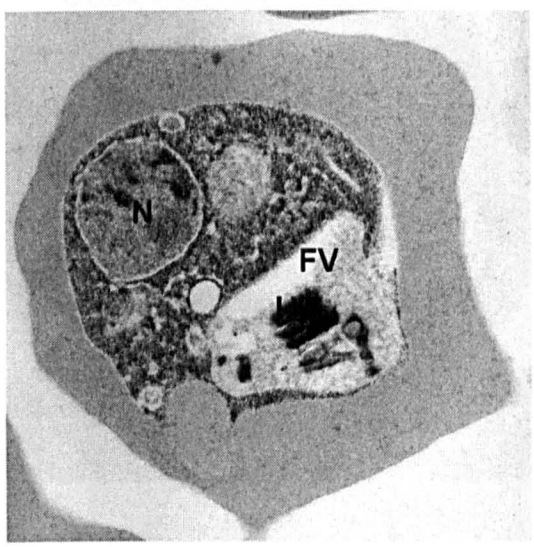


Figure 3.7. X 15500 magnification.
29 hrs post invasion

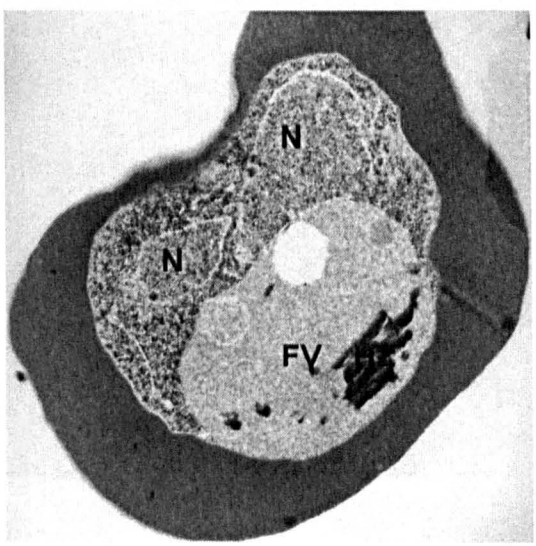


Figure 3.8. X 15500 magnification.
32hrs post invasion

3.2.3.2. Electron micrograph showing the effect of ARM (IC20) on *P.falciparum* (K1 isolate).

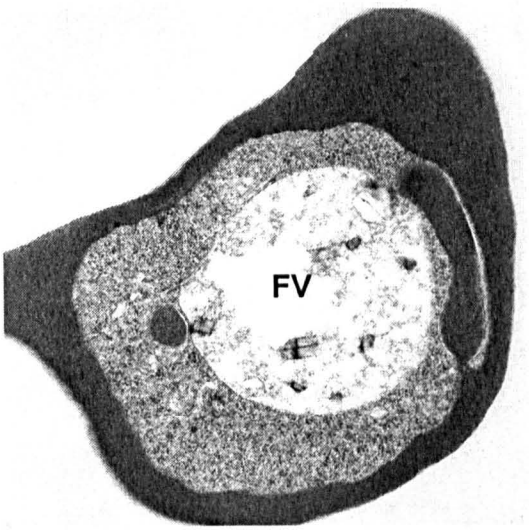


Figure 3.9. X 21000 magnification.
1 hr post drug exposure

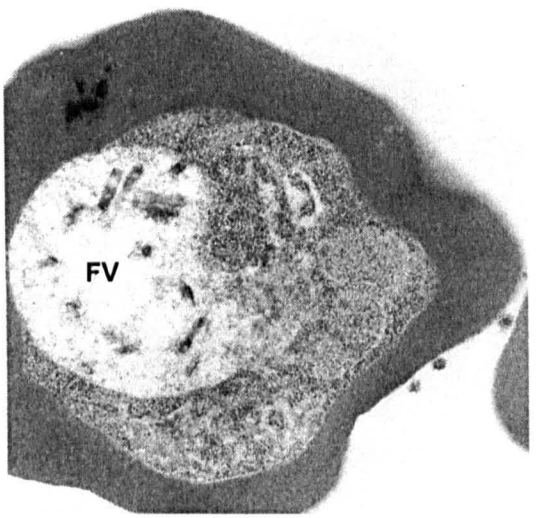


Figure 3.10. X 21000 magnification.
3 hr post drug exposure

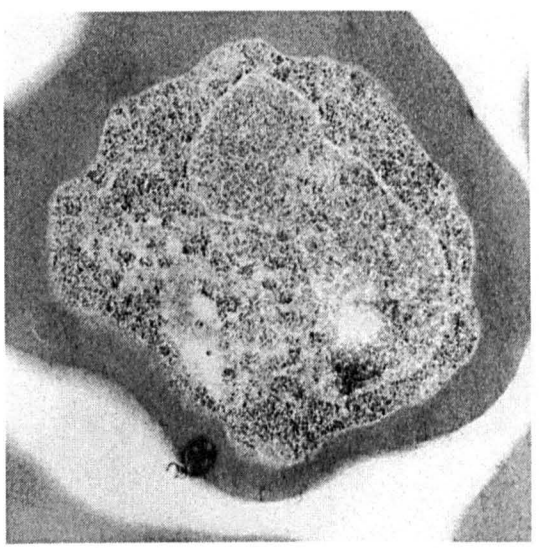


Figure 3.11. X 21000 magnification.
5 hr post drug exposure

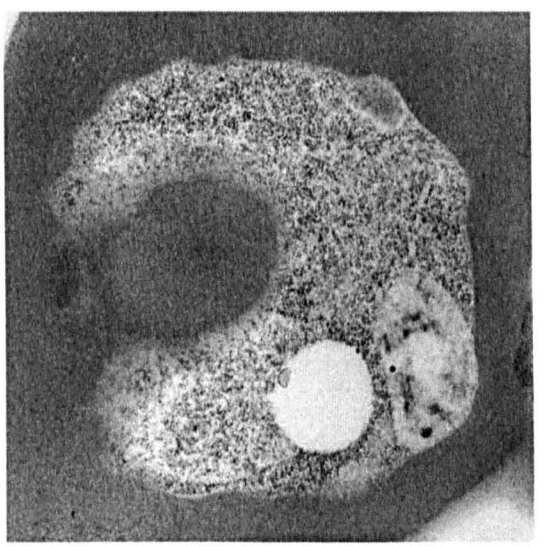


Figure 3.12. X 15500 magnification.
24 hr post drug exposure.

3.2.3.3. Electron micrographs showing the effect of ARM (IC 50) on *P.falciparum* (K1 Isolate).

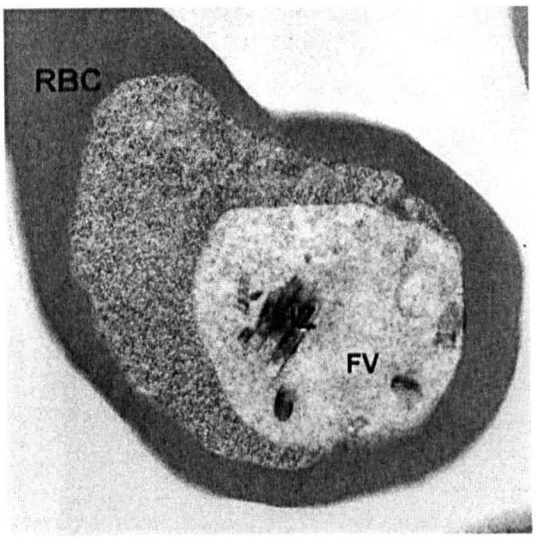


Figure 3.13. X 21000 magnification.
1 hr after exposure to drug.

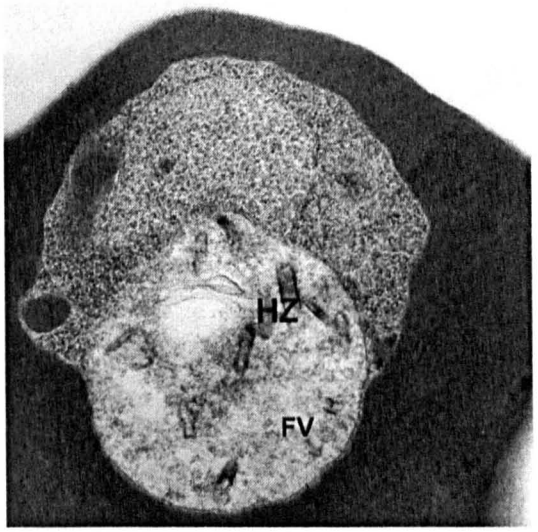


Figure 3.14. X 21000 magnification.
3 hr after exposure to drug.

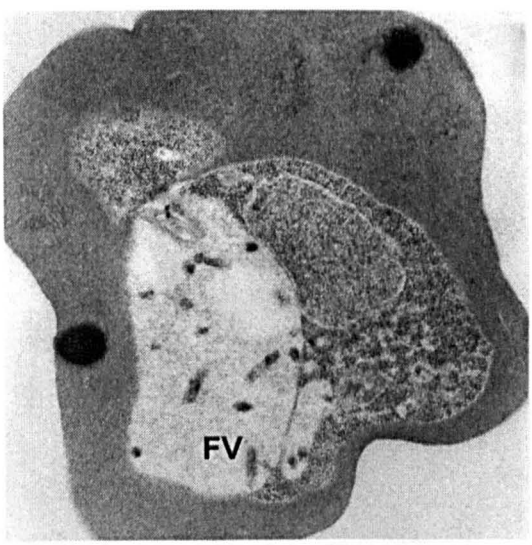


Figure 3.15. X 21000 magnification.
5 hr after exposure to drug.

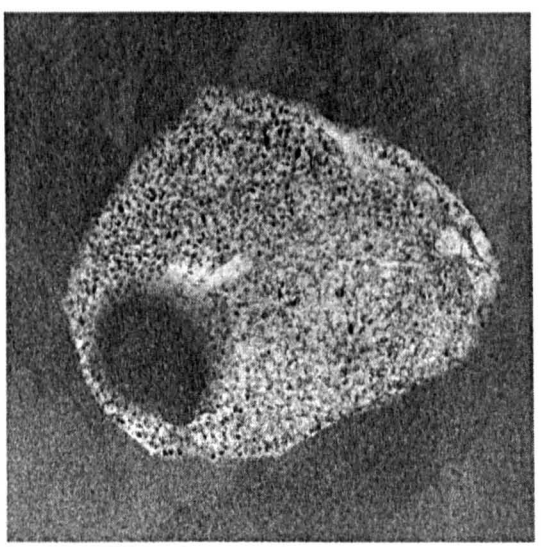


Figure 3.16.X 39000 magnification.
24 hr after exposure to drug.

3.2.3.4. Electron micrograghs showing the effect of ARM (IC 90) on *P.falciparum* (K1 isolate).

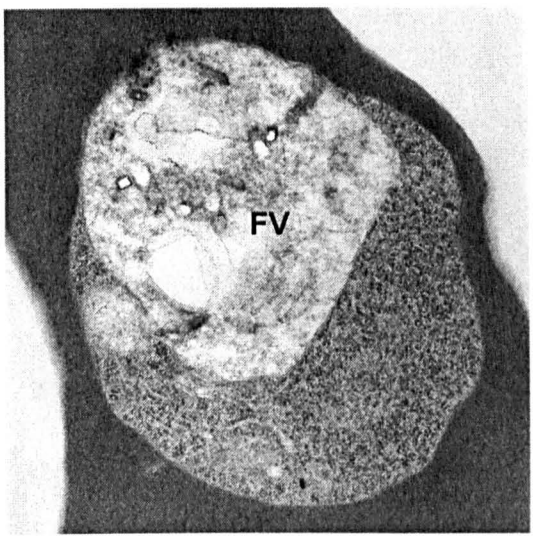


Figure 3.17.X 21000 magnification.

After 1 hr of exposure to drug.

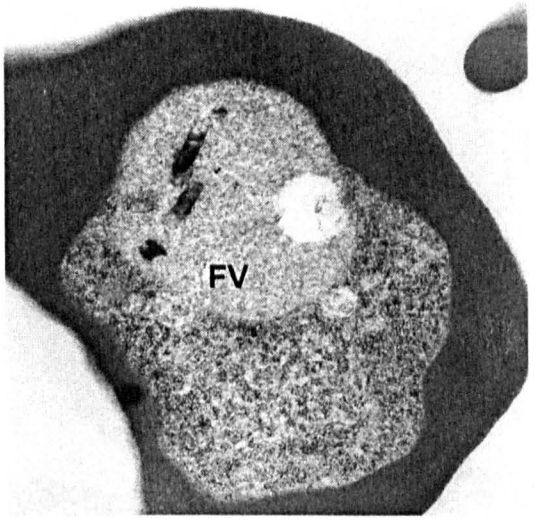


Figure 3.18. X 21000 magnification.

After 3 hr of exposure to drug

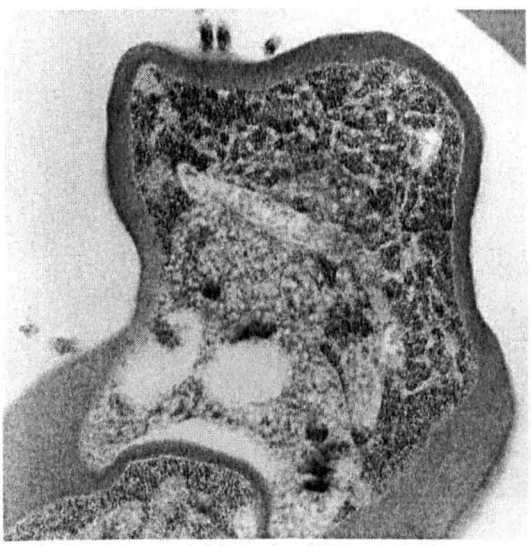


Figure 3.19. X 21000 magnification.

After 5 hr of exposure to drug.

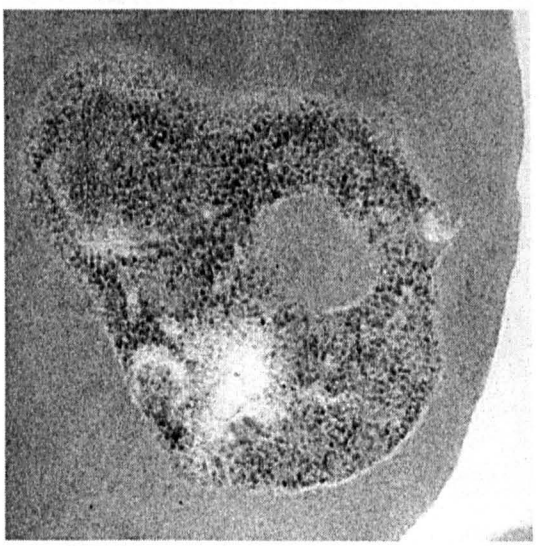


Figure 3.20.X 39000 magnification.

After 24 hr of exposure to drug

3.2.3.5. Electron micrographs showing the effects of LMF (IC 20) on *P.falciparum* (K1 isolate).

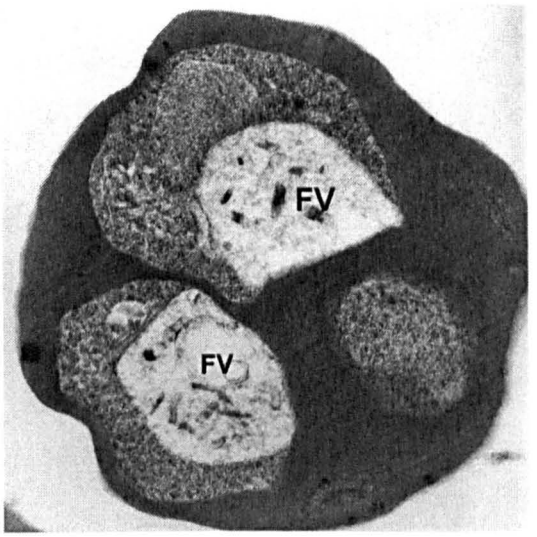


Figure 3.21. X 15500 magnification.
1 hr after exposure to drug.

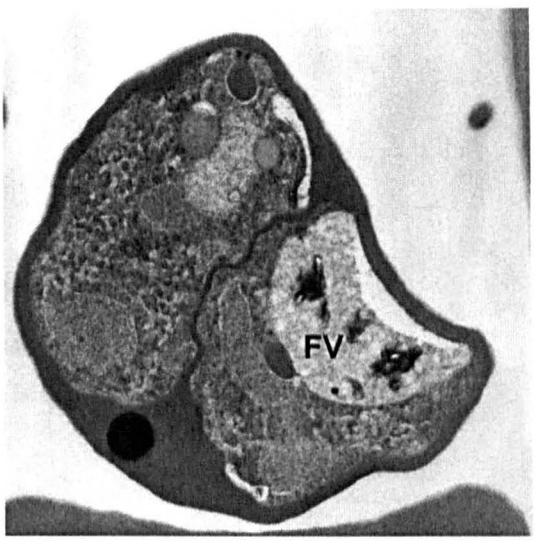


Figure 3.22. X 8900 magnification.
3 hr after exposure to drug.

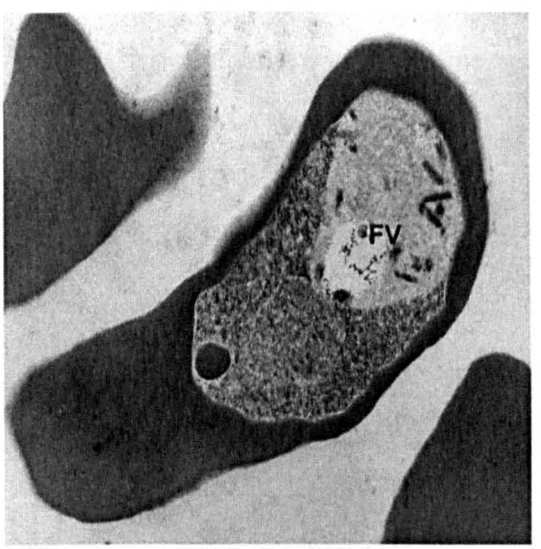


Figure 3.23. X 11500 magnification.
5 hr after exposure to drug.

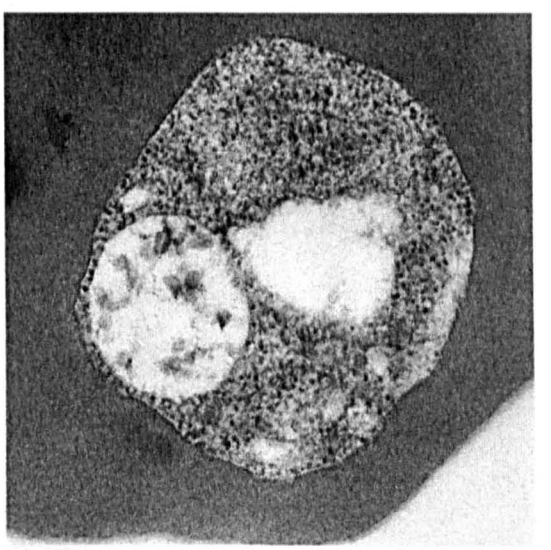


Figure 3.24. X 28500 magnification.
24 hr after exposure to drug.

3.2.3.6. Electron micrographs showing the effects of LMF (IC 50) on *P.falciparum* (K1 isolate).

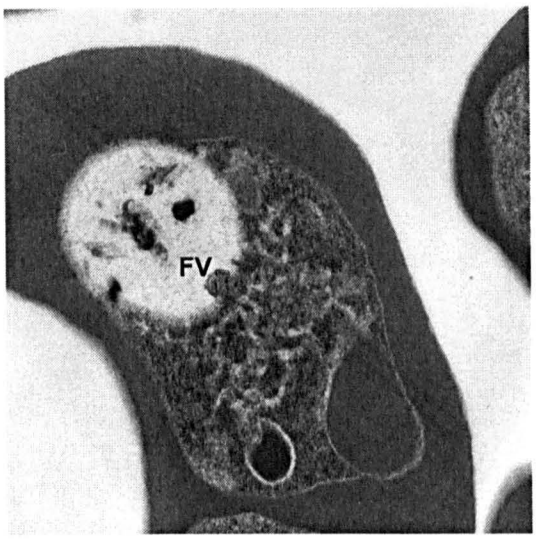


Figure 3.25. X 8900 magnification.
3 hr after exposure to drug.

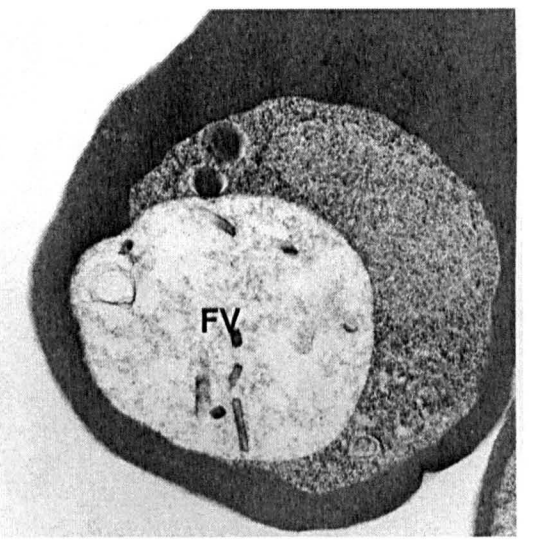


Figure 3.26. X 21000 magnification.
5 hr after exposure to drug.

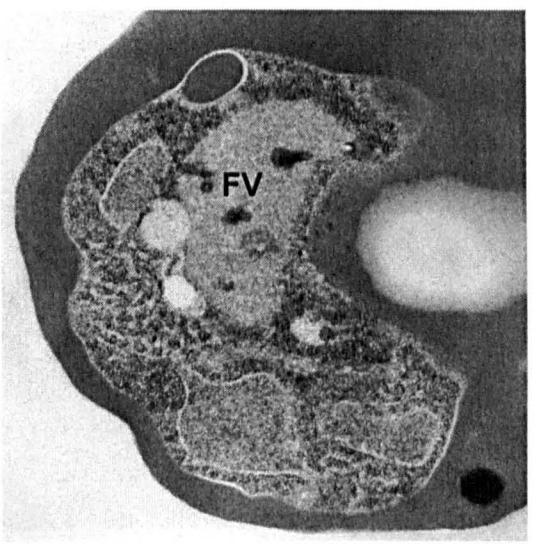


Figure 3.27. X 21000 magnification.
8 hr after exposure to drug.

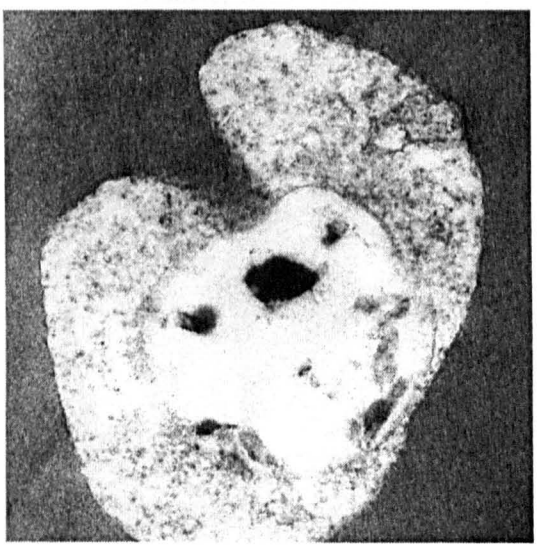


Figure 3.28. X 39000 magnification.
24 hr after exposure to drug.

3.2.3.6. Electron micrograph showing the effects of LMF (IC 90) on *P.falciparum*.

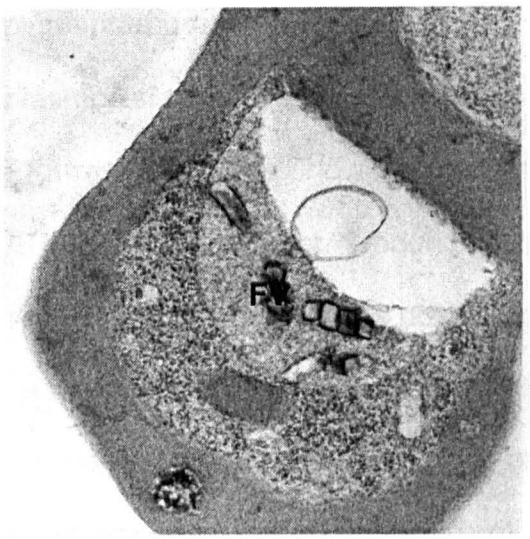


Figure 3.29. X 28500 magnification.
After 1 hr of exposure to drug.

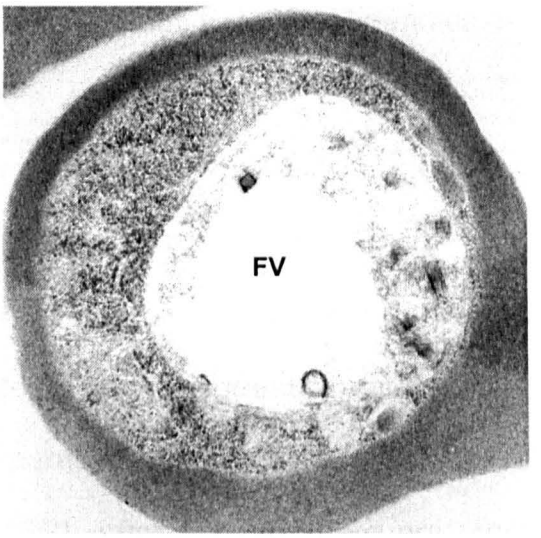


Figure 3.30.X 28500 magnification.
After 3 hr of exposure to drug.

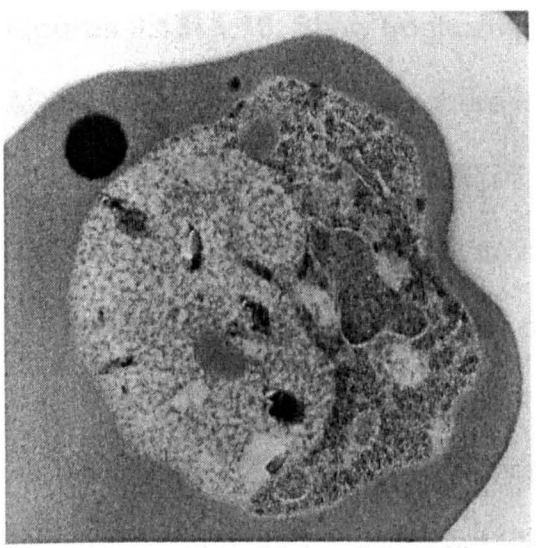


Figure 3.31. X 21000 magnification.
After 5 hr of exposure to drug.

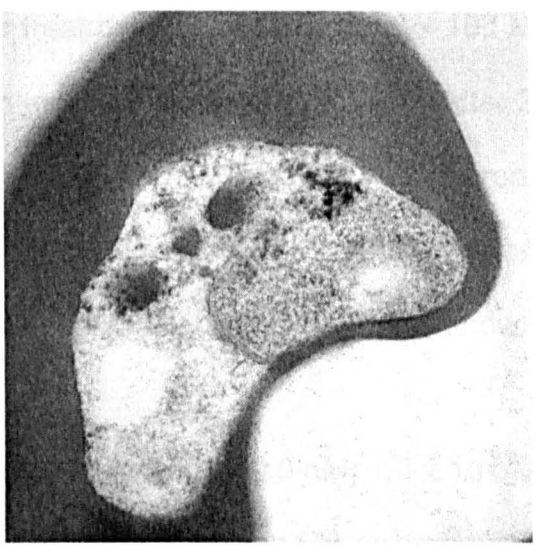


Figure 3.32.X 21000 magnification.
After 24 hr of exposure to drug.

Figures 3.5- 3.8.

Control electron micrographs show *P. falciparum* trophozoites with normal non-dilated food vacuoles (FV) and increasing amounts of crystalline haemozoin (HZ) pigment and granular material filling the parasitophorous vacoule. N denotes the plasmodial nucleus.

Figures 3.9- 3.12. Show trophozoites after treatment with (4 nM) 4.0 x 10^{-9} M ARM (IC_{20}), and after 1 hour of exposure there is some swelling of the food vacoule (FV). After 3 hours post exposure there is again enlargement of the food vacuole and the haemozoin pigment is scattered with less granular material filling the food vacuole compared to control parasite. There is also noticeable enlargement of the endoplastic reticulum (ER). After 5 hours there are gross degenerative changes involving both the nucleus and the cytoplasm as in figs. 3.11 & 3.12.

Figures 3.13- 3.16. Show trophozoites after treatment with (6.0 nM) 6.0 x 10^{-9} M ARM (IC_{50}), and after 1 hour there is again swelling of the FV, and ER. After 3 hours, there is progressive FV & ER enlargement, and HZ pigment is scattered. After 5 hours gross degenerative changes involving both the nucleus and the cytoplasm have occurred, indicative of severe *Plasmodial* cell damage (as shown in figures. 3.15 & 3.16).

Figures 3.17- 3.20 show trophozoites after treatment with (10.0 nM) 1.0 x 10^{-8} M ARM (IC_{90}), and shows significant FV swelling after 1 hour and ER enlargement. After 3 hours, there is shrinking of FV with less HZ pigment granules, and gross

distension of the ER. After 5 hours there is overt necrosis and disintegration of the parasite (as shown in figs. 3.19 & 3.20).

Figures 3.21- 3.24 show trophozoites after treatment with (80.0 nM) 8.0 x 10^{-8} M.LMF (IC_{20}). There is some minimal FV enlargement becoming more apparent after 5 hours (figure. 3.23). After 24 hrs there is FV distension and cytoplasmic lytic vacoules in some *Plasmodial* cells (figure. 3.24).

Figures 3.25- 3.28 show trophozoites after treatment with (200.0 nM) $2x10^{-7}$ M LMF (IC_{50}). After 3hours, there is progressive FV enlargement, gradual reduction in HZ pigment granules, and the matrix in the FV becomes increasingly granular. After 24 hrs the *Plasmodial* cell is grossly damaged with an almost empty FV as the only recognisable structure in some cells (figure. 3.28.).

Figures 3.29- 2.32 show trophozoites after treatment with (340.0 nM) 3.4 $x10^{-7}$ M LMF (IC_{90}). There is FV enlargement seen after 1 hour post exposure and there is gradual reduction in HZ pigment granules and enlargement of the ER (Figs. 3.29 & 3.30). After 5 hours the FV begins to shrink, with sparse HZ granules and is more electron-lucent, and the ER is more prominent (Fig. 3.31.). After 24 hrs post-treatment, the parasite is grossly damaged and intracellular organelles cannot be identified.

3.3. DISCUSSION

Co-artemether, shows potential being one of the most effective antimalarials currently available for multi-drug-resistant *falciparum* malaria [276, 369, 370, 424, 425, 429, 540]. Little information however is available on the mechanism(s) of drug action of its components. This part of the study provides some evidence on possible cellular targets of ARM and LMF action in *P.falciparum* parasites.

The sensitivity studies clearly showed that ARM and its active metabolite, dihydro-artemisinin (DHA) have very potent antimalarial activity *in-vitro* against both CQ sensitive and CQ resistant isolates as reported by many others [13, 366, 374, 541-543]. LMF was however is less potent but effective, and an inverse relationship between parasite sensitivity to CQ and sensitivity to LMF was observed. A similar observation has been documented with other class II blood schizonticides such as mefloquine, halofantrine, and quinine, [215, 544-548], this may be indicative of a common mechanism of action. ARM displays a very rapid inhibition effect evident after 1hr of exposure which is irreversible after 5 hr exposure at IC_{50} and IC_{90} concentrations. LMF is a less potent antimalarial and is relatively slow in action, with effects observed after about 3hrs of drug exposure which are irreversible effects after 8-12 hours exposure at IC_{50} and IC_{90} drug concentrations. Sub-optimal concentrations, IC_{20} and IC_{10} have an initial effect but as expected there is significant residual parasite growth. The sensitivity of the test for direct *in-vitro* effects of the drug on parasite growth and viability is directly comparable to the *in-vivo* drug concentration [549], and therefore in the clinical setting such suboptimal concentrations have to be avoided.

Previous studies have shown that the endoperoxide bridge is critical for artemisinin compounds' activity [187, 375, 550]. Endoperoxides are reported to undergo reductive cleavage which requires the presence of low-valency transition metals, probably iron (II) in the reduced form of haemin (ferrous haem [ferroprotoporphyrin IX, Fe(II)PPIX]), or exogenous non-haem iron(II) to generate oxygen-centred (O-centred) radicals [342]. The O-centred radical, as a potent hydrogen-abstracting agent, generates C-centred radicals by intramolecular hydrogen atom abstraction. The resultant C-centred radicals are then thought to react with biomolecules. The endoperoxide bioactivated radicals have been documented to interact with haem and haemozoin [327, 551], but it is argued that these interactions per se may not explain the endoperoxides' killing action, rather the associated free radical damage of membranes is implicated [183, 327, 340, 551, 552]. Studies on the ultrastructure of *P.berghei in-vivo* have shown artemisinin causing changes in the limiting and other membranes of the parasite, together with alterations in ribosomal organization and endoplasmic reticulum of the parasite within 30 min of administration to the host mice [331]. Ye and colleagues [538], report injury to the membrane structures being a pathognomonic feature of artemisinin action on the *P.falciparum* parasite, along with formation of the autophagic vacuoles containing no pigment, while that of CQ results in the formation of autophagic vacuoles containing pigment and the appearance of food vacuole like-structure. Other studies on the ultrastructure of *P.falciparum in-vitro* [330], revealed concentration of the drugs in the parasite food vacuole and mitochondria, and changes occurring in the mitochondria,

nuclear and food vacuole membranes after 2 h of artemisinin and DHA administration. The later studies were however, carried out with greater than tenfold higher concentrations than the therapeutically relevant drug concentrations. Toshiro et al [553] also carried *in-vitro* ultrastructural studies at still higher concentrations of artemether of 50 - 100 nM on *P. falciparum,* and observed that artemether causes extensive injury to the cellular and subcellular membranous structures of *P.falciparum* and suggested lipid peroxidation as the mechanism of toxicity. Morphological studies of effects of ARM on *P.falciparum* in an owl monkey (*Aotus trivirgatus*) model attributed the rapid onset of drug action to mitochondrial damage. They suggested this was the primary target causing impairment of oxidative phosphorylation. Changes in membranous structures including FV were considered secondary to mitochondrial damage. Thomas Akompong and colleagues [554] later documented contrasting findings indicating that artemisinin had no effects on the food vacuole ultrastructure or its contents at pharmacologically effective concentrations. They suggested that the drug alters a subset of the membrane transport properties of the tubovescicular membrane network and the parasitophorous vacuolar membrane, which are thought to be involved in the delivery of essential extracellular nutrient solutes to the parasite however activity was not blocked by inhibitors of the tubovescicular membrane network. Results from some earlier study however had showed that artemisinin had no effect on phase transitions in artificial membranes and inferred that the antimalarial effect of artemisinin is not attributed to the damaging lipid structure of parasite membranes [555]. In these present studies, we clearly

observe changes in the food vacuoles as part of the initial changes after exposure of *P.falciparum* parasites to therapeutic concentrations of ARM The IC_{50} of ARM in this K1 isolate of *P.falciparum* is 6.0 nM while that of LMF is 120 nM, and parasite cells were treated with 4 – 10 nM ARM and 88 – 213 nM LMF and hence these studies were conducted with therapeutic concentrations of these drugs. Electron microscopy results show that ultra-structural cellular damage correlates with drug concentration and time of exposure to drug, and thus use of very high doses as used by other workers may have distorted the morphological alterations.

