School of Pharmacy

Pharmacodynamic Studies of Antimalarial Drugs

in a Murine Malaria Model

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DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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ABSTRACT

Murine malaria models have proved to be a valuable preclinical tool, particularly in the development of new concepts in the research of human malaria. *Plasmodium berghei* (*P. berghei*), is the most extensively studied and manipulated rodent parasite and as a laboratory model, is largely selected for studies relating to developmental biology of parasites and investigation into new and innovative drug therapies. Whilst direct extrapolation from rodent biology to human malarias should be generally avoided, murine malaria models may contribute a greater understanding of important characteristics for antimalarial drug development and drug efficacy studies. However, there is currently a paucity of murine pharmacological data available for both commonly used, and emerging, antimalarial therapies. The findings of the studies in this thesis are seen as an important contribution to the preclinical knowledge of the investigated drugs which to date, have not been adequately studied.

The aim of the thesis was to investigate the efficacy, pharmacokinetic and/or pharmacodynamic properties of various antimalarial drugs, in a *P. berghei* murine malaria model. Specific aims were to:

(i)   Evaluate the pharmacodynamic effects of dihydroartemisinin (DHA) in asplenic *P. berghei* infected mice.

(ii) Investigate the pharmacokinetic and pharmacodynamic properties of single dose piperaquine (PQ) in healthy and *P. berghei* infected mice.

(iii) Investigate the extended antimalarial effect of PQ concentrating on drug efficacy, re-inoculation outcomes and parasite viability.

(iv) Evaluate the pharmacokinetic and pharmacodynamic properties of single and multiple doses of chloroquine (CQ) in healthy and *P. berghei* infected mice.

Using an asplenic model of *P. berghei* malaria, the efficacy of single doses of DHA (0, 10, 30 and 100 mg/kg) were evaluated in uninfected and *P. berghei* infected, intact and asplenic mice. Haematology, liver biochemistry and histopathology were

performed to investigate the responses of key organs to malaria infection. Whilst overall efficacy of single dose DHA in asplenic mice was shown to be similar to intact mice, the rate of parasite recrudescence after parasite nadir (20 h at all doses studied) was significantly higher in the asplenic mice, particularly at higher doses (30 and 100 mg/kg DHA). Histopathology of the liver and associated blood chemistries, demonstrated an increased stimulation of liver function during malaria infection in asplenic mice, when compared to intact mice.

Whilst studying the pharmacokinetic and pharmacodynamic responses of PQ in the *P. berghei* malaria treatment model, particular focus was placed on (i) pharmacodynamic properties of single doses of PQ (0, 10, 30 and 90 mg/kg PQ phosphate (PQP)); (ii) pharmacokinetic parameters of PQ in healthy and *P. berghei* infected mice; (iii) efficacy of combined doses of 10 mg/kg PQP and 30 mg/kg DHA. Single dose administration of PQP resulted in a median survival time of 4, 10 and 54 days after doses of 0, 10 and 30 mg/kg PQP, respectively, while mice receiving a single 90 mg/kg dose showed a medium survival time exceeding 60 days (experimental endpoint). Pharmacokinetic analysis determined the elimination half-life of PQ in healthy and *P. berghei* infected mice was 18 and 16 days, respectively. Furthermore, extrapolation of PQ concentrations suggested that at 60 days the plasma drug concentration would be ineffective at suppressing the *P. berghei* infection (<10 μg/L). Combination of PQP and DHA resulted in a significantly lower parasite nadir (22 ± 12 fold) than for either drug given individually.

Given that high dose PQP (90 mg/kg) demonstrated extended antimalarial efficacy, further invetsigations were pursued on drug efficacy, re-inoculation outcomes and parasite viability after a single 90 mg/kg dose of PQP. Investigation showed that after initial dosing, PQ concentrations were not adequate to suppress parasitaemia after 25 days. Furthermore, although viable parasites were present up to 90 days after drug administration, once these viable parasites were passaged into naive mice they were found to be generally resistant to PQ when exposed to the drug for a second time. Overall, PQ was found to have a substantial antimalarial effect in this model with this effect appearing to be sufficient for a host immunological

response to be established thus resulting in the long term survival of *P. berghei* infected mice.

Although CQ is widely used in preclinical animal studies, there is a paucity of comprehensive pharmacokinetic data of CQ in animal models. In this thesis robust pharmacokinetic and pharmacodynamic data of CQ is presentated after single and multiple dose administration of CQ in the *P. berghei* malaria model. The pharmacokinetics of desethyl-CQ (DECQ), the major active metabolite of CQ, were estimated. Pharmacodynamic data demonstrated that parasite nadir was reached 79 h after a single dose of 60 mg/kg CQ, with all mice developing parasite recrudescence. Multiple dose (5 x 50 mg/kg CQ; dosed every 24 h) administration resulted in parasitaemia falling below the limit of detection. Despite a short period of recrudescence (between 10 and 24 days after initial dose), parasitaemia remained undetectable until the experimental end point (35 days after the initial dose). Pharmacokinetic analysis determined an elimination half-life of 46.6 h in healthy mice and 99.3 h in malaria-infected mice (single dose data; non-compartmental analysis). The mean rate of formation of DCQ from CQ was 0.63 ± 0.55 h^{-1} with a formation half-life of 1.7 ± 1.0 h.

Consequently, the drug efficacy, pharmacodynamic and pharmacokinetic data included in this thesis demonstrates that the current *P. berghei* murine malaria treatment model can be used as a valuable preclinical conceptual tool for the investigation of antimalarial drugs such as DHA, PQ and CQ.

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ABBREVIATIONS

ACT	artemisinin combination therapy
ALP	alkaline phosphatase
ALT	alanine transaminase
AO	acridine orange
AQ	amodiaquine
ARC	Animal Resource Centre
ART	artesunate
AST	aspartate transaminase
AUC	area under the curve
BC	before Christ
CI	confidence interval
CL	clearance
CL/F	apparent clearance
CQ	chloroquine
DECQ	desethylchloroquine
DHA	dihydroartemisinin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid (anticoagulant)
ED_{50}	effective dose 50%
ED_{90}	effective dose 90%
ELISA	enzyme-linked-immunosorbent serologic assay
Fig.	figure
FOV	field of view
g	gravitational force
G6PD	glucose-6-phosphate dehydrogenase
Gran	granulocytes
h	hour(s)
Hb	haemoglobin
HCL	hydrochloric acid

HCT	haematocrit
HPLC	high performance liquid chromatography
HRP	histidine-rich protein
IC_{50}	concentration of drug required to inhibit growth by 50%
IES	inter-endothelial slits
IgG	Immunoglobulin G
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
KH_2PO_4	potassium dihydrogen phosphate
L	litre(s)
LC-MS	liquid chromatography-mass spectrophotometry
LD_{50}	lethal dose 50%
LOD	limit of detection
LOQ	limit of quantification
Lymph	lymphocytes
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean cell volume
MDR	multidrug-resistant
MIC	mean inhibitory concentration
min	minute(s)
mg/kg	milligrams per kilograms
Mono	monocytes
mm	millimetres
MPV	mean platelet volume
MW	molecular weight
NaCl	sodium chloride
NaOH	sodium hydroxide
NHMRC	National Health and Medical Research Council
P. berghei	*Plasmodium berghei*
P. chabaudi	*Plasmodium chabaudi*

PCR	polymerase chain reaction
PCT	parasite clearance time
PD	pharmacodynamic
P. falciparum	*Plasmodium falciparum*
Pf EMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PK	pharmacokinetic
P. knowlesi	*Plasmodium knowlesi*
pLDH	lactate dehydrogenase
P. malariae	*Plasmodium malariae*
P. ovalae	*Plasmodium ovalae*
PQ	piperaquine
PQP	piperaquine phosphate
PRBC	parasitised red blood cells
QC	quality control
RBCC	red blood cell count
RBCDW	red blood cell distribution width
RDT	rapid diagnostic test
RESA	ring-infected erythrocyte surface antigen
RNA	ribonucleic acid
s.c.	subcutaneous
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
s.d.	standard deviation
sec	second(s)
SPF	specific pathogen free
$t_{1/2}$	elimination half-life
TBW	total body weight
UV	ultraviolet light
V	volume of distribution
V/F	apparent volume of distribution
WBCC	white blood cell count
WHO	World Health Organization
wk	week

CHAPTER ONE

INTRODUCTION

1.1 MALARIA

The transmission of malaria from the mosquito vector was first described by Ronald Ross in 1898, at which time his discovery was described as 'important as the discovery of America' (386). Notwithstanding the massive strides in biology and medicine in the past 100 years, malaria still poses the greatest threat of all known parasites to human health (386, 577, 584). Despite an increased awareness of the disease, improved access to antimalarial drugs and global economic development, more people die from malaria today than 40 years ago with an estimated incidence of infection increasing 2-3 fold over the last 35 years (197, 386). Furthermore, with increasing drug resistance as well as changes in world climate, malaria is returning to areas from which it had previously been eradicated and is now entering new areas such as Eastern Europe and Central Asia (197, 386).

Malaria is caused by transmission of single-celled protozoan parasites of genus *Plasmodium* from an *Anopheles* mosquito vector, to a suitable vertebrate host. Whilst there are over 200 species of *Plasmodium*, only five species have the capability to cause malarial illness in humans. *Plasmodium falciparum* (*P. falciparum*)*, P. vivax, P. ovalae and P. malariae* are all human specific parasites which cause both serious (*P. falciparum*) and milder forms (*P. vivax, P. ovalae and P. malariae*) of the disease (26, 85, 382, 573). More recently a fifth human species *P. knowlesi* has being identified which can cause human infections from zoonotic transfer from macaques monkeys (*Macaca fascicularis*) (112, 247). At the present time the most dominant parasite is *P. falciparum* which accounts for 40-60% of malaria cases worldwide and >95% of all malaria deaths (421). In tropical Africa, where *P. falciparum* is the dominant pathogen, it is estimated that a child will die every 12-30 sec from malaria (386).

1.1.1 Epidemiology

The 2008 World Malaria Report (580) estimated that in 2006 there were 3.3 billion people at risk of malaria infection of whom 247 million had a reported malaria infection, resulting in nearly 1 million deaths. Those most at risk from fatal malarial infections included children under the age of 5, pregnant women and the immunocompromised (580). It has been suggested that reported figures from the World Health Organization (WHO) could be an underestimation of the annual burden of this parasitic disease (458).

Malaria is endemic throughout the tropical areas of sub-Saharan Africa, Southeast Asia, the Pacific Islands, India and Central and South America (Fig. 1.1)(382, 579, 580). In 2008, 109 countries were declared to be malaria endemic with 45 of these countries falling within the WHO African region (579, 580). Statistically, this equates to approximately 40% of the world's population being under threat from this parasitic disease (26, 386).

* China, Indonesia, Philippines, Solomon Islands, Sudan, Vanuatu and Yemen have subnational elimination programmes.

Figure 1.1   Distribution of malaria transmission in 2007 (580). Worldwide distribution of malaria-free and malaria-endemic countries including those that are currently in phases of malaria control, pre-elimination, elimination and prevention of reintroduction (580).

In Southeast Asia, particularly along the borders of Thailand with Myanmar and Cambodia, *P. falciparum* is multidrug resistant with chloroquine (CQ), sulphadoxine-pyrimethamine (Fansidar) and mefloquine monotherapies all ineffective and quinine therapy rapidly losing potency (Fig. 1.2)(578). In sub-Saharan Africa CQ resistance to falciparum malaria is widespread and antifolate resistance is rapidly developing (26).
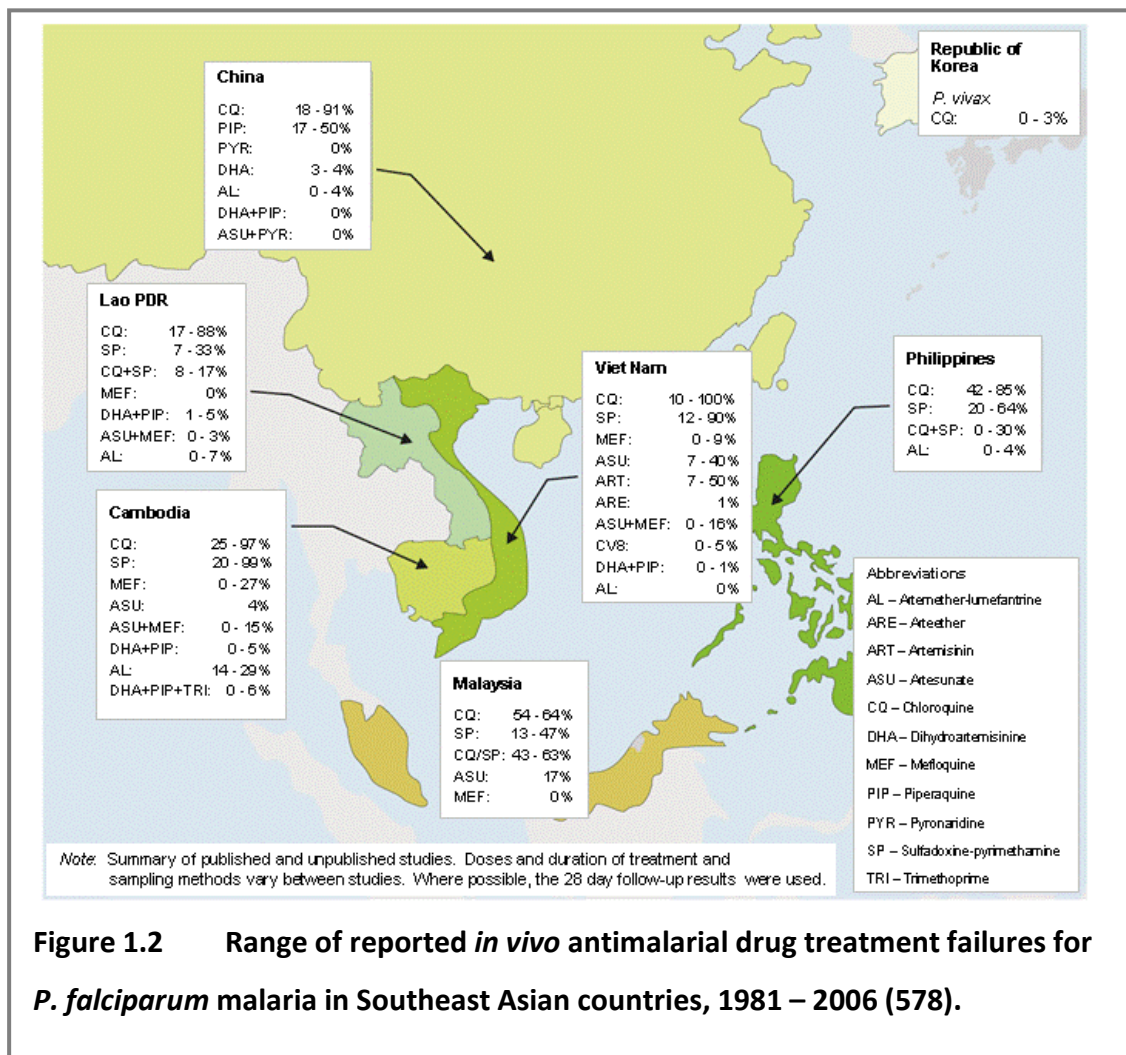
Figure 1.2   Range of reported *in vivo* antimalarial drug treatment failures for *P. falciparum* malaria in Southeast Asian countries, 1981 – 2006 (578).

1.1.2   Pathophysiology and aetiology

1.1.2.1 Historical background

The symptoms of a disease resembling malaria were first described over 4,000 years ago in the *Nei Ching* (The Canon of Medicine), the ancient Chinese medical writings edited by Emperor Huang Ti in 2700 BC (341). Features of the disease, later named

mal'aria, Italian for bad air, became widely recognized in 4th century BC after Hippocrates characterised the clinical symptoms and complications of this seasonal intermittent fever (181, 341). By 6th century BC the symptoms of malarial fever, which were attributed to the bites of certain insects, were extensively described in the *Susruta*, a Sanskri text (341). During the same time period Roman writers associated the incidence of malarial fever to swampy regions (341, 442). Despite the continued characterization of disease processes, the cause of malaria was not isolated until 1880 (73, 341, 442).

In November 1880, Charles Laveran, microscopically observed the exflaggelation of a male gametocyte which led to the conclusion that the causative agent of malaria was a protozoan parasite (73, 341, 442). During the next 10-20 years, four different human species of malaria parasite were identified and named by Italian and American scientists (*P. vivax* and *P. malariae*, Giovanni Grassi and Raimondo Fileeti, 1890; *P. falciparum*, William Welch, 1897; *P. ovalae*, John Stephens, 1922) (442).

Perhaps the greatest advancement in the study of malaria was the discovery of the role of the *Anopheles* mosquito in the transmission of malaria. On 20th August 1897 Ronald Ross found the malaria parasite within the stomach tissue of an Anopheline mosquito that had fed on a malaria-infected patient four days earlier (73, 341). In further malaria research Ross showed the transmission of malaria parasites between birds demonstrating that the mosquito acted as an intermediate host for the avian malaria. After feeding mosquitoes with blood from infected birds he showed that the malaria parasite developed in the mosquito stomach and later migrated to the salivary glands, allowing the mosquito to then infect other birds during subsequent blood meals (341). The complete sporogenic cycles of *P. falciparum, P. vivax and P. malariae* were soon after described by a team of Italian scientists led by Giovanni Grassi (1898-1899). Thus, the process of malaria transmission was established (341).

1.1.2.2 Life cycle

The genus *Plasmodium* may be identified taxonomically by the presence of protozoa with two forms of asexual division, schizogony and sporogony, and a single stage of sexual division. Schizogony is the phase of parasitic asexual division and maturation occurring within the vertebrate host, while sporogony is the sexual and asexual and divisional stage occurring with a mosquito vector (446). In order to successfully complete the parasitic life cycle (Fig. 1.3) two hosts, the definitive and intermediate hosts, must be present.

The female *Anopheles* mosquito is the sole vector of *Plasmodium* parasites and whilst there are over 600 species of *Anopheles* mosquitoes worldwide, only 60 species have been found to transmit malaria (382, 421). The schizogonic cycle is initiated when a malaria-infected mosquito takes a blood meal from a person, releasing on average 15-20 (26), but up to 100 sporozoites (422), into the blood circulation from the salivary glands. After entering the bloodstream the sporozoites take about 30-45 min to travel to the liver where they invade hepatocytes and begin to multiply. This cycle, referred to as the pre-erythrocytic cycle, takes approximately 6-14 days and results in the production of merozoites. Growth and division in the liver takes approximately 6, 6, 10 and 15 days for *P. falciparum*, *P. vivax*, *P. ovalae* and *P. malariae,* respectively (382, 386). In *P. vivax* and *P. ovalae* infections some sporozoites appear to develop for the first 24 h at which stage they become dormant, as single-celled forms known as hypnozoites, which can remain in the hepatocytes for months to years until they are reactivated causing a relapsed malaria infection (382, 386, 421).

At the conclusion of the pre-erythrocytic cycle, the host liver cells burst releasing thousands of merozoites into the blood circulation which attach to and invade erythrocytes within 20 sec of release (319, 386). This process initiates the erythrocytic cycle. Within the erythrocytes the parasites begin to again multiply resulting in erythrocyte (red blood cell) rupture 48–72 h later, depending on parasite species. Following erythrocyte rupture, both merozoites and pyrogenic materials are released resulting in both an increase in parasite biomass and malarial

Figure 1.3   The life cycle of human malarial parasites (85). The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host ❶. Sporozoites infect liver cells ❷ and mature into schizonts ❸, which rupture and release merozoites ❹. (Of note, in *P. vivax* and *P. ovalae* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony Ⓐ), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony Ⓑ). Merozoites infect red blood cells ❺. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites ❻. Some parasites differentiate into sexual erythrocytic stages (gametocytes) ❼. Blood stage parasites are responsible for the clinical manifestations of the disease.