The most distinctive ultrastructural changes following administration of ARM were swelling of the food vacoule (FV) occurring as early as 1 hour post exposure, followed by less marked progressive reduction in haemozoin pigment and endoplastic reticulum enlargement. These findings concur with the earlier reports that artemisinins may at least in part, exert their effect by interfering with the *Plasmodial* haem metabolic machinery [163, 346, 352, 550, 556], probably by inhibition of digestive vacuole proteolytic activity of malarial parasite.

LMF is a class II blood schizonticide, and the currently accepted view is that LMF exerts its antimalarial effect as a consequence of its interaction with ferriprotoporphyrin IX [557]. Ultrastructural results in these studies show that LMF morphological changes are evident after 3hours with significant changes being the FV enlargement with gradual loss of pigment granules. These observations suggest that LMF possibly has common targets with other quinoline drugs [519, 520, 528, 558-562] i.e. *Plasmodial* parasite feeding process. LMF is structurally

and physico-chemically related to the aryl amino group of antimalarial drugs, including quinine, mefloquine, and halofantrine. These similarities together with the reminiscent morphological features may further support a common mechanism of action of these drugs.

These Electron Microscopic (EM) results suggest that the *Plasmodial* digestive vacuole is a primary target for both ARM and LMF, however the damage to the FV may follow different mechanism(s) for the two drugs. Changes in the FV are evident even with the lowest drug concentration (IC_{20}) used for both drugs. The *Plasmodium* digestive vacuole plays a pivotal role in the metabolism of the parasite. The food vacuole accommodates a number of biochemical activities such as haemoglobin degradation, haem crystallisation, amino acid transport, oxygen radical detoxification, accumulation and efflux of small molecules, maintenance of physiological vacuolar pH gradients (acidification) and generation of free toxic iron. Any of these processes are vulnerable to the effects of these drugs, but which of these processe(s) is responsible for the toxicity of these drugs to the *Plasmodial* cell? The progressive reduction in haemozoin pigment is observed with the two drugs but is more pronounced with the LMF treated parasites which may suggest implication of the haemoglobinolytic pathway. These findings are in line with the notion that LMF complexes with haem and has effects on haemozoin formation. The morphological differences displayed by LMF compared to CQ may relate to LMF being a class II schizonticide as opposed to CQ which is a class I schizonticide. ARM and DHA on the other hand are thought to use Fe^{++} catalytically to generate carbon centred radicals and are

therefore different from LMF, but their activation may be dependent on Fe^{++} derived from haemoglobin catabolism.

Although the Electron Microscopy morphological alterations suggest possible involvement of the haemoglobinolytic pathway, EM per se is unable to provide evidence of a definitive mechanism of action of these drugs. Further work is done in the subsequent chapters to supplement these observations.

CHAPTER 4

THE ROLE OF INTRACELLULAR IRON IN THE MECHANISMS OF ARTEMETHER AND LUMEFANTRINE ANTIMALARIAL ACTION.

4.0. INTRODUCTION

Initial work in chapter three implicates the *Plasmodial* food vacuole as a common intra-cellular target for both ARM and LMF, however, this evidence is inadequate for a definitive mechanism of action. This organelle is the cellular site where metabolic processes that are critical for the survival of the parasite are reported to take place [139]. These include haemoglobin degradation, haem bio-crystallization, maintenance of acidification, and release of free iron among other processes. During the hemoglobinolytic process, most of the haem that is released is crystallized into haemozoin. There is evidence that a fraction of this haem loses its iron [563]. This process has been suggested as a potential source for the parasite's ferro-proteins, notwithstanding that non-haem iron may be essential for parasite growth. Work done by previous researchers suggest that haem plays a critical role in the mechanism of action of artemisinins [187, 352, 375] and other antimalarial drugs, including 4-aminoquinolines such as chloroquine [285, 286], quinoline methanols such as mefloquine [512, 564], and phenanthrene methanols such as halofantrine [512, 564]. This data coupled with the observations described in chapter 3 have prompted an investigation of the role of intracellular iron in the mechanism of action of ARM and LMF.

The trace element iron is essential for the rapid proliferation of the parasite, where it is required for growth and replication, in particular for the synthesis and function of iron-containing proteins such as ribonucleotide reductase [565]. It is assumed that malaria parasites akin to other microorganisms obtain their iron in two general ways; by non-specific, low-affinity transport, requiring relatively large amounts of iron outside the cell: and by specific, high-affinity transport, which can accumulate iron even when it is present in small amounts or sequestered by a competing ligand like transferrin [566].

The sources of iron available to intraerythrocytic *Plasmodia* are however debatable [567-572], and several possible sources have been suggested including: (a) plasma transferrin bound iron; (b) iron derived from erythrocyte host ferritin; (c) a labile intra-erythrocytic iron pool, and; (d) iron derived from the catabolism of host haemoglobin in the food vacuole of the parasite. There is stronger evidence in favour of the last two sources [173, 573-577]. Loria and colleagues recently reported that in *Plasmodium falciparum* infected erythrocytes only about one third of the haem is polymerized to form haemozoin. The remainder appears to be degraded by a non-enzymic process which leads to an accumulation of iron in the parasite [513].

The mechanism of action of artemisinin antimalarials is postulated to be dependent on the presence of iron. These compounds contain a unique endoperoxide bridge which is essential for their antimalarial activity [187, 578-

580]. The presence of intraparasitic haem and iron is thought to activate the artemisinin compounds into free radicals and other electrophilic intermediates which then alkylate specific malaria target proteins [183, 184, 187]. The interaction of artemisinin and haem is thought to account, at least in part, for the selectivity of the drug since high levels of haem are acquired by the parasite as a consequence of haemoglobin catabolism [157]. Previous work done on artemisinin and arte-ether has shown that iron chelation antagonizes the antimalarial effect of these drugs *in-vitro* and also prevents the toxic effects of artemisinin in mice [327], suggesting that iron rather than haeme per se plays a role in the mechanisms of action and toxicity of artemisinin.

LMF is also postulated to target the parasite-based machinery for host cytosol digestion and hemoglobin catabolism. LMF is a class II aryl amino alcohol blood schizontocide structurally related to quinine, mefloquine, and halofantrine. LMF antimalarial action is attributed to its interaction with haeme [557].

In these studies three iron chelators (deferoxamine-hydroxyethyl starch (DFO-HES), (deferoxamine) desferrioxamine (DFO), and CP94.HCl), each of which have different permeability properties, were used as probes to investigate the importance of iron to the survival of the parasite, and subsequently it's role in the mechanism of action of ARM and LMF. Previous work has shown that Iron chelator treatment of erythrocytes infected with *Plasmodium falciparum* selectively interferes with iron-dependent metabolism of malaria parasites and inhibits their development [200]. Antimalarial iron chelators are postulated to act variously, implicating several potential targets in the *Plasmodial* cell. The

antimalarial modes of action of iron chelators hinges on their ability to penetrate the parasite and sequester their iron.

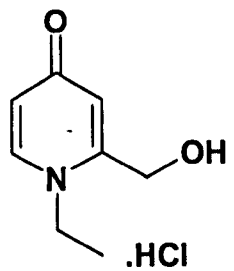
Deferoxamine-hydroxyethyl starch (DFO-HES) is a large molecule, a conjugate of deferoxamine (DFO) and hydroxyethyl starch (HES). The iron-binding properties of DFO are virtually unchanged after the attachment of a bio-compatible polymer like hydroxyethyl starch (HES) [581]. DFO-HES unlike desferroxamine, it is unable to cross the erythrocyte membrane [582]. DFO-HES was used in this study to selectively chelate the extracellular iron. Desferrioxamine per se also has poor ability to penetrate mammalian cells including normal erythrocytes, but is capable of entering parasitized cells [582] and in this study DFO was employed to study effects of chelation of intraerythrocytic iron. On the other hand CP94.HCl is a small molecule which is highly lipid soluble and thus easily penetrates both erythrocyte and *plasmodial* cell membranes. CP94.HCl was assumed to have access to both the intraerythrocytic and parasite-associated iron.

Materials and Methods

Reagents

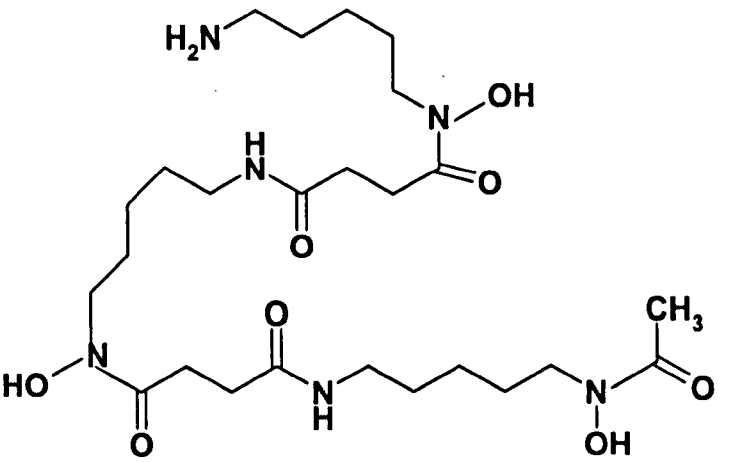
CP94.HCl and deferoxamine-hydroxyethyl starch (DFO-HES) were a gift received from Dr. W. Graham McLean, Department of Pharmacology Therapeutics, Liverpool University, Liverpool UK. The deferoxamine (desferrioxamine B, DFO) used in this work was purchased from Sigma.

The chemical structures of the iron chelators, CP94.HCl and deferoxamine (DFO) are shown below.



CP94.HCl

(a)



Desferrioxamine (DFO)

(b)

Figure. 4.1.

4.1.2. Parasite maintenance

The K1, CQ resistant parasite, was used in these experiments maintained in culture as described in chapter 2, section 2.1.6.

In-vitro antimalarial activity of different antimalarials (ARM, DHA, LMF and CQ) and iron chelators (CP94.HCl), desferrioxamine (DFO) and deferoxamine-hydroxyethyl starch (DFO-HES).

The dose dependent effects of each drug on parasite viability was assessed using a modification of the method described by Desjardins [439] as described in detail in chapter 2, section 2.2.4.

4.1.3. Effects of chelator on the antimalarial activity of ARM, DHA, LMF and CQ in vitro.

The effects of the highly lipid soluble chelator, CP94.HCl on the antimalarial activity of ARM, DHA, and LMF, using CQ as a positive control was assessed by isolobologram analysis as described in chapter 2, section 2.3.2.

4.2. RESULTS.

The IC_{50} for each drug used is reported in chapter 3. The IC_{50} for each iron chelator is presented in **table 10** and figure 4.2. The membrane permeability of each chelator is in the following order: CP 94.HCl is the most lipophilic and also bears the smallest molecular structure (refer to figure 4.1); desferrioxamine (DFO) is less lipophillic and has a fairly large molecular structure (refer to figure. 4.1); and deferoxamine-hydroxyethyl starch (DFO-HES) is not lipophilic, and is the least membrane permeable. DFO-HES also bears the largest structure of the three chelators used in these studies. Of the iron chelators, CP94.HCl was the most effective antimalarial with an IC_{50} of 1.25×10^2 μM; desferrioxamine (DFO)

had intermediate antimalarial activity with an IC_{50} of 9.5 X 10^2 μM, and HES70-DFO had the least antimalarial activity with an IC_{50} of 1.3 X 10^4 μM.

The most lipid soluble and active iron chelator, CP94.HCl was chosen to investigate the importance of iron in the activity of each antimalarial drug using isobologram analysis. Representative isobolograms are shown as shown in figures 4.3 – 4.6 The plots for CP94.HCl against DHA (figure 4.3.) and ARM (figure 4.4.) are strongly convex upwards, indicating antagonism. A similar observation albeit less marked was obtained when CP94.HCl was used in combination with CQ (positive control) (figure 4.6.). The *in vitro* combination of CP94.HCl with LMF was less convincing and appeared additive (figure 4.5.).

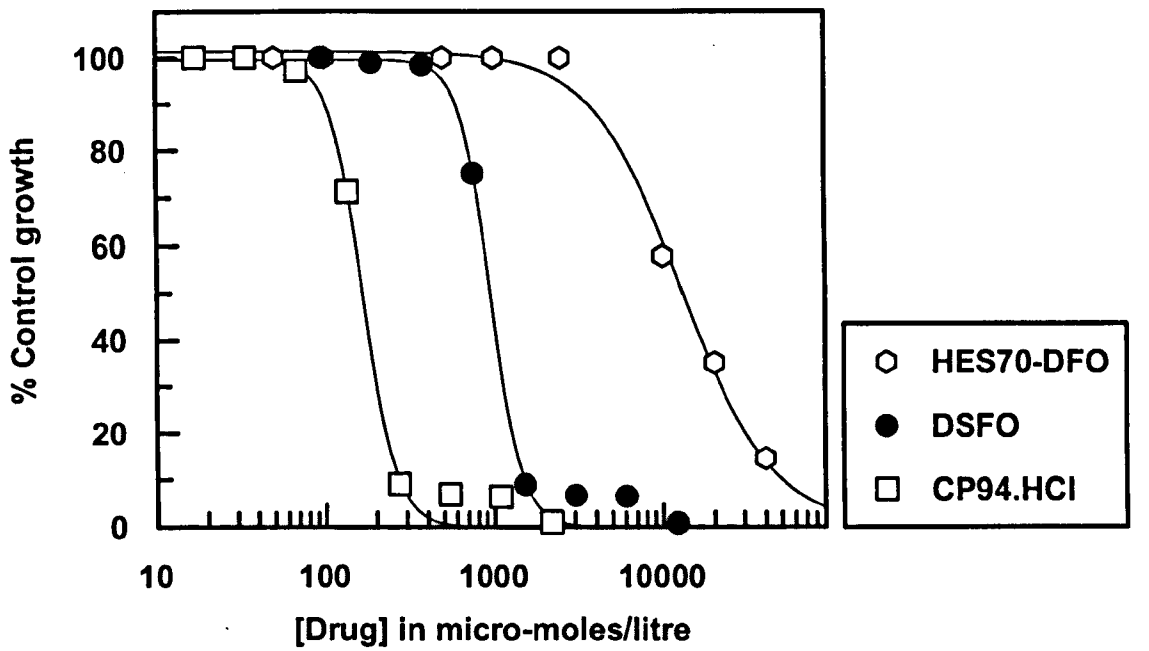


Figure 4.2. The antimalarial activities of different iron chelators (HES70-DFO, DFO and CP 94.HCl) tested against *Plasmodium falciparum* (K1) in vitro, using standard sensitivity assays. These curves are representative of 3 separate assays.

IRON CHELATOR	IC_{50} (μM)
CP 94.HCl	$1.25 \times 10^2 \pm 12.5$
DSFO	$9.53 \times 10^2 \pm 43.6$
HES70-DFO	$1.29 \times 10^4 \pm 772.$

Table 10. Shows in-vitro sensitivities of K1 (CQ resistant strain) of *Plasmodium falciparum* parasites to iron chelators of differing lipophilicity. The data represents mean ± sd of IC50 values (nM) derived from at least 3 different assays.

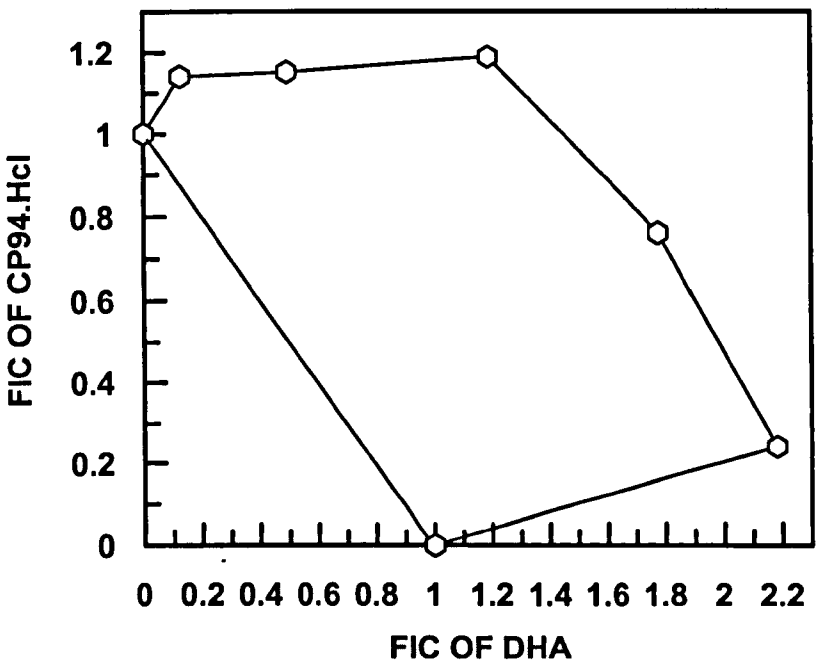


Figure 4.3. Representative isobole for the interaction between CP 94.HCl and dihydro artemisinin (DHA). Drugs were tested against *P. falciparum* (K1) malaria *in-vitro*, using standard isobole assays. Experiments were performed on at least 3 independent settings.

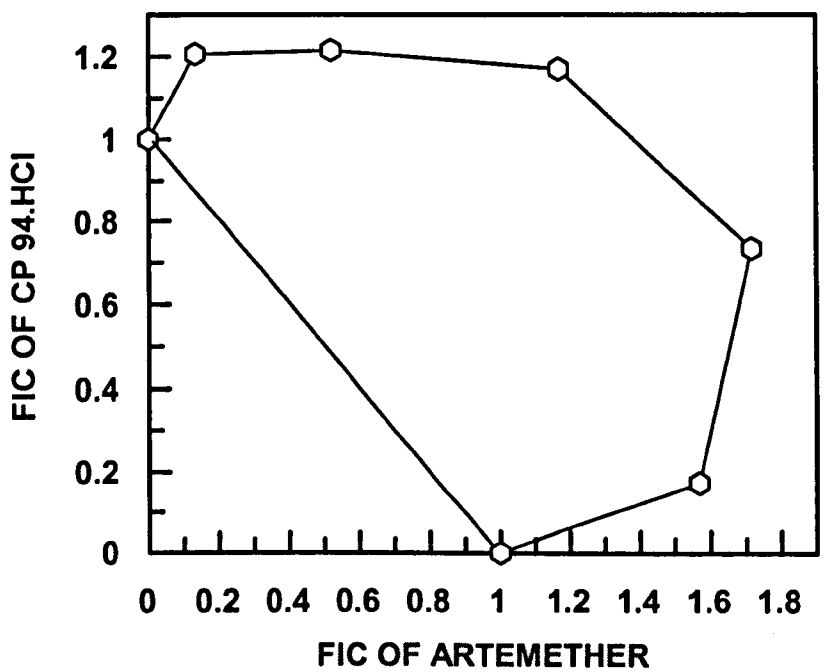


Figure 4.4. Representative isobole for the interaction between CP 94.HCl and artemether. Drugs were tested against *P. falciparum* (K1) malaria *in-vitro*, using standard isobol assays. Experiments were performed on at least 3 independent settings.

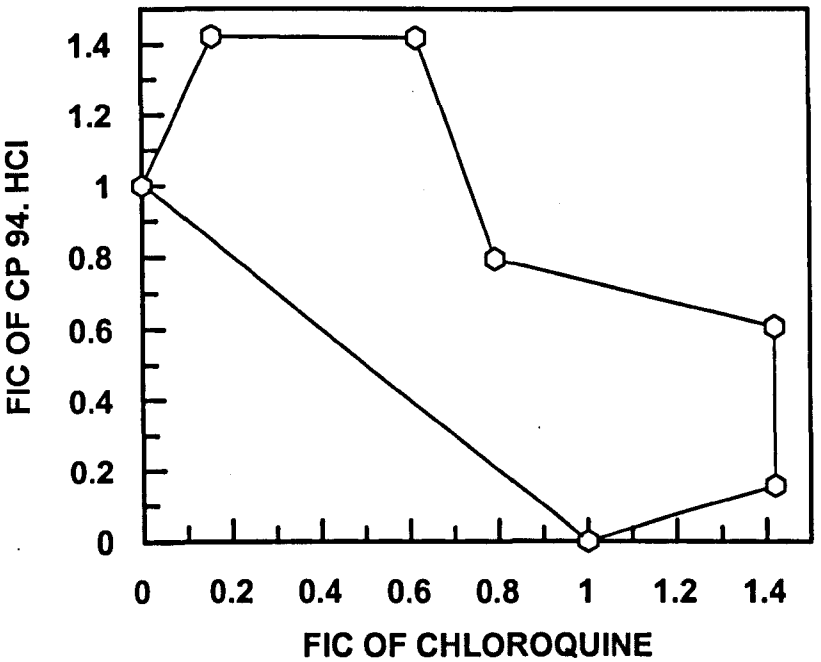


Figure 4.5. Representative isobole for the interaction between CP 94.HCl and lumefantrine. Drugs were tested against *Plasmodium falciparum* (K1) malaria *in vitro*, using standard isobol assays. Experiments were performed on 3 independent occasions.

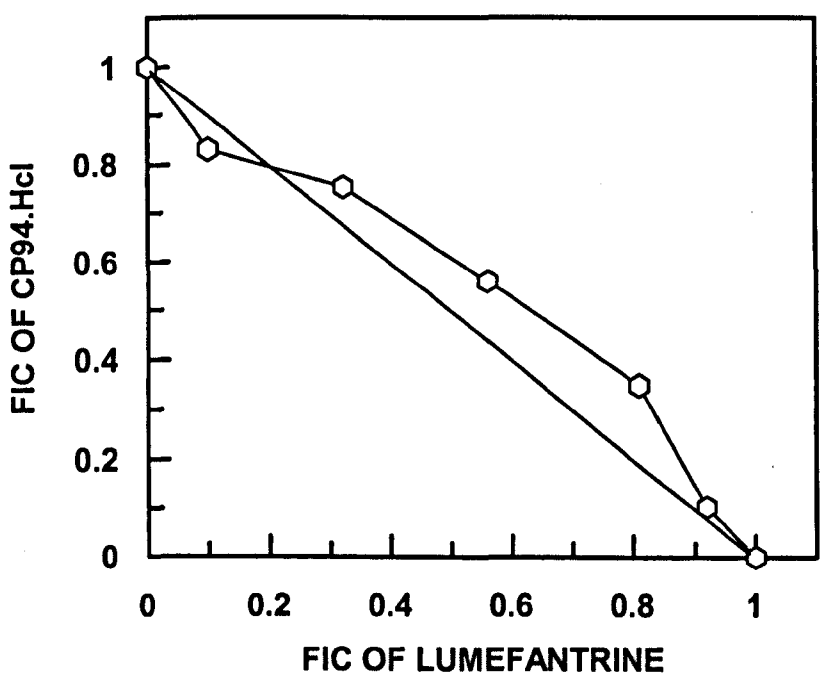


Figure 4.6. Representative isobole for the interaction between CP 94.HCl and chloroquine. Drugs were tested against *Plasmodium falciparum* (K1) malaria *in vitro*, using standard isobol assays. Experiments were performed on 3 independent occasions.

4.3. DISCUSSION

Akin to other living organisms, iron is essential to the malaria parasite for its fundamental vital activities like growth and multiplication, and iron deprivation threatens its survival. The capacity of readily exchanging electrons makes iron not only essential for fundamental cell functions, but also a potential catalyst for chemical reactions involving free-radical formation and subsequent oxidative stress and cell damage [174, 583-588]. These reactions involving generation of reactive species may be part of the normal physiological pathways occurring in the cell, such as signal transduction, cell signalling and redox regulation of cell proliferation and apoptosis [589], or they may be involved in the bio-activation of exogenously administered agents such as artemisinin derivatives [187] and other antimalarials.

In this study three iron chelators (CP94.HCl, desferrioxamine, and deferoxamine-hydroxyethyl starch) were used either alone or in combination with different antimalarial drugs, as probes to investigate the importance of intracellular iron in ARM and LMF mechanisms of action. The choice of iron chelators was based on the understanding that the antimalarial effect of iron chelators is attributed to their interaction with a labile iron pool within parasitised erythrocytes, and that increasing their affinity to iron, coupled with increased lipophilicity improves their antimalarial activity [574]. CP 94.HCl is a highly permeant anionic iron chelator, and like other highly lipophilic 3-hydroxypyrimidin-4-ones [590], displays good antimalarial activity and is thought to affect a wide range of *Plasmodial* parasite

developmental stages (un-published data). CP94.HCl was used in these experiments to achieve selective deprivation of intracellular and free parasite-associated iron, given its highly permeant property. Conversely, high molecular weight deferoxamine-hydroxyethyl starch (DFO-HES), a conjugate of deferoxamine (DFO) and hydroxy ethyl starch (HES) [581], is not taken up by *P. falciparum*-infected erythrocyte and therefore has no effect on intracellular iron [327]. DFO-HES was used in order to achieve selective extracellular iron deprivation. Hydrophilic and poorly permeant desferrioxamine (DFO) is thought to be taken up by *P. falciparum*-infected erythrocyte but is poorly taken up into the parasite. DFO thus has less antimalarial activity, albeit its antimalarial activity has been demonstrated *in-vitro* [532, 572, 591-600] and *in-vivo* [532, 572, 574, 575, 591-593, 601, 602].

The IC_{50} values for the iron chelators used in this work (presented in table 4.1. and figure 4.2.) are comparable to those reported by earlier researchers [590]. The antimalarial activity of the iron chelators has been shown to increase with increasing lipophilicity of iron chelators, as reflected in the present data. CP94.HCl, the most lipophilic and potent antimalarial of the three iron chelators was chosen to investigate *Plasmodial* intacellular iron. This highly lipophilic iron chelator is expected to access, bind and sequester intracellular iron. It is intracellular iron that is targeted and also the iron fraction that is important to parasite survival.

The data presented here (figures 4.3 & 4.4.) indubitably demonstrate that the effects of DHA and ARM on the parasite are grossly antagonized by CP94.HCl. This provides confirmatory evidence that intracellular iron is necessary in the mechanism of action of ARM and its active metabolite DHA as antimalarials. The 'chelatable' intracellular iron is responsible for the bio-activation of artemether but the source of this iron is not yet clear. Findings from several researchers support the involvement of haem-bound iron and these include: (1) Iron chelators have been shown to bind directly to haem [603]; (2) the alkylation process of the bio-activated artemisinin products has been shown to involve haem [327], and alkylated haems have been documented to release free iron [604] and this freed iron is likely to be made available to the direct action of the chelator; (3) chloroquine, which binds haem avidly, has an antagonistic effect to the antimalarial effect of artemisinin when used in combination *in vitro*.[380, 605]; (4) artemisinin has also be shown to inhibit chloroquine-mediated clumping of haemozoin pigment granules [325]; (5) parasite derived haemozoin when incubated with [^{14}C] artemisinin has been shown to concentrate the drug [327]; and (6) a chloroquine-resistant strain of murine malaria, *Plasmodium berghei*, which is devoid of haemozoin, is highly resistant to artemisinin [325], In sharp contrast, human chloroquine-resistant *P.falciparum* which contain haemozoin is fully sensitive to artemisinin [606] however it unclear whether it is the iron in the haem or iron from the haem that accounts for this difference.

Previous work has also shown that the iron catalysed bioactivation of artemisinin compounds results in generation of highly reactive carbon-centered free radicals

together with other electrophilic intermediates [187, 324, 340, 341, 580, 607] which kill the parasite by alkylating essential malarial protein(s) [187, 550, 579]. Asawamahasakda and colleagues have shown that specific parasite associated proteins are alkylated when cultures are incubated in physiological concentrations of dihydroartemisinin in a model system [608]. Similar proteins are alkylated by other antimalarial endoperoxides, but the inactive analogs such as deoxyarteether do not display this effect [608].

Yikang et al [609] however, demonstrated in a model chemical system that traces of non-haem iron in the presence of cysteine can also cleave artemisinin effectively, and that the transient carbon centered radical formed can covalently bond to the ligand at iron through a sulfur atom.

The interaction of CP94.HCl with LMF appeared additive, and this was unexpected and is in contrast to CQ (positive control) which displayed antagonism. These findings may suggest that either LMF forms a very tight complex with hematin (ferri-protoporphyrin IX) making it unavailable to the iron chelator, or LMF and CP94.HCl may be acting on different targets and the 'chelatable' intracellular iron may not be critical in the mechanism of action of LMF.

The widely accepted notion is that both CQ and LMF exert their antimalarial activity by interacting with hematin (ferri-protoporphyrin IX) derived from the proteolytic degradation of hemoglobin in the parasite food vacuole [557]. Although there is sufficient evidence supporting the central role of haemoglobin

degradation in the mechanism of action of 4-aminoquinolines, quinoline methanols and phenanthrene methanols [283], there is need to investigate further its role in the mechanism of action of LMF. There is also need to establish whether the source of the iron necessary for the bio-activation of ARM is from the haemoglobin degradation pathway. This forms the basis of the next chapter.

CHAPTER 5

THE HAEMOGLOBINOLYTIC PATHWAY: A PRIME TARGET FOR ARTEMETHER AND LUMEFANTRINE ANTIMALARIAL ACTION AGAINST PLASMODIUM.FALCIPARUM.

INTRODUCTION

Data from the preceding chapter sturdily implicates the intracellular iron as essential for the action of ARM, but the definite source of this iron remains unclear. It is also hypothesized that LMF akin to other quinolines exerts its antimalarial activity by interacting with hematin (ferri-protoporphyrin IX) derived from the haemoglobin degradation pathway. This back ground prompted an investigation into the role of the haemoglobin degradation pathway in the mechanism of action of both ARM and LMF, and this forms the basis of the present chapter.

The human malaria parasite, *Plasmodium falciparum*, degrades an estimated 60-80% of its host cell hemoglobin during its trophozoite-stage [610]. Host cell haemoglobin, present at 340 mg/mL (5 mM) in the erythrocyte cytosol is believed to be the principal source of nutrients for the *Plasmodial* cell, supplemented by imported extracellular nutrients. *Plasmodium* have limited capacity for *de novo* amino acid synthesis [190], however haemoglobin degradation is capable of providing all essential amino acids with the exception of isoleucine (absent in haemoglobin) and a limited supply of methionine, cysteine, glutamic acid, and

glutamine. Haemoglobin degradation may also serve the role of creating physical space within the erythrocyte which is required to accommodate the growing malaria parasite [611].

5.0.1. The *Plasmodium* Food Vacuole.

The *Plasmodium* food vacuole is a complex organelle that is evolutionally well adapted for haemoglobin metabolism. The food vacuole is in some ways analogous to the secondary lysosomes of mammalian cells [612]. This vital structure of *Plasmodium falciparum* is the site of hemoglobin degradation, haem crystallization, detoxification of oxygen radicals, acidification, generation of free iron, peptide transport, and antimalarial drug accumulation [139, 613].

5.0.2. Potential sites of parasite vulnerability in the haemoglobin degradation pathway of *P.falciparum* and their relevance to the mechanism of action of ARM and LMF.

In erythrocytic parasites, proteases have been reported to play an important role in the degradation of haemoglobin by trophozoites, the rupture of erythrocyte by mature schizonts, and the invasion of erythrocytes by free merozoites. *Plasmodial* proteases are appealing candidates for drug development because they play vital roles in parasite metabolism which are central to the growth and maturation of the parasite. Haemoglobin degradation is a semi-ordered metabolic process [611] that seems to involve multiple proteases. This catabolic process is preceded by endocytosis of host haemoglobin from the erythrocyte cytosol. This

is then trafficked to an acidic food vacuole where proteolysis reportedly begins. Currently, four food vacuole proteases have been well characterized. Two homologous aspartic proteases namely plasmepsin I and II, appear to initiate the degradative process where they are responsible for the initial cleavage of native haemoglobin molecule in a highly conserved hinge region [156, 614]. Two other proteins, histo-aspartic protease (HAP) and plasmepsin IV (PM IV) which are homologous to the well characterized plasmepsin I and II [149, 150, 615-619] have been identified and they are thought to localize to the food vacuole and to participate in haemoglobin degradation [620] but their precise role is yet to be established. A fairly typical papain-family cysteine protease, falcipain-2 [612, 621] cleaves relatively large globin fragments. A metallo-protease, falcilysin, acts much further downstream cleaving much smaller peptides. The resulting small globin peptides are then presumably transported to the parasite cytosol. The final steps of hydrolysis of globin to free amino acids probably involve a cytosolic aminopeptidase [611, 622]. Inhibitors of each of these proteases have potent antimalarial effects [149, 157, 283, 612, 621, 623].

The biosynthesis of the plasmepsins is believed to be identical although plasmepsin I is synthesized and processed to the mature form soon after the parasite invades the erythrocyte, while plasmepsin II synthesis is delayed until later in development [149]. The proplasmepsins are type II integral membrane proteins that are transported through the secretory pathway before cleavage to the soluble form. Proplasmepsin maturation is presumed to require acidic

conditions and compounds that block this processing/maturation process are expected to be potent antimalarials. Examples of plasmepsin processing inhibitors include the tripeptide aldehydes N-acetyl-L-leucyl-L-leucyl-norleucinal (ALLN) and N-acetyl-L-leucyl-L-leucyl-methional (ALLM) which reversibly inhibit *Plasmodial* aspartic proteinases [149]. Mature plasmepsin I is selectively inhibited by Roche compounds such as Ro40-4388, Ro61-7835, Ro61-9379 [152], while membrane permeant inhibitor, l-transepoxy-succinyl-leucylamido-(4-guanidino) butane (E64) is a non selective inhibitor for *Plasmodial* cysteine protease [160, 283, 624-627]. When incubated with cultured *P. falciparum* parasites, cysteine and aspartic protease inhibitors have been shown to exhibit synergistic effects in blocking parasite metabolism and development [623].

Haem (Fe(III)PPIX) crystallization is a parasite-specific process that enables the detoxification of haem following its release in the digestive vacuole during hemoglobin degradation. This represents both an essential and a unique pharmacological drug target [628]. Based on the previous discussion of the haemoglobin degradation pathway it can be postulated that the quinoline antimalarials appear to act by preventing sequestration of this toxic haem while the artemisinin type compounds require the haem iron for their bio-activation (refer to figure. 1.5, chapter 1).

Aryl-amino alcohols are weak monoprotic bases under physiological conditions, and are lipid soluble at neutral pH (in contrast with 4-aminoquinolines like

chloroquine and amodiaquine which although weak bases are diprotonated and hydrophilic (but also lipid soluble) at neutral pH). The widely accepted hypothesis is that the antimalarial quinolines work by blocking the crystallization of toxic haeme (Fe(II)PPIX) released during haemoglobin proteolysis in intraerythrocytic *Plasmodium falciparum* in the food vacuole [158, 281, 352, 353, 562, 564, 629, 630]. This is achieved by forming a drug-FPIX complex capable of exerting toxicity within the parasite which cannot take part in the crystallization process. Whether this mechanism explains the mode of action of both type-1 quinolines like chloroquine and type–2 quinoline drugs like LMF, remains an issue of debate [512]. Recent work however from this research group [283, 286] provides substantial evidence that the interaction with haem is central to the activity of quinoline containing antimalarials.

The artemisinin-type compounds are reported to have activity throughout the asexual intra-erythrocytic schizogonic cycle in addition to gametocytocidal effects [204, 631]. The mechanism of artemisinin action is still poorly understood, but the consensus of several research groups is that reductive cleavage of the intact peroxide bridge by free intraparasitic iron and ferrohaeme ferrous-protoporphyrin IX (Fe(II)PPIX) generates C-centred radicals. These highly reactive species, or downstream reactive species generated from them, then kill the parasite by alkylating parasitic biomolecules such as essential malarial protein(s) [183, 184, 187, 344, 345, 580, 632, 633]. Alternative mechanisms have also been proposed such as the generation of a hydroperoxide following heterolysis of the C3-O2

bond of the artemisinin 1,2,4-trioxane pharmacophore and consequent hydroxylation or oxidation of parasitic biomolecules [342]; and the involvement of endoperoxides in the inhibition of haem polymerization [352, 353].

In order to formally test these hypotheses we have used selective inhibitors of the haemoglobin degradation pathway to block the generation of FPIX. If this process is central to drug action these inhibitors should antagonize the action of both the quinoline and the artemisinin drugs. Using a range of protease inhibitors including the specific inhibitor of *Plasmodial* plasmepsin I, Ro40-4388; a plasmepsin processing inhibitor N-acetyl-L-leucyl-L-leucyl-norleucinal (ALLN); and a nonselective inhibitor of cysteine proteinase, E64, we have probed the dependence of ARM and LMF action on efficient haemoglobin degradation. Previous work has undoubtedly shown that chloroquine exerts its antimalarial activity via such a haem dependent mechanism [283, 286, 287, 556, 634, 635], and so chloroquine was used as a positive control in this work

5.1. MATERIALS AND METHODS

5.1.1. Drugs used in the study.

N-acetyl-L-leucyl-L-leucyl-norleucinol (ALLN) and *trans*-epoxy-L-leucyl-amido-(4-guanido)-butane (E64) were purchased from Sigma, Dorset, United Kingdom. Roche40-4388, were obtained from Hoffmann-la Roche, Basel, Switzerland. For other drugs used, as in chapter 4.

5.1.2. Parasite isolates and cultivation.

Both the CQ-resistant isolate of *Plasmodium falciparum* K1 and the CQ-sensitive isolate HB3, were used for the *in vitro* sensitivity assays, but only K1 isolate was used for the drug combination assays. The parasites were maintained as described in **section 2.1**.

5.1.3. In Vitro sensitivity assays.

(As described in chapter 2, section 2.2.4).

5.1.4. Drug combination assays.

Prior to investigating the interaction between LMF, DHA, ARM and the inhibitors of haemoglobin degradation (Inhibitor of processing of plasmepsins I and II; plasmepsin I inhibitor roche40-4388 and cysteine proteinase inhibitor E64) the IC_{50} value for each compound was established as described in section 2.2.4. This information was then used to determine the drug concentrations needed for isobologram analysis. A working solution was prepared from a stock solution of each drug with a concentration determined such that the IC50 of each drug would occur around the fourth serial dilution. Combinations of the working solutions were prepared in constant ratios of 0:10, 1:9, 3:7, 5:5, 7:3, 9:1, and 10:0. Each combination was then serially diluted and processed as for the standard sensivity assay. The fractional inhibitory concentration (FIC; FIC=IC_{50} of the drug in combination /IC_{50} of the drug when used alone) of each drug was calculated and plotted as an isobologram [442].

5.2. RESULTS

DRUGS	K1 (nM ± SD)	HB3 (nM ± SD)
DHA	2.01 ± 0.02	1.12 ± 0.04
ARM	8.36 ± 0.32	2.8 ± 0.1
LMF	123.41 ± 11.77	279.23 ± 9.71
CQ	140.22 ± 13.4	13.6 ± 4
Roche40-4388	248.5 ± 8.6	264 ± 24.3
ALLN	143.7 ± 8.6	145 ± 23.2
E64	10150 ± 632	11200 ± 1460

Table 11. In-vitro sensitivities of HB3 (CQ sensitive strain) and K1 (CQ resistant isolate) of *Plasmodium falciparum* parasites to DHA, ARM, LMF, CQ, Roche40-4388, ALLN and E64.The data represents mean ± sd of IC50 values (nM) derived from at least 3 different occasions

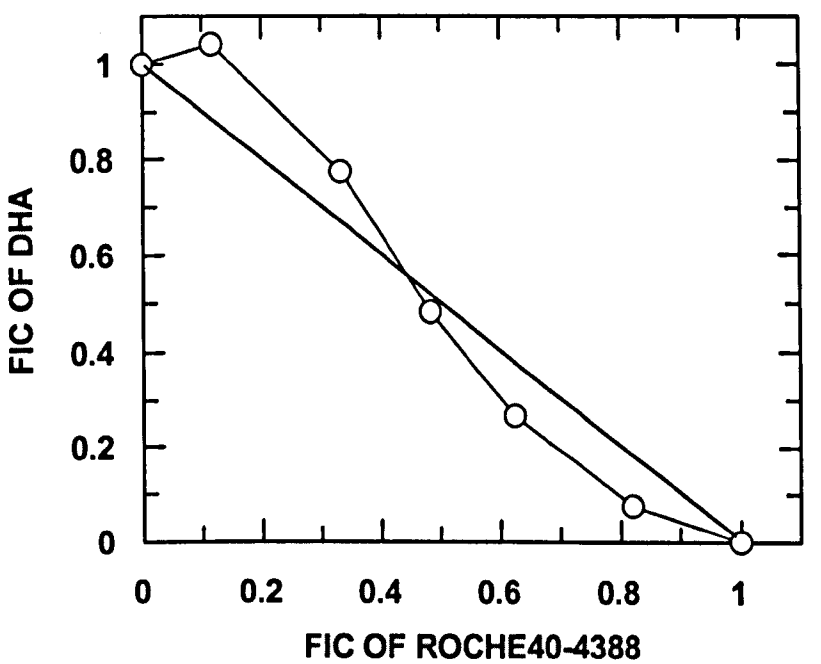


Figure 5.1. Representative isobologram demonstrating the interaction between DHA and the plasmepsin 1 inhibitor Roche40-4388. These studies were performed on at least 3 separate occasions using the K1 isolate of *P.falciparum*.

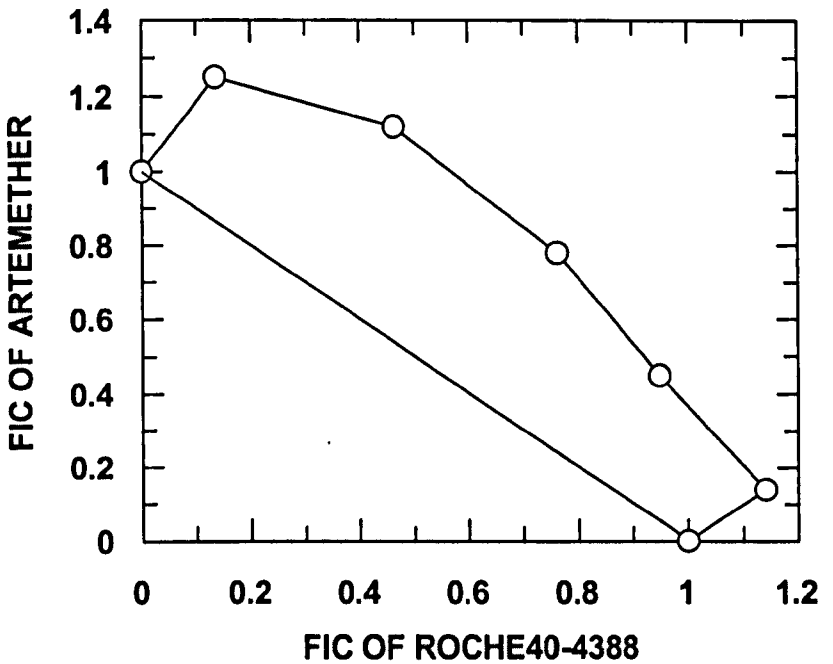


Figure 5.2. Representative isobologram demonstrating the interaction between artemether and the plasmepsin 1 inhibitor Ro40-4388 in combination. These studies were performed on at least 3 separate occasions using the K1 isolate of *P.falciparum*.

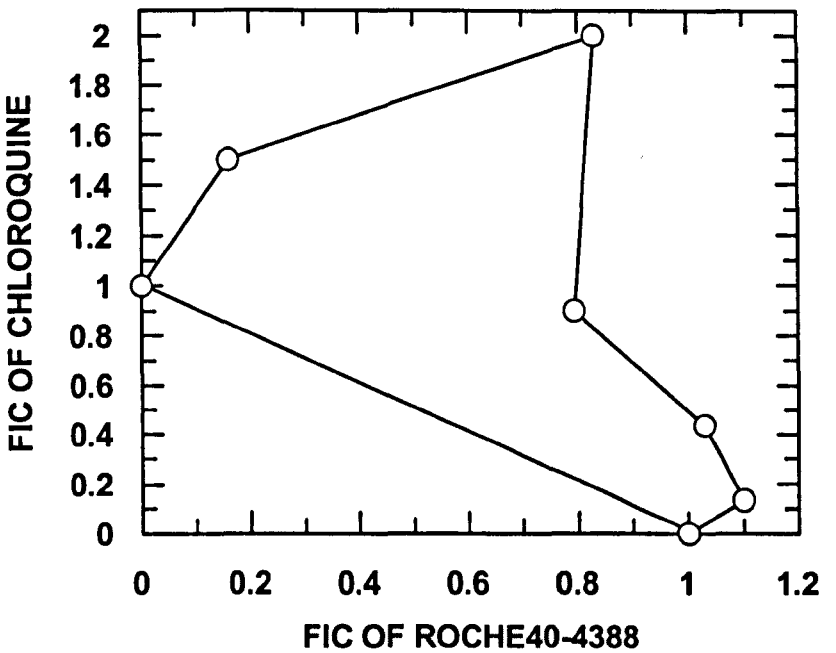


Figure 5.3. Representative isobologram demonstrating the interaction between chloroquine and the plasmepsin 1 inhibitor Ro40-4388 in combination. These studies were performed on at least 3 separate occasions using the K1 isolate of *P.falciparum*.

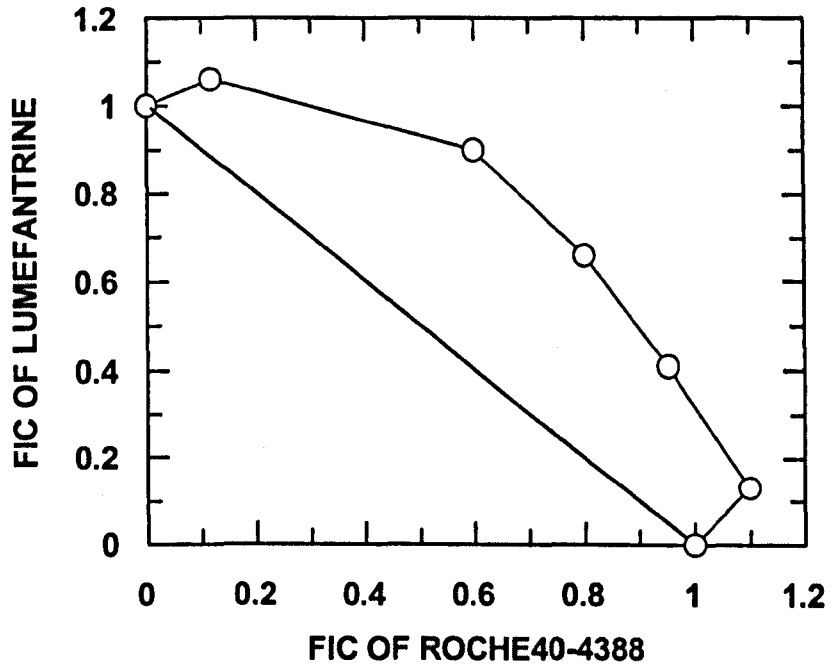


Figure 5.4. Representative isobologram demonstrating the interaction between lumefantrine and the plasmepsin 1 inhibitor Ro40-4388 in combination. These studies were performed on at least 3 separate occasions using the K1 isolate of *P.falciparum*.

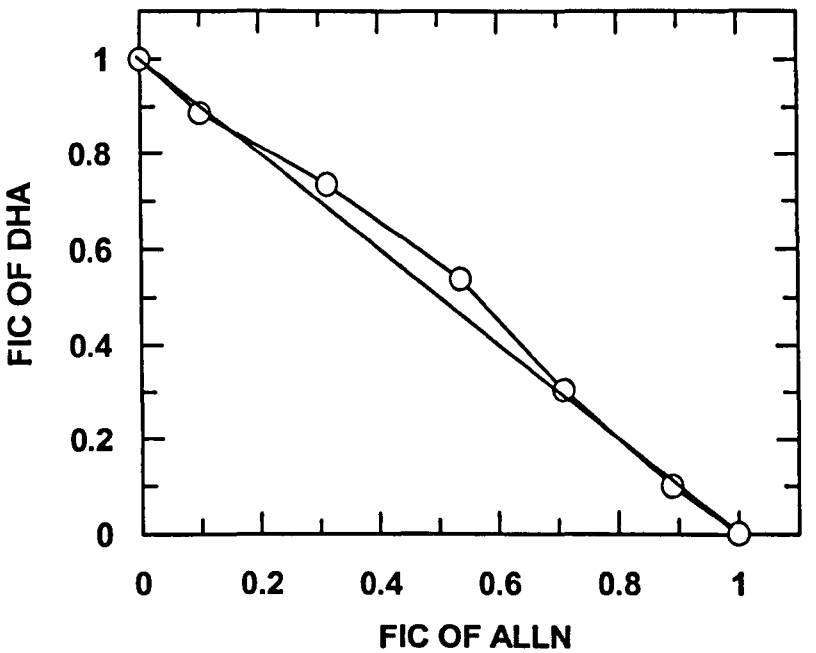


Figure 5.5. Representative isobologram demonstrating the interaction between DHA and the plasmepsin processing inhibitor ALLN in combination. These studies were performed on at least 3 separate occasions using the K1 isolate of *P.falciparum*.

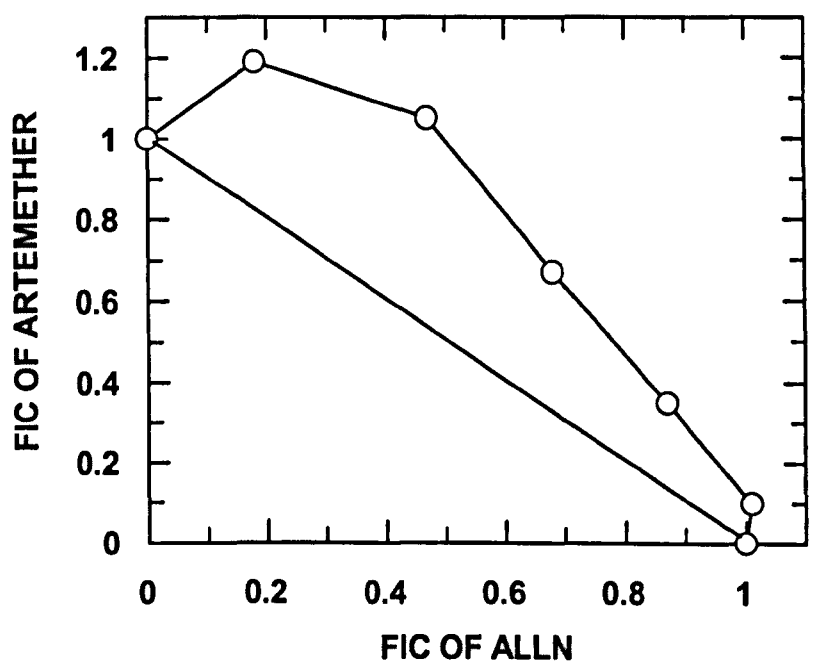


Figure 5.6. Representative isobologram demonstrating the interaction between artemether and the plasmepsin processing inhibitor ALLN in combination. These studies were performed on at least 3 separate occasions using the K1 isolate of *P.falciparum*.

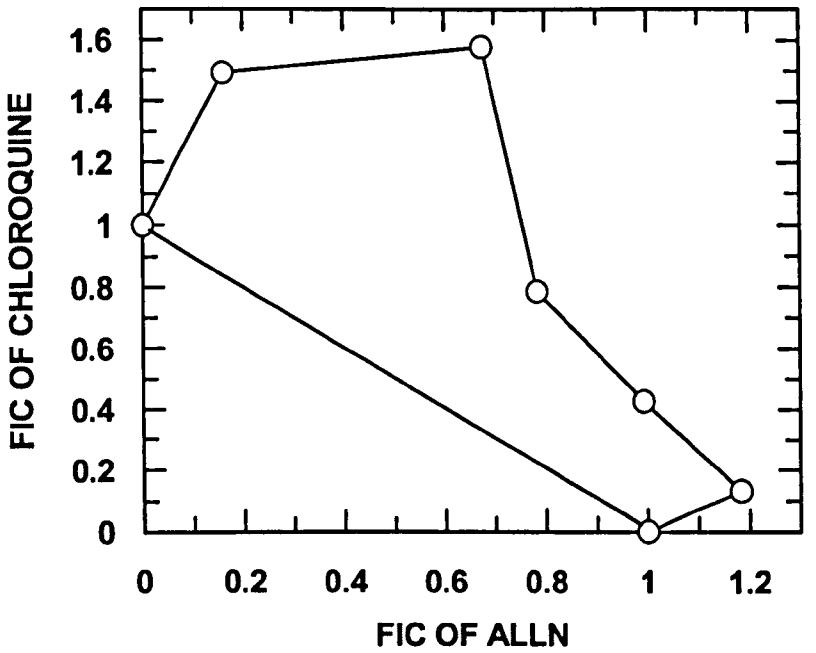


Figure 5.7 Representative isobologram demonstrating the interaction between chloroquine and the plasmepsin processing inhibitor ALLN in combination. These studies were performed on at least 3 separate occasions using the K1 isolate of *P.falciparum*.

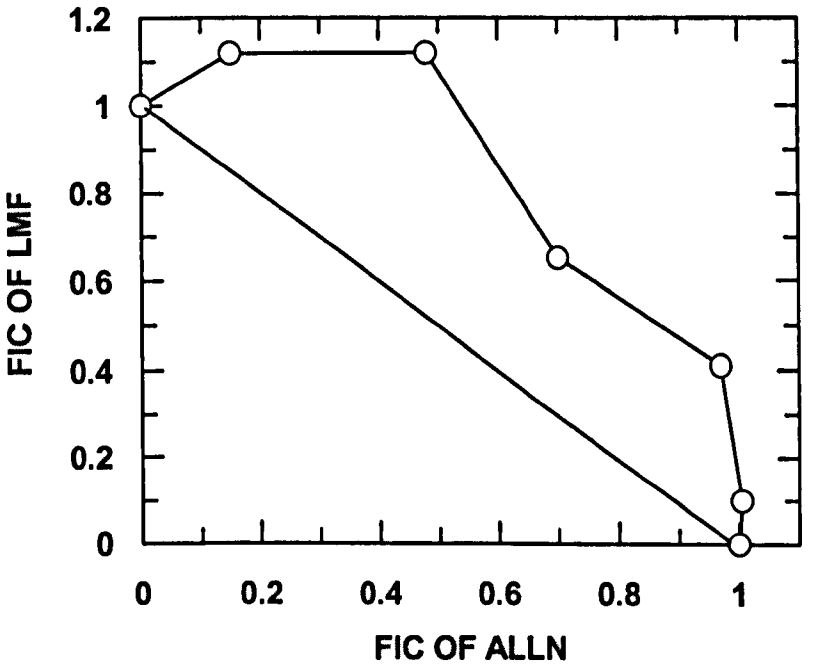


Figure 5.8. Representative isobologram demonstrating the interaction between lumefantrine and the plasmepsin processing inhibitor ALLN in combination. These studies were performed on at least 3 separate occasions using the K1 isolate of *P.falciparum*.

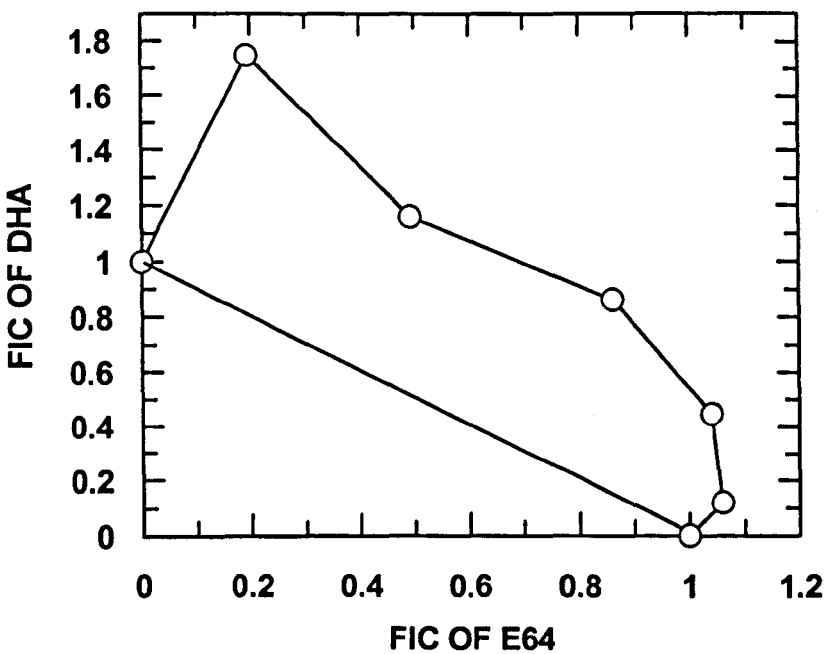


Figure 5.9. Representative isobologram demonstrating the interaction between DHA and the cysteine protease inhibitor E64 in combination. These studies were performed on at least 3 separate occasions using the K1 isolate of *P.falciparum*.

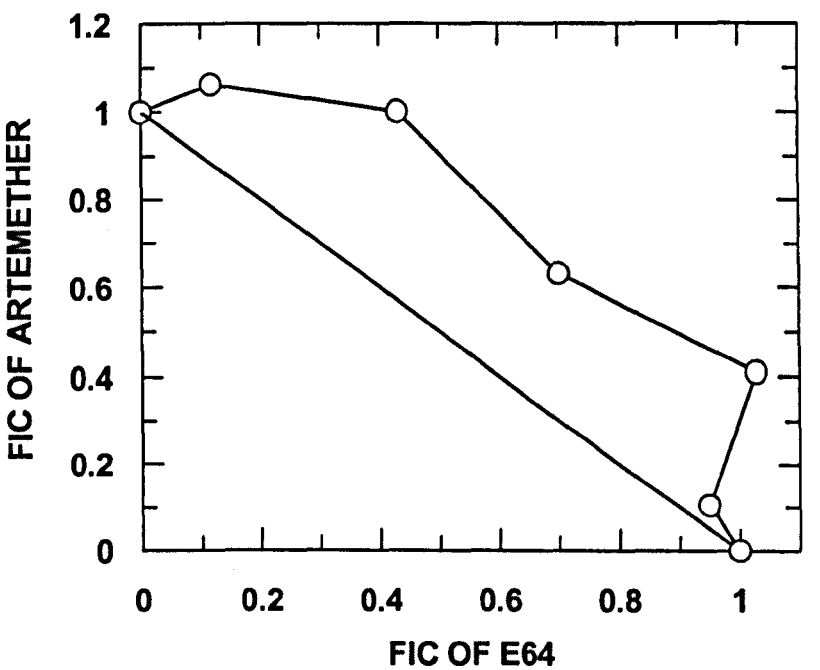


Figure 5.10. Representative isobologram demonstrating the interaction between artemether and the cysteine protease inhibitor E64 in combination. These studies were performed on at least 3 separate occasions using the K1 isolate of *P.falciparum*.

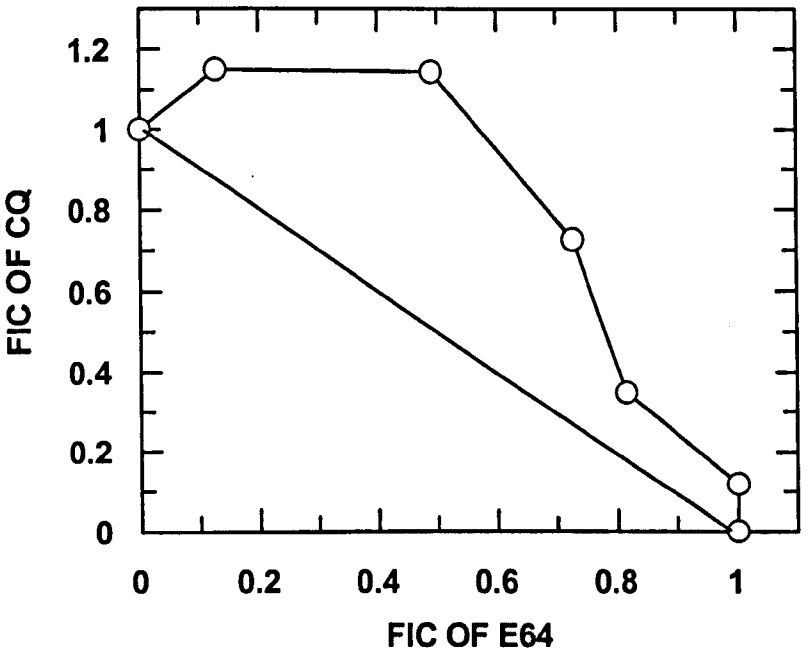


Figure 5.11. Representative isobologram demonstrating the interaction between chloroquine and cysteine protease inhibitor E64 in combination. These studies were performed on at least 3 separate occasions using the K1 isolate of *P.falciparum*.

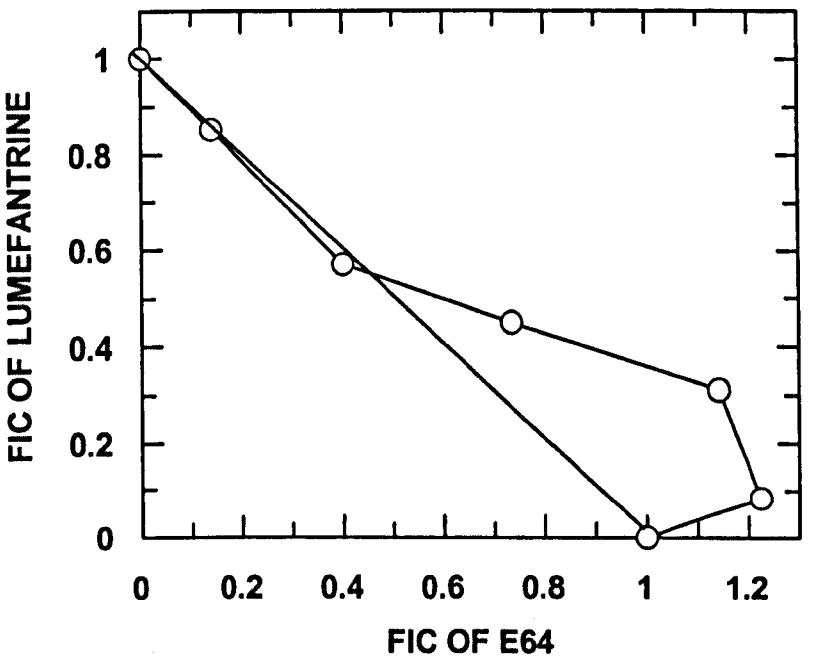


Figure 5.12. Representative isobologram demonstrating the interaction between lumefantrine and cysteine protease inhibitor E64 in combination. These studies were performed on at least 3 separate occasions using the K1 isolate of *P.falciparum*.

5.2. RESULTS.

5.2.1. In vitro sensitivity of the parasites to antimalarial drugs and proteinase inhibitors.

Table 5.1. shows all the IC_{50} data for the antimalarial activities of drugs DHA, ARM, LMF and CQ and for the proteinase inhibitors Ro40-4388, ALLN and E64 against CQ-resistant isolate K1 and CQ sensitive isolate HB3. Both Ro40-4388 and ALLN inhibited parasite growth at nano-molar (nM) concentrations though ALLN is more potent than Ro40-4388 as indicated by the IC_{50} values. Cysteine proteinase inhibitor E64 inhibited *Plasmodial* growth at micro-molar concentrations and was the least potent of the three proteinase inhibitors used in this study.

5.2.2. The interaction between antimalarials (DHA, ARM, LMF, and CQ) and proteinase inhibitors.

Figures 5.1- 5.12 show representative isobolograms for antimalarial drug-proteinase inhibitor combinations. In confirmation of previous observations the antimalarial activity of CQ was profoundly antagonized by the specific inhibitor of plasmepsin 1 (figure 5.3), the inhibitor of protease processing (figure 5.7) and the cysteine protease inhibitor (figure 5.11). ARM activity was antagonized similarly but to a lesser extent than CQ (figures 5.2, 5.6, and 5.10). In contrast the active metabolite of ARM, DHA displayed addition as inhibition of plasmepsin 1 activity directly (figure 5.1) or indirectly (figure 5.5) but the interaction with E64 was

profoundly antagonistic (figure 5.9). LMF activity was modestly antagonized plasmepsin 1 inhibitor (figure 5.3) and plasmepsin processing inhibitor (figure 5.7) but interacted additionally with E64. These patterns of interaction were qualitatively identical on at least 3 repeat experiments.

5.3. DISCUSSION

Plasmodium falciparum like other haematophagous parasites is dependent on host cell haemoglobin degradation as a vital source of nutrients for successful growth and development. Haemoglobin degradation may also facilitate *Plasmodial* growth within the erythrocyte by creating space for the fast growing parasite [611]. This degradative process has remarkable implications for antimalarial chemotherapy, albeit the basis for haemoglobin degradation and the biochemical mechanism of this process is still poorly understood. The *in-vitro* sensitivity data in table 5.1 shows the antimalarial potential of protease inhibitors such as Ro40-4388 and ALLN (with the exception of that of E64 which is micro-molar concentrations), which compares quite well with the potency of established antimalarials. The potency of these protease inhibitors as indicated by the inhibition of parasite growth represented by IC_{50} values, clearly demonstrates the relevance of the haemoglobinolytic pathway to the survival of the *Plasmodial* cell. A number of *in vitro* studies have demonstrated that inhibitors of *Plasmodial* proteases have potent effects against cultured malaria parasites [152, 160, 573, 625, 627].

Haemoglobin catabolism occurs principally in the in the acidic digestive vacuole and involves several proteases of which the aspartic proteases (plasmepsin I and II) and the cysteine protease (falcipain-2) play an important role. Homologous plasmepsin I and II have been localized to the *P.falciparum* food vacuole, have acidic pH optima and have similar biochemical functions [156, 614, 615, 619]. The plasmepsins are synthesised as proenzymes, which are type II integral membrane proteins (unlike other typical proforms of aspartic proteases which are soluble) that are transported through the secretory pathway before cleavage to mature soluble forms under acidic conditions [149]. The synthesis and processing of both plasmepsins is greatest in trophozoites (which corresponds with period of maximum haemoglobin degradation) and is presumably active in young ring-stage parasites [149]. The tripeptide aldehyde, ALLN used in this work inhibits the cleavage of both proenzymes (PMI & PMII) and is also reported to inhibit cysteine proteases including calcium activated cysteine proteases (calpains) [149]. In addition the processing inhibitors may block endocytosis [149]. The non-specific nature of ALLN may explain its greater potency relative to the specific mature plasmepsin I inhibitor Ro40-4388. Previous work has shown that ALLN and Roche40-4388 inhibits growth at nano-molar concentrations [152, 283] and the results of this work are quite comparable.

Falcipain-2 is a papain-family cysteine protease [612, 621] with a cathepsin L-like substrate specificity. The activity of falcipain-2 is also most prominent in trophozoites, the erythrocytic stage during which most haemoglobin degradation

occurs [621] and has again been localized to the parasite food vacuole, the site of haemoglobin degradation [156, 621]. Falcipain-2 inhibitors block the hydrolysis of haemoglobin by cultured *P.falciparum* parasites, causing the accumulation in the food vacuole of large quantities of undegraded globin and a subsequent block in parasite development [612, 636]. E64, a non specific cysteine protease inhibitor was therefore used in this work as another probe to investigate the dependence of the various antimalarials on efficient degradation of haemoglobin. E64 has previously been shown to reduce the formation of haemozoin via an inhibition of haemoglobin degradation [354, 612].

These data show as others have shown [152, 283] that the antimalarial activity of CQ is profoundly inhibited by inhibitors of the haemoglobonolytic pathway; plasmepsin processing inhibitors (figure 5.7), specific plasmepsin I inhibitor (figure 5.3) and cysteine protease inhibitor (figure 5.11). There is substantial evidence that the binding of CQ to ferriprotoporphyrin IX forming FPIX-CQ complex is the principal driving force for CQ uptake in *Plasmodium falciparum* [287, 634]. Specific proteinase inhibitors that block the degradation of hemoglobin and stop the generation of FPIX also inhibit drug uptake. The marked antagonism displayed by specific plasmepsin 1 inhibitor, Ro 40-4388 on the antimalarial activity of CQ is attributed to Ro 40-4388 blocking haemoglobin cleavage with consequent reduction in FPIX formation. Plasmepsin processing inhibitor, ALLN prevents proplasmepsin processing and consequently indirectly prevents formation of FPIX and also inhibits *plasmodial* endocytosis. Inhibitors of cysteine proteases like the epoxide E64 also results in marked antagonism with

CQ (figure 5. 11) probably because the interaction culminates in the build up of un-degraded haemoglobin and consequently prevents FPIX formation. CQ is thought to exert it's antimalarial activity by antagonising the sequestration of toxic haem moieties and build up of toxic (FPIX) CQ complex [637]. Based on these findings chloroquine was a used as a positive control for the experiments in this study to investigate the role of haemoglobin degradation in the mechanisms of action of the DHA, ARM and LMF.