The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal ❽. The parasites' multiplication in the mosquito is known as the sporogonic cycle Ⓒ. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes ❾. The zygotes in turn become motile and elongated (ookinetes) ❿ which invade the midgut wall of the mosquito where they develop into oocysts ⓫. The oocysts grow, rupture, and release sporozoites ⓬, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle ❶.

symptoms of fever and anaemia (26, 382, 386). This asexual erythrocytic cycle usually continues until it is either controlled through immune responses, drug therapy or until host death. The morbidity and mortality associated with malaria are derived primarily from the erythrocytic cycle (386).

After several erythrocytic cycles a yet unidentified trigger diverts the development of certain intraerythrocytic merozoites into sexual forms known as gametocytes. The male and female gametocytes are taken up by a mosquito and in the mosquito midgut they mature as gametes (26, 382, 386). The sporogonic cycle is initiated through the fertilization of the gametes producing zygotes which within 24 h matures to a motile ookinete. The ookinetes burrow through the mosquito midgut wall to encyst on the basal lamina where, within the developing oocyts, there are many miotic divisions resulting in the formation of sporozoites. When the infective oocysts rupture sporozoites are released at which time they migrate through the haemocoel to the salivary glands completing the sporogonic cycle, approximately 7 to 30 days after gametocyte ingestion (depending on the host, infective parasite species and environmental conditions (26, 382, 386).

1.1.2.3 Malaria and the immune system

Clinical immunity to severe non-cerebral falciparum malaria usually occurs after one or two infections, however, immunity against mild disease takes much longer to acquire (200, 519). In malaria endemic regions, children born to immune mothers are protected against malaria infections, although they may be exposed to infections, for the first 6 months after birth by maternal antibodies transferred through breast milk (200, 369, 454, 519). During this period of time the immune system in the infants compiles a repertoire of specific humoral and cellular immune responses against the infecting parasites (519). However, as exposure to maternal antibodies wanes, the child will have a period of 1 to 2 years of increased susceptibility to malaria infections before they are able to acquire active immunity (369).

Innate immunity, the immunity not associated with specific antigens, may be classified into genetically based resistance and cell mediated mechanisms (455). During malaria infections genetically based resistance mechanisms may influence the progression of disease through the impairment of merozoite invasion of erythrocytes, reduction in the growth of parasites within an erythrocyte, impaired liberation of merozoites from schizonts and the reduction in vitality of merozoites after they are released from the rupturing schizont (309, 455). Cell mediated mechanisms are responsible for the phagocytosis of merozoites or parasitised erythrocytes by neutrophils, monocytes or macrophages, and through the production of cytotoxic molecules (i.e. cytokines or nitric oxide) which are produced by various immunological cells against both the free parasites or parasitised erythrocytes (103, 455, 519). Whilst the innate immune system, comprising mainly dendritic cells, monocytes and macrophages, natural killer cells, and T cells, is an important defence mechanism against malaria infections it also plays a very important role in shaping the adaptive immune response to blood-stage malaria (519).

In malaria endemic regions a person will often be exposed to a number of malaria infections during childhood. Surviving the malaria infection may result in the development of a state of immunity, referred to as acquired immunity, where a low level parasitaemia is maintained whilst remaining asymptomatic (369, 393). Acquired immunity, immunity developed in response to foreign antigens in the body, is both species- and stage- specific and results from the generation of specific antibodies to several variant antigenic proteins, most notably *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1), which are produced by trophozoite and schizont stage parasites and expressed on the surface of parasitised erythrocytes (301, 369, 393, 455). However, as there are over 50 genes that encode PfEMP-1 molecules, acquired immunity to *P. falciparum* arises after multiple infections. Whilst acquired immunity does not prevent future re-infection with *P. falciparum,* the inflammatory response to the parasites, which causes the acute febrile symptoms, are limited and mechanisms to kill parasites or inhibit parasite replication are enhanced (25, 455).

The difficulties that confront immunologists studying host responses of human malarias has led to the use of experimental models of rodent malaria (159). Although it is readily acknowledged that murine models cannot accurately reflect all aspects of human infections, there is commonality between the immune responses to malaria parasites in humans and rodents (159). Studies in both humans and mice have demonstrated an important role of dendritic cells (274, 370, 395, 519, 585) and T cells (103, 196, 369, 393), among others, for the formation and maintenance of malarial immunity (159).

1.1.3   Diagnosis of malaria

Clinical diagnosis of malaria is imprecise but in many cases is the basis of therapeutic care for patients presenting with fever in malaria endemic areas, particularly if laboratory support is not available or delayed (225, 565). Due to the life-threatening nature of the disease, the correct and timely diagnosis of malaria infection is critically important (16, 52, 197, 225).

Diagnosis of malaria infection as a cause of disease is multifactorial and includes both the presence of parasites in the blood and clinical symptoms of infection (576). The detection of parasites on a blood film does not always indicate the cause of disease, as children who are indigenous to an endemic area may continually have low level parasitaemias, however, this does not present as symptomatic disease (197). Diagnosis also plays an important role in patient management and follow-up chemotherapy. Apart from detecting parasites in the blood, laboratory testing is also imperative to detect signs of poor prognosis (i.e. haemoglobin levels, blood glucose, lactate and the presence of protein or free haemoglobin in the urine) or to guide the methods of chemotherapy (glucose-6-phosphate dehydrogenase (G6PD)) (225). Despite the obvious need for improvement of malaria diagnosis, this area remains the most neglected aspect of all malaria research and development (565).

Whatever the method used, a diagnostic test should be able to correctly differentiate between individuals who are infected with malaria and those that are

not (16, 197, 298). Consequently, the validity of the test is usually determined on its sensitivity and its specificity (225).

1.1.3.1 Presumptive diagnosis

In the vast majority of malaria endemic countries, national poverty ensures that laboratory support cannot be relied on for the diagnosis and treatment of malaria (225). Clinical diagnosis is the least expensive thus most commonly used diagnostic method and often is the basis for patient self-treatment (565). Although malaria infection has a number of distinct clinical symptoms, the overlapping of malaria symptoms with other tropical diseases (including influenza, pneumonia, viral hepatitis or typhoid) impairs its specificity (225, 565). In areas of high malarial endemicity fever is most often related to malaria infection therefore, the vast majority of patients presenting with fever will be presumptively diagnosed and treated with antimalarials (565).

1.1.3.2 Blood examination for malaria parasites

Light microscopic examination of Giemsa stained blood films is the most widely practiced and most appropriate diagnostic instrument for parasite detection (59, 197, 576). Examination of a correctly prepared blood film allows an inexpensive, yet definitive, diagnosis of malaria infection including speciation and quantification (59, 225, 565). Thick and thin blood films should be prepared and appropriately stained. The most commonly used stain used in the field setting is Giemsa stain (225).

Disadvantages of light microscopy as a diagnostic method are that the processes of blood collection, slide preparation, staining and reading can be time-consuming and the microscopist requires adequate training and supervision to ensure accuracy and consistency (59). Whilst availability of microscopic diagnosis has shown to reduce drug use in some clinical trials (248), in clinical practice the results may be disregarded by the clinicians who tend to favour presumptive diagnostic techniques (43, 59).

To increase the sensitivity and specificity of malaria diagnosis, modifications to simple microscopy have been made. The quantitative buffy coat method (QBC^{TM}, Becton-Dickinson) works by staining parasites with acridine orange stain (59, 275). Blood is placed in microhaematocrit tubes precoated in acridine orange stain which are then centrifuged. Any parasites that may be present in the blood will spin at a specific density and be easily identifiable at a predetermined position on the microhaematocrit tube (41, 59).   Advantages of this system are that it requires less training to operate and read the tests compared to normal Giemsa stained blood films, as well as taking less time to complete diagnosis compared to light microscopy. Under ideal conditions, the QBC system has been found to be slightly more sensitive than routine light microscopy for the diagnosis of malaria (41, 275), however, its disadvantages of higher cost, requirement of electricity, specialized equipment and supplies as well as a decreased specificity for species identification has led to the QBC system not being routinely incorporated into diagnostic laboratories (59).

1.1.3.3 Detection of malarial antigen

Rapid diagnostic tests (RDTs) detect malarial antigen in small quantities of blood (5–15 µL) by immunochromatographic assay with monoclonal antibodies directed against the target parasite antigens that are abundant in the blood during a malaria infection (325, 334, 565). A positive result on an antigen detection assay suggests infection with malaria parasites (225). Current antigen detection methods diagnose active malaria infections, primarily *P. falciparum*, rapidly and reliably in an easy to use immunochromatographic kit which does not need a microscope for diagnosis. The two predominant antigen testing kits commercially available today are based on the detection of the malarial antigen Histidine-Rich Protein 2 (HRP-2) or the enzyme parasite Lactate Dehydrogenase (pLDH) (292, 299).

1.1.3.4 Serology

Serological testing methods have been used for the detection of malaria infection since the early 1960s, when indirect fluorescent antibody tests (IFAT) and indirect haemagglutination assays (IHA) were described. A disadvantage of serological

testing methods is that as they detect antimalarial antibodies, current and past infections cannot be differentiated and the tests have limited value in the treatment or management of malaria infections. Therefore the only reliable use of these testing procedures is for the detection of negative serological assays, thus demonstrating that the patient does not have malaria (225).

1.1.3.5 Molecular Methods

The application of DNA or RNA hybridization via polymerase chain reaction (PCR) based probes, to malaria diagnosis has several advantages over traditional methods, although the feasibility of implementing into the field setting is limited. However, it may have a place as a research tool to monitor malaria control programs, or to perform quality control checks on microscopic diagnosis or to determine the distribution of important genes, particularly those associated with drug resistance (225, 327). Recent publications (206, 325, 575) have suggested that PCR is able to detect blood parasitaemias of less than 0.00002%, if performed under the best possible conditions. This level of parasitaemia is theoretically the detection of a single parasite in an entire sample, although this is rarely achieved (225). A detection of a parasitaemia of 0.00002%, which equates to 5 parasites per µL blood, is a detection threshold at least five times lower than that achieved by a thick blood film performed in optimal conditions (i.e. 0.0001%), assuming that an experienced scientist has spent at least 10 min examining 100 fields of view (205, 206, 325). Whilst in field conditions the sensitivity of PCR may only be comparable or slightly better than examination of a thick blood film by a trained microscopist (206), the specificity of PCR is generally considered to be better than microscopy, particularly if the patient has a mixed infection (225).

1.1.3.6 Value of diagnostic methods

In most situations, the 'gold standard' for individual diagnosis is microscopic examination of thick and thin blood films (197, 576). However, there are situations when this form of diagnosis is not ideal (225). In areas of high malarial endemicity, clinical diagnosis alone is usually the only feasible and cost-effective method for the recommendation of first-line antimalarial treatment. For example, in a country that

has an annual health budget of US$2 per person, it is difficult to justify diagnostic assays costing more than US$1 if the cost of treatment is only US$0.04 (225). Unfortunately, all modern diagnostic tests are likely to cost at least US$1 and the use of less costly microscopy requires a health care infrastructure and expertise that may not exist in the field setting. When microscopy is not available, the use of dipstick antigen tests may be of value, particularly in areas of low endemicity where infection usually coincides with infection (225, 325).

1.1.4   Enumeration of malaria parasites

It has long been established that there is a strong correlation between parasite density and the severity of malaria (116, 163, 225). It is currently considered that any *P. falciparum* infection above 250,000 parasites/µL (approximately 5% of erythrocytes) should be regarded as a severe parasitaemia requiring urgent antimalarial treatment and emergency care (507, 509). The presence of *P. falciparum* schizonts in peripheral circulation may also be taken as a sign of severity as due to sequestration mechanisms, mature *P. falciparum* parasites are rarely visualized in the peripheral circulation (225).

The enumeration of parasites serves as a useful guide for the clinical management of infection and furthermore provides an valuable guide for the success of clinical trials and epidemiological studies (205, 298). Thick and thin blood films are routinely used to monitor infection, to determine the development of resistance to antimalarial drugs and to serve as an important information base in the development of appropriate national and international drug policies (298, 390). Whilst observation of thick and thin blood films remain the first choice of enumerative studies (325), with several methods of parasite density estimation (151, 193, 205, 298, 367), no standard procedure exists for counting parasites on a thick blood film (565).

In 1910, Ross and Thompson described a method of parasite enumeration where thick blood films were prepared, using a measured volume of blood, dehaemoglobinised and then stained. Parasite density was then determined by

thoroughly searching the entire volume of blood using a sliding stage (427). This method was further modified by Earl and Perez (151) who used a fixed volume of blood in a pre-established area. This varied from the methods described by Ross and Thompson where blood volume was not restricted to a specific area. In the field and clinical laboratory three different techniques are routinely employed; (i) determining the percentage of infected erythrocytes (from a thin blood film), (ii) determining the number of parasites per white blood cell (WBC) or (iii) determine the number of parasites in a field of view (145, 298).   Despite the method used for parasite enumeration, all determined values can be transformed into an index to determine the number of parasites per volume of blood. This is determined by assuming that a constant number of erythrocytes, leukocytes or microscopic fields in that volume of blood (i.e. 5,000,000 erythrocytes, 8,000 leukocytes or 400 microscope fields) per microlitre (µL) of blood (193, 298). Although these indices appear crude, differences between the methods are not important as they would rarely vary by more than a factor of 1 (145).   However, variability in blood film preparation techniques and the method employed for parasite enumeration accounts for the majority of variability observed in parasite counts (145, 260). For example, a large difference in parasitaemia is observed when comparing counting of parasites against leukocytes on a thick blood film and against erythrocytes on a thin blood film (354). Such variability could significantly affect research results and outcomes (565).

For the accurate determination of parasite density the number of parasitised erythrocytes are determined in a thin blood film (193). To ensure accuracy, at least 100,000 erythrocytes should be counted (193). A drawback of this enumeration technique is that it is time-consuming and therefore not a feasible method for large-scale field studies. As a result two indirect methods, using a thick blood film, are currently employed for the determination of parasite density (193).   The first method determines parasite density by counting the number of parasites per leukocyte then multiplying the figure by 8000, the average white blood cell count per µL. The second indirect method counts the average number of parasites per high powered field of view with parasite density calculated from this value and the

estimated volume of blood in the thick blood film (193, 510). Although these two indirect methods are substantially quicker alternatives for the determination of parasite density, disadvantages of these methods are related to the accuracy of parasite counts. The first method will be inaccurate if the total white blood cell count differs markedly from the assumed value of 8,000 leukocytes per µL, as can occur if the patient has an underlying bacterial infection, inflammation or as seen in children with malaria (193, 311). The second method will be inaccurate if the thick smears are prepared incorrectly or the volume of blood is not known (193, 510).

In a comparative study conducted by Greenwood et al. (193) it was demonstrated that there was little difference between the two indirect enumeration methods using thick blood films . 'True' malaria parasite density was determined by counting the number of parasitised erythrocytes within 100,000 erythrocytes then multiplying this percentage by the total red blood cell count (RBCC), determined using a Coulter Counter. Parasite density was then determined using both of the indirect thick blood film methods and the results compared to the 'true' malaria density. They concluded that although both indirect methods were reasonably accurate and allocated levels of parasitaemia within broad bands, it was easier and slightly faster to determine parasite density using the high powered fields of view method (193).   This was due to the fact that only parasites needed counting, compared to the other method where both parasites and leukocytes needed to be enumerated. Although the parasite density results determined in both of these indirect methods were assessed to be acceptable for the clinical management of malaria cases, for an accurate parasite enumeration the percentage parasitaemia should be determined using the thin blood film method (193).

A number of attempts to rationalize and define the reliability of microscopic diagnosis of malaria in the field and laboratory have been described. In 1966, Raghavan used a statistical approach with special reference to the efficacy of cross-checking procedures in malaria eradication programs (403). It was found that thick blood film examinations, using the standard 100-field of view criteria, could only detect with "reasonable certainty" parasite densities of 44 parasites in 1,000 thick

blood film fields. However, as it was not made clear what a 'standard film' was, it made it difficult to draw any definitive conclusions from this study. In an attempt to standardize counting techniques, Dowling and Shute (145) calculated the average thickness of thick and thin blood films that they used in comparative studies. Through these comparative studies they concluded that whilst a high proportion of parasites were lost during film dehaemoglobinization, a parasite count as low as one parasite per μL could be detected after 10 min close examination of a thick blood film. Whilst this is not equated to the number of fields examined, it is similar to procedures using a detection threshold of four or more parasites identified after 6 minutes of thick blood film examination (367).

A further consideration, when determining parasite density as a measure of clinical success, is that without understanding the behaviour of the sequestered population of *P. falciparum* parasites it is difficult to obtain a reliable measure of treatment success (129, 191). This is an important factor in the clinical setting as parasite sequestration is responsible for the majority of severe malaria pathology (184, 191). In an attempt to overcome this shortcoming in parasite enumeration of *P. falciparum* infections, a mathematical model was proposed by Gravenor et al. (191) which allowed the number of sequestered parasites to be estimated using sequential peripheral blood films. However, despite the predicted value of such a simple model in providing insight into the success of antimalarial therapy against all blood stages of *P. falciparum* parasites, it is acknowledged that the parasitaemias determined will only be a conceptual parasite density (129, 130, 191).

1.1.5 Antimalarial drug resistance

The antimalarial drug CQ was first introduced into the clinical setting in 1945 but within 12 years CQ-resistant falciparum malaria was reported in Southeast Asia and South America (546, 566). By the late 1980s, the Thai-Cambodain and Thai-Myanmar borders were declared to be multidrug-resistant (MDR) areas with a prevalence of mefloquine and sulfadoxine-pyrimethamine resistant falciparum parasites (566). Today, drug resistance has emerged to all classes of antimalarial

drugs, including evidence of the recent emergence of resistance to artemisinin derivatives in Southeast Asia (143, 348) (Table 1.1).

Table 1.1 **Dates of introduction and first reports of antimalarial drug resistance.** *[Table modified from data presented in Wongsrichanalai et al, (566)]*

Antimalarial drug	Introduced (genuine clinical use)	First reported resistance	Difference (years)	Refs
Quinine	1632	1910	278	(376)
Chloroquine	1945	1957	12	(547)
Proguanil	1948	1949	1	(57, 376)
Sulfadoxine-pyrimethamine	1967	1967	0	(57, 376)
Mefloquine	1977	1982	5	(567)
Atovaquone	1996	1996	0	(291)
Artemisinins	1980	2008	28	(143, 348)

Antimalarial resistance is defined as "the ability of a parasite to survive in the presence of concentrations of drug that normally destroy parasites of the same species or prevent their multiplication" (540). Drug resistance in malaria depends on the ability of the parasite to respond, through innate genetic diversity, to adverse conditions (540, 557). It is acknowledge that the problem of antimalarial resistance has emerged as a result of the indiscriminate use of antimalarials leading to the development of high levels of resistance in parasites through selective pressure (574). The goal of malaria chemotherapy is therefore to use the antimalarial drugs in such a way that the selection process is minimized, thus extending the therapeutic life of the drug (540, 551).

Many factors contribute to the development and spread of drug resistance including characteristics of the drug itself (dosing, drug pressure, pharmacokinetics, cross-resistance), human host factors (host immunity, maintenance of resistant parasite reservoir), parasite characteristics (genetic mutations, transmission level) and vector and environmental factors (vector affinity of parasites) (59, 234, 361, 372, 376, 551, 562, 566). Characteristics of the drug may be perhaps the most important determinants for drug resistance, particularly the drug elimination half-

life (208). Drugs with long elimination half-lives, such as mefloquine and piperaquine, may exert substantial residual selection on new infections contracted after treatment of the primary infection when the drug persists at subtherapeutic concentrations in the plasma (566). Subtherapeutic dosing also results in an increased risk of the emergence of resistant forms because any residual parasites may proliferate (44). As a result the therapeutic index may increase beyond the maximum dose tolerated, with a manifestation in the occurrence of drug resistance (566).