The interaction of plasmepsin processing inhibitors (figure 5.6), specific plasmepsin I inhibitor (figure 5.2) and cysteine protease inhibitor (figure 5.10) with the antimalarial activity of artemether showed antagonistic interaction reminiscent of those obtained with CQ, but of lesser magnitude. These observations suggest that FPIX must be important in the bioactivation process of ARM, perhaps the iron (II) in the reduced form, ferrous haem [ferroprotoporphyrin IX, Fe(II)PPIX]. It is also worth noting that the interaction of highly lipid soluble iron chelator, CP94.HCl (discussed in chapter 4) with artemisinin compounds, namely ARM and DHA displayed very profound antagonism. This suggests that although Fe(II)PPIX is important, free chelatable intracellular iron (II) possibly non haem iron II plays a more important role in the bioactivation process of artemisinin compounds.

In contrast to the interaction of ARM with protease inhibitors, the active metabolite of ARM, DHA displayed addition with inhibition of plasmepsin 1 activity directly (figure 5.1) or indirectly (figure 5.5) but the interaction with E64 was strongly antagonistic (figure 5.9). In this case the antimalarial activity of DHA

is not antagonized by Ro40-4388 and ALLN because there is likely to be a limited amount of Fe^{2+}which is sufficient to catalyse the bioactivation of DHA. Cysteine protease inhibitor, l-transepoxy-succinyl-leucylamido-(4- guanidino) butane (E64) has been shown to cause irreversible inhibition of haemoglobin degradation with consequent accumulation of undegraded haemoglobin and swelling of the *Plasmodial* food vacuole. This is likely to interfere with the acidification process in the food vacuole and consequently limit formation of Fe2+ which is necessary for endoperoxide bioactivation.

LMF activity was modestly antagonized by the plasmepsin 1 inhibitor (figure 5.3) and plasmepsin processing inhibitor (figure 5.7). The antagonism observed here is possibly due to reduced formation of FPIX. The interaction between E64 and LMF is unexpectedly less than what is observed with E64 in combination with CQ. These findings suggest that although LMF is thought to be dependent on effective formation of FPIX from the haemoglobinolytic pathway, this may only be part of the mechanism of action of this drug.

So far the findings suggested that both ARM and LMF implicated the plasmodial food vacuole is a common target, and that haemoglobin degradation has a central role in the mechanism of action of both drugs. However, the available data was insufficient to fully explain the mechanism of action of both drugs. There was therefore need to investigate other possible mechanisms and targets for both LMF and ARM, and in the following chapters a molecular approach is used.

CHAPTER 6

A PROTEOMIC STRATEGY TO INVESTIGATE DRUG ACTION IN *PLASMODIUM FALCIPARUM*: CHANGES IN PROTEIN EXPRESSION PROFILES FOLLOWING TREATMENT WITH ARM AND LMF.

6.0 INTRODUCTION

This chapter describes a global investigation of the differences in protein expression profiles between drug treated and untreated *P.falciparum* parasite populations, aimed at improving our understanding of the parasite response to either ARM or LMF drug pressure. The proteome provides global analysis of protein expression from a genome under a given set of conditions [454, 638]. The proteome, unlike the genome, is not a fixed feature of an organism. It is dynamic and changes with the developmental state of a cell and the environmental conditions under which an organism is subjected to [448] for example drug treatment. This experimental strategy was employed in an attempt to unravel the complex molecular processes and underlying perturbation of physiological function that follows exposure of parasites at therapeutically relevant drug concentrations for appropriate exposure times. In short this is an attempt to describe the parasites response to lethal drug exposure at the protein level.

A two platform strategy was employed based on (a) two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) to separate proteins coupled with gel imaging software to identify qualitative and semi-quantitative alterations in

protein expression patterns followed by (b) mass spectrometry, to formally characterize the protein repertoire involved in the processes associated with drug toxicity in *Plasmodium falciparum* following ARM and LMF treatment. The high-resolution two-dimensional gel electrophoresis [444] using immobilized *p*H gradients (IPG-Dalt) [445] enabled the resolution and array of *Plasmodial* proteins extracted sequentially from a total parasite protein preparation [446]. Subsequent analysis of the visualised protein expression patterns was performed using image analysis software, PDquest™. The general workflow for this proteomic study is summarized in **Fig. 6.1** and the background to these methodologies and investigations is detailed in **chapter 1, section 1.5.**

Work discussed in previous chapters enabled characterization of the time required to evoke irreversible drug toxicity in *P.falciparum* following ARM and LMF treatment *in-vitro*. Although this work clearly implicated the *Plasmodial* food vacuole and in particular the haemoglobinolytic pathway as a common chemotherapeutic target for both drugs, this type of analysis could never reveal the full complexities of drug action. It was therefore expedient that we undertake this form of investigation in order to implicate specific protein targets in the mechanism of action of these two antimalarial agents. Using the K1 parasite isolate, a comparative proteomic approach (control versus drug treated) has been performed to develop proteomic maps under these predetermined drug exposure conditions

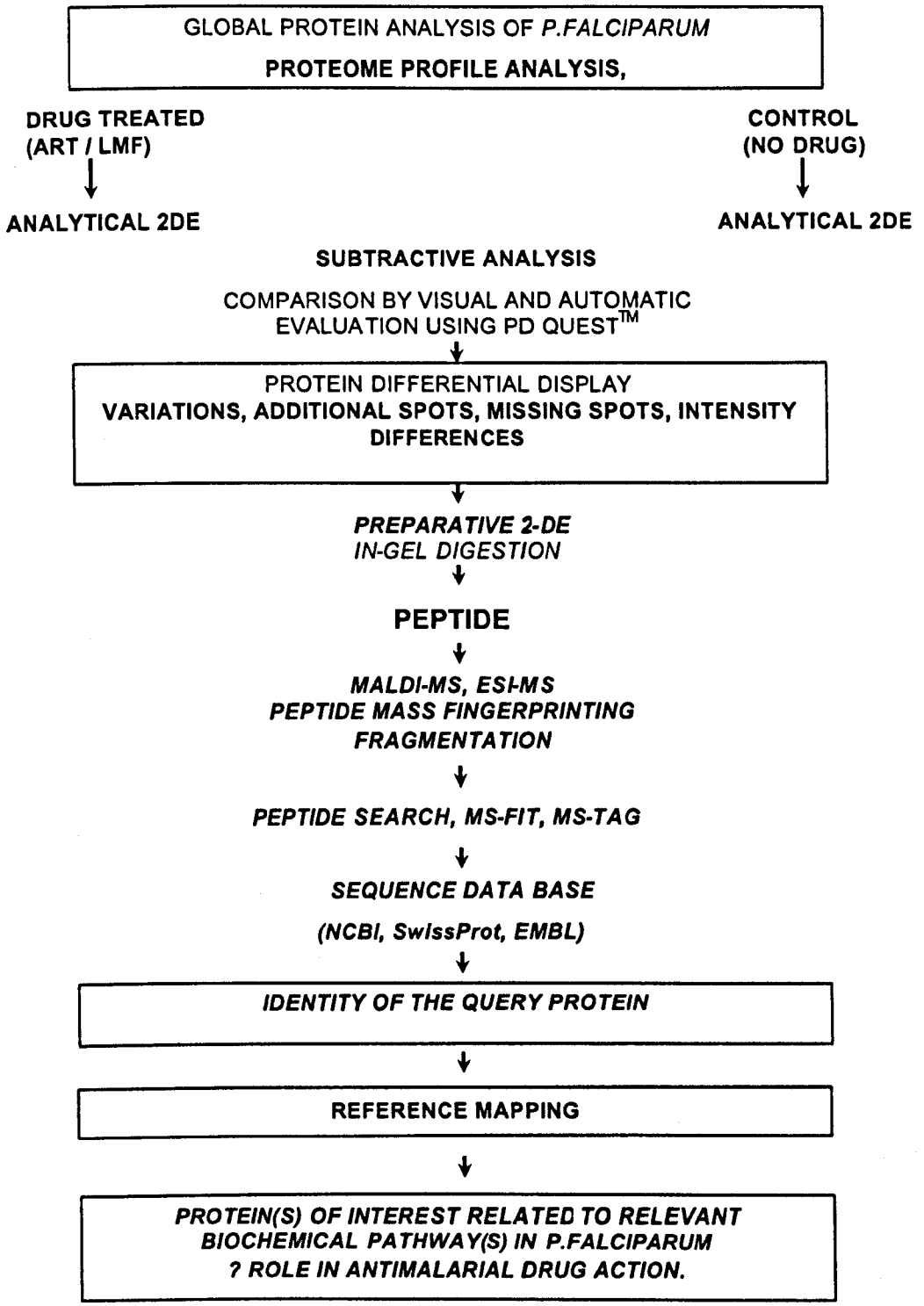


Figure 6.1. **Proteome analysis. Strategies used in this study to detect and analyse *P.falciparum* proteins implicated in ARM & LMF antimalarial action. Sections in italics form the basis of chapter 7.**

Proteins are the primary targets for most therapeutic agents and there is evidence that artemisinin derivatives react with malarial proteins, the Translationally Controlled Tumor Protein (TCTP) being an example [333, 348]. The mechanism of action of LMF is postulated to be related to that of CQ [557] and it is again assumed to implicate cellular protein targets. Pandey [352] has also argued that the artemisinin compounds exert their antimalarial activity by a mechanism similar to that of CQ but this view is not generally supported. The binding of CQ to FP is thought to precipitate a series of toxic events in the parasite food vacuole by inhibiting FP crystalisation [165] and possibly preventing FP destruction by glutathione-dependent [639] and peroxidative [513] mechanisms. CQ is concentrated at least 1000-fold inside the digestive organelle of the malaria parasite [640]. The major mechanisms postulated to account for this CQ accumulation include trapping of charged CQ molecules in the acidic food vacuole or active uptake of the CQ by a specific transporter however there is now compelling evidence in favour of CQ binding to an intracellular receptor as the principle driving force [512]. In keeping with this it is suggested that the two proteins implicated in CQ resistance the *Plasmodial* P-glycoprotein homolog-I (Pgh-I) and the CQ resistance transporter (PfCRT) modulate quinoline uptake either directly by transporting drugs in and out of the food vacuole or indirectly by contributing to the generation of a pH or electrochemical gradient.

The principal aim of this part of the thesis (chapters 6 and 7) is to define the biochemical pathways that are perturbed as a consequence of drug treatment. It is assumed that this will help in unraveling the complexities of drug action and

will highlight critical points in the parasites biochemistry that represent potential novel drug targets.

6.1. METHODS

6.1.1. Cell culture and treatment.

This was performed as described in **section 2.6.1**.

6.1.2. Sample preparation (protein extraction) and total protein determination in sample extracts.

These were performed as described in **sections 2.6.2, 2.6.3** and shown schematically in **figure 6.2.**

6.1.2.1. Sequential Protein Extraction Protocol

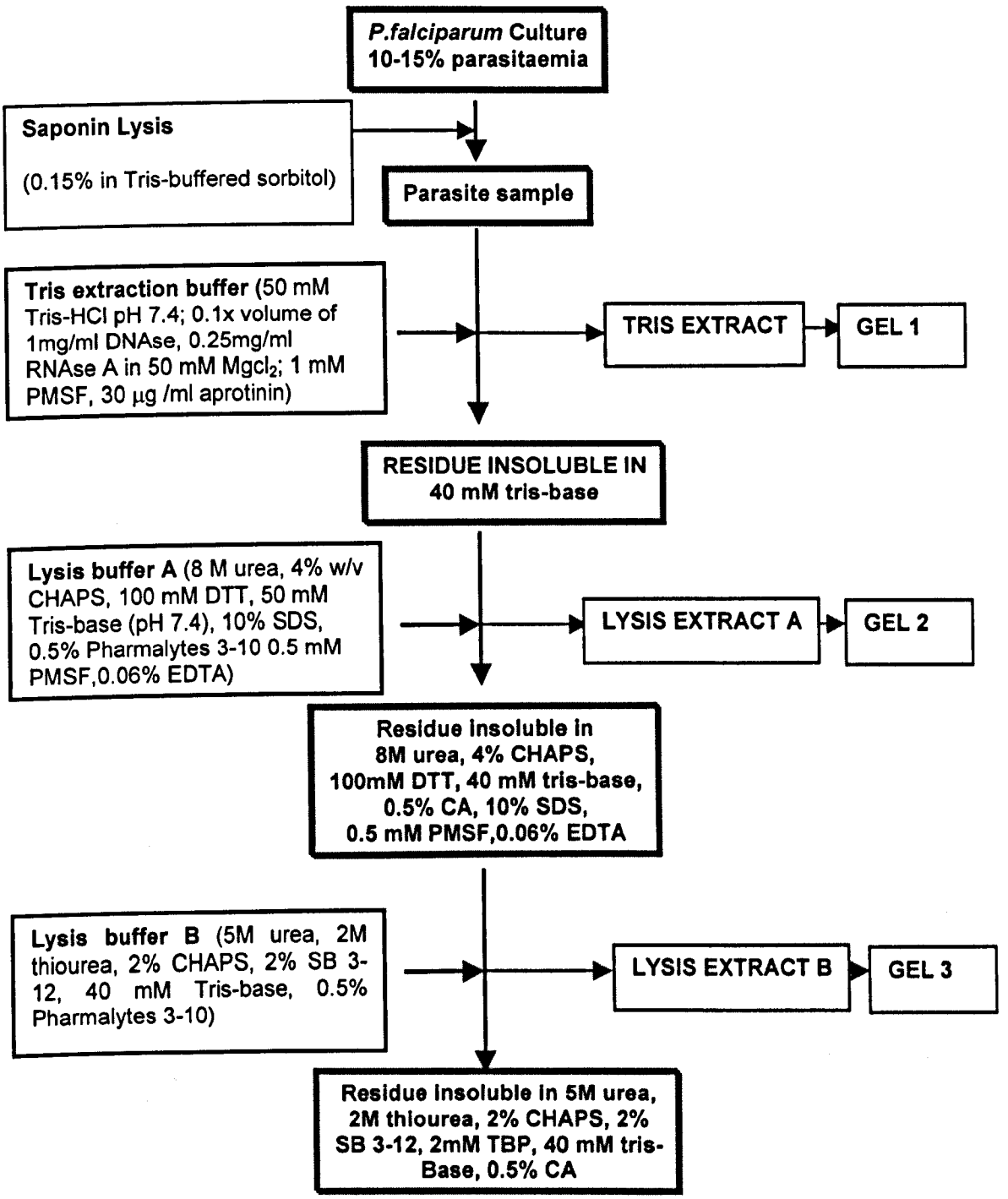


Figure 6.2. Schematic illustration of the 3 step differential extraction protocol based on the concept of differential protein solubility in solubilisation solutions of increasing strength, a modification of that described by M. P. Molloy et al [446]. The Tris extraction buffer solution and lysis solutions A & B denote the 3 solubilisation solutions used. Attempt to do the fourth extraction using SDS was not successful.

6.1.3. Preparation of 2-D gels

All manipulations were performed wearing low protein gloves, in a clean work area. The microfuge tubes and all utensils used were rinsed with 50% v/v MeOH/0.1% v/v TFA solution and allowed to dry. Details of the procedure are presented in **sections 2.6.2-7.** The analytical gels were stained with silver nitrate. Preparative gels for MS were stained with colloidal Coomassie Brilliant Blue G-250 [641].

6.1.4. Gel analysis.

The scanned gels were processed with PDQuest^{TM} software as described in detail in section **2.6.9.** Differential analysis was performed using PDQuest^{TM} version 6.2.1 software tools on a set of four replicate gels per sample. Experimental variations such as intensity differences were analysed with the scatter analysis and normalization of gels was performed. This software process compensates for non-expression related variation in protein spot intensity, which is important for accurate quantitation. Analysis sets were generated to create and study groups of proteins that display treatment related abundance changes. This was performed by measuring the qualitative or quantitative changes in protein expression. Only protein groups present in at least three of the four gels were considered for differential analysis. The analysis window covered the p*I* range from 3-10 and the molecular mass range from 5-100 kDa. (Overlapping narrow range immobilized pH gradients were not used in this work).

For identification of proteins by mass fingerprint analysis (discussed in detail in chapter 7) a software match was performed between analytical silver-stained

gels and preparative gels. This allowed precise position correlation of the spots to be excised for further analysis.

6.2. RESULTS

An example of a typical analytical gel is shown in **figure 6.3.** Analytical gels loaded with 100 µg of protein (standardized through out the study) were used for gel spot analysis. Proteins were separated between pH 3 and 10 using a linear IPG strip for the first dimension and between 10 and 100 kDa in vertical SDS PAGE gel for the second dimension. In this control Tris extract more than 800 individual silver stained parasite proteins can be resolved.

Figure 6.4 shows a typical micro-preparative gel. The micro-preparative gels were loaded with a larger amount of protein (300 µg as opposed to 100 µg) compared to that used for analytical gels and they were stained with Coomassie blue stain. Proteins were again separated between pH 3 and 10 using a linear IPG strip for the first dimension and between 10 and 100 kDa in the vertical SDS PAGE gel for the second dimension. In this control gel more than 500 individual Coomassie stained parasite proteins can be resolved. This gel was run with a protein extract obtained from a single step extraction using the enhanced extraction solution (5 M urea, 2 M thiourea, 2 % w/v SB 3-12, 2 mM TBP, 0.5% v/v ampholytes *p*H 3-10).

Figure 6.5 Shows examples of three different 2 D gels (A, B, & C) which are representative gel maps of proteins separated from the three different sequential

extracts as schematically represented in figure 6.2. Gel A represents gel spots of Tris soluble proteins, extracted with Tris buffer (50 mM Tris-HCl) and these aqueous soluble proteins are assumed to mainly represent cytosolic *Plasmodial* proteins. Gel B represents proteins soluble in a moderately severe extraction buffer (Lysis buffer A, which comprises of 8 M urea, 4% w/v CHAPS, 100 mM DTT, 50 mM TRIS-base (pH 7.4), 10% SDS, 0.5% Pharmalytes 3-10 0.5 mM PMSF,0.06% EDTA). These are assumed to include moderately bound membrane *Plasmodial* proteins and also some cytosolic proteins. Gel C represents proteins soluble in a more rigorous extraction buffer (Lysis buffer B, which comprises of 5M urea, 2M thiourea, 2% CHAPS, 2% SB 3-12, 40 mM Tris-base, 0.5% Pharmalytes 3-10). These are assumed to be mainly tightly bound membrane proteins. As clearly demonstrated in this figure the three gels A, B, & C display a different array of protein spots suggesting that the extracts contain different *Plasmodial* protein complements, although there are spots common to more than one extract and about 10 spots (highlighted in gel C of figure 6.5) appear in all the three gels.

Figures 6.6, 6.8, 6.9, 6.10, 6.11 & 6.12 represent match-sets of 2-D gels generated using PDQuest™ computer software. Gel A in these match-sets represents the gel standard or average gel. It is a synthetic gel image from the spots of the four members (Gels B, C, D, & E) of the match-set generated by Pdquest™ and it represents all spot data in the match-set. It is based on a template chosen from one of the match-set members. The match-sets were

generated on both untreated (controls) and drug treated (ARM or LMF) 2-D gel separated parasites proteins from both Tris extract and the first lysis extract. Gel match-sets were not generated for the second lysis extract because the gel protein arrays of the drug treated samples were not significantly different from the untreated parasite proteins on analysis.

Figures 6.7a & 6.7b are scattergrams performed on the control Tris match-set **(Fig. 6.6)**. They clearly show that there is good protein spot correlation between gels that were prepared and run under similar conditions.

Figure 6.13 shows gel standards (average gels) A, B, & C generated by PDQuest^{TM} for both controls (untreated), and drug treated ARM or LMF Tris soluble *P.falciparum* proteins respectively. These were generated from four member gels in each group run under standardized conditions. Quantitative analysis of protein spots that were present in both untreated and treated groups was performed; there was a general overall reduction in protein expression after ARM treatment with 13 spots showing at least a three fold reduction in expression. Only 5 proteins showed a more than three fold increase in spot intensity following ARM treatment. Conversely there was general increase in protein expression following LMF treatment with 33 spots displaying at least a 3 fold increase in spot intensity. These findings suggest clear differences in the mechanism of action of these two drugs.

Figure 6.14. Shows the gel standard for the control lysis gel match-set (the four member gels are not displayed in this window). This master gel represents spot data from all the four member gels and shows about 500 protein spots separated between PH 3 – 10 and Molecular weight 10 – 100 kDa.

Figure 6.15. Shows gel maps A, B, & C for both controls (untreated) and drug treated ARM or LMF lysis soluble *P.falciparum* proteins respectively. Quantitative analysis of protein spots that were present in both untreated and treated groups was performed and these results demonstrate a general trend towards down regulation of many proteins in the high pH region (8-10) in both ARM and LMF treated samples. 10 spots in the ARM treated group showed at least a 3 fold increase in protein intensity while 16 displayed at least 3 fold reduction in protein intensity (mainly alkaline proteins). In comparison 6 protein spots showed at least a 3 fold increase in protein intensity and five proteins a reduction of at least 3 fold in protein intensity following LMF treatment. **Figure 6.16** is a sample of the PDQuest™ pictorial presentation (bar chart) of the quantitative differences in gel intensity between the individual control (untreated) gels versus the treated (ARM or LMF) gels.

Figure 6.17. Represents the gel standard map of ARM treated lysis buffer A (8 M urea, 4% w/v CHAPS, 100 mM DTT, 50 mM TRIS-base (*p*H 7.4), 10% SDS, 0.5% Pharmalytes 3-10, 0.5 mM PMSF,0.06% EDTA) soluble *P.falciparum* proteins, generated from four member gels. It demonstrates qualitative

differences (presence or absence of protein spots possibly representing the switching on and off of protein expression profiles) in ARM treated compared to controls (with no drug). These data show significant qualitative differences between the two groups. The spots present in both control and ARM treated are denoted by the green spots without annotations. 42 spots (numbered 1-42) were present only in the ARM treated group (red spots) and 26 spots (numbered 43-69) were only present in the control group. **Figure 6.18** shows the equivalent qualitative differences following LMF treatment in a similar lysis extract. These data show the spots present in both groups (LMF treated versus control) in green, and 73 spots (red spots, numbered 1-73) are shown to be present only in the LMF treated. There were no spots observed in the control group that were not also present in the LMF treated group.

Figure 6.19 gives a sample of the Two-dimensional electrophoretic protein pattern of 2-D SDS-PAGE standards run separately. These were used for the determination of the *p*H and molecular weight ranges for our 2-D gel system and for constructing calibration curves for calculating the relative p*I* and M_r of unknown *Plasmodial* proteins. 5 µL of BIO-RAD 2-D SDS-PAGE standards were run according to the method of Klose [461] and silver stained.

6.2.1. Analytical and Micro-preparative Gel Samples represented in figures 6.3 and 6.4 respectively.

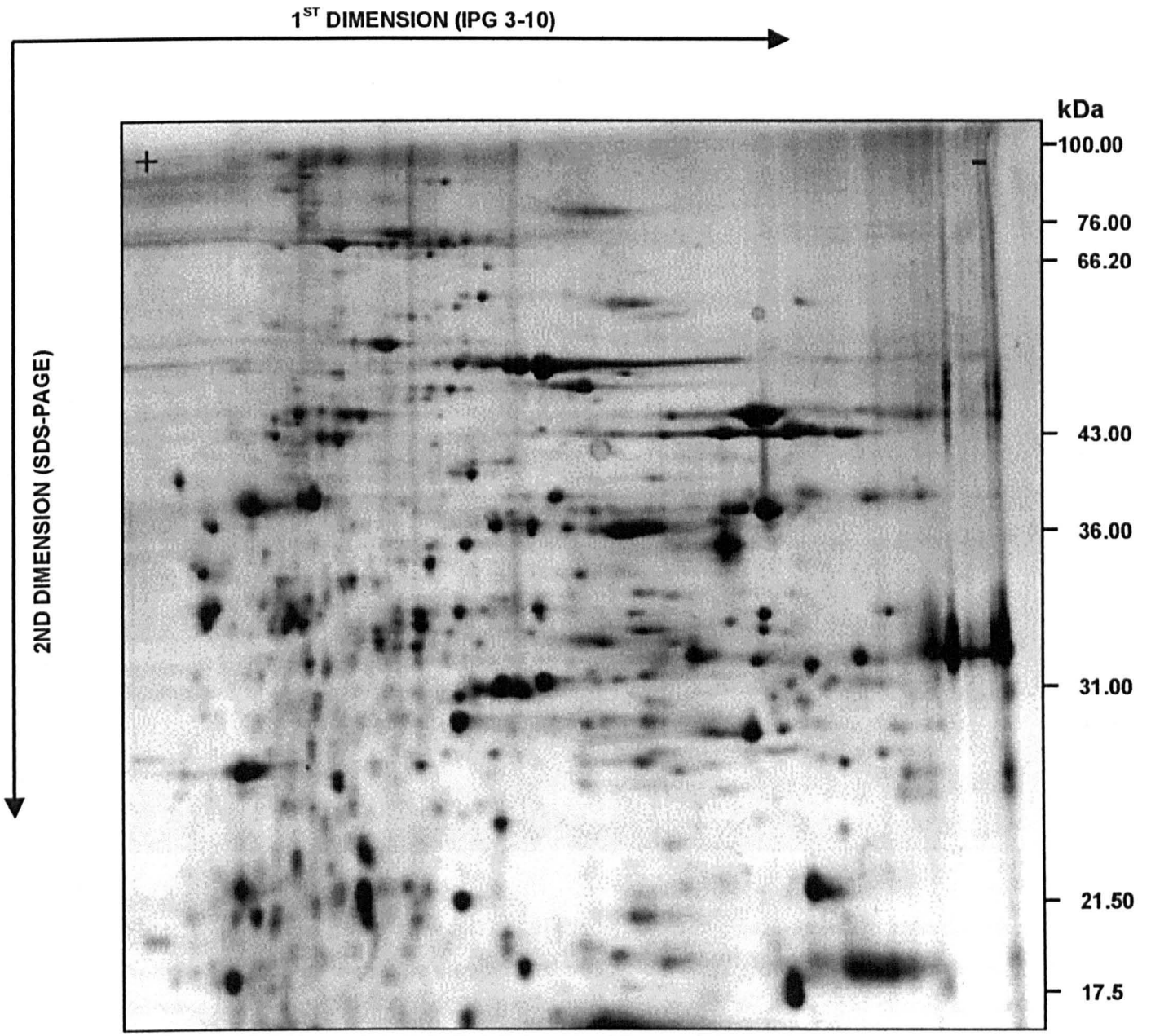


Figure 6.3. Analytical 2-D gel of Tris soluble *P.falciparum* proteins (K1 isolate). First dimension, ready-made IPG 3-10 (8 h at 3000 V max.) separation distance, 130 mm; 100 µg of sample was loaded onto IPG gel by in-gel rehydration. Second dimension, vertical SDS-PAGE (12.5% T constant); silver stain.

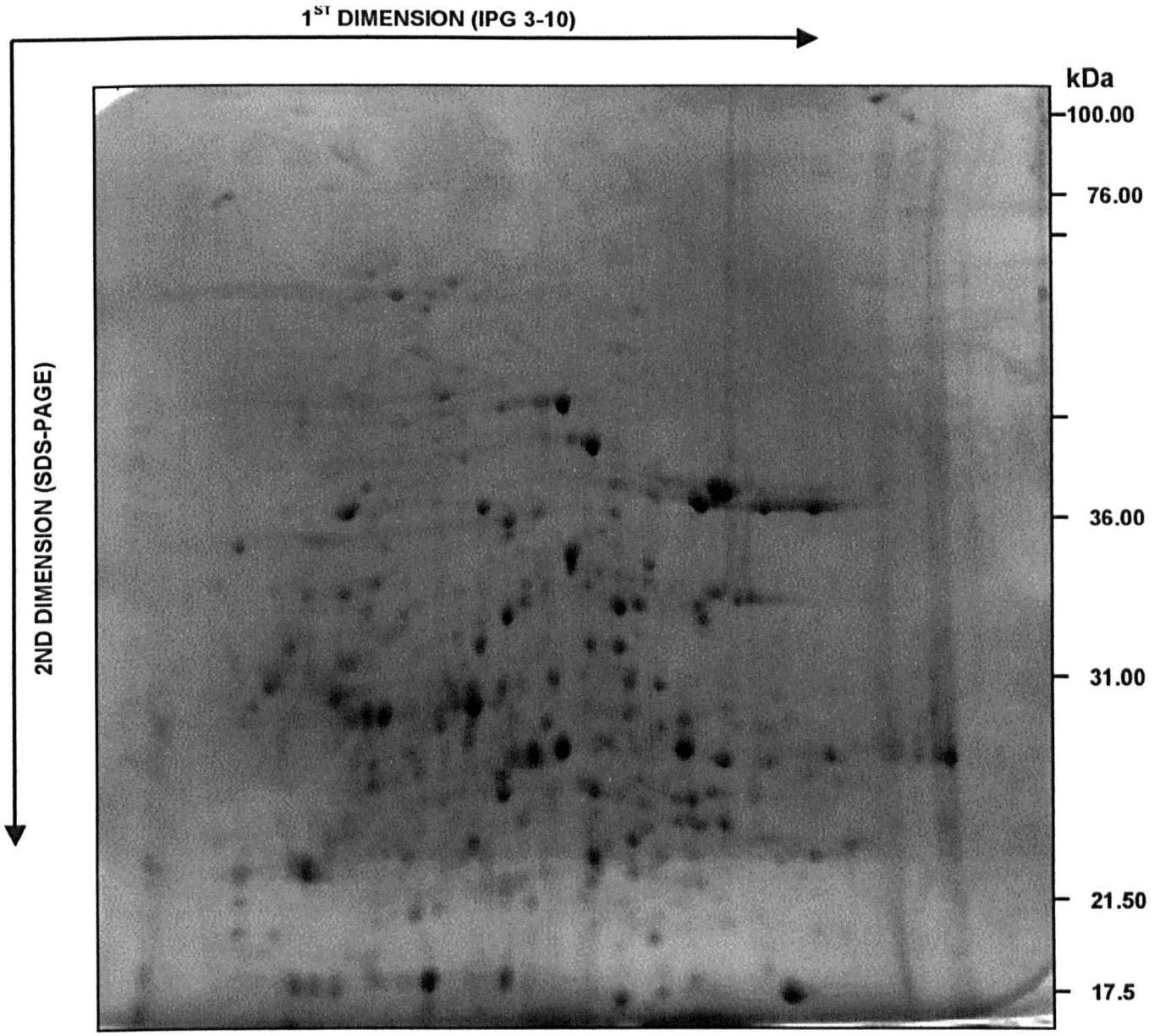


Figure 6.4. **Micro-preparative 2-D gel of *Plasmodium falciparum* protein extract obtained from a single step extraction using the enhanced extraction solution, 5 M urea, 2 M thiourea, 2 % w/v SB 3-12, 2 mM TBP, 0.5% v/v ampholytes *p*H 3-10. First dimension, IPG 3-10 (8 h at 3000 V max.) separation distance, 130 mm; 300 µg of sample was loaded onto IPG gel by in-gel rehydration. Second dimension, vertical SDS-PAGE (12.5% T constant). Colloidal Coomassie blue stain.**

6.2.2. 2-D gel protein display of sequentially extracted Plasmodial proteins.

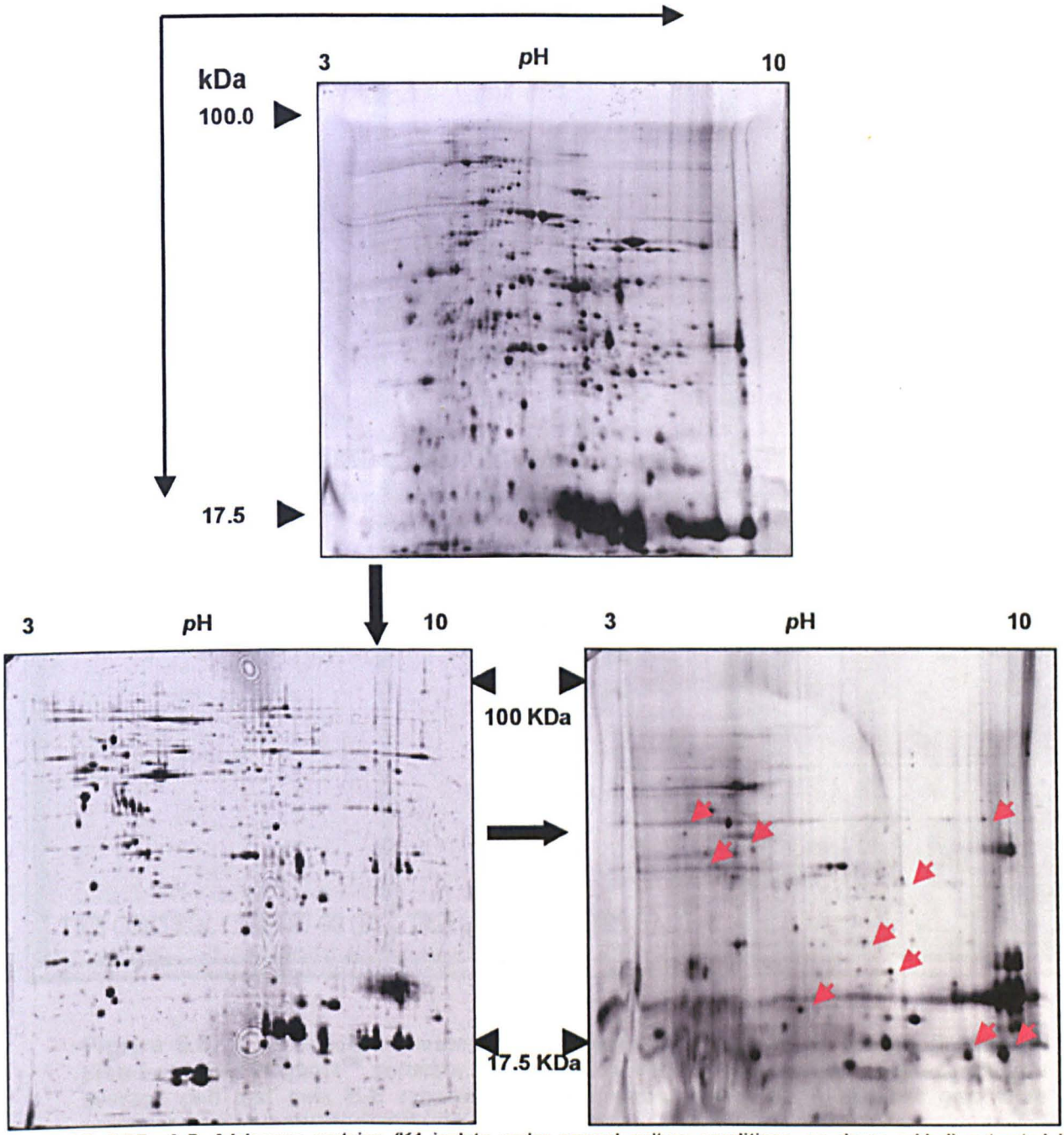


Figure 6.5: 2-DE of *P. falciparum* proteins (K1 isolate under normal culture conditions, no drugs added) extracted by differential solubilisation of the *Plasmodial* cell lysate (refer figure 6.2). *First dimension*: Isoelectric focusing in an immobilized *p*H gradient *p*H 3-10 in a 13 cm long gel strip. *Second dimension*: SDS PAGE in a 12.5% homogenous gel. Silver stained. Gel A=TRIS soluble proteins (mainly cytosolic proteins), Gel B=Lysis buffer A (8 M urea, 4% w/v CHAPS, 100 mM DTT, 50 mM TRIS-base (pH 7.4), 10% SDS, 0.5% Pharmalytes 3-10 0.5 mM PMSF,0.06% EDTA) soluble proteins (moderately bound membrane proteins), Gel C =Lysis buffer B ((5M urea, 2M thiourea, 2% CHAPS, 2% SB 3-12, 40 mM Tris-base, 0.5% Pharmalytes 3-10) soluble proteins (tightly bound membrane proteins). The protein spots highlighted in Gel C are uniquely present in all three gel extracts.