The assessment of *P. falciparum* susceptibility to antimalarial drugs is commonly assessed through the use of an *in vivo* therapeutic response test (465, 572), although *in vitro* assays measuring the intrinsic sensitivity of *P. falciparum* from the inhibition of growth or schizont maturation are also used (59, 547, 566). Whilst both testing methods have their place in clinical assessment of parasite resistance, it is often observed that results obtained from *in vitro* and *in vivo* testing methods are not always comparable (415). An advantage that an *in vivo* assessment method has over *in vitro* testing is that it takes into account host factors such as immunological regulation of infection. Furthermore, pharmacokinetic data may be warranted to differentiate between true resistance and failure to achieve adequate drug concentrations, an observation that is limited to *in vivo* models (551, 566).

1.2   MURINE MODELS FOR CHEMOTHERAPY

1.2.1 Murine malaria models

1.2.1.1 Isolation of murine parasites and their implementation into the research laboratory

A notable contribution to malaria chemotherapy research occurred in 1948 when the Belgian parasitologist Ignace Vincke reported the presence of a *Plasmodium* species in African thicket rats in the Belgian Congo (now Zaire) (241, 382, 527). The isolated parasite, later named *Plasmodium berghei*, was found to be infective not only in the natural host but also in laboratory rats, mice and hamsters (382) and as a result soon became an integral component of antimalarial drug discovery and drug development (33, 368, 382, 502).

Three other rodent specific species, *P. vinckei, P. yoelii* and *P. chabaudi*, along with their subspecies (Table 1.2), were subsequently identified (261, 267, 416), with the majority of these parasites now used for chemotherapy research (241, 382, 433). The rodent malaria parasites were found to be analogous to the malaria parasites infecting humans and primates in the most essential aspects of structure, physiology and life cycle and as a consequence have proved to be valuable in studies on the activity and stage specificity of drugs, and the molecular biology and genetics of drug-resistance (241, 243, 382).

Historically, the main contribution of the murine model has been in the screening of new antimalarial compounds, which can be clearly seen by comparing statistics of drug discovery both before and after the introduction of the murine malaria model (382). During World War II (1939–1945) the only antimalarial drug screening model was the avian model, which in itself was problematic due to the vast differences in parasite life cycle compared to human malarias (382). However, the *P. gallinaceium* (avian) model was soon superceeded by the introduction of the rodent model which proved to be a far superior drug screening model. This was demonstrated as between 1963 and 1990 over 300,000 compounds were screened in rodent models in comparison to only 4,000 compounds using avian models (372, 382).

Table 1.2   Murine *Plasmodium* species used for chemotherapy research

(adapted from (241, 382))

Species	Subspecies	Isolate strain	Place of origin	Extent of use
P. berghei (Vincke and Lips, 1948)		Keyberg 173	Zaire (Katanga)	+++++
		NH65	Zaire (Katanga)	++
		ANKA	Zaire (Katanga)	++
		RLL	Zaire (Katanga)	-
		SP11	Zaire (Katanga)	+
P. yoelii	*P.y.yoelii* (Landau and Killick-Kendrick, 1966)	17X	Central African Republic	++
			Brazzaville	
				-
	P.y. killicki (Landau et al. 1968)		S.W. Nigeria	
		N67		+++
	P.y. nigeriensis (Killick-Kendrick, 1973)		Zaire (Katanga)[*]	
				++++
	P. yoelii ssp. NS			
P. vinckei	*P.v. vinckei* (Rodhain, 1952)		Zaire (Katanga)[*]	+
	P.v. brucechwatti (Killick-Kendrick, 1975)		S.W. Nigeria	-
	P.v. lentum (Landau et al. 1970)		Brazzaville	-
	P.v. petteri (Carter and Walliker, 1977)		Central African Republic	+
			E. Camaroon	
	P. vinckei ssp.			-
P. chabaudi	*P.c. chabaudi* (Landau, 1965)	AS	Central African Republic	++
			Brazzaville	
	P.c. adami (Carter and Walliker, 1977)			-

1.2.1.2 Life cycle

The parsite life cycle of rodent and human malarias are remarkably similar, a feature not shared with all species of malaria (82). The main difference in life cycle between the rodent and human forms is the time taken to complete asexual schizogony which takes 18-24 hours in the rodent malarias and 48-72 hours in the human species (82, 241, 382). There are also slight differences in the timing of each stage of life cycle of the rodent malaria strains, with the main differences seen in the chronobiological characteristics of length of schizogonic cycle, rhythm and time of schizogony, degree of synchronicity, the age of erythrocyte invaded and the pathogenicity (268, 382). Table 1.3 provides a summary of the major features of the life cycle of each of the four rodent *Plasmodium* species compared to human malarias in general.

Table 1.3   Different characteristics of the four rodent malaria parasites compared to human parasites *(241, 382)*

	P. berghei	*P. yoelii*	*P. chabaudi*	*P. vinckei*	Human parasites
Merozoites per schizont	12–18	12–18	6–8	6–12	8–16
Reticulocyte preference	Yes	No	Yes	Yes	Yes/No
Synchronous infection	No	No	Yes	Yes	Yes/No
Optimum temperature range for sporogony (^{o}C)	19–21	23–26	24–26	24–26	>26
Sporozoite infection time (days)	13–14	9–11	11–13	10–13	Temperature dependant
Duration of pre-erythrocytic schizogony (h)	48–52	43–48	50–58	60–72	6–15 days
Duration of asexual blood stage (h)	22–24	18	24	24	48–72
Developmental time of gametocytes (h)	26–30	27	36	27	48 h–12 days

1.2.1.3 Scientific contribution of murine malaria models

Despite the advantages of studying human malaria parasites for determining the clinical applicability of new antimalarial treatment regimens or drug candidates, experimental models of murine plasmodia are increasingly employed in an attempt to understand the developmental biology of malaria parasites, parasite-host interactions and the pathology of human malarias (13, 269, 278). These models have also proved to be an important investigational tool in the discovery and development of vaccines, antimalarial compounds and their therapeutic regimens (81, 82, 222, 241, 268, 423). Furthermore, it has been suggested that the selection and use of a suitable animal model will generally reduce the time required for the development of the drug/vaccine prior to its evaluation in humans (401, 539).

Areas where the study of murine models has contributed to the overall understanding of *Plasmodium* parasites include;

- (Ultra-structural) morphology of the different life cycle stages (12, 294, 445)
- Genetics of malaria parasites (81, 530, 531)
- Function of malaria specific proteins, including candidate antigens for drug targets (315, 473, 523)
- Parasite-host interactions in the mosquito (141, 204, 340, 411)
- Immunity to malaria (269, 392, 466)
- Vaccine development (222, 353, 391)
- Drug development and resistance (371, 372, 423)

Although simian malaria is most comparable to human parasite strains, the study of murine malaria parasites has several advantages over the more complex simian models. Generally, murine models are inexpensive and easy to develop and maintain (268). Large-scale dissective and interventional studies may also be performed in murine models while such techniques are not ethical nor practical in humans or non-human primates (486). It is also well recognized that the use of simian models for the general screening of antimalarial therapies requires specialized skills and financial resources that are adequate to cover the high cost of

animals and drugs.   More importantly, the ethical considerations in such cases demand that the use of high order experimental animals should be restricted (382).

When using murine models, selection of rodent malaria species and mouse strain is an important variable to be considered during experimental design and interpretation (162). The individual species and strains of plasmodia have been well characterised and all differ significantly in their degree of infection, lethality and synchronicity, which can affect experimental results. For example, *P. berghei* and *P. vinckei* produce lethal infections whilst *P. yoelii, P. chabaudi adami, P. chabaudi chabaudi* and *P. vinckei petteri* all produce self-resolving infections (162, 487). Furthermore, the course of infection may also vary depending on the selected mouse strain (162).

1.2.2 *Plasmodium berghei*

Experimental murine malaria models exploit all four rodent parasites, although *P. berghei* is the most comprehensively studied and manipulated parasite (162, 268, 487, 539).   As a laboratory model, 37 animal species have been found to be susceptible to *P. berghei* after artificial inoculation with infected blood (590). The majority of these species belong to the rodent family, however, African fruit bats and new-born rabbits have also been found to be susceptible (502, 590). In 1965, Meir Yoeli presented a paper at the Royal Society of Tropical Medicine and Hygiene meeting (590) describing the segregation of each animal species into a number of 'groups' based on the course and outcome of the *P. berghei* infection in the inoculated host. These are described below as presented in the original paper.

a)   A fulminating and fatal course terminating in death within 7 to 14 days, accompanied by high and rising parasitaemia. The white mouse and the baby albino rat fall into this category.

b)   A more prolonged clinical course, terminating in the death of the host within 2-3 weeks and accompanied by a rising parasitaemia. The golden hamster (*Mesocricetus auratus*) belongs to this category.

c) Chronic and latent course with spontaneous recovery. Rarely, death from the infection and fluctuation in parasitaemia. The adult albino rat is typical of this group.

d) Mild and transient *P. berghei* infection with low parasitaemia and complete recovery.

e) Short-lived survival of inoculated parasites in an alien host, without power of multiplication. Detection of 'static' infection by blood sub-inoculation into susceptible hosts.

Within the laboratory, *P. berghei* is used to infect hamsters, rats and mice (502). When involvement of sporogonic and/or the exo-erythrocytic stages are sought, the susceptible mosquito vector *Anopheles stephensi*, is widely employed (400, 526). As a laboratory model, *P. berghei* is largely selected for studies relating to developmental biology of parasites, molecular genetics studies and most importantly for investigation into new and innovative drug therapies (241, 268, 502). Specific characteristics that differentiates *P. berghei* from the other rodent malarias include:

- Preferentially invades reticulocytes (119, 502, 539).
- Largely asynchronous in laboratory rodents (241).
- Results in a lethal infection within 1 to 3 weeks in most laboratory models; although a large number of environmental factors influence the virulence and pathology of infection (384, 406, 430, 448, 502).
- Cerebral effects may be observed in selected rodent species (216, 486).

The morphology of asexual *P. berghei* parasites have been described as very similar in characteristics to those of the human malaria *P. vivax* (501) (Fig. 1.4). The ring stage is usually uni-nucleate and contains a large food vacuole (241, 501, 502). As the parasite matures to the trophozoite stage the food vacuole is seen to significantly reduce in size resulting in a parasite that is non-vacuolate, slightly amoeboid and containing fine granules of black pigment (241, 501, 502, 527). Further maturation sees the trophozoite develop a number of merozoites (6 to 20).

In heavy infections, the presence of multiple parasitised cells is not uncommon (241, 502).

hpi	characteristics	morphology
0-4	invasion (merozoites)	
4-8	intracellular growth	
8-12	intracellular growth	
12-18	intracellular growth	
18-20	schizogony sexuality manifest	
20-22	sexual dimorphism not visible	
22-23	schizogony completed	
23-26	sexual dimorphism visible	
26-27	gametocyto-genesis completed	
30-48	stable gametocytemia	
48-57	degeneration of gametocytes	

Figure 1.4   **Morphology of erythrocytic stages of *P. berghei* (241, 322)**

1.2.2.1 In vivo models

For the majority of murine malaria research albino laboratory mouse strains have generally been used (382). Although some areas of murine research, including those studies observing immunological responses of murine malaria, use specific inbred strains of mouse such as BALB/c or C56BL/6 (17, 99), random outbred Swiss

mice are suggested to be acceptable for the majority of murine malaria research providing the mice are appropriately housed, nourished and kept free of concomitant pathogens (372, 382). The gender and age of laboratory mice is also thought to be an important consideration for chemotherapy studies (192, 372, 596). It is suggested that male mice weighing between 18 and 20 g are ideal for routine studies as female mice may demonstrate variable responses to chemotherapy depending on their time of oestrus cycle (372). Mice also tend to become less susceptible to *P. berghei* infections with advancing age whilst young mice that weigh under 18 g are hard to handle compared to slightly older and heavier animals (192, 372, 596).

Within the laboratory, mice can be infected with rodent parasites through a number of techniques, as infection via mosquito transmission and artificial inoculation with infected blood are both successful inoculation methods (502). When infection via mosquito transmission is desired, the laboratory mice are placed within a restricted area with mosquitoes burdened with infective sporozoites (have fed on infectious blood at least 14 days prior). The mosquito bite is expected to transfer 20 to 50 sporozoites into the host (large variability between parasite species and strain) resulting in a model including both liver and blood stage infection as well as maintaining gametocyte production (241, 382, 502, 526).

In comparison, mice can be inoculated by direct blood passage from infected mice with high parasitaemia either through intravenous (i.v.) or intraperitoneal (i.p.) injection, with an inoculum of blood containing all asexual blood stages (schizonts, trophozoites and ring-forms) (502). Early inoculation studies comparing these two routes of infection suggested that i.p. injection was as suitable as i.v. injections as an inoculation technique (498, 502), with the majority of parasites injected penetrating the peritoneal wall and entering the blood stream within one minute of i.p. injection (58). Whilst more recent studies have suggested that i.v. injection may be a more accurate route of inoculation (as when using i.p procedures it is estimated that only 10% of injected parasites will survive and enter the bloodstream)(241), other inoculum factors must be considered with inoculum size

being one of the most important (207). In order to prevent fluid overload in the mouse, which may lead to severe illness or death of the animal, injection of fluid volumes must be carefully considered. Although a more direct method of fluid delivery, i.v. inoculation is hindered as no more than 0.2 mL should be injected into the adult mouse (> 12 weeks of age) (207). As the majority of mice used for experimental research are usually between the age of 5–9 weeks, the maximum injectable fluid volume would be smaller. Therefore, i.p. injections are commonly used in mice as fluid volumes up to 2 mL can be injected into the abdominal cavity, with a low risk of detrimental effects to the animal (207). Therefore, if using artificial inoculation methods the route of infection must be carefully considered, depending on inoculum size and dilution volume (502).

1.2.2.1.1 Chemotherapy testing procedures

Since the introduction of *P. berghei* parasites into laboratory animals, several methods have been used for testing the response of erythrocytic infections of *P. berghei* to antimalarial drugs (372). In 1950, Thurston (498) established the first of the standard methods for testing antimalarial activity against blood stage *P. berghei*. This initial method was then further modified by a number of researchers resulting in the development of five additional drug screening methods, of which the "Rane test" and "Peters 4-day test" are the most commonly used (49, 86, 89, 132, 176, 198, 360, 378, 381, 387) .

1.2.2.1.1.1 Thurston test for activity against blood forms

The Thurston test is based on the studies by Curd et al (120) and Tonkin and Hawking (505) for antimalarial activity in the avian *P. gallinaceum* malaria model. Albino mice, weighing between 15 and 20 g, were inoculated, by i.p. injection, with *P. berghei* infected blood containing between 5-15 million parasites on Day 0. All mice received the same parasite inoculum. Test drugs, of which doses were weight adjusted, were given orally once daily for four days, commencing three to four hours after parasite inoculation. Parasitaemia was monitored on the fifth and seventh day by Giemsa stained thin blood films. Parasitaemia was determined by the percentage of parasitised erythrocytes and the geometric mean was taken for

each group of mice. The minimum effective dose was defined as the smallest dose that gave a mean infection level of <1% on the fifth day (120, 498, 505).

In 1953, Thurston published a modification of her initial method (499) in which the mice were given a smaller initial parasite inoculum of approximately 1 million parasitised erythrocytes, resulting in a parasitaemia that ranged between 1-5% on the fifth day. The minimum effective drug dose was defined as the smallest dose that reduced the parasitaemia to 2% of that of the control mice (372, 499).

Although the Thurston test provided a good model for determining drug activity against *P. berghei* (498-501), certain limitations were identified. The first of these was the measurement of drug activity which, when described by Thurston was the minimum effective dose that gave <1% infection on the fifth day and later the smallest dose that reduced parasitaemia to 2% of that of the control mice. This form of measurement was deemed to be ineffective for comparison of the activity of different drugs hence the development and adaptation of the ED_{50} (Effective Dose 50%: 50% suppression of parasitaemia when compared to untreated controls) by Rollo in 1952 (418).   The use of an ED_{50} and later ED_{90} (373) as a measurement and determinant of drug efficacy remain in use for drug activity studies (162). A second limitation identified by Peters (373) in 1965 was that the Thurston test monitored parasite suppression and was not a true therapeutic test. Therefore, further modifications took place (498) so that drug therapy was not initiated until parasitaemia reached 10% (373). However, perhaps the main disadvantage of the modified Thurston method was the increased time required for the operator to count blood films as inoculation methods often resulted in a chronically low infection for prolonged periods of time (499, 501).

1.2.2.1.1.2 Warhurst bio-assay techniques

In an attempt to overcome the interpretation problems caused by host immunity associated with the Thurston test, Warhurst (536) and Warhurst and Folwell (537) described a bio-assay technique which overcame previously noted problems of host immunity and copious numbers of thin blood films. In this test, a group of 10 mice

were infected with a standard 10^7 parasitised erythrocyte suspension (erythrocytes suspended in ice-cold 50% calf serum and Ringer solution) and then divided into two groups of five after infection. One to 3 h after infection one group of mice receive a single dose of drug whilst a placebo injection was given to the other group of 5 mice. A second group of 10 animals were treated identically except that at the time of infection, the mice received an inoculum that was one-tenth that received by the first 10 animals. The mean period of time required to reach a 2% parasitaemia for each group of mice was plotted on a logarithmic scale and the mean growth rate was determined through comparison of the two inoculum sizes (537).

1.2.2.1.1.3 Thompson test for suppressive curative activity

In the early 1960s, Schneider became interested in developing a compound that would give a human long lasting protection against malaria, after oral administration (372). In an attempt to test the suppressive activity of the new compounds, a 21-day parasite free test was designed (372). The purpose of this testing procedure was to treat all experimental animals on Day 0 with the test compound, and then infect groups of animals at Day 7, 14 and 28. Daily blood films were prepared and mice were monitored for a period of 21 days after parasite inoculation. At Days 7 and 21 blood was drawn from the donor mouse and injected into 2 naive mice to test for parasite presence and/or viability (372). This testing procedure was reported to be extremely sensitive and enabled the differentiation between a true schizogonic drug and one that simply inhibited parasitaemia (372).

A modified version of Schneider's 21-day parasite free test was used with success in the development of cycloguanil pamoate by Thompson et al. (495, 496) which was moved to human volunteer studies and demonstrated antimalarial protection for more than one year with sensitive *P. vivax* and *P. falciparum* strains (106, 107).

1.2.2.1.1.4 Rane in vivo screening system

The Rane *in vivo* screening system was developed by Leo Rane at the Walter Reed Army Institute of Research (WRAIR) as a tool to assess the efficacy of antimalarial compounds that entered their research and development program (372, 382).

This testing system used albino mice that were infected with a standard 10^6 inoculum of *P. berghei* parasites by i.p. injection. Three days after infection, mice were treated with a single dose of drug and animals observed daily for survival or death (372, 382). Untreated control mice usually died 6 to 7 days after inoculation. The drug was considered to be active, and underwent further investigation, if the treated animals survived for at least twice as long as the untreated control mice. Any mice that survived longer than 60 days without exhibiting symptoms were said to be cured, whilst animals that died between day 2-5 of drug administration were said to have died from drug toxicity (372). The minimum effective dose was compared to the maximum tolerated dose (i.e. the dose that produced no more than 1 in 5 toxic deaths) with dose levels titrated downwards to give an indication of the therapeutic index of the drug (382). This simple test system allowed over 25,000 compounds to be examined per year with all promising drugs further investigated using methods such as the '4-day test' to obtain more accurate information on their activity (365, 382, 503).

An advantage of the Rane method is that as it monitors animal survival and death as an indicator of drug activity, an increased number of compounds may be studied in a short period of time (382). However, a general limitation of this method is that the drug efficacy of certain antimalarial compounds, such as proguanil, may have an immediate effect on parasitaemia but does not affect animal survival (372). Therefore, if drug success is dependent on animal survival time, some active antimalarial compounds may be overlooked.