6.2.3. Matchsets of 2-D gels generated using PDQuest™ representing sets of gels run under standardized conditions.

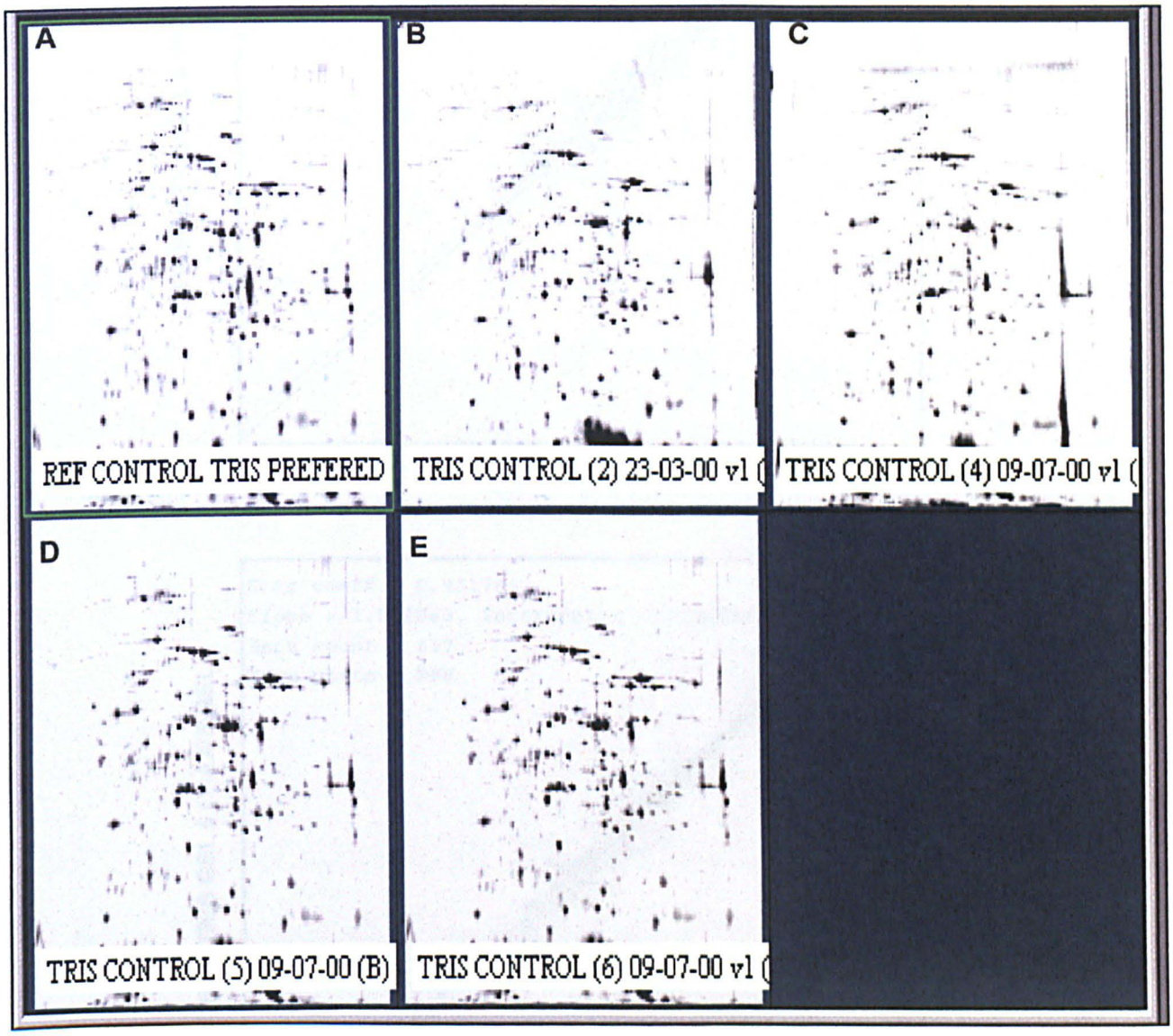


Figure 6.6. A gel matchset created from 4 control 2-D gels of TRIS soluble *Plasmodial* proteins using PDquest™ software. Gel A represents the gel standard (representative / average gel) and gels B-E represent the 4 member gels. The 4 member gels were processed and run under standardized conditions.

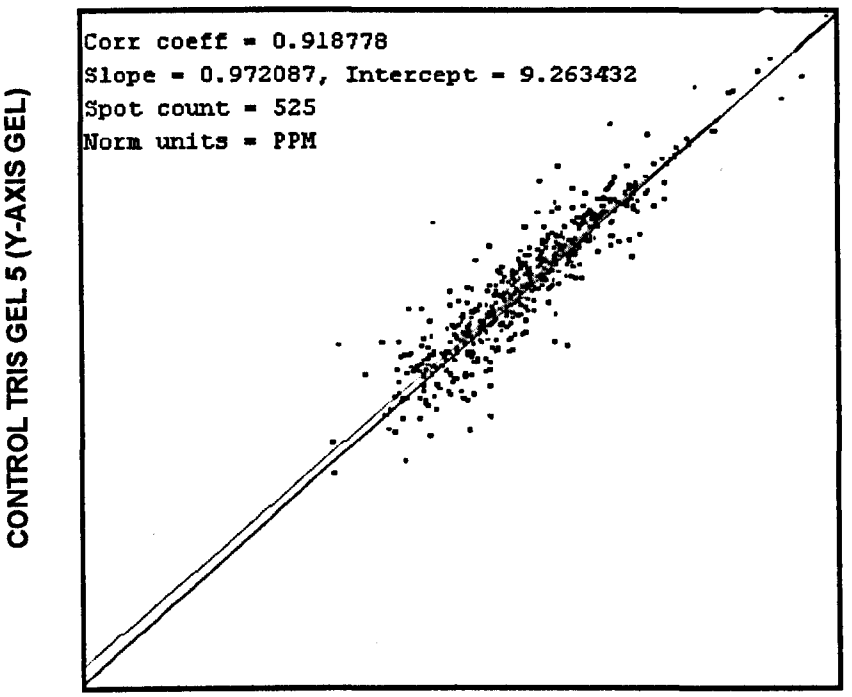


Figure 6.7a

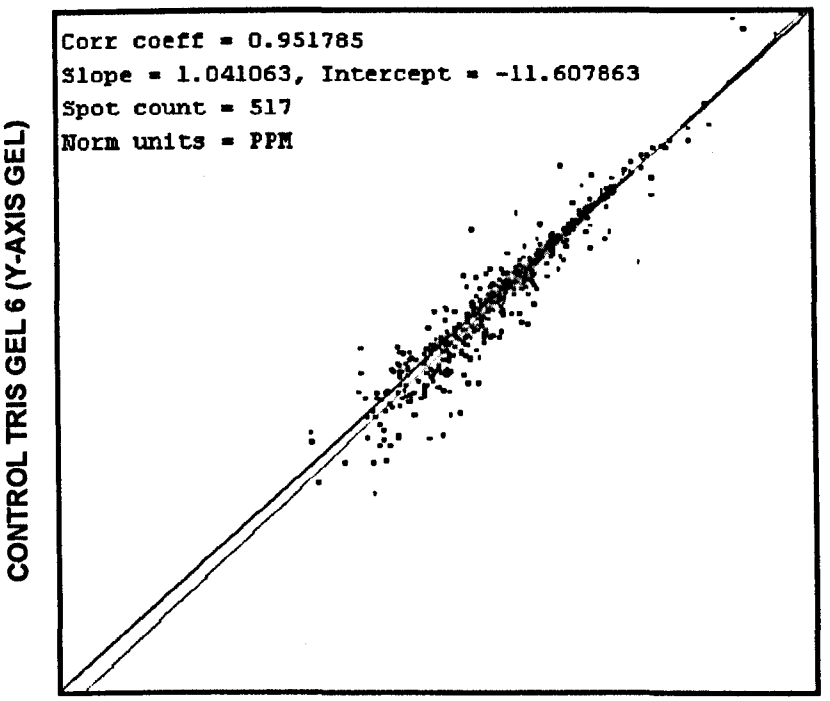


Figure 6.7b

Figures 6.7a &b Show scattergrams demonstrating the extent of correlation of protein spots between the gels included in the Control TRIS match set (figure 6.6.) prepared and run under similar conditions. Some minor differences present were averaged in the reference gel.

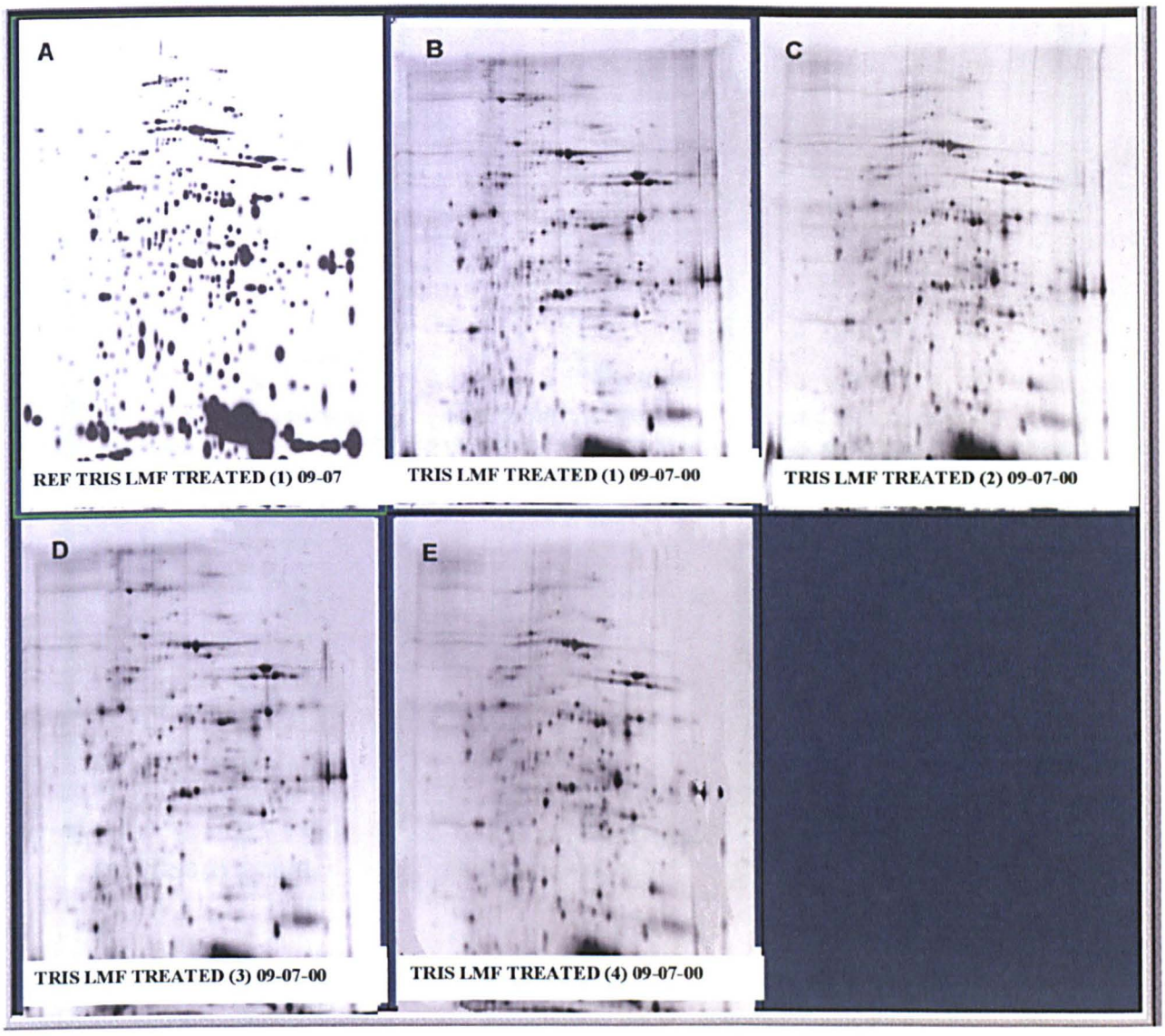


Figure 6.8. A gel matchset created from four 2-D gels of Tris soluble *Plasmodial* proteins harvested following exposure of the parasites to LMF $_{IC90}$ for 6 hr. Gel A represents the gel standard (representative / average gel) and gels B-E represent the 4 member gels. The 4 member gels were processed and run under standardized conditions.

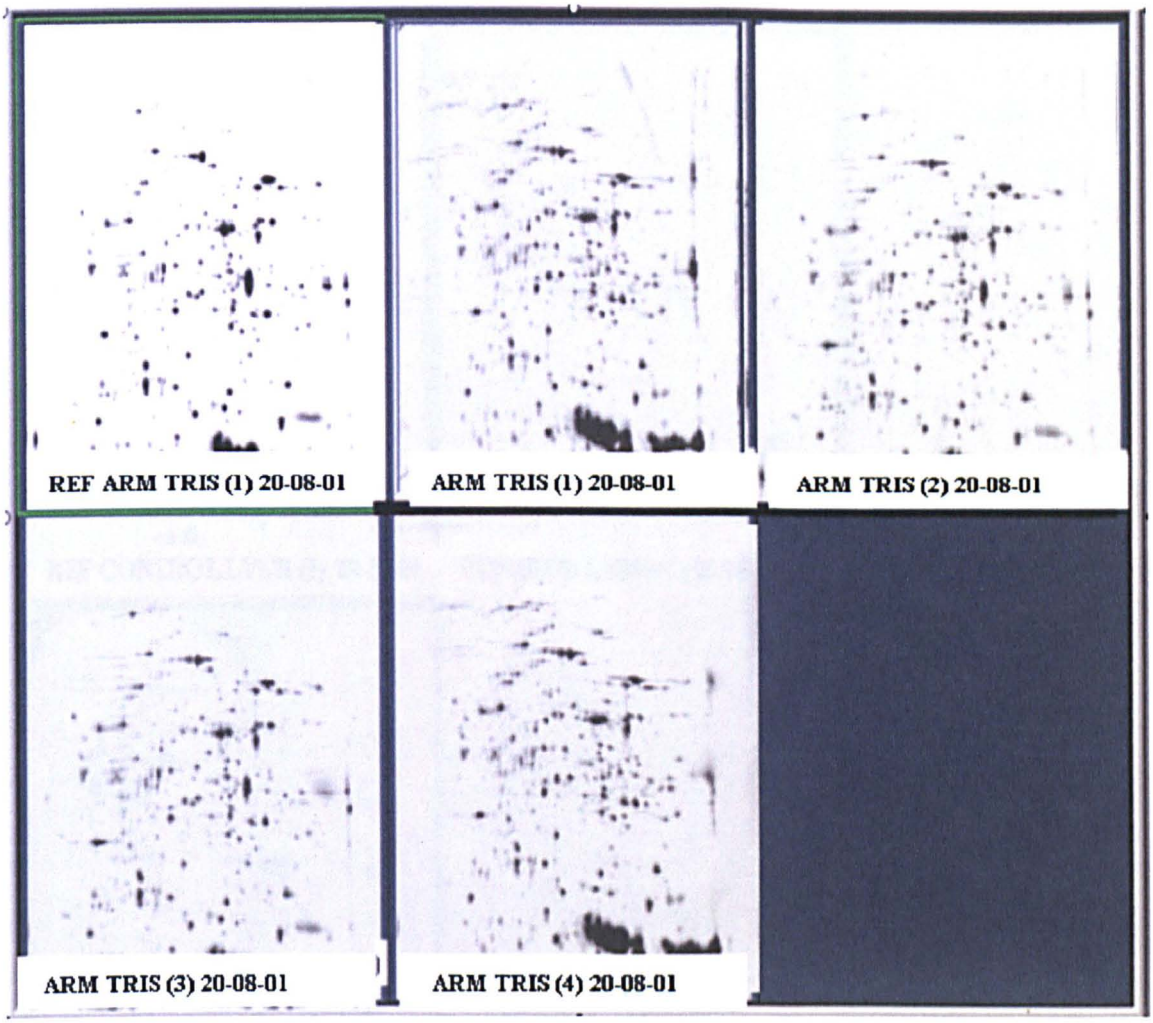


Figure 6.9. A gel matchset created from four 2-D gels of tris soluble *Plasmodial* proteins harvested following exposure of the parasites to ARM $_{IC90}$ for 5 hr. Gel A represents the gel standard (representative / average gel) and gels B-E represent the 4 member gels. The 4 member gels were processed and run under standardized conditions.

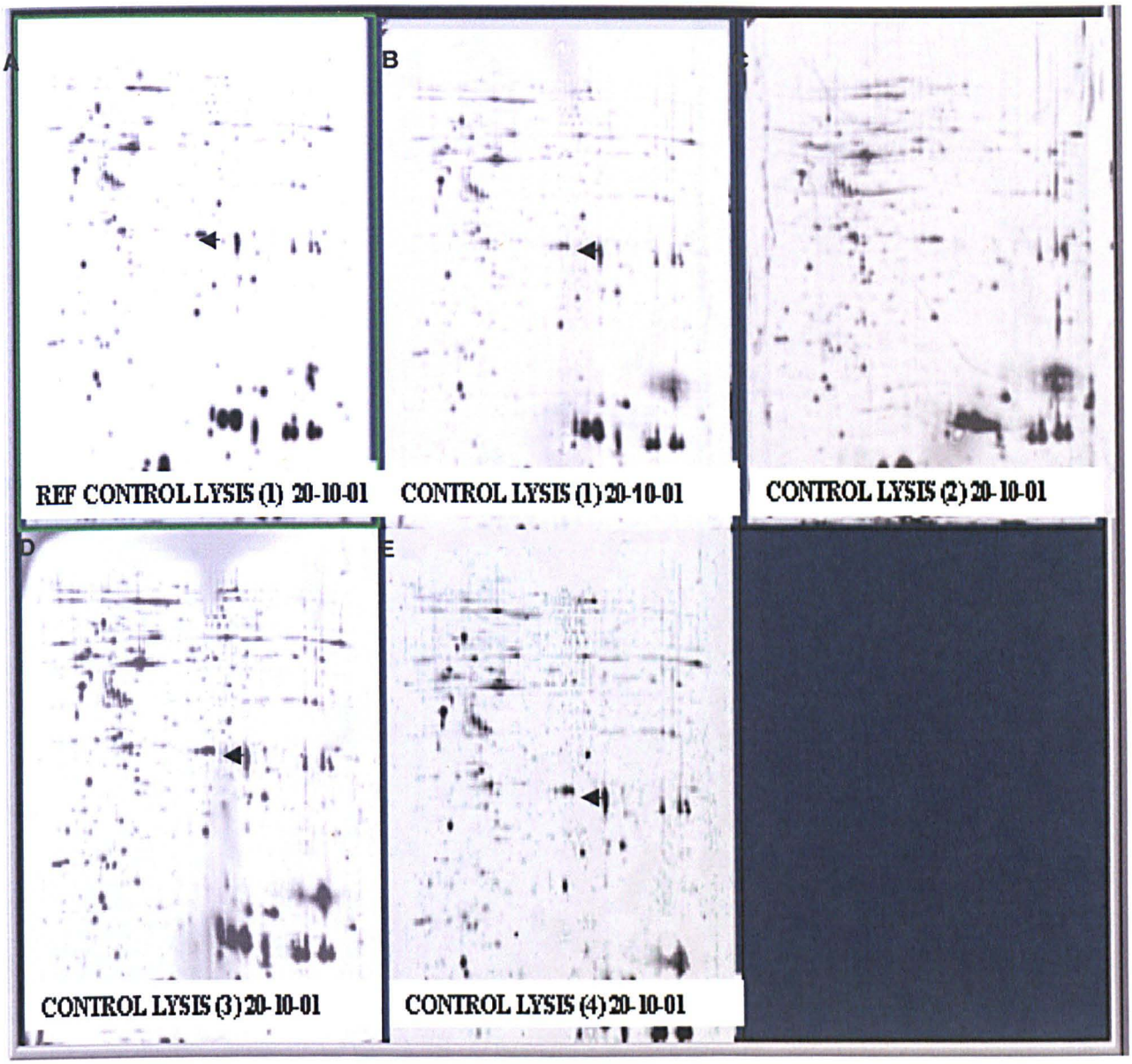


Figure 6.10. A gel matchset created from 4 control 2-D gels of lysis solution A (refer to figure 6.2) soluble *Plasmodial* proteins. Gel A represents the gel standard (representative / average gel) and gels B-E represent the 4 member gels. The 4 member gels were processed and run under standardized conditions. (Note: the arrows points to an internal standard which was absent in gel C)

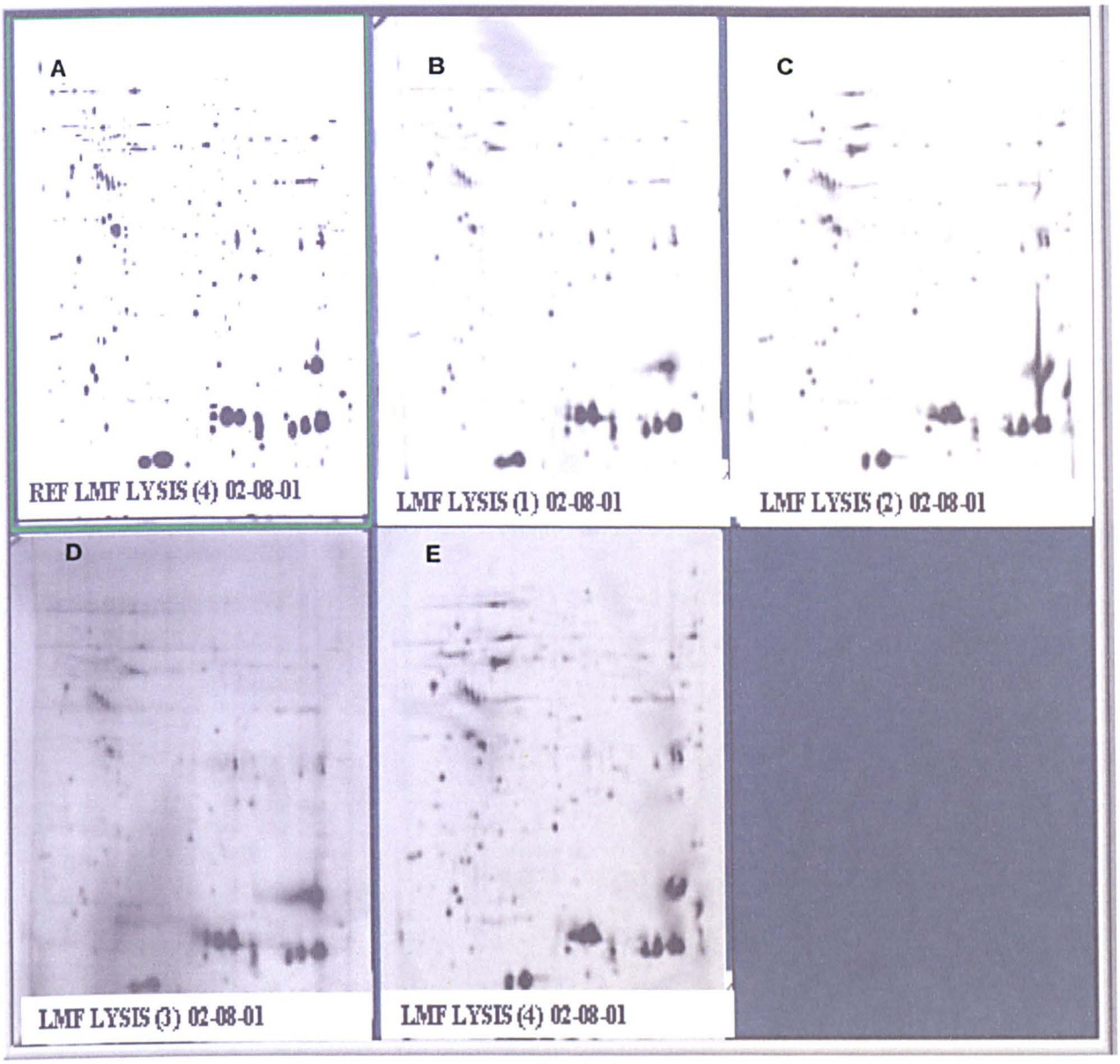


Figure 6.11. **A gel matchset created from four 2-D gels of lysis solution A (refer to figure 6.2) soluble *Plasmodial* proteins harvested following exposure of the parasites to LMF IC_{90} for 6 hr. Gel A represents the gel standard (representative / average gel) and gels B-E represent the 4 member gels. The 4 member gels were processed and run under standardized conditions.**

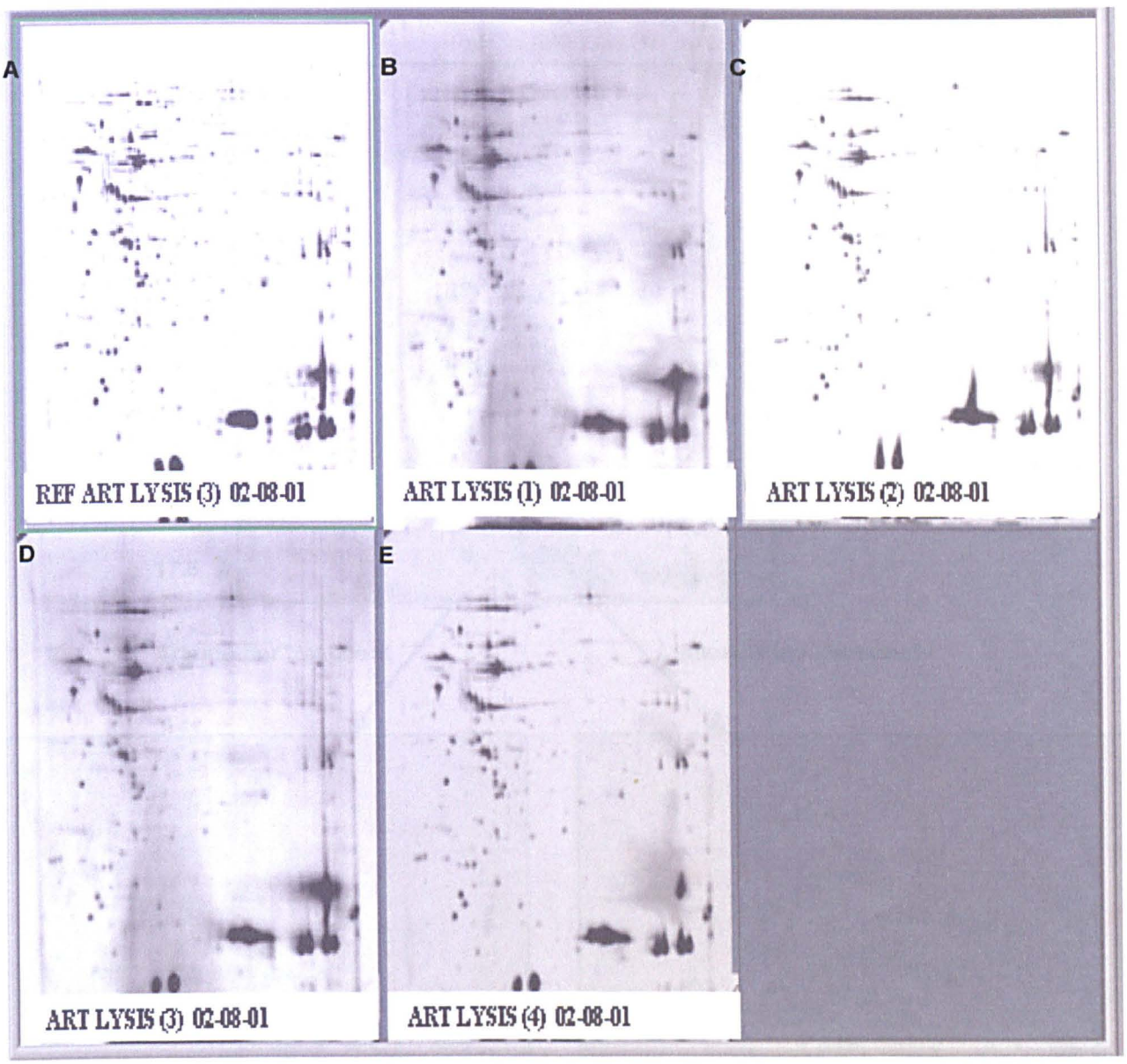


Figure 6.12. **A gel matchset created from four 2-D gels of lysis solution A (refer to figure 6.2) soluble *Plasmodial* proteins harvested following exposure of the parasites to ARM IC_{90} for 5 hr. Gel A represents the gel standard (representative / average gel) and gels B-E represent the 4 member gels. The 4 member gels were processed and run under the standardized conditions.**

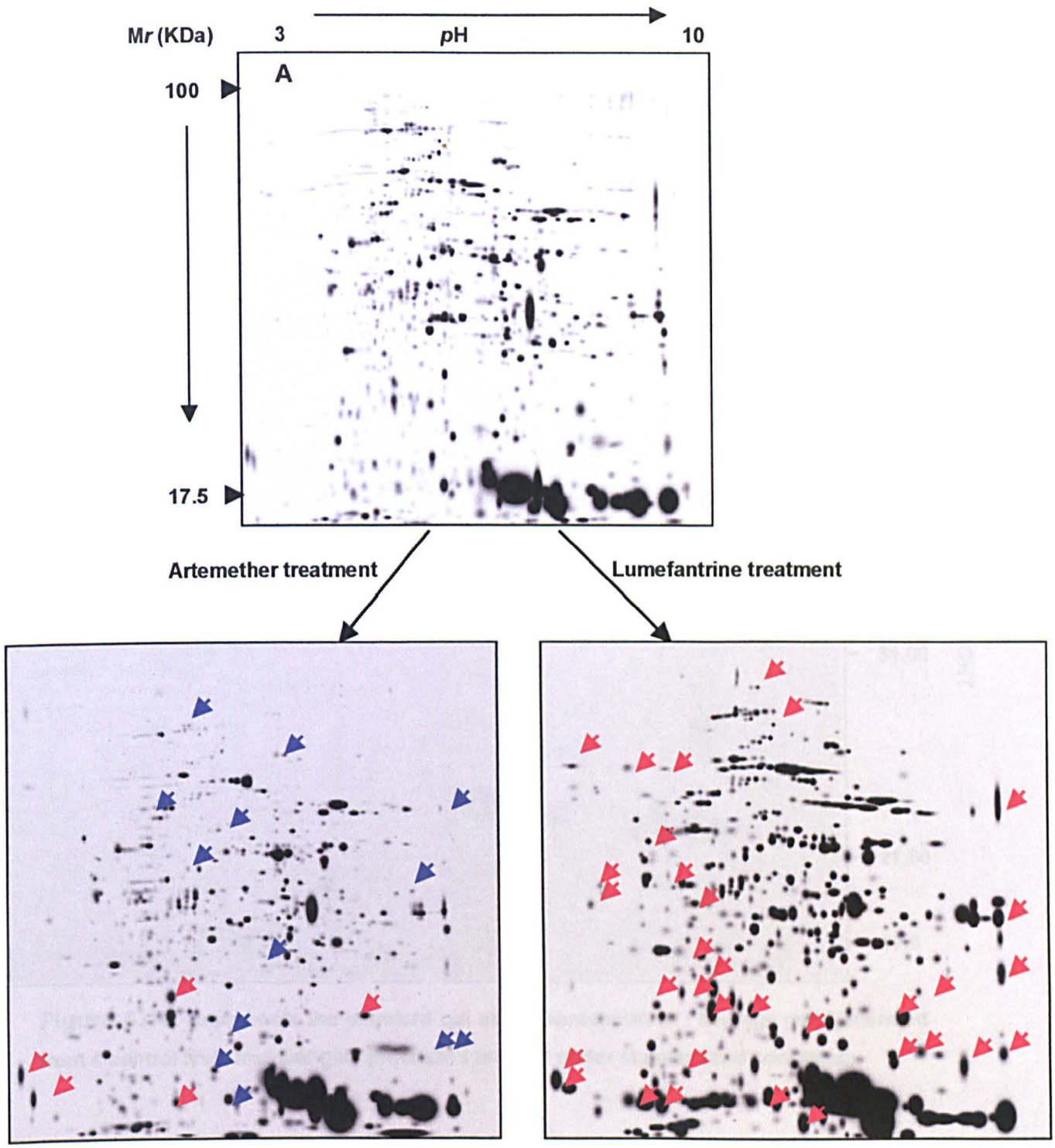


Figure 6.13. A, B, and C denote gel standard maps for control (no drug), ARM and LMF treated Tris soluble *P.falciparum* proteins respectively, generated from at least four member gels. Proteins found over or under expressed in at least three member gels represented in the standard gel maps are high lighted with arrows. The red arrows denote over expressed and the blue arrows denote under expressed proteins.

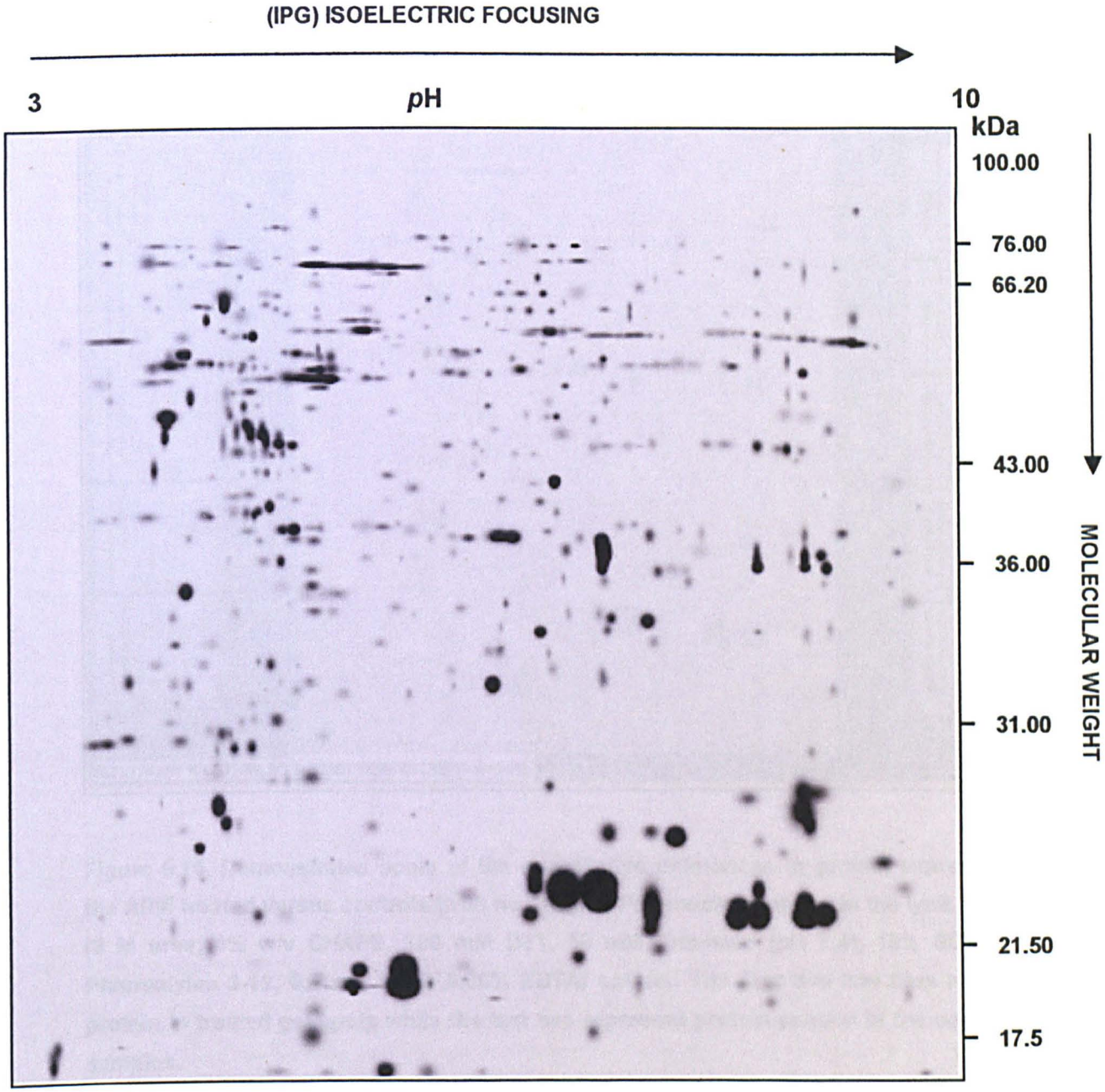


Figure 6.14. Represents the standard gel map (representative / average gel) generated from 4 control lysis member gels processed and run under standardized conditions.

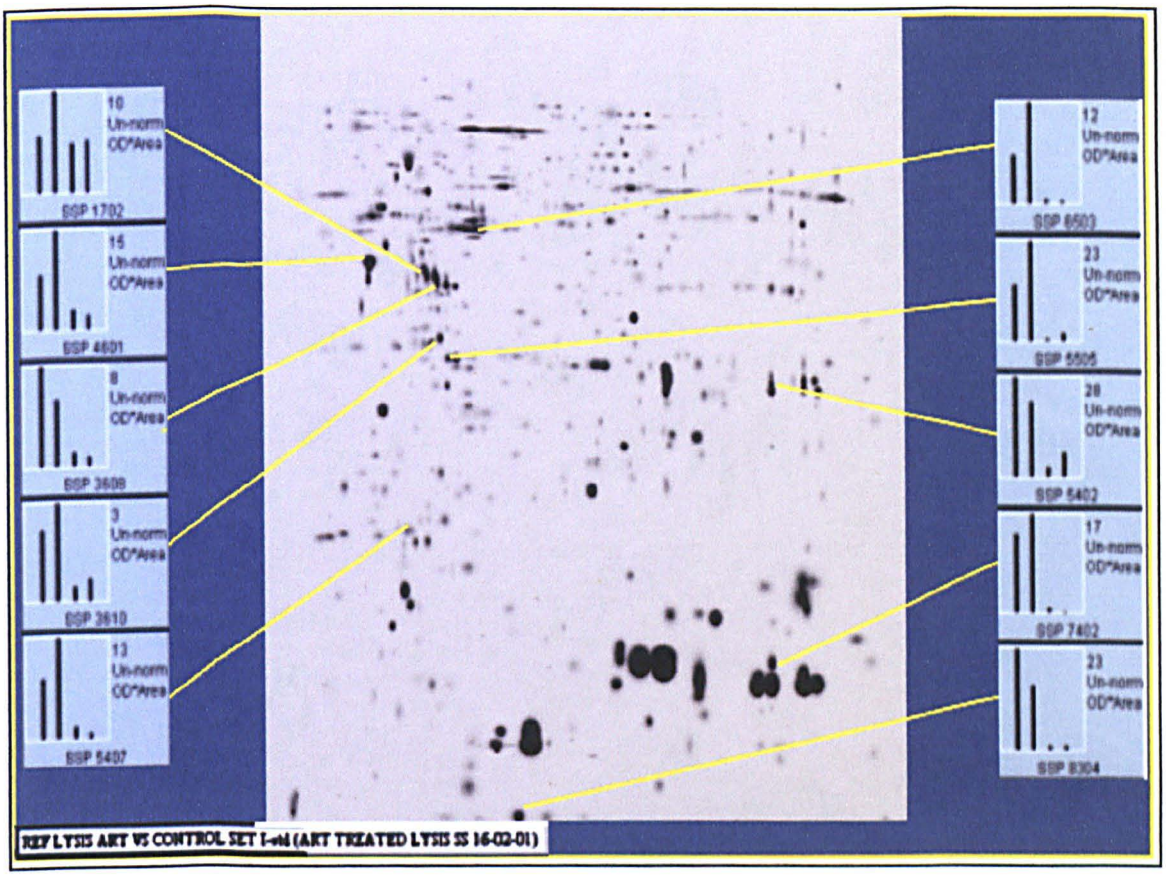


Figure 6.16. Demonstrates some of the quantitative differences in protein expression in the ARM treated versus controls (with no drug) of *Plasmodial* proteins in the lysis buffer A (8 M urea, 4% w/v CHAPS, 100 mM DTT, 50 mM Tris-base (*p*H 7.4), 10% SDS, 0.5% Pharmalytes 3-10, 0.5 mM PMSF,0.06% EDTA) extract. The first two line bars represent protein in treated gel spots while the last two represent protein content in the control gel samples.

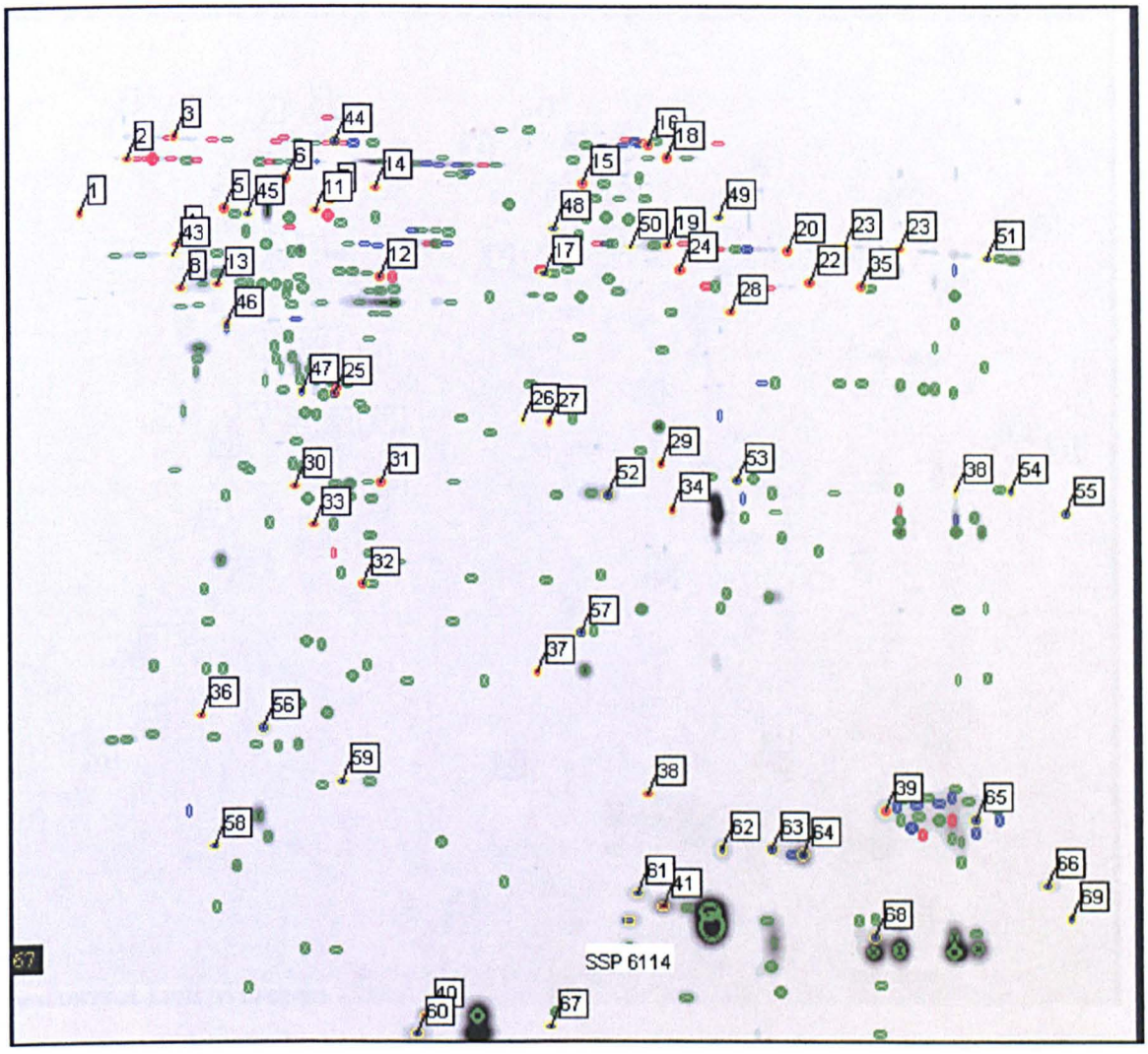


Figure 6.17. Gel standard map of ARM treated lysis buffer A (8 M urea, 4% w/v CHAPS, 100 mM DTT, 50 mM TRIS-base (*p*H 7.4), 10% SDS, 0.5% Pharmalytes 3-10, 0.5 mM PMSF,0.06% EDTA) soluble *P.falciparum* proteins, generated from four member gels. It demonstrates qualitative differences in ARM treated compared to controls (with no drug). Green spots=spots in both control and ARM treated; Red spots (numbered 1- 42) =present only in ARM treated. Blue spots (numbered 43-69) = present only in the controls.

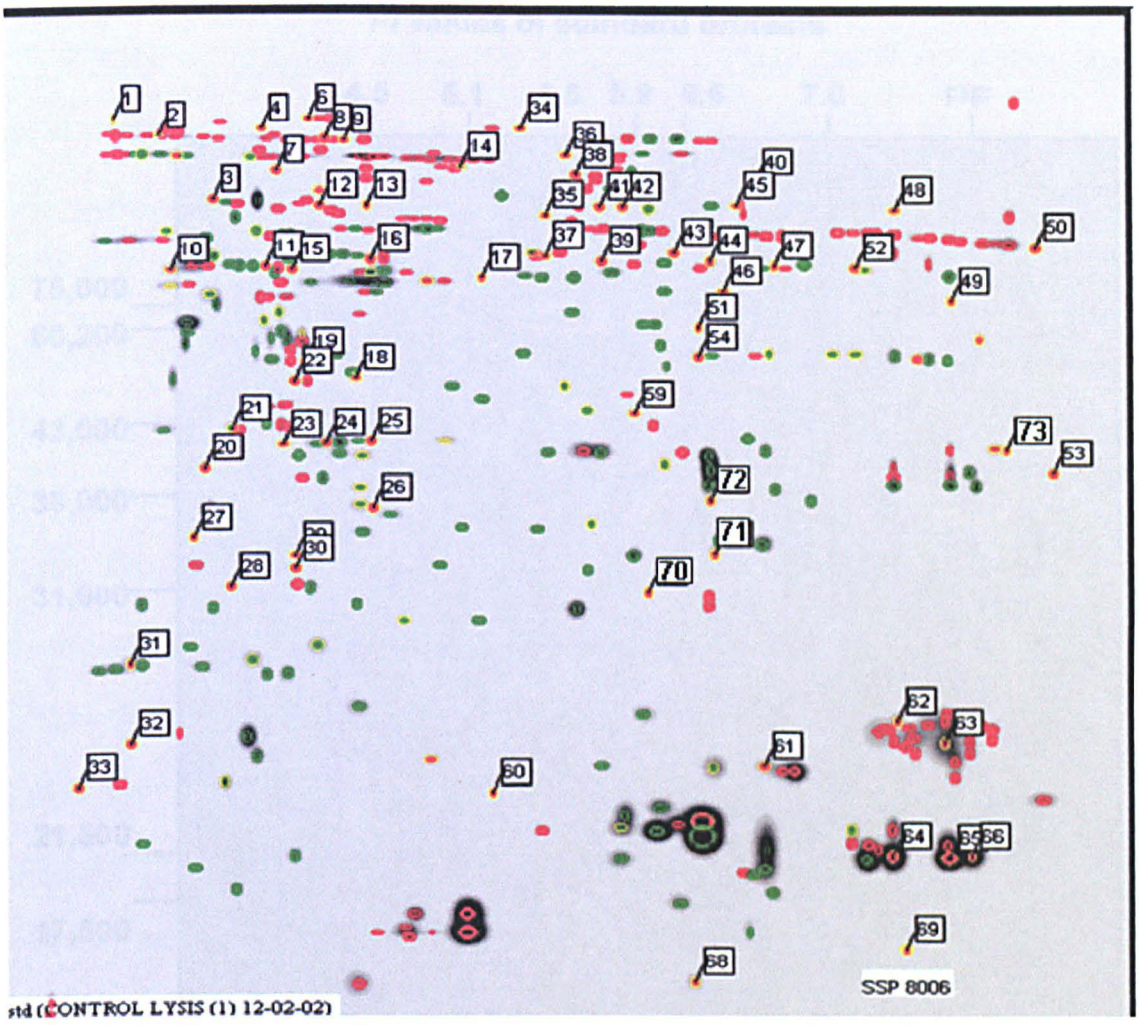
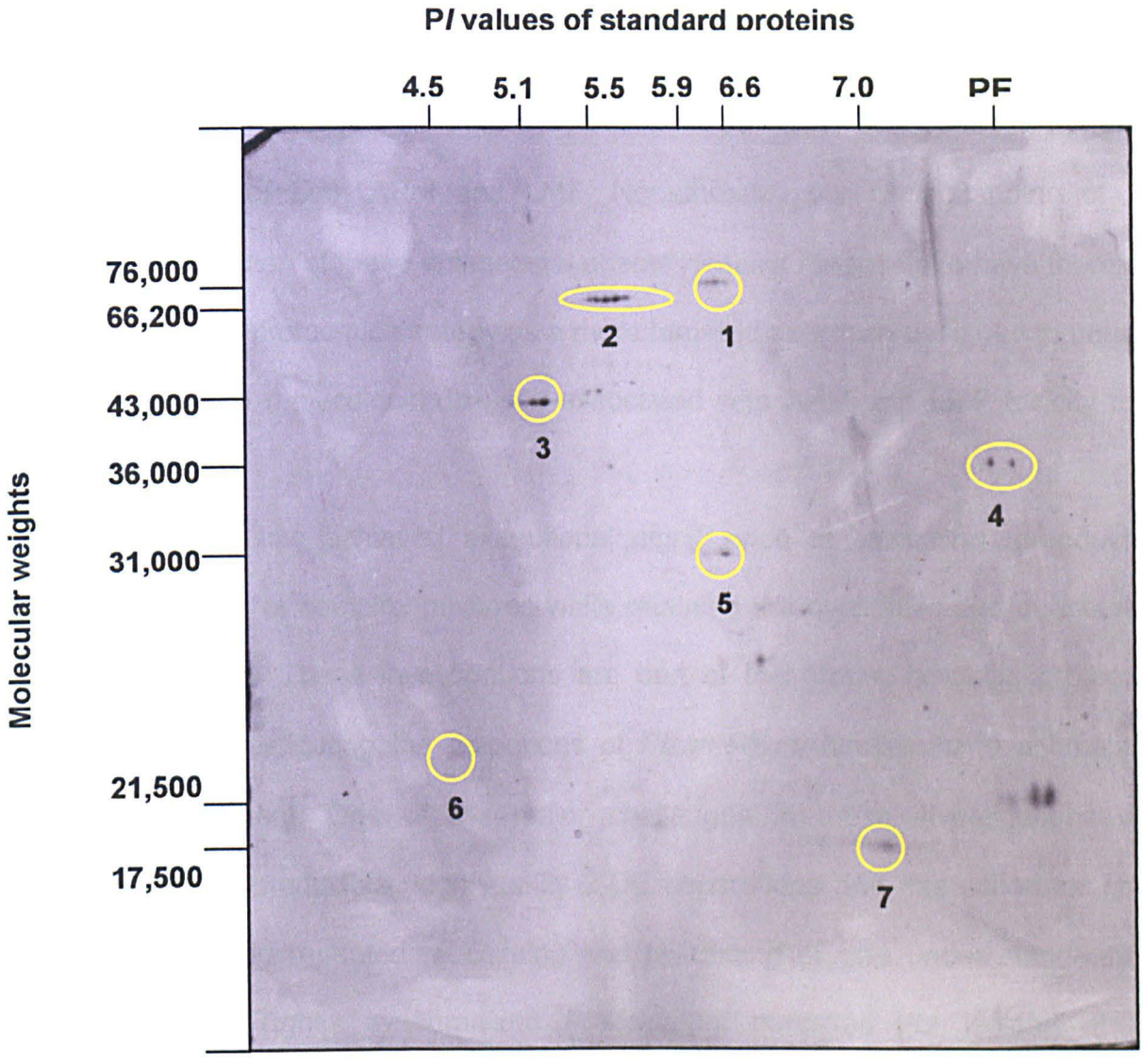


Figure 6.18. Gel standard map for LMF treated lysis (buffer A (8 M urea, 4% w/v CHAPS, 100 mM DTT, 50 mM Tris-base (pH 7.4), 10% SDS, 0.5% Pharmalytes 3-10, 0.5 mM PMSF,0.06% EDTA)) soluble *P.falciparum* proteins, generated from four member gels. It demonstrates qualitative differences in LMF treated compared to controls (with no drug). Green spots =spots in both control and LMF treated; Red spots (numbered 1- 73) =present only in LMF treated. No spots were observed only in the control group



Protein Spots: 1. Conalbumin; 2. Albumin; 3. Actin; 4. GAPDH; 5. Carbonic anhydrase; 6. Trypsin inhibitor; 7. Myoglobin.

Figure 6.19: Two-dimensional electrophoretic protein pattern of 2-D SDS-PAGE Stardands that were used for the determination of the *p*H and molecular weight ranges for our 2-D gel system and for constructing calibration curves for calculating the relative p*I* and M_r of unknown *Plasmodial* proteins. 5 µL of BIO-RAD 2-D SDS-PAGE standards were run according to the method of Klose [461] and silver stained. The isoelectric points of BSA and muscle actin are altered by urea in the standards buffer.

6.3. DISCUSSION

Work done in the earlier chapters has incontrovertibly implicated the *Plasmodial* food vacuole, principally the haemoglobinolytic pathway, as a common target for the action of both ARM and LMF. Nonetheless, our understanding of the definitive action of these antimalarial agents remains meager. We have therefore employed a proteomic strategy as a more targeted approach designed to unravel fundamental molecular processes associated with ARM and LMF toxicity to *P falciparum.*

2-D PAGE has achieved exceptional significance in producing reproducible fractionation of complex mixtures while retaining the qualitative and quantitative relationships. These investigations are part of the ground-breaking proteomic projects investigating the responses of *Plasmodium falciparum* to antimalarial drug treatment. One of the major challenges in these investigations was obtaining reproducible, high quality 2-DE separations and this called for great precision and repeated processing and re-running of gels under standardized conditions. Tightly synchronized *P.falciparum* parasites (K1 isolate) at the trophozoite stage 20-26 hr post invasion were used, with the same batch being maintained throughout these studies. This careful control of all experimental conditions has resulted in these highly consistent and reproducible results.

It would be impossible to analyse the total proteome of an organisms such as *P.falciparum*, with a potential protein repertoire in excess of 6000, in a single 2D gel array. In order to reduce this complexity with these protein samples the extracted parasite proteins were pre-fractionated prior to electrophoretic analysis.

In these studies a modification of the Molloy et al protocol [446] based on the differential extraction approach was used. This approach is based on a straightforward model of sequentially extracting proteins by exploiting differential solubility in solubilising solutions of increasing strength [642] and integration of highly solubilising conditions into the isoelectric focusing (IEF) sample solutions [446] (**refer to figure 6.2**). **Figure 6.5** shows 2-D gels from *Plasmodial* proteins sequentially extracted with increasingly rigorous solubilising solutions. The three resultant gels A, B, and C in **figure 6.5** evidently display different protein patterns. This confirms the value of the sequential extraction approach which allowed the identification of some low expression proteins which otherwise would have been masked by the more abundant proteins in a single extraction approach. Gel A in **figure 6.5** represents the first extract (the aqueous fraction) of Tris-base soluble proteins and is assumed to predominantly represent cytosolic proteins [446]. The addition of the Tris base has an added advantage of [446] minimizing proteolysis [446] of the extracted proteins. Gel B in **figure 6.5** represents the second and more stringent extract of proteins soluble in lysis buffer solution A (8 M urea, 4% w/v CHAPS, 100 mM DTT, 50 mM Tris-base (*p*H 7.4), 10% SDS, 0.5% Pharmalytes 3-10) 0.5 mM PMSF,0.06% EDTA) which is an adaptation of the conventional solubilisation solution. This fraction comprises of the mild-moderately bound membrane proteins and some cytosolic proteins. The most rigorous extract is represented in gel C in **figure 6.5**, and was obtained with enhanced solubilisation conditions, lysis solution B (5M urea, 2M thiourea, 2% CHAPS, 2% SB 3-12, 40 mM Tris-base, 0.5% Pharmalytes 3-10). This

fraction denotes some of the tightly bound membrane proteins. Attempts to solubilise the final residue with SDS sample solution as documented by Molloy et al [446] was repeatedly associated with unsuccessful IEF (separation in the first dimension). This was attributed to the high ionic strength of SDS making it unable to maintain the native charges of the *Plasmodial* proteins thereby interfering protein mobility during IEF.

Figure 6.3 shows an example of an analytical gel of principally cytosolic, Tris soluble *Plasmodial* proteins separated by 2-DE and visualized by silver staining. In comparison **Figure 6.4** shows a sample of a micro-preparative gel of *Plasmodial* protein extract performed in a single enhanced extraction and visualized with Coomassie blue dye staining. This later gel format was later processed for mass spectrometric characterization of protein spots. (Evidently some of the low abundance proteins are not visualized in **figure 6.4** because this sample was prepared in one step without prior enrichment.)

The *Plasmodial* cell is a living cell and as such there is a continual and programmed dynamic flux in the parasites biochemistry. This dynamic state is reflected in the proteome. Although a proteome can theoretically consist of several thousand proteins, not all proteins are likely to be expressed at the same time within a living cell. Consequently a proteome map, unlike a static gene map, is defined by life-cycle stage and environmental conditions. This dynamic state of the proteome makes it technically difficult to create a proteome map like the static gene map. To overcome these problems, parasite material used in these experiments was obtained from cultures which had undergone two consecutive

rounds of synchronization. A series of drug treated *Plasmodial* samples were always compared with a series of replicate controls (without drug). This reduced problems of inter-gel variations bearing in mind that the process of preparing, loading and running 2 D gels is complex and inherently imprecise and therefore the processing of multiple gels to produce gel standards (master maps) with truly representative features was thought to reduce non expression related variability. This also made identification of specific qualitative and quantitative changes in protein expression easier to confirm. In these studies gel analysis was implemented using PD Quest^{TM} computer software. With this software matched gel sets were generated (as described in **section 2.6.9**). **Figures 6.6; 6.8; 6.9; 6.10; 6.11,** and **6.12** represent gel match sets that were generated from at least 4 member gels processed and run under standardized conditions. The standard gel generated from these match-sets formed the basis for the comparison of parasite protein populations under differing experimental conditions. In these studies gels have been used to identify and categorize specific qualitative and quantitative differences in protein expression profiles in control parasites populations compared with either ARM or LMF treated parasites. The high level of reproducibility between gels is confirmed from visual inspection of the match-sets and from the linearity of the scattergrams (**figures 6.7 a. & b).**

On average, between 510 -900 spots were observed in the gels loaded with the Tris soluble extract, 200-500 with the second extract (lysis buffer A soluble proteins) and 100-200 in the third and most severe extract, when stained with routine silver staining procedures. A couple of protein spots in the Tris extract gel

were also present in the first lysis extract gel (Figure 6.5) suggesting that some of the cytosolic proteins were in both extracts and/or the Tris extract also contained some mildly bound *Plasmodial* membrane proteins. About 10 unique proteins (high lighted in gel C of **figure 6.5)** were present in all the three *Plasmodial* protein extract gels. In these investigations it is apparent that only a small fraction of the expected *Plasmodium falciparum* proteome is visualized.

Despite this the results from this work show clear alterations in overall protein expression profiles between the three groups i.e. ARM & LMF treated compared with the control group which were reflected in the Tris extract. **Figure 6.13**.shows quantitative differences in the protein expression following treatment with either ARM or LMF. Treatment with ARM appears to down regulate a number of cytosollic proteins as indicated by the blue arrows in gel map B of figure 6.13. Conversely treatment with LMF appears to be associated with up regulation of a number of proteins as indicated by the red arrows in gel C of figure 6.13. Only proteins that displayed more than a 3 fold difference in expression were highlighted. Qualitative analysis of this Tris fraction was difficult because of a large number of proteins arrayed very close together and therefore no definite switching on and off of proteins could be demonstrated in this extract. Use of a narrow range PH IPG strip would probably have helped resolve the protein spots better but this was not done in these experiments. There are noteworthy quantitative differences following either ARM or LMF treatment in the acidic part of the gel (*p*H 3.5-6) following gel separation of proteins extracted under the first lysis extraction conditions. In addition there was a trend towards a down

regulation of many proteins in the more alkaline electrophoretic ranges (figure 6.15). A sample of PDQuest™ pictorial representation of the quantitative differences in gel spot intensity is shown in **figure 6.16.** Although a few proteins from the first lysis extract were upregulated following LMF treatment there was no down regulation in any of the proteins migrating in the more alkaline side of the gel (**figure 6.15**). The third extract (product of more rigorous lysis extract) did not show any striking qualitative nor quantitative differences in the protein expression profiles between the drug treated and the controls. This was in part due to difficulties in handling this fraction which gave very inconsistent results.

In addition to these quantitative differences in protein expression drug treatment also resulted in significant qualitative alterations. These differences may relate to actual switching in protein expression patterns although the possibility of drug treatment induced protein modifications, resulting in altered electrophoretic movement, cannot be ruled out at this time. **Figures 6.17** and **6.18** show qualitative differences in the first lysis protein extract following ARM or LMF treatment respectively. The results in **figure 6.17** clearly show that 26 proteins are down regulated to an extent below the detectable level using these stained gels as indicated by circles 43-69. Alternatively 42 proteins were up regulated or expression switched after ARM treatment as indicated by circles 1- 41 in **figure 6.17.** In addition **figure 6.18** shows the equivalent qualitative differences in the first lysis extractable proteins after LMF treatment. 73 protein spots which were present in the LMF treated group were not present in the control group, which could possibly be to upregulation of some of these proteins. There were very few

protein spots switched on after LMF treatment (i.e. present in treated but not control gels).

The differences in protein expression profiles in the ARM treated as compared to the LMF treated parasite populations provide compelling evidence to suggest that these two drugs trigger different killing mechanisms/parasite responses. This may occur despite the fact that they may be acting within a common organelle such as the food vacuole and may exploit common biochemical pathways such as the haemoglobinolytic pathway.

These differentially expressed proteins were further processed for potential identification using mass spectrometry and this further definitive characterization forms the basis of chapter 7.

CHAPTER 7

CHARACTERISATION OF POTENTIAL THERAPEUTIC TARGET PROTEINS FOR ARM AND LMF ACTION ON *PLASMODIUM FALCIPARUM*; A MASS SPECTROMETRIC APPROACH.

7.0. INTRODUCTION.

The preceding chapter demonstrated clear and reproducible differences in the protein expression profiles of both ARM and LMF treated parasite populations compared with the untreated group, using two-dimensional gel electrophoresis (2-DE) and gel image analysis. These observations prompted an investigation to further understand the relevance of these differentially expressed proteins within a biochemical context. The current chapter focuses on attempts to formally characterize the separated constitutive *Plasmodial* proteins by mass spectrometry. Proteins with expression patterns exhibiting alteration on drug treatment have been clustered according to relatedness within specific biochemical processes. This type of proteome profiling has been used previously to study drug toxicity mechanisms and has played a key function in the discovery of novel molecular mechanisms for a number of drugs [643-649] and clinical conditions [453].

In an analogous fashion disease-specific proteins (DSPs) have also been identified by this strategy. Furthermore these have been shown to be useful as clinical markers to monitor disease progression or response to therapy. Finally as the vast preponderance of drug targets are proteins, their identification and characterization using the global strategy employed here has the potential to

uncover novel therapeutic targets which could not be predicted from any other type of analysis.

Mass spectrometry was used in this work to characterize the 2 D gel separated proteins. Mass spectrometry (MS) is currently the micro-analytical technique of choice for the characterization of proteins at the amounts available from 2-D gels. It is a much more productive and sensitive micro-characterization technique than Edman sequencing [488]. In addition, MS also has the power to characterize virtually any type of post-translational modifications.

Matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) represent the two complementary techniques used to achieve an unambiguous identification of proteins and protein mixtures. MALDI is a high-throughput technique suitable for rapid identification of large numbers of proteins. This was the method employed in this work. The comparison of high accuracy fingerprinting against a sequence database provides the most likely protein hits. Sometimes MALDI peptide mapping may be insufficient for the identification of proteins due to differential detection of peptide moieties and due to the high degree of redundancy of the present genomes. It is therefore recommended that MALDI mapping is combined with nano-electrospray tandem mass spectrometry [488, 650]. We were unable to use the ESI technique in this work because of time limitations. ESI is more labour intensive and lengthy, but provides additional structural information through fragmentation of the selected ion in the mass analyzer. ESI also has an added advantage of being readily coupled to several

separation techniques, allowing chromatographic fractionation of complex protein mixtures by eluting each component at different retention times.

7.1. METHODOLOGY:

7.1.1. Characterisation of protein spots by MALDI-MS

: As described in detail in section 2.6.8.

7.2. RESULTS:

Figure 7.1. Indicates a typical example of a 2-D gel of Tris soluble *P.falciparum* proteins of the K1 isolate. It shows the array of proteins separated in the first dimension using ready-made IPG 3-10 (8 h at 3000 V max.) separation distance, 130 mm and second dimension using a vertical SDS-PAGE (12.5% T constant) gel. This gel map shows protein spots that have been characterized by MALDI-MS in this study. These proteins are numbered 1-51 and are also listed in **Table 12.** Spots A-I were used as internal standards to calibrate our gel system and they represent: A. Conalbumin ; B. Albumin; C. Actin; D. Carbonic anhydrase; E Trypsin inhibitor; F. Myoglobin; G. GAPDH; H. Beta haemoglobin; I. DNase artifact.

Figure 7.2 Indicates a representative 2-D gel of first lysis extract (assumed to represent moderately bound *Plasmodial* membrane proteins) of the K1 isolate. This gel was run and calibrated under standardized conditions as indicated in **figure 7.1.** This gel map shows protein spots that have been characterized by

MALDI-MS in this study and these are numbered 1-50 and they are also listed in **Table 13.**

Figure 7.3. Denotes a sample of MALDI spectra used in this study to identify proteins separated by 2D electrophoresis. The red boxes show examples of the MALDI spectra obtained from the protein gel spots, while the blue boxes show the identified proteins along with their amino acid sequences (Phosghoglycerate Kinase and Fructose- Bisphosphate Aldolase are given here as examples)

Table 12. Shows Tris soluble proteins that have been characterized by MALDI-MS. The data presented here shows that the following proteins were differentially expressed. Following LMF treatment the following proteins were up-regulated by at least 2 fold:

(a) *Glycolytic enzymes*: Enolase (2-phospoglycerate dehydratase) (2-phospho-D-glycerate hydro-lyase), Phosphoglycerate kinase, Fructose-biphoshate aldolase, Aldolase (AAA29716), Chain A, Fructose-1,6-biphosphate Aldolase, and glyceraldehyde-3-phosphate dehydrogenase.

(b) *Membrane* associated calcium binding protein

(c) *Enzymes involved in amino acid metabolism*: Ornithine amino transferase and ADP Ribosylation factor

(d) *Stress related proteins*: T-compex protein 1 (Heat Shock Protein 60).

(e) *Lysosomal* function protein, Trophozoite cysteine proteinase precursor

None of the down regulated proteins following LMF treatment were characterized.

In contrast following ARM treatment the following proteins were down regulated by at least 2 fold:

(a) *Glycolytic enzymes*: Enolase (2-phospoglycerate dehydratase) (2-phospho-D-glycerate hydro-lyase), Phosphoglycerate kinase, Fructose-biphoshate aldolase, Aldolase (AAA29716), Chain A, Fructose-1,6-biphosphate Aldolase, and glyceraldehyde-3-phosphate dehydrogenase.

(b) Enzymes involved in *Plasmodial* cell rescue against oxidative insults: Peroxidoxin & Thioredoxin.