1.2.2.1.1.1 Peters "4-day test"

The '4-day test' , as described by Peters (379), comprises two distinct parts: (a) a preliminary '4-day test' for the detection of suppressive activity and (b) the '4-day test' for quantitative assessment of blood schizontocidal activity (382).

a) Preliminary '4-day test' for detection of suppressive activity: Groups of male albino mice are inoculated with either 2 x 10^6 *P. berghei* N parasites or a similar inoculum of *P. chabaudi* AS. All mice are then given a fixed dose of 30 mg/kg [once daily for 4 consecutive days beginning on the day of infection]. For each parasite species one group of mice receive drug administration via subcutaneous (s.c.) injection whilst the other group receives drug through oral administration. Parasitaemia is determined through preparation of peripheral blood film on the fifth day (first day after conclusion of treatment), to determine qualitatively the presence and degree of activity of the screening dose (379).

b) '4-day test' for quantitative assessment of blood schizontocidal activity: Male albino mice are inoculated with 10^6 parasitised erythrocytes (of either *P. berghei* or *P. chabaudi* strain) by i.v. administration. Following inoculation, all mice are dosed once daily for 4 consecutive days using the dosing range determined in the preliminary test (as described above). Each tested compound may be administered s.c., i.p. or by any other desired route. The parasitaemia is determined on the fifth day after inoculation and the ED_{50} and ED_{90} values are determined from a semi-logarithmic time-density plot. The degree of cross-resistance (I_{50} or I_{90}) is determined by comparing the drug activity in sensitive and resistant parasite strains using the following formula:

$$\text{Index of cross resistance} = \frac{ED_{50}/ED_{90} \text{ of resistant line}}{ED_{50}/ED_{90} \text{ of sensitive parent strain}}$$

Compounds shown to possess suppressive activity can be further tested at the dose ranges determined in the preliminary '4-day test' for detection of suppressive activity and including one dose step further than the minimum fully suppressive dose, for suppressive curative activity and influence on survival times as outlined in the Thompson test (1.2.2.1.1.3) (378-380, 382, 384).

1.2.2.2 In vitro studies

In vitro interactions between antiplasmodials, as represented by isobolograms, provide useful activity data for clinical studies. However, they do not necessarily determine the efficacy of a combination in the host, since this also depends on pharmacokinetic characteristics. Synergy, indifference (addition), and antagonism are the expected outcomes of drug-drug interactions (164).

In 1976, Trager and Jensen (508) described an *in vitro* culture method in which human malaria parasites could be maintained in continuous culture, whilst still remaining infective to Aotus monkeys. Today, parasites from both human malaria and experimental animals are used successfully in continuous culture for a wide variety of malaria research facets, in particular drug development, assessment of drug combinations and parasite sensitivity studies (46, 54, 63, 79, 88, 126, 224, 242, 243, 288, 349, 405, 417, 459, 516, 568).

1.2.2.2.1 Methods for evaluation of drug combinations

Among the techniques employed in the evaluation of the combination of two antimicrobials potentially exhibiting synergy, are the checkerboard technique and the time-killing curve method (235, 302). The checkerboard or fractional inhibitory concentration (FIC) technique employed a methodology similar to that utilized for the determination of the minimum inhibitory concentration (MIC), the lowest concentration of drug inhibiting growth after 24 h of incubation. The combination is said to have a synergistic effect if there is a 4 fold reduction in the MIC of each of the agents tested alone (358). In the time-killing curve method, the reduction of a fixed inoculum over 24 h exposure of combination antimicrobials is compared with the effect of each agent used alone (235). In this method the measure of success is

determined by calculating the IC_{50}, which is the concentration of drug required to inhibit growth of the parasite by 50% compared to untreated controls (349, 488).

1.2.2.2.2 Isobolograms

The study of the presence, type, and degree of interaction between biologically active agents is highly relevant to many research areas including pharmacology, immunology and toxicology (302). The construction of isoboles (Fig. 1.5) is a classic procedure of the analysis of interactions between agents (164, 302). The isobole method has been used for the evaluation of synergy and antagonism in many fields and requires experimental data for agents used alone and in different dose combinations at equi-effective levels (126, 164, 302, 475). The construction of isobolograms has several disadvantages. Finding a combination of drugs that would produce a given effect is a time-consuming task. In addition, isobolograms can only be used to evaluate interactions at this effect level (302).

Figure 1.5. Isobolograms demonstrating antagonistic (a) and synergistic (b) drug combinations in the rodent *P. berghei* model. *(Taken from (459))*
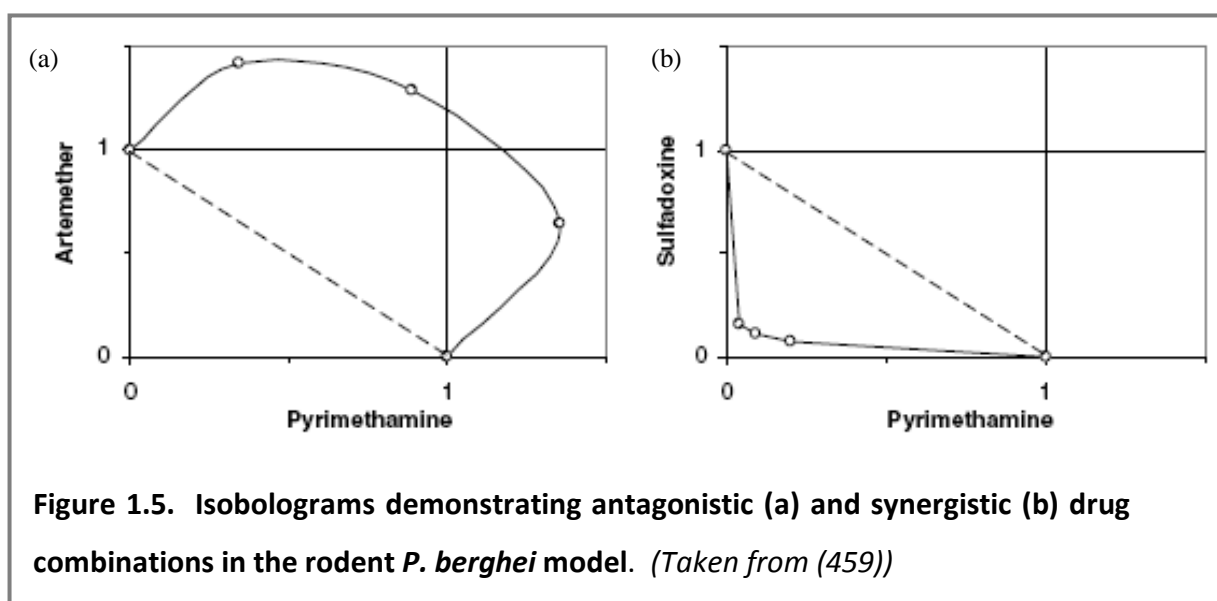
However, isobolograms have also proven to be a useful method of evaluating antimalarial drug combinations in *in vivo* models such as the *P. berghei* murine malaria model. Whilst discrepancies in isoboles prepared from *in vitro* and *in vivo* data may occur, this is not of major concern as *in vivo* parasites are exposed to variable levels of drugs and metabolites, compared to the direct antiparasitic activity observed with *in vitro* parasite cultures (459). This method of antimalarial

compound assessment has been successful in several *P. berghei* murine models (89, 459).

1.2.2.3 In vivo versus in vitro studies

The majority of primary drug screening *in vitro* methods, as well as investigation into their modes of action, are performed using *P. falciparum* parasites in culture. Nevertheless, the study of the action of drugs in rodent *Plasmodium* models remains an important step for *in vivo* studies of the activity of new molecules, drug associations and the setting up of new therapeutic strategies (268).

Rodent malaria models are widely used for screening new drugs for their antimalarial activity *in vivo* (243, 378). The study of drugs in rodent-malaria models can provide valuable information about stage specificity of the drugs, routes of administration and development of resistance (243). The *P. berghei* rodent model has been developed to allow the determination of the effect of drugs on the erythrocytic schizogony both *in vivo* and *in vitro* cultures (242, 323, 379, 384, 417, 430, 500-502, 525). Synchronized short-term cultures of blood stages are easily established (242) in which antimalarial efficacy and stage specific activity of drugs can be accurately assessed and compared with the *in vivo* antimalarial activity (243).

Comparisons of the antimalarial activities from different studies are hampered when different *Plasmodium* species and different methods are used. *In vitro* and *in vivo* studies using the same species are therefore valuable in determination and comparison of the antimalarial activity of drugs (243, 324). For example, studies with a number of dihydroartemisinin (DHA) derivatives showed good activity against *P. falciparum* in cultures, but were ineffective against *P. berghei* in rodents (281). It is unknown whether variation in susceptibility of various parasite species, differences in stability of drugs between culture and host or other factors in the host are responsible for the observed difference in antimalarial activity (281). Variation in the antimalarial activity of a drug between studies can also be influenced by differences in culture methods, routes of administration and solvents,

which hamper comparison of results from laboratories using different techniques (243). Standardized experiments and combination *in vitro* and *in vivo* studies with the same species are therefore essential for the accurate comparison of the antimalarial efficacies of different drugs (243). This can be observed in a series of experiments conducted by Janse et al. (243), who demonstrated a good comparison of the in vitro and in vivo antimalarial activity of artemisinin in *a P. berghei* model.

Traditional *in vitro* measurements such as the MIC are used to predict outcome of antimicrobial therapy. While these measurements are a good indication of the potency of an antimicrobial, they do not provide the type of information necessary to determine the optimal drug dose or dosing interval (18). The MIC test provides information about a drug concentration at a single time point. However, the MIC does not elucidate the effect of varying drug concentrations over time or whether there may be effects that persist after drug exposure. Both *in vitro* and animals studies have been used to determine the impact of drug concentration on the rate and extent of antimicrobial killing (18). Both *in vitro* and animal studies can also determine the impact of drug exposure on organism growth after drug exposure. However, only *in vivo* animal models are able to determine the time course of activity at the site of infection and the potential impact of host immune factors on drug activity (18, 278).