(c) *Protein synthesis and Nucleic acid metabolism*: ADP-Ribosylation factor, Adenosylhomocysteinase (S-adenosyl-L-homocysteine hydrolase).

(d) *Parasite respiration related proteins*: Malonyl CoA-acyl carrier protein transacylase precursor.

(e) *Others*: Glutamate rich protein (CAB64136) ? surface antigen protein.

Table 13. Shows first lysis soluble proteins that have been characterized by MALDI-MS. The data presented here shows proteins that were differentially expressed.

Consequent to LMF treatment the following groups of proteins were up-regulated by at least 2 fold:

(a) *Ca-dependent. metabolism proteins*: Membrane-associated calcium-binding protein

(b) *Haem metabolism proteins*: Putative aspartic proteinase; HAP protein

(c) *Heat shock stress & protein folding proteins*: Heat shock 90 KD, and HSP60

(d) *Others*: Cytoadherence related protein DBL alpha protein, Nucleic acid metabolism protein AP endonuclease 1, and merozoite surface protein.

No under expressed proteins consequent to LMF treatment were characterized.

Following ARM treatment the following groups of proteins were over expressed by at least 2 fold:

(a) *Calcium dependent metabolism protein*: Membrane-associated calcium-binding protein

(b) *Heme metabolism related protein*: Putative aspartic proteinase; HAP protein

(c) *Heat shock stress & protein folding proteins*: Heat shock 90 KD, and HSP60

(d) *Protein metabolism related proteins*: Serine/threonine protein phosphase pp5, and Eukaryotic peptide chain release factor

Following ARM treatment the following groups of proteins were under expressed by at least 2 fold in the first lysis fraction.

(a) *Glycolytic enzymes*: Enolase (2-phospoglycerate dehydratase) (2-phospho-D-glycerate hydro-lyase), Phosphoglycerate kinase, Fructose-

biphoshate aldolase, Aldolase (AAA29716), Chain A, Fructose-1,6-biphosphate Aldolase, and glyceraldehyde-3-phosphate dehydrogenase.

(b) Enzymes involved in *Plasmodial* cell rescue against oxidative insults: Peroxidoxin & Thioredoxin.

Among the total 101 proteins that were characterized, 63 proteins were not differentially expressed. These included:

(a) Surface proteins e.g. (AF188190) polymorphic antigen, Merozoite surface antigen 2 precursor (MSA-2) (allelic form 3), (M83792) trophozoite antigen, (AY054816) erythrocyte membrane protein 1, and (AJ408333) Apical Membrane Antigen 1.

(b) Integral Membrane proteins: e.g. PfEMP1 protein, (AJ311567) putative Cg2 protein, kappa domain, and (AF239725) CG9,

(c) Haemoglobin: Haemoglobin delta chain

(d) Folate metabolism related proteins: e.g. dihydropteroate synthetase

(e) Structural/cytoskeletal proteins: e.g. Actin I (P10988),

(f) Choline transport related proteins: e.g. phosphocholine cytidylyltransferase

(g) Cell cycle proteins: e.g. Lipoamide acyltransferase,

(h) CGMP-dependent metabolism: e.g. putative Rab2 GTPase,

(i) Parasite haem synthesis: e.g. delta-aminolevulinic acid dehydratase (ALAD)

(j) Others with unknown functions: e.g., thrombospondin-related protein, and hypothetical protein in calmodulin 5'region

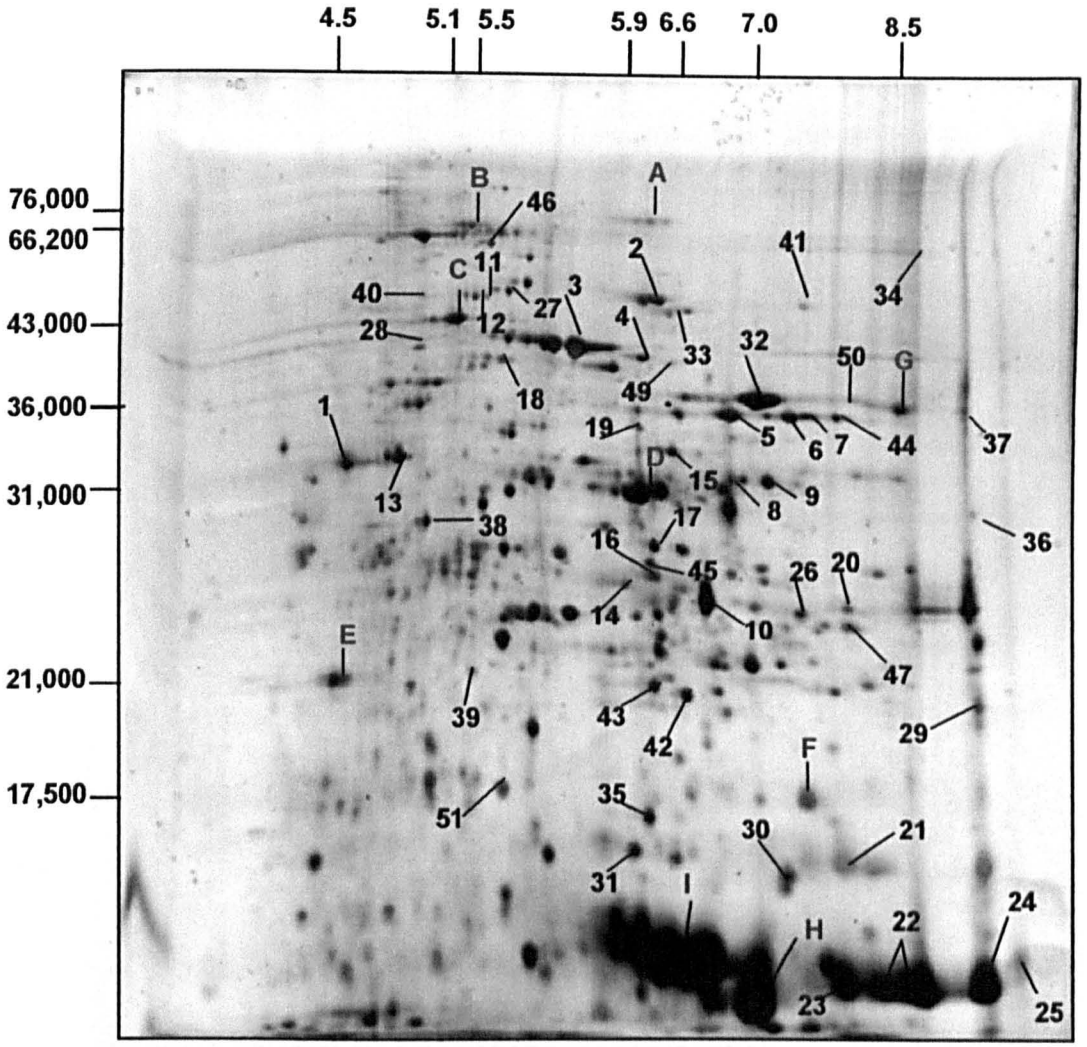


Figure 7.1. 2-D gel of Tris soluble *P.falciparum* proteins (K1 isolate). First dimension, ready-made IPG 3-10 (8 h at 3000 V max.) separation distance, 130 mm; 100 µg of sample was loaded onto IPG gel by in-gel rehydration. Second dimension, vertical SDS-PAGE (12.5% T constant); silver stain. The numbered spots were characterized by MALDI-MS and are listed in Table 7.1. Spots A-I were used as internal stardards: A. Conalbumin; B. Albumin; C. Actin; D. Carbonic anhydrase; E Trypsin inhibitor; F. Myoglobin; G. GAPDH; H. Beta haemoglobin; I. DNase artifact.

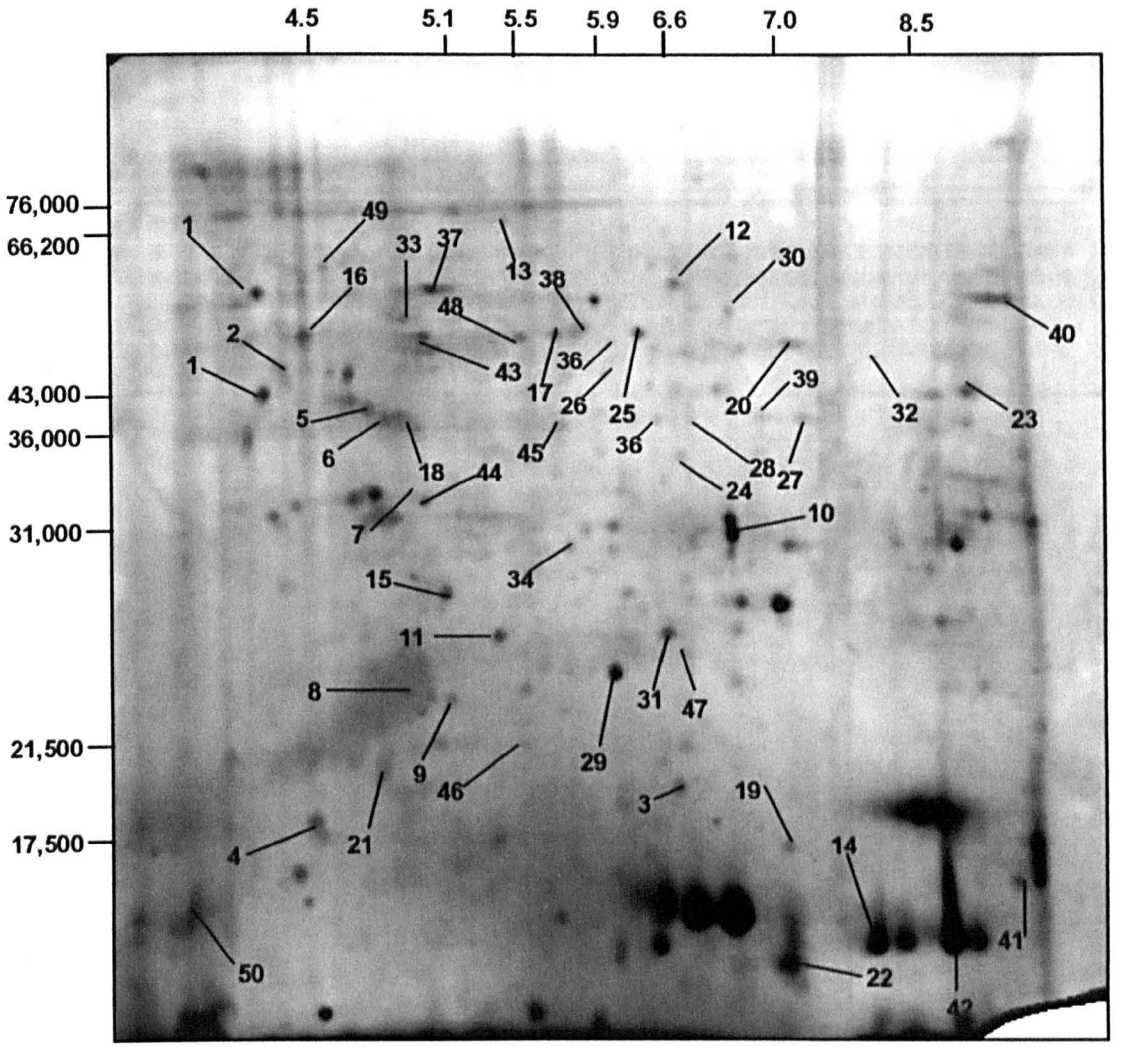
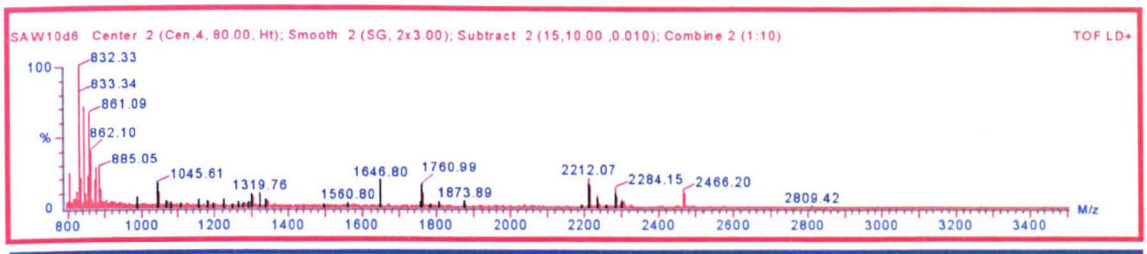
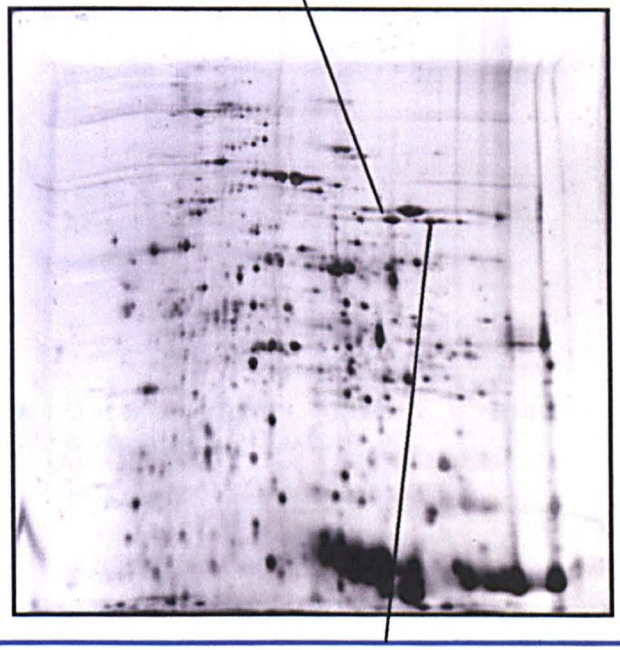


Figure 7.2. 2-D gel of Lysis buffer A soluble *P.falciparum* proteins (K1 isolate). [Lysis buffer A (8 M urea, 4% w/v CHAPS, 100 mM DTT, 50 mM Tris-base (pH 7.4), 10% SDS, 0.5% Pharmalytes 3-10 0.5 mM PMSF,0.06% EDTA)]. **The numbered spots were characterized by MALDI-MS and are listed in Table 7.2.**



>PGK_PLAFA (P27362) PHOSPHOGLYCERATE KINASE (EC 2.7.2.3).MLGNKLSISDLKDIKNKKVLVRVDFNVPIENGIIKDTNRITATLPTINHLKKEGASKIILISHCGRPDGLRNEK YTLKPVAETLKGLLGEEVLFLNDCVGKEVEDKINAAKENSVILLENLRFHIEEEGKGVDANGNKVKANKEDVEKF QNDLTKLADVFINDAFGTAHRAHSSMVGVKLNVKASGFLMKKELEYFSKALENPQRPLLAILGGAKVSDKIQLIKN LLDKVDRMIIGGGMAYTFKKVLNNMKIGTSLFDEAGSKIVGEIMEKAKAKNVQIFLPVDFKIADNFDNNANTKFVTD EEGIPDNWMGLDAGPKSIENYKDVILTSKTVIWNGPQGVFEMPNFAKGSIECLNLVVEVTKKGAITIVGGGDTASL VEQQNKKNEISHVSTGGGASLELLEGKELPGVLALSNK



>ALF_PLAFA (P14223) FRUCTOSE-BISPHOSPHATE ALDOLASE (EC 4.1.2.13) (41 KMAHCTEYMNAPKKLPADVAEELATTAQKLVQAGKGILAADESTQTIKKRFDNIKLENTIENRASYRDLLFGTKGLG KFISGAILFEETLFQKNEAGVPMVNLLHNENIIPGIKVDKGLVNIPCTDEEKSTQGLDGLAERCKEYYKAGARFAKWR TVLVIDTAKGKPTDLSIHETAWGLARYASICQQNRLVPIVEPEILADGPHSIEVCAVVTQKVLSCVFKALQENGVLLE GALLKPNMVTAGYECTAKTTTQDVGFLTVRTLRRTVPPALPGVVFLSGGQSEEEASVNLNSINALGPHPWALTFSY GRALQASVLNTWQGKKENVAKAREVLLQRAEANSLATYGKYKGGAGGENAGASLYEKKYVY

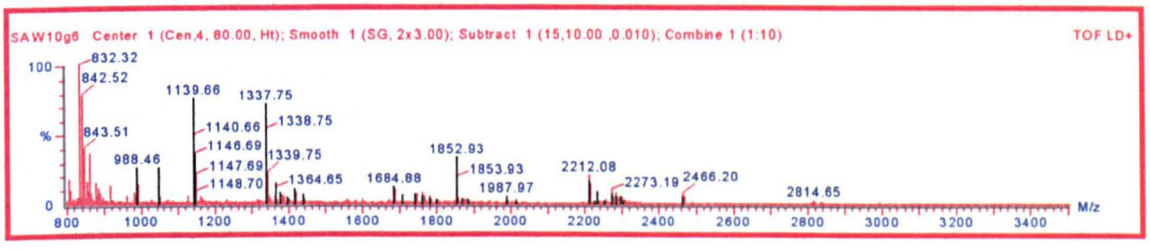


Figure 7.3. Sample of MALDI spectra that identify proteins separated by 2D electrophoresis. Trypsin enzymatic cleavage of a mixture of proteins gives a mixture of proteins whose masses are analyzed in a data bank with computer support.

Spot No.	Description /(Genbank Accession) / PID	Function	Calculated pI value	Nominal mass (Mr)	Matched peptides	Sequence Coverage (%)	Score
1* Å	(M77834) membrane associated calcium-binding protein (AAB49899); gi/1899003	Ca-dep. metabolism	4.49	39350	8	25.36	45
2*®	Enolase (2-phospoglycerate dehydratase) (2-phospho-D-glycerate hydro-lyase) (Q27727);	Glycolysis	6.21	48673	13	43.27	35
3*	Ornithine aminotransferase (ornithine-oxo-acid aminotransferase) (Q07805);	Amino acid catabolism	6.47	46074	9	19.57	26
4*	Ornithine aminotransferase (ornithine-oxo-acid aminotransferase) (Q07805);	Amino acid catabolism	6.75	46025	5	11	27
5*	Phosphoglycerate kinase (P27362); gi/129926	Glycolysis, fatty acid metabolism	7.63	45398	5	12	55
6*®	Fructose-biphoshate aldolase (A44942); gi|419934	Glycolysis	8.5	40080	7	28.46	33
7*	(J03084) Aldolase (AAA29716); gi/160572	Glycolysis	8.57	39246	9	29.5	58
8*®	(AF030440) glyceraldehyde-3-phosphate dehydrogenase (AAD10249); gi/4103985	Glycolysis	7.59	36614	8	28.57	37
9*®	Phosphoglycerate kinase (P27362); gi/129926	Glycolysis, fatty acid metabolism	8.1	45398	11	35.34	33
10*	Triosephoshate isomerase (TIM) (Q07412); gi|586112	Glycolysis	6.01	27917	12	22	56

Spot No.	Description /(Genbank Accession) / PID	Function	Calculated pI value	Nominal mass (Mr)	Matched peptides	Sequence Coverage (%)	Score
11	(NC_000521) predicted using hexExon; MAL3P2.12 (PF0215c), Hypothetical protein; (NP_473176) gi|16805148	Unknown ?Cell cycle	5.62	53325	6	11	32
12	(NC_000521) predicted using hexExon; MAL3P2.12 (PF0215c), Hypothetical protein; gi|16805148	Unknown	5.62	53325	7	11	20
13	(AF188190) polymorphic antigen (AAF04099); gi/558073	Surface antigen	4.65	43290	7	19	48
14	Merozoite surface antigen 2 precursor (MSA-2) (allelic form 3); gi|1709115	Surface antigen	6.22	28875	5	13	16
15	(M83792) trophozoite antigen; gi|160618	Surface antigen	6.57	39861	5	14	18
16®	(AB020595) 1-cys peroxidoxin; gi|4996210	antioxidant	6.31	25148	5	17	20
17	Hypothetical protein in calmodulin 5'region; gi|140325	Unknown	6.37	29306	5	19	28
18	Ribonucleoside-diphoshate reductase small chain (ribonucleotide reductase R2 subunit); gi|1710398	Nucleic acid metabolism	5.37	40570	4	18	21
19	(AF061150) merozoite surface protein 1; gi|3821993	Polymorphic surface protein (codes for a major asexual blood antigen)	6.36	39119	5	11	23
20	(NC_000910) RAB GTPase; gi|16804999	cGMP-dep. metabolism	8.83	26655	5	16	17
21	(AF221805) PfEMP1 protein (AAF36645); gi/7109140	Integral membrane protein (cytoadhesion)	8.46	13031	6	28	31

Spot No.	Description /(Genbank Accession) / PID	Function	Calculated pI value	Nominal mass (Mr)	Matched peptides	Sequence Coverage (%)	Score
22	Haemoglobin delta chain HDHU; gi|70353	Oxygen transport/ Heme metabolism	7.97	15914	6	33	48
23	(AF084607) erythrocyte membrane protein 1 type ap; gi|3695247	Surface, Cytoskeleton	8.45	14026	5	21	21
24	(AF329575) merozoite surface protein 2; gi|13345549	Surface, Cytoskeleton	9.11	11556	6	48	28
25	(X02542) unidentified open reading frame out of phas rame with lac z (CAA26390); gi/871500	Unknown	9.71	14574	3	22	30
26	(AF250161) dihydropteroate synthetase	Folate metabolism	7.81	26001	3	18	29
27®	Adenosylhomocysteinase (S-adenosyl-L-homocysteine hydrolase)	Nucleic acid metabolism	5.64	53858	6	18	28
28	(AY044180) merozoite surface protein 3 precursor (AAK94780); gi/15375031	Polymorphic surface protein	4.58	41139	5	15	35
29	Hypothetical protein C0855w (T18512); gi/7494269	Unknown	9.45	18346	5	27	30
30	(AF133860) DBL alpha protein(AAD33621; gi|4928518	Unknown ?cytoadherence	7.92	12841	5	35	43
31	(AY054816) erythrocyte membrane protein 1; gi|19109032	Surface, Cytoskeleton	6.17	14576	5	35	26
32*Å	(NC_000910) T-complex protein 1 (HSP60 fold superfoldfamily) (NP_473055); gi/16805026)	Heat shock stress, Protein folding	7.47	60752	5	10	22
33	(AJ408333) Apical Membrane Antigen 1; gi|13810374	Surface antigen	6.16	50218	5	23	35

Spot No.	Description /(Genbank Accession) / PID	Function	Calculated pI value	Nominal mass (Mr)	Matched peptides	Sequence Coverage (%)	Score
34*	Trophozoite cysteine proteinase precursor (TCP) (P25805); gi/118152	Lysosomal function	8.6	66838	6	9	21
35	(AJ311567) putative Cg2 protein, kappa domain; gi|13446660	Trans-membrane protein ? linked to drug resistant malaria	6.06	17005	4	23	32
36	(AF239725) CG9 (AAF59949); gi/7329219	Trans-membrane protein localizes to the parasite digestive vacuole	9.08	31082	4	16	27
37	(NC_000910) rifin (NP_473127); gi6805098	Clonally variant proteins expressed on surfaces of RBCs infected with *P.f.* ? cytoadherence	9.04	35233	5	20	31
38	(NC_000521) predicted using hexExon; MAL3P2.28 (PFC0295c), 40s ribosomal protein; (gi/16805164)	Protein synthesis	4.9	30715	4	12	57
39	(AJ005572) hypothetical protein (CAA06601); gi/3093383	Unknown	4.83	21859	5	24	37
40®	(AJ269899) glutamate rich protein (CAB64136)	surfaantigen	4.51	47731	5	18	34
41®	(AF237571) malonyl CoA-acyl carrier protein transacylase precursor (AAK83684); gi/15080868	Respiration	8.71	46275	5	15	33
42®	(AF225977) thioredoxin peroxidase-1 (AAF67110); gi:7677316	Antioxidant (Cell rescue, defence, senescence and death)	6.65	21793	5	22	22
43*Å	ADP-Ribosylation factor (Q94650); gi|3182916	Protein synthesis	5.83	20899	5	46.67	53

Spot No.	Description /(Genbank Accession) / PID	Function	Calculated pI value	Nominal mass (Mr)	Matched peptides	Sequence Coverage (%)	Score
44*®	Chain A, Fructose-1,6-biphosphate Aldolase (3319034)	Glycolysis	8.35	39949	6	16	42
45	Hypothetical protein in calmodulin 5'region	Unknown	6.37	29306	5	19	28
46	(AY054983) Serine threonine protein phosphatase PP5 (AAL15170)	Protein phosphorylation	5.13	69153	7	10	35
47	(AJ38271) Rab 18. GTPase (CAD27350)	cGMP-dep. metabolism	8.11	23148	4	18	22
48	(AF180426) methionine adenosyltransferase (AAG02013); gi/9927542	Methionine metabolism (*S*-adenosylmethionine synthesis), principal methyl donor and precursor for polyamines	6.28	44816	6	17	31
49*®	Enolase (2-phoshoglycerate dehydrogenase). 2-phospho-D-glycerate hydro-lyase (Q27727); gi/3023709	Glycolysis	6.21	4831	5	12	55
50	(U73195) MO15-related protein kinase Pfmrk (AAD55782); gi|1695919	Unknown	7.49	37964	5	12	28
51*Å	ADP-Ribosylation factor; G1/3182916	Protein synthesis	6.1	20899	6	48.67	26

Table 12. Tris soluble *Plasmodial* proteins characterized by MALDI-MS.

* denotes proteins that are over expressed in LMF treated parasite extracts
Å denotes proteins that are over expressed in ARM treated parasite extracts
® denotes proteins that are under expressed in ARM treated parasite extracts

Spot No.	Description/(Genbank Accession) / PID	Function	Calculated pI value	Nominal mass (Mr)	Matched peptides	Sequence Coverage (%)	Score
1*Å	(M778334) membrane-associated calcium-binding protein (AAB49899); gi/1899003.	Ca-dep. metabolism	4.49	39350	11	29.74	62
2	(AY044180) merozoite surface protein 3 precursor (AAK94780); gi/15375031	Polymorphic surface protein	4.59	41142	6	18	38
3	(AF008998) PfEMP1; gi|2645477	membrane protein (cytoadhesion)	6.34	20795	5	19	26
4	(U91650) merozoite surface antigen 2; gi|4028690	Surface antigen	5.06	18862	6	21	21
5	(AF221817) PfEMP1 protein; gi|7109164	membrane protein (cytoadhesion)	8.6	14167	6	33	23
6	(AF061141) merozoite surface protein 1; gi|3821975	Surface antigen	7.10	39825	5	11	18
7Å	(NC_000521) predicted using hexExon; MAL3P2.28 (PFC0295c), 40s ribosomal protein; (gi/16805164)	Protein synthesis	4.9	30715	5	11.2	49
8	Ribonucleoside-diphoshate reductase small chain (ribonucleotide reductase R2 subunit); gi|1710399	Nucleic acid metabolism	5.59	37348	5	11	21
9	(AF061141) merozoite surface protein 1; gi|3821975	Surface antigen	7.01	39825	5	11	18
10Å	Triosephoshate isomerase (TIM) (Q07412); gi|586112	Glycolysis	6.01	27917	5	20	46
11	(AF030694) CG6; gi|2642501	Unknown	6.57	32091	4	13	19

Spot No.	Description/(Genbank Accession) / PID	Function	Calculated pI value	Nominal mass (Mr)	Matched peptides	Sequence Coverage (%)	Score
12Å	(NC_000910) T-complex protein 1 (HSP60 fold superfamily); gi|16805026	Heat shock stress & Protein folding	7.47	60752	5	13	26
13Å	(AY054983)serine/ threonine protein Phosphase pp5; gi/17223793	Protein phosphorylation	5.13	69153	7	18	35
14	(AY054881) erythrocyte membrane protein 1; gi|19109533	a variant antigen of the malaria parasite	8.59	14922	5	33	27
15	MAL3P2.28 (PFC0295c), 40S ribosomal protein; gi/16805164.	Protein synthesis	4.9	30715	5	17.80	57
16	Actin I (P10988) ; gi/113224	Structural/ Cytoskeleton	5.27	41816	11	31.91	31
17*	(NC_000521) predicted using hexExon; MAL3P7.39 (PFC1040w), Hypothetical protein, len: 434 aa; gi|16805314	Unknown	5.84	51496	5	12	20
18	(AF315035) phosphocholine cytidylyltransferase; gi|12964703	Choline transport (intracellular trafficking)	5.02	42631	5	12	26
19	(AF191051) apical membrane antigen 1; gi|6581012	Surface, Cytoskeleton	6.9	16200	5	18	24
20*Å	(AJ009990) putative aspartic proteinase; HAP protein; Gi/4584228	Heme metabolism	8.05	51661	6	15	70
21*Å	Heat Shock 90 KD protein protein homolog.; gi|123670	Heat shock stress & Protein folding	5.5	21954	5	22	25

Spot No.	Description/(Genbank Accession) / PID	Function	Calculated pI value	Nominal mass (Mr)	Matched peptides	Sequence Coverage (%)	Score
22	(AF221817) PfEMP1 protein; gi|7109164	Integral membrane protein (cytoadhesion)	8.60	14167	5	33	25
23*	(NC_000521) band 7-related protein; gi|16805265	Unknown	9.4	43057	5	15	20
24	MAL3P2.3 (PFC0170c) Lipoamide acyltransferase; Gi/16805139	Cell cycle	5.44	50958	5	31.64	30
25Å	(NC_000910) eukaryotic peptide chain release factor; Gi/16805009	Protein synthesis	6.58	48034	6	21.92	57
26	(AF180426) methionine adenosyltransferase; gi|9927542	Protein synthesis (methionine metabolism)	6.28	44816	6	17	31
27	(U73195) MO15-related protein kinase Pfmrk; gi|1695919	Unknown	7.49	37964	4	8	24
28	(AF069296) replication factor C3; gi|11559500	Unknown	6.67	39207	4	13	26
29Å	ADP-Ribosylation factor; G1/3182916	Protein synthesis	6.1	20899	5	46.67	24
30Å*	(NC_000910) T-complex protein 1 (HSP60 fold superfamily); gi|16805026	Structural/ Cytoskeleton	7.45	60752	6	23.4	22
31	(AJ308736) putative Rab2 GTPase (CAC34627)	cGMP-dep. metabolism	6.33	24394	3	19	41
32	(AF071408) MAP2 kinase; gi|11559502	Unkown	7.95	54990	6	16.89	20
33	(NC_000910) hypothetical protein; gi|16805054	Unknown	5.05	64535	5	14	29

Spot No.	Description/(Genbank Accession) / PID	Function	Calculated pI value	Nominal mass (Mr)	Matched peptides	Sequence Coverage (%)	Score
34	(AJ249365) delta-aminolevulinic acid dehydratase (ALAD); gi|6688975	Parasite haem synthesis	5.8	28516	5	23	27
35	MAL3P3.19 (PFC0400w), 60S Acidic ribosomal protein P2; Gi/16805185	Protein synthesis	4.49	28158	5	20	49
36	(AJ408333) Apical Membrane Antigen 1 (CAC34774)	Integral membrane antigen	6.16	50218	7	20	37
37*A	Heat shock protein; gi/311895	Heat shock stress & Protein folding	5.1	72359	7	29.45	40
38	(NC_000521) putative inorganic pyrophosphatase; gi|16805247	Unknown	5.87	45204	5	17	27
39*	(AF061131) merozoite surface protein 1; gi|3821955	Surface protein	6.69	39578	5	16	20
40*	(AF326063) AP endonuclease 1 (AAK01930); gi/12711643	Nucleic acid metabolism	9.09	57672	5	11	35
41*	(AF133886) DBL alpha protein (AAD33647); gi/4928570	? Cyto-adherence	9.28	15396	5	35	28
42	Haemoglobin delta chain HDHU; gi|70353	Oxygen transport/ Heme metabolism	7.97	15914	6	33	48
43	Merozoite surface antigen precursor; Gi/323125	Surface antigen	5.2	45795	9	28	33
44	(X53019) erythrocyte membrane-associated antigen; gi|9795226	Antigen (membrane Integral protein)	5.18	35970	5	10	25
45	Ribonucleoside-diphosphate reductase small chain (Ribonucleotide reductase R2 subunit); gi|1710399	Nucleic acid metabolism	5.59	37348	5	12	22

Spot No.	Description/(Genbank Accession) / PID	Function	Calculated pI value	Nominal mass (Mr)	Matched peptides	Sequence Coverage (%)	Score
46*Å	Heat Shock 90 KD protein homolog.; gi|123670	Unknown	5.5	21954	4	18	21
47	(AF366355) erythrocyte membrane protein 1; gi|17063562	a variant antigen of the malaria parasite	6.42	23546	6	14	26
48	MAL3P2.3 (PFC0170c) Lipoamide acyltransferase; gi|16805139	Protein biosynthesis	5.44	50958	5	7	31
49	(AB006336) thrombospondin-related protein; gi|3273257	Unknown	4.88	63369	5	12	20
50	(AF404260) erythrocyte membrane protein 1; Gi/15825293	a variant antigen of the malaria parasite	8.45	14443	6	11.27	26

Table 13. Lysis buffer A soluble *Plasmodial* proteins characterized by MALDI-MS.

* denotes proteins that are over expressed in LMF treated parasite extracts
Å denotes proteins that are over expressed in ARM treated parasite extracts
® denotes proteins that are under expressed in ARM treated parasite extracts

7.3. DISCUSSION

Proteins play a central function in all cellular processes in living cells including the *Plasmodial* cell such as: cellular organization (cytoskeletal elements); as enzymes and carrier molecules involved in fundamental physiological processes including metabolic pathways, intracellular trafficking and recognition processes etc. Consequently it is reasonable to predict that proteins are putative targets for chemotherapeutic agents directed against the parasite. Investigations performed in the preceding chapter showed significant differences in protein expression profiles after treatment of parasite populations with either the ARM or LMF as compared to the controls. Additionally, there were demonstrable differences in the 2 D gel protein spot patterns between the ARM treated and LMF treated parasite populations. This suggests differences in the mechanism of action of the two drugs and in the specific cellular processes that are activated by treatment with each individual drug.

The data in this chapter represents an initial attempt to characterize some of the parasite proteins of interest i.e. proteins that are differentially expressed and some control proteins which are neither over nor under expressed, as identified from 2-DE gels. The protein spots of interest were in-gel digested with trypsin, the resulting peptides were extracted from the gel piece and the peptide molecular masses were determined by MALDI-TOF-MS followed by analysis and data base interrogation via Masslynx coupled with Mascot data base searching algorithm. Peptide Mass Fingerprint (PMF) was selected as the preferred

analytical tool for protein identification in these studies because the technique is fast and straightforward and therefore appropriate given the study duration and the enormous number of protein spots that were implicated. Trypsin was used for in-gel digestions because it is most commonly used [487, 498, 651] and displays a high level of specificity cleaving the proteins very explicitly after lysine and arginine residues. Trypsin digestion results in quite small peptides, which can be efficiently eluted from the gel. In addition, trypsin has been acknowledged to produce autoproteolytic fragments which can be used for internal calibration of the mass spectra [651], nonetheless in these studies adrenocorticotrophic hormone (ACTH) was used to calibrate the mass spectra to ensure better standardized conditions.

A probability based protein identification tool was used for database searches with MassLynx along with Mascot, both of which support mass fingerprinting, sequence queries and MS/MS ion searches. With the MALDI mass spectrometer (M@LDI, Micromass) that was used throughout this work, peptide mass fingerprints were obtained semi-automatically and the resultant mass lists searched against non-redundant protein database (Swiss-Prot/Trembl) using ProteinLynx 3.4 (Micromass) software. However, due to limited successful *Plasmodium falciparum* hits in the data base accessed with ProteinLynx at the time, all searches were repeated manually with Mascot using the NCBInr database for *Plasmodium falciparum* taxonomy. The search criterion used was a minimum of five matching peptides with peptide mass tolerance (error window on

experimental peptide mass values) of ± 0.1 Da, the mass values were monoisotopic, and the protein mass was unrestricted. The sequence coverage for the identified proteins in both sets of gels analysed (Tris and Lysis extract proteins) varied from 7- 48%, while the probability based on Mowse score varied from 17- 70 for the identified proteins (Tables 7.1 & 7.2). 50% of the identified proteins from the Tris soluble extract and about 40% of the lysis soluble extract had sequence coverage ≥ 20%. Spots 18, 26, 36, 38, & 47 in table 7.1 and spots 11, 27, 28, 31, & 46 in table 7.2 had less than 5 peptides and coverage less than 20%. However these have been included in the listing because although the spots were faint (low total protein mass) PMF results were highly consistent from two matching gels spots from separate gels. In addition spots 25 & 35 in table 7.1 had 3 & 4 matching proteins respectively but these are quite small proteins (low molecular weights) and were found with sequence coverages more than 20% for each of them. By and large, with the exclusion of the proteins with less than 5 matching proteins listed above, a sum of 88 proteins were identified from both extracts. Of the 88 identified proteins 49% had sequence coverage ≥ 20% and 24% had probability based Mowse score ≥ 40%. Additionally, 8 out of the 43 proteins from the first extract **(Table 12)** were identified in 2 different spots, while 4 out of the 45 proteins and 3 out of the 45 protein from the second extract **(Table 13)** were identified in 2 and 3 different spots correspondingly. This indicates that different isoforms of the same protein were present.

Mascot uses a probability based Mowse score. The Mowse Score is denoted by the expression -10*Log(P), where P is the probability that the observed match is

a random event. Protein scores greater than 47 are considered significant ($p<0.05$) [652, 653].

An intriguing observation from these studies was that ARM treated parasites were associated with increased expression of a membrane associated calcium binding protein of 3 fold. In addition the LMF treated parasites also showed a trend towards an increase in this protein of 2 fold. This may reflect a role for calcium deregulation in parasites as they die under drug pressure. Such alteration in calcium physiology has been suggested in previous studies. Artemisinin derivatives [333, 348, 654] have been reported to interact with the malaria protein referred to as Translationally Controlled Tumor Protein (TCTP). This protein has been located by immuno-fluorescence and immuno-electron microscopy to both the cytoplasm and food vacuole [333, 348]. Reminiscent of other TCTPs, the *P. falciparum* protein binds to calcium [333, 348]. It is speculation to assume that the 39.4 KDa membrane associated calcium binding protein identified here is linked with the mechanism of action of endoperoxides via a calcium regulatory activity perturbation. Importantly TCPT was not a protein identified in this initial albeit limited protein analysis.

Both ARM and LMF treatment are coupled with increased expression of heat shock proteins (hsps) e.g. heat shock 90 KD protein homolog and T-complex protein 1 (HSP60), this could have been predicted as part of the normal parasite response to chemical/toxic stress. Reactive oxygen species (ROS) generating systems, of which endoperoxides are an example, have been documented to induce expression of several eukaryotic genes including stress proteins among

others [655]. The biological role of these proteins in malaria is not fully understood but it is possible that they provide protection to the parasite under stress by either binding to the toxic molecules directly, by binding damaged proteins to flush them out of the parasite [656] or by binding vital cell proteins as a means of protection.

ARM treated parasites have 2.5-fold reduction in their expression of thioredoxin, 1-cyst peroxidoxin, ribonucleoside-diphoshate reductase (small chain), adenosylhomocysteinase, glutamate rich protein and malonyl CoA-acyl carrier protein transacylase precursor. Thioredoxin and peroxidoxin are acknowledged proteins involved in cellular antioxidant systems [657-664]. It is recognized that *P. falciparum*-infected erythrocytes are under considerable oxidative stress [173, 583, 665-672] consequently efficient antioxidant systems are a prerequisite to ensure parasite development within the host cell. Intuitively if the peroxides exert their antimalarial effects by exerting oxidative stress [173] antioxidant defence levels might be expected to increase. This is not what was observed and may indicate the irreversible loss of these intact biologically active antioxidant molecules under drug pressure as the system becomes overwhelmed with reactive species. Notably the peroxides form stable adducts with their protein targets which may have increased their rate of protein turnover. An alternative explanation could be that the electrophorectic mobility of some of the proteins within the gel may have altered through drug adduct formation (such drug/protein adducts may also have prevented successful Mascot data base searches in

some instances). Thioredoxin and glutathione redox systems represent two powerful means which the parasite employs to detoxify reactive oxygen species [665, 671]. Interestingly a recent gene knock out study has demonstrated that thioredoxin reductase is essential for parasite survival highlighting the importance of this biochemical pathway. Perhaps the loss of thioredoxin reflects a key marker of oxidatively induced parasite death [673].

Both ribonucleoside-diphosphate reductase, adenosylhomocysteinase are involved in nucleic acid metabolism. These too appear to be potential targets for ARM action with consequent inhibition of nucleic acid synthesis. The reduction of ribonucleotides to deoxyribonucleotides, the precursors of DNA, is catalyzed by ribonucleotide reductase (RNRase), and this is a rate-limiting reaction in DNA replication and repair. RNRase is a cell-cycle-regulated, iron requiring allosteric heterodimeric enzyme. It is thought to be a target of iron chelation therapy [200, 201]: deprivation of iron from the enzyme results into inhibition of DNA synthesis and parasite death. DNA synthesis inhibition per se doesn't explain the broad stage specificity of action of ARM on the ring and early schizont stages, however it is likely to be part of the multiple protein targets responsible for ARM toxicity to the *Plasmodial* cell.

Treatment with LMF resulted in a clear and noteworthy upregulation in the glycolytic pathway. Glycolytic enzymes including enolase (2-phospoglycerate dehydratase) (2-phospho-D-glycerate hydro-lyase), phosphoglycerate kinase, fructose-biphosphate aldolase, triosephosphate isomerase (TIM) etc were increased more than 2 fold. This was especially noticeable in the Tris soluble,

cytosolic fraction. In complete contrast, with the exception of Triose phosphate isomerase enzyme, ARM resulted in a reduction in the expression profiles of the glycolytic enzymes analysed. It is unclear why the effects on glycolysis should be drug dependent. One argument would be that ARM kills parasites so rapidly that the parasite has no time to mount an adequate compensatory response whereas the LMF kill effect may be more protracted, triggering increased glycolytic activity as part of a general stress response. Previous studies with schistosomes [674] has shown that the ARM-damaged schistosomes also have decreased activities of a number of enzyme systems, including those involved in glycolysis. It could be argued that the reduction in glycolytic enzyme expression seen with ARM treatment is a principal element of the drug action. However, this raises serious questions as to why these drugs show little host toxicity or activity in other cellular systems. It would be essential to demonstrate selective Fe II bio-activation of ARM (or DHA) to reactive species by cytosolic parasite extracts compared to host cells in order to investigate this further.

Both drug treatments resulted in an up regulation of histo-aspartic protease HAP protein expression. HAP was recently characterized and localize to the food vacuole. The protein is implicated in haemoglobin degradation [620] but it's precise role is yet to be established. This interpretation is in conformity with the earlier observations in chapter 5 that the haemoglobin degradation my have a central role in the mechanism of action of both ARM and LMF.

Other proteins showing changes in expression profiles include parasite enzymes involved in protein metabolism like eukaryotic peptide chain release factor,

serine/threonine protein phosphatase PP5, MAL3P2.28 (PFC0295c), 40s ribosomal protein and ADP ribosylation factor, which appear to be up regulated following ARM treatment.

The observations made in these investigations suggest that both ARM and LMF have multiple direct or indirect intracellular targets with the peroxide appearing to exert a broader range of effects than the LMF. Moreover, what has been shown is only the 'tip of the iceberg'. Only a small percentage of the proteins undergoing changes in expression have been identified in this initial study. There are numerous problems when studying the global proteomes of a certain cell or organism. Most of these are associated with 2-DE, which is known to have systemic bias against very large, very small, low abundance and hydrophobic proteins [448, 675]. It is argued that only proteins expressed at medium to high levels can be detected from the 2-DE gels [492]. In these investigations a differential solubilisation approach [446] was used but still the total number of proteins visualized by silver staining of the gels from the three *Plasmodial* cell extracts, only added up to a maximum of 1700 protein spots which is far below the projected figure from genomic studies. There is still a plethora of information yet to be gleaned from this proteomic strategy.

In conclusion the study begins the proteomic analysis of drug action in *P. falciparum.* There is clearly much more work to be done before we obtain a complete readily interpretable picture.

CHAPTER 8

GENERAL DISCUSSION

8.1. INTRODUCTION

Regardless of the considerable headway made in malaria control over the past decade, malaria remains a grave problem, particularly in sub-saharan Africa, where about 90% of clinical cases occur. Malaria, either alone or in combination with other diseases, is estimated to kill between 1.1 and 2.7 million people worldwide each year and over 2400 million people remain at risk [3]. The ever-increasing population of multi-drug resistant *Plasmodium falciparum* is doubtlessly one of the major factors contributing to the persistence, and resurgence of malaria [676-680]. The limited number and type of antimalarial drug available has significantly fuelled the development of drug resistance [681]. To assuage this problem there are two plausible approaches: development of drugs with novel modes of action, or use of combinations of currently available drugs that have independent modes of action, in particular artemisinin-based combinations [278, 682, 683]. An important feature of such combinations will be the absence of significant levels of resistance at the time of their deployment in combination.

The combination strategy, because of its effectiveness in the treatment of multi-drug resistant infections, is well accepted in the treatment of tuberculosis and AIDS. The malaria community has embraced combinations only recently and

their use has been advocated using an artemisinin as the basis for such combinations [416, 684]. Combination of drugs with independent modes of action is theoretically predicted to prevent the emergence of resistance to both drugs and increase the chances of parasite killing. This is because the probability that an infected patient will have parasites that are resistant to both drugs is greatly reduced (the product of the probability of resistance to either drug alone). The development of an ARM-LMF fixed combination (co-artemether) is part of this initiative. It is argued that ARM causes a rapid and substantial reduction in parasite biomass with the remaining parasites being killed by exposure to high concentrations of the accompanying LMF.

In view of the acceptance of the combination approach, the ARM-LMF combination might be a potential alternative first line in the treatment of multi-drug malaria. There is, nonetheless, an astonishingly poor understanding of how ARM and LMF kill the *Plasmodium falciparum* malaria parasites. The studies which make up this thesis aim at improving our understanding of the cellular and molecular mechanisms associated with co-artemether (ARM and LMF) drug induced parasite toxicity. Importantly these aspects have been investigated at therapeutically relevant drug concentrations and appropriate drug exposure times.

8.2. BIO-CHEMICAL INVESTIGATIONS ON CELLULAR MECHANISMS OF ARM AND LMF ACTION

8.2.1. Baseline *in-vitro* drug sensitivity studies and electron microscopic ultrastructural studies.

The parasite sensitivity studies undoubtedly show that ARM and its active metabolite, dihydro-artemisinin (DHA) have very potent antimalarial activity *in-vitro* against both CQ sensitive and CQ resistant isolates as variously documented [13, 366, 374, 541-543]. Conversely, LMF was less potent but effective. An inverse relationship between parasite sensitivity to CQ and sensitivity to LMF was observed analogous to findings with other class II blood schizonticides such as mefloquine, halofantrine, and quinine, [215, 544-548]. This may be indicative of a common mechanism of action between these drugs.

Time dependent drug sensitivity assays were performed to define the duration of drug exposure required to achieve irreversible parasite toxicity to either ARM or LMF. The results from these assays demonstrated that ARM has a very rapid effect on growth inhibition evident within 1hr of exposure with maximal irreversible growth inhibition achieved after 5 hr of exposure to IC_{50} and IC_{90} drug concentrations. These IC values corresponded to 5.7 ± 0.32 and 7.7 ± 0.38 nM (mean values ± SD) respectively. LMF was shown to be less potent with a relatively slower action, with effects observed after about 3hrs of drug exposure and maximal irreversible growth inhibition achieved after 8-12 hours of exposure to drug at IC_{50} and IC_{90} drug concentrations corresponding to 123.41 ± 11.77 and

212.73 ± 12.45 nM respectively. On basis that the sensitivity of the test for direct *in-vitro* effects of the drug on parasite growth and viability is directly comparable to the *in-vivo* drug concentration [549] it was assumed that the concentrations of ARM and LMF in these studies were therapeutically relevant. Indeed these circulating concentrations are readily achieved after the current coartem dosing regimen.

Parallel investigations were then performed using these predetermined therapeutically relevant drug concentrations and appropriate exposure times to carefully observe the series of cellular events that follow exposure of *Plasmodium falciparum* to either ARM or LMF. Ultra-structural cellular changes were serially observed using electron microscopy over a post exposure period of 24 hrs, relating the changes to the growth inhibition investigations. The rate and extent of cellular damage with the two drugs correlated well with growth inhibition data. Earlier studies on the ultrastructure of *P.falciparum in-vitro* (discussed in detail in chapter 3) observed that ARM causes extensive injury to the cellular and subcellular membranous structures of *P.falciparum* majorly involving the mitochondria and suggested lipid peroxidation as the possible mechanism of toxicity. These studies were however, carried out with greater than tenfold higher concentrations (50-100nM) of artemether than the therapeutically relevant drug concentrations (less than 10nM). The observations reported in this thesis were done at therapeutically relevant drug concentrations and exposure times. The ultrastructural changes implicated the *Plasmodial* digestive vacuole to be a

common intra-cellular target for both ARM and LMF action. Obvious changes included progressive swelling of the food vacuole and dispersion of the haemozoin pigment (especially in the LMF treated) with a probable disruption of membrane integrity in the ARM treated parasites. These findings suggested the possible Involvement of the haemoglobin degradation pathway known to occur in the parasite food vacuole. Given that EM per se was incapable of providing ample evidence for a definitive mechanism of action of these drugs further work was done to probe the possible involvement of the haemoglobin metabolism.

8.2.2. The role of intracellular iron in ARM and LMF action.

The food vacuole is the cellular location for some metabolic processes that are vital for the survival of the parasite [139]. These include the related processes: haemoglobin degradation, haem bio-crystallization, maintenance of acidification, and release of free iron. Most of the haem released from haemoglobin catabolism is crystallized into haemozoin. A fraction of this haem is suggested to be a potential source of iron for the malaria parasite's ferro-proteins albeit non-haem iron may also be essential for parasite growth. It is assumed that haem plays a critical role in the mechanism of action of artemisinins [187, 352, 375] and other antimalarial drugs, including 4-aminoquinolines such as CQ [285, 286], quinoline methanols such as mefloquine [512, 564], and phenanthrene methanols such as halofantrine [512, 564].

Three iron chelators: (deferoxamine-hydroxyethyl starch (DFO-HES), desferrioxamine also called deferoxamine (DFO), and CP94.HCl) which have different permeability properties, were used as probes to investigate the importance of iron to the survival of the parasite, and subsequently to investigate its role in the mechanism of action of ARM and LMF. DFO-HES being a large molecule, a conjugate of deferoxamine/ desferrioxamine (DFO) and hydroxyethyl starch (HES) was assumed to selectively chelate the extracellular iron. DFO per se (unconjugated) has intermediate membrane permeability, with poor ability to penetrate mammalian cells including normal erythrocytes but is capable of entering parasitized cells [582]. The small and highly lipid soluble molecule CP94.HCl, easily penetrates both erythrocyte and *plasmodial* cell membranes. CP94.HCl was assumed to have access to both the intraerythrocytic and parasite-associated iron. CP94.HCl demonstrated the most potent antimalarial effect of the three iron chelators with an IC_{50} of 125 ± 12.5 nM compared to 953 ± 43.6 and 12900 ± 772 nM for DFO and DFO-HES respectively. CP94.HCl was therefore was used in the subsequent drug combination sensitivity assays in probing the importance of *Plasmodial* intracellular iron in ARM and LMF action.

The results of these combination assays undoubtedly demonstrated that the effects of DHA and ARM on the parasite are grossly antagonized by CP94.HCl. This provides confirmatory evidence that intracellular iron is necessary in the mechanism of action of ARM and its active metabolite DHA as antimalarials. The source of this iron is however not clear although haemoglobin degradation is the most likely contributor.

Conversely, the interaction of CP94.HCl with LMF appeared additive. This was as expected from a drug thought to act via a haeme based mechanism although this contrasts with CQ (which displayed antagonism) which is also thought to target haem. These findings may be attributed to the fact that either LMF forms a very tight complex with hematin (ferri-protoporphyrin IX) making it unavailable to the iron chelator, or LMF and CP94.HCl may be acting on different targets and the 'chelatable' intracellular iron may not be critical in the mechanism of action of LMF.

8.2.3. The haemoglobinolytic pathway: A prime target for ARM and LMF action against *Plasmodium falciparum*.

The haemoglobin degradation pathway is of critical relevance in terms of antimalarial chemotherapy, albeit a process which is still poorly understood. Investigations using a range of different protease inhibitors including: Ro40-4388 (specific plasmepsin 1 inhibitor), ALLN (plasmepsin processing inhibitor) and E64 (cysteine protease inhibitor) were performed as probes to test the significance of the haemoglobinolytic pathway in the antimalarial mechanisms of ARM and LMF action. The potency of these protease inhibitors as inhibitors of parasite growth represented by IC_{50} values in their own right demonstrates the importance of the haemoglobinolytic pathway to the survival of the *Plasmodial* cell.

The interaction of the plasmepsin processing inhibitor (ALLN), a specific plasmepsin I inhibitor (Ro40-4388) and a cysteine protease inhibitor (E64) with

ARM demonstrate clear antagonism reminiscent of data obtained with CQ, albeit of lesser magnitude. These observations imply that the generation of FPIX must be important in the bioactivation process of ARM, possibly the catalysed by iron (II) in the reduced form, ferrous haem [ferroprotoporphyrin IX, Fe(II)PPIX]. However, the magnitude of the antagonism between ARM with the protease inhibitors is much less than was observed with the highly lipid soluble iron chelator, CP94.HCl. The interaction of this iron chelator with either ARM or DHA displayed a very profound antagonism. This may suggest that although Fe(II)PPIX is important, free chelatable intracellular iron (II) possibly from non haem iron II plays a more important role in the bioactivation of these artemisinin compounds.

In contrast to the interaction of ARM with protease inhibitors, the interactions with DHA were clearly additive in combination with Ro40-4388 and ALLN, but profoundly antagonistic with E64. These observations with DHA seem to confirm the absolute requirement of free Fe^{2+} for its bio-activation. Presumably even the small amounts of catalytically active free iron are sufficient. The Cysteine protease inhibitor, l-transepoxy-succinyl-leucylamido-(4- guanidino) butane (E64) has been shown to cause irreversible inhibition of haemoglobin degradation with consequent accumulation of un-degraded haemoglobin and swelling of the *Plasmodial* food vacuole. It is unclear how this effect antagonises the actions of DHA but presumably such an effect would compromise the normal endocytic pathway which might indirectly influence the availability of Fe2+.

Both ALLN and Ro40-4388 produced a modest inhibition of LMF antimalarial activity. The antagonism observed here is most probably due to reduced formation of FPIX as is the case with CQ. The interaction between E64 and LMF was however unexpectedly less than what is observed with E64 in combination with CQ. These observations suggested that although LMF is believed to be dependent on effective formation of FPIX from the haemoglobinolytic pathway, there may be other aspects to its mechanism of action.

These findings do confirm the haemoglobinolytic pathway as being central to the mechanism of action of both ARM and LMF. The available data however was not sufficient to fully explain the mechanism of action of both drugs in a truly definitive manner. This prompted further investigations using a molecular approach.

8.3. A PROTEOMIC STRATEGY TO INVESTIGATE DRUG ACTION: CHANGES IN PROTEIN EXPRESSION PROFILES FOLLOWING TREATMENT WITH ARM AND LMF.

A molecular approach was attempted to complement the preceding cellular analyses which could never reveal the complete complexities of drug action. This involved use of comparative proteomics to study differences in protein expression profiles between drug-treated (ARM or LMF) and untreated *P.falciparum* parasite populations. This was an endeavor to investigate the global parasites' response to lethal drug exposure at the protein level.

This strategy involved use of the two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) to separate parasite extracted proteins, use of PDQuest™ gel imaging software to identify qualitative and semi-quantitative alterations in protein expression patterns, and finally the use of mass spectrometry to formally characterize the proteins of interest. MALDI mass spectrometry and comparison of high accuracy fingerprinting against the available *P.falciparum* sequence database was used to obtain the most likely protein hits.

The results of these investigations showed clear alterations in overall protein expression profiles between the three groups i.e. ARM & LMF treated compared with the untreated parasites (control group). These differences in protein expression profiles in the ARM treated as compared to the LMF treated parasite populations provide convincing evidence to imply that there are differences in the mechanism of action of the two drugs and in the specific cellular processes that are activated by exposure to them.

An attempt was made to formally characterize the proteins of interest i.e. proteins that are differentially expressed together with a number of control proteins which are neither over nor under expressed, as identified from 2-DE gels. A total of 101 gel separated protein spots were characterized. These identified proteins were then clustered according to relatedness within specific biochemical processes.

Specifically the proteins up regulated following ARM treatment were: the membrane associated calcium binding protein (calcium dependent protein), the haemoglobin metabolism related enzyme histo-aspartic protease (HAP), the protein metabolism related protein (serine/threonine protein phosphase pp5, and Eukaryotic peptide chain release factor) and heat shock proteins (hsps) e.g. heat shock 90 KD protein homolog and T-complex protein 1 (HSP60). Conversely the following proteins were down regulated: a majority of the identified glycolytic enzymes; Cellular anti-oxidant enzymes (thioredoxin and peroxidoxin); and nucleic acid metabolism proteins (ribonucleoside-diphoshate reductase and adenosyl homocysteinase).

LMF treatment was also associated with increased expression of a number of glycolytic enzymes, membrane associated calcium binding protein; amino acid metabolism related enzymes (ornithine amino transferase and ADP ribosylation factor); stress related proteins (T-complex protein 1 (heat shock protein 60)) and lysosomal function protein, trophozoite cysteine proteinase precursor, heame metabolism proteins: putative aspartic proteinase; HAP protein; heat shock stress & protein folding proteins (heat shock proteins); and others like cyto-adherence related protein DBL alpha protein and nucleic acid metabolism protein (AP endonuclease 1). None of the proteins down regulated by LMF treatment were characterized.

These investigations suggest that both ARM and LMF have multiple direct or indirect intracellular targets with the endoperoxide seeming to present a broader range of effects than the LMF. The data described here is only the 'tip of the iceberg'. Only a small proportion of the proteins undergoing changes in expression have been identified in this initial study. There is still a surfeit of information yet to be gleaned from this proteomic strategy.

In order to obtain a complete readily interpretable picture of the intricacies associated with LMF and ARM action much more work is needed. It is possible that the relatively long time exposure to drug is detecting down-regulation of some proteins or dying stages of the parasite. Earlier time points may be relevant and informative for future investigations. These investigations only mark the beginning of the proteomic analysis of drug action on *Plasmodium falciparum*.

8.4. CONCLUSION

The work covered in this thesis has evidently shown that:

(1) The *Plasmodial* digestive vacuole is a common intra-cellular target for both ARM and LMF action.

(2) Intracellular iron is necessary in the mechanism of action of ARM and its active metabolite DHA as antimalarials. In addition, although Fe(III)PPIX is important, free intracellular iron (II), Fe^{2+} possibly from non haem iron II plays a more important role in the bioactivation of artemisinin compounds such as ARM and DHA.

(3) Haemoglobin degradation is central to the mechanism of action of both ARM and LMF.

(4) The proteomic studies suggest that both ARM and LMF have multiple direct or indirect intracellular targets with the endoperoxide seeming to present a broader range of effects than the LMF.

The endoperoxides appear to implicate different biochemical pathways like the glycolytic pathway, calcium dependent metabolism, cellular anti-oxidant systems, haemoglobin metabolism, protein, and nucleic acid metabolism. LMF seems to predominantly implicate the haemoglobin degradation and the glycolytic pathway.

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APPENDIX 1

SOLUTIONS AND BUFFERS

A. Lysis solution A

(8 M urea, 3% CHAPS, 1 % SDS, 40 ml)

	Final concentration	Amount
Urea (FW 60.06)	8M	19.2 g
CHAPS	3% (W/V)	1.2 g
Tris base (FW 121.1)	40 mM	0.194 g
DTT	13 mM	0.16 g
Ampholytes	2 % (V/V)	800 µL
SDS (FW 288.38)	1 % (W/V)	0.4 g
PMSF	0.5 mM	
EDTA		0.024 g
Glycerol	10 % (V/V)	4 ml
Double distilled H_20		to 40 ml

Store in 1.0 ml aliquots at $^{-}20\ ^{0}C$.

B. Lysis solution B

	Final concentration	Amount
Urea (FW 60.06)	8M	19.2 g
CHAPS	4% (W/V)	1.6 g
Tris base (FW 121.1)	40 mM	0.194 g
Double distilled H_20		to 40 ml

Prepare fresh or store in aliquots at $^{-}20\ ^{0}C$.

Rehydration stock solution without IPG Buffer[1]

(8 M Urea, 2% CHAPS, bromophenol blue, 25 ml)

	Final concentration	Amount
Urea (FW 60.06)	8 M	12 g
CHAPS	2% (w/v)	0.5 g
Bromophenol blue	trace	(a few grains)
Double distilled H_2O		To 25 ml

Store in 2.5 ml aliquots at $^{-}20\ ^{0}C$.

[1] DTT and IPG Buffer are added just prior to use. Add 7 mg DTT per 2.5 ml aliquot of rehydration stock solution. (2% IPG Buffer (50 µl per 2.5 ml) is recommended for Multiphor II IEF system for pH ranges 4-7 L, 3-10 L, or 3-10 NL).

Rehydration stock solution with IPG Buffer[1]

(8 M Urea, 2% CHAPS, 2% IPG Buffer, bromophenol blue, 25 ml)

	Final concentration	Amount
Urea (FW 60.06)	8 M	12 g
CHAPS	2% (w/v)	0.5 g
IPG Buffer	2% (v/v)	500 µl
Bromophenol blue	trace	(a few grains)
Double distilled H_2O		to 25 ml

Store in 2.5 ml aliquots at $^{-}20\ ^{0}C$.

[1] DTT is added just prior to use. Add 7 mg DTT per 2.5 ml aliquot of rehydration stock solution.

D. SDS equilibration buffer[1]

(50mM Tris-Cl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue, 200 ml)

	Final concentration	Amount
1.5 M Tris-Cl, pH 8.8	50 mM	6.7 ml
Urea (FW 60.06)	6 M	72.07 g
Glycerol (87% v/v)	30% (v/v)	69 ml
SDS (FW 288.38)	2% (w/v)	4.0 g
Bromophenol blue	trace	(few grains)
Double distilled H_2O		to 200 ml

Store in 40 ml aliquots at $^{-}20\ ^{0}C$.

[1] This is a stock solution. Prior to use DTT 100 mg or Iodoacetamide 450 mg per 10 ml SDS equilibration buffer is added.

E. 4X Resolving gel buffer

(1.5 M Tris-Cl pH 8.8, 1000 ml)

	Final concentration	Amount
Tris base (FW 121.1)	1.5 M	181.5 g
Double distilled H_2O		750 ml
HCl (FW 36.46)		Adjust to pH 8.8
Double distilled H_20		to 1000 ml

Filter solution through a 0.45 µm filter.

Store at 4 ^{0}C

F. 10% SDS

	Final concentration	Amount
SDS (FW 288.38)	10 % (w/v)	5.0 g
Double distilled H_2O		to 50 ml

Filter solution through a 0.45 µm filter.

Store at room temperature.

F. 10% Ammonium persulphate

	Final concentration	Amount
Ammonium persulphate (FW 228.20)	10%	0.1 g
Double distilled H_2O		to 1.0 ml

Fresh ammonium persulphate "cracles" when water is added. If it does not, it is advisable to replace it with fresh stock [Berkelman, 1998 #3140]. Prepare just prior to use.

G. Gel storage solution

(0.375 M Tris-Cl pH 8.8, 0.1% SDS 200ml)

	Final concentration	Amount
4X resolving gel buffer (solution E)	1X	50 ml
10% SDS	0.1%	2 ml
Double distilled H_2O		to 200 ml

Store at 4 ^{0}C

I. SDS electrophoresis buffer[1]

(25 mM Tris, 192 mM glycine, 0.1% SDS, 5 litres)

	Final concentration	Amount
Tris base (FW 121.1)	25 mM	15.1 g
Glycine (FW 75.07)	192 mM	72.1 g
SDS (FW 288.38)	0.1% (w/v)	5.0 g
Double distilled H_2O		to 5000 ml

Store at room temperature.

[1] Because the pH of this solution does not require being checked, it can be made up directly in large reagent bottles up to 20 litres at a time and stored at room temperature [Berkelman, 1998 #3140].

K. Agarose sealing solution

	Final concentration	Amount
SDS electrophoresis buffer		100 ml
Agarose	0.5%	0.5 g
Bromophenol blue	trace	a few grains

Add all ingredients into a 500 ml Erlenmeyer flask. Swirl to disperse. Heat in microwave oven on low until the agarose is completely dissolved. Store at room temperature.

L. Phosphate Buffered Saline (X10 PBS); 0.1 M; pH 7.4

	Amount
Disodium hydrogen phosphate (Na_2HPO_4)	11. g
Sodium dihydrogen phosphate (NaH_2P0_4)	3.0 g
Sodium Chloride (NaCl)	85. 0 g
Distilled Water	Make up to 1000 ml

Store at room temperature

K. Tris-Buffered Sorbitol Solution (10 mM Tris / 250 mM sorbitol pH 7.0)

	Final concentration	Amount
Tris base (FW 121.1)	10 mM	0.121 g
Sorbitol	250 mM	
Double distilled H_2O		750 ml
HCl (FW 36.46)		Adjust to pH 7.0
Double distilled H_20		to 1000 ml

Filter solution through a 0.45 µm filter.

Store at 4 ^{0}C

APPENDIX 2

APPARATUS AND REAGENTS

Equipment used for Proteomics work.

2117 Multiphor II Electrophoresis Unit, (Pharmacia biotech)
2219 Multitemp II Thermostatic Circulator (Pharmacia biotech)
BIO-RAD Power Pac 3000
GS-710 calibrated imaging densitometer (BIO-RAD)
Hoefer®SE 600 standard dual cooled gel electrophoresis units (Pharmacia biotech)
Immobiline DryStrip Kit (comprising of dry strip reswelling tray, electrodes, Immobiline strip aligner and IEF electrode strips (electrode wicks))
MALDI mass spectrometer (M@LDI, Micromass)
Polypropylene microcentrifuge tubes (500μL) and (500 μL)
Rotor mixer
Silver staining kit (BIO-RAD)
Ultrasonic processor (Vibra Cell™)
Vacuum concentrator

Reagents

α Cyano-4-Hydroxycinnamic acid (Sigma)
Acetonitrile HPLC grade (Sigma)
Acrylamide: *N,N'*;Methylene-bis-acrylamide, ultra pure grade (30%) 37.5: 1 (Anachem)
ACTH Clip 18-39 (Adenocorticotrophic hormone)
Agarose (Sigma)
Alcohol dehydrogenase (Sigma)
Ammonium bicarbonate (sigma)
Ammonium persulphate (Ammonium peroxydisulfate) (Sigma)
Aprotinin (Protease inhibitor) (Sigma)

Artemether (donation from WHO)

BIO-RAD 2D-SDS-PAGE Standards

BIO-RAD protein assay dye reagent concentrate

Bromophenol blue (3′,3″,5′,5″-Tetrabromophenol sulfonate phthalein)

Calcium chloride (sigma)

CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate) (Sigma)

Deionised water (dH_20)

Deoxyribonuclease 1 (DNase), (Sigma)

Dihydro-artemisinin (donation from WHO)

Di-Sodium hydrogen orthophosphate (BDH)

Dry strip cover fluid (Mineral oil), (Sigma-aldrich)

D-Sorbitol (Sigma)

DTT (Dithiothreitol) (Sigma)

EDTA Ethylenediamine-tetraacetic acid (Dipotassium salt) (Sigma)

Ethanol HPL grade (BDH)

Formaldehyde, 37 WT.% solution in water (Aldrich)

Glacial Acetic Acid (Sigma)

Glycerol (Sigma-Aldrich)

Glycine (Aminoacetic acid) (Sigma)

Immobiline DryStrips (precast polyacrylamide gels, (T=4%, C=3%),Linear pH gradient 3-10, separation distance 13 cm)

Iodoacetamide (Sigma-Aldrich)

Lumefantrine (Benflumetol), (Norvatis Pharma Ag Basel)

Pharmalyte pH 3-10

PMSF (Phenylmethanesulfonyl fluoride) (Sigma)

Potassium dihydrogen orthophosphate (BDH)

Potassium Ferricyanide (BDH)

Ribonuclease (RNase), (Sigma)

Saponin (from Quillaja bark) (Sigma)

Silver nitrate (Sigma-Aldrich)

Sodium bicarbonate (Sigma)

Sodium Carbonate (Sigma)

Sodium dodecyl sulphate (BDH)

Sodium hydrosulfite (Sigma)

Sodium thiosulfate (Sigma)

TEMED (*N,N,N',N'*-tetramethyl-ethylenediamine), (Sigma)

Thiourea (Thiocarbamide) (Sigma)

Triflouro acetic acid (Sigma)

Triton X-100 (non-ionic surfactant) (BDH)

Trizma®base (Tris [hydroxymethyl] amino methane), (sigma)

Trizma®hydrochloride (Tris [hydroxy methyl] amino methane hydrochloride), (Sigma)

Trypsin, modified sequencing grade (Promega V5111)

Urea (99.5%) (Sigma)