Animal model studies have a distinct advantage over both *in vitro* models and clinical trials in the ability to discern which pharmacokinetic/pharmacodynamic dosing index is most closely associated with efficacy (18). Furthermore, whilst studies with bacteria, tissue culture, and computer simulation have been found to provide useful information for therapeutic studies, testing on animals is required in order to attain results influenced by the complexities of a living organism (407).

1.2.3   Murine malaria as a model for human infections

There are a large number of animal models that provide investigators with the ability to characterise the pharmacokinetics and pharmacodynamics of antimalarial drugs (268, 382, 407). However, murine models are the most versatile of the

animal models in which to study the interactions between host and the erythrocyte stage of parasite, the analysis of molecular interactions and precise pathological changes (134, 278, 433). Attractive characteristics of the murine model include the fact that it is (i) inexpensive and easy to maintain, (ii) the mouse immune system is well characterised and (iii) large scale intervention studies of a nature not permissible in humans, may be performed (487). While there may be pharmacokinetic and immune state differences between different animal models, appropriate pharmacodynamic analyses most often account for these differences and allow comparison of data among studies possible (18).

Although mouse models may contribute to the understanding of the pathogenesis of complications of malaria, the human disease is not replicated in full (269). This may be seen in particular in the study of cerebral malaria, in which several murine models have been developed (83, 121, 134, 486). Whilst many similarities between human and murine cerebral malaria can be identified, particularly in regard to pathological lesions (such as sequestration, cerebral hemorrhage or infarction) and clinical symptoms (such as ataxia, fitting and coma) (134, 293), there are also distinct differences in the disease progression. A criticism of several of the current cerebral models (such as *P. berghei* ANKA in C57BL/6 mice) is that these murine models are characterised by monocyte adherence to blood vessels (23, 486) although more recent models have found the presence of parasite sequestration in the brain which more closely mimics *P. falciparum* infection (23, 214). However, recent studies in African children have also identified that those who have died from cerebral malaria also show the adherence of mononuclear cells in the brain (278, 394). Therefore, for future studies the use of *P. berghei* ANKA in BALB/c mice may be a more appropriate clinical model given that both infected erythrocytes and mononuclear cells were found to sequester in the brain, thus more closely resembling the human disease (214, 278). Furthermore, one of the major differences in the pharmacokinetics between animals and humans is that the rate of drug elimination is faster in animals. This is especially true in small rodents, which are commonly used in animal infection models. In general, the half-life of antimicrobial drugs is 6 to 9 fold longer in humans than in mice (18).

Whilst mouse models are not exact replicas of the human infection and disease, they do provide a useful insight into disease pathogenesis, complications of infection, interaction of the host immune system and treatment therapies (269, 278).   Whilst direct extrapolation from rodent biology to human malarias should be done with caution, and might not be applicable in all situations, each of the four rodent malaria species has similar characteristics to the human malarias and which makes them suitable for parallel study (75, 80, 269).   Therefore, a suitable murine model may contribute valuable data, and a greater understanding, of specific disease processes, therapeutic responses and the identification of potential targets for therapeutic intervention, all important characteristics for drug development and drug efficacy studies (1, 269, 278, 433).

1.3 ANTIMALARIAL DRUGS

1.3.1 Introduction

In the current clinical setting there are at least 10 defined classes of antimalarial drugs which are used for the prophylaxis and chemotherapy of malaria including arylaminoalcohols, 4-aminoquinolines, dihydrofolate reductase inhibitors, 8-aminoquinolines, antibiotics, sesquiterpene lactones and naphthoquinones (42, 250, 300, 423, 535, 540, 555). Prophylactic drugs are used to prevent the establishment of the *Plasmodium* parasite either in the liver (primaquine) or blood (mefloquine), whilst malaria chemotherapies are used to suppress and clear parasitaemia and achieve clinical cure for current infections (540).

1.3.1.1 Classification of antimalarial drugs

Conventional antimalarial drugs are generally classified based on either the stage of parasite that they target or on chemotherapeutic actions (236). In consideration of these factors, antimalarials are classified as blood schizontocides, tissue schizontocides, gametocytocides or sporontocides.

1. *Blood schizontocides* – this class of drug act on the asexual erythrocytic forms of all *Plasmodium* species are used to achieve clinical or suppressive cure. The majority of all commonly used antimalarials fall into this category eg. chloroquine, quinine, mefloquine, pyrimethamine, amodiaquine, proguanil, sulfadoxine, tetracyclines, artemisinin derivatives (39, 236, 266, 539) (Fig. 1.6).

2. *Tissue schizontocides* – this class of antimalarials is used either for prophylaxis or for the prevention of relapse of *P. vivax* or *P. ovalae* by eliminating developing tissue schizonts (primary tissue forms) or latent hypnozoites (secondary exoerythrocyic forms). Examples of tissue schizontocides include primaquine, pyrimethamine and proguanil (39, 236, 266, 539) (Fig. 1.6).

3. *Gametocytocides* – These antimalarials act by destroying the sexual forms of the parasite in the blood thereby preventing transmission of infection to the mosquito. Chloroquine and quinine have gametocytocidal activity against *P. vivax* and *P. malariae*, but not against *P. falciparum*. Primaquine is a commonly used gametocyctocide for *P. falciparum* infections (39, 236, 266, 539). Recent investigations have also provided evidence that the artemisinin drugs also demonstrate gametocytocidal activity against *P. falciparum* (213, 398, 574) (Fig. 1.6).

4. *Sporontocides* – This class of antimalarials acts by preventing development of oocysts and sporozoites in the infected mosquito, thus preventing further transmission of infection. Primaquine, pyrimethamine and proguanil are all classed as sporonticides (236, 266, 539) (Fig. 1.6).
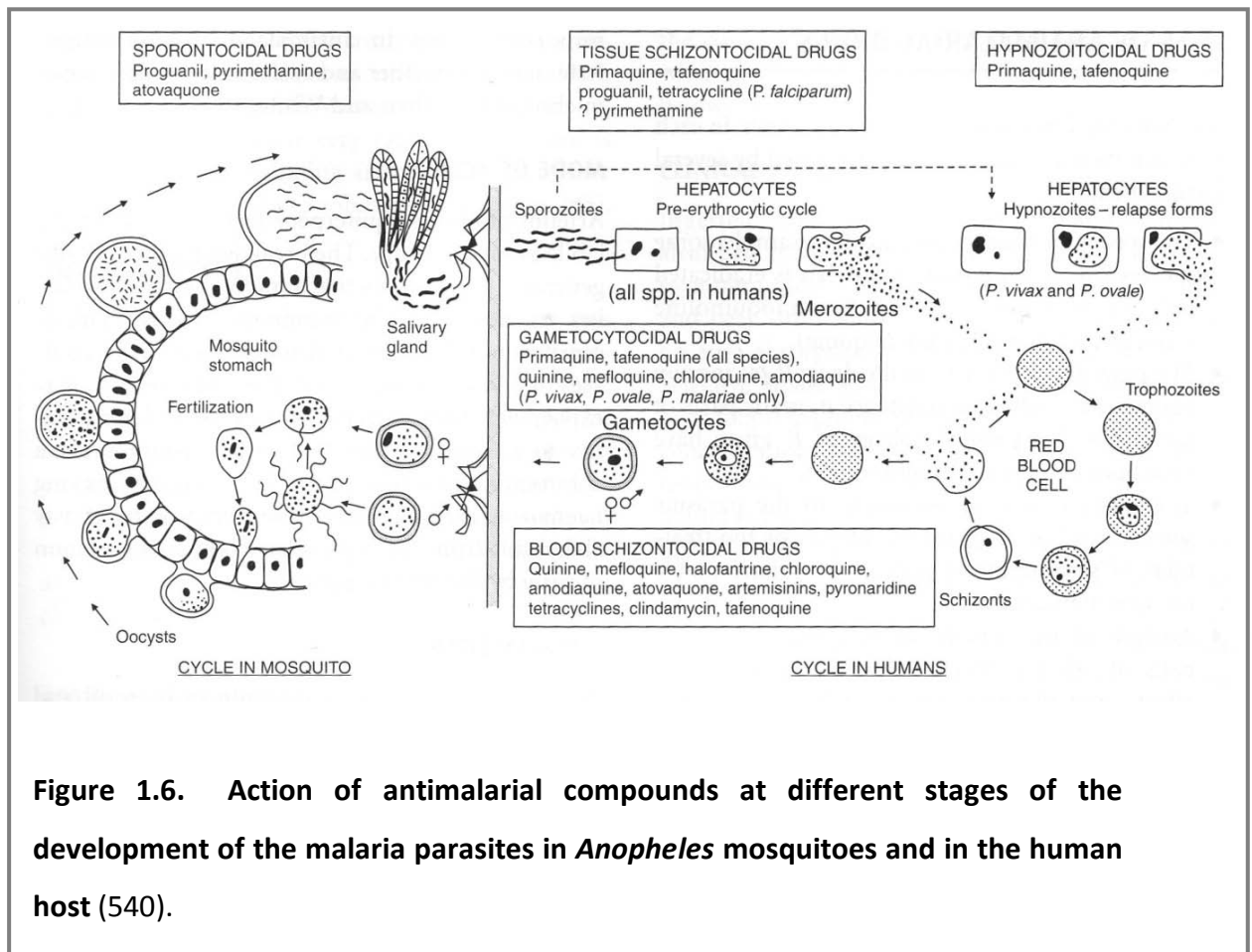
Figure 1.6. Action of antimalarial compounds at different stages of the development of the malaria parasites in *Anopheles* mosquitoes and in the human host (540).

1.3.1.2 Determinants of drug effectiveness

When selecting an antimalarial drug for clinical therapy, a number of factors much be considered. These include assessment of the patient's age, the degree of immunity (if any), the likely pattern of susceptibility to antimalarial drugs, drug pharmacokinetics, parasite burden, and the cost and availability of those drugs (39, 555). All of these factors prove to be important determinants in the selection of a suitable treatment option. The main issues related to drug selection are predominantly related to drug availability, adherence to therapy and drug resistance patterns (39, 44, 186, 555).

1.3.2 Artemisinins

Artemisinin, more traditionally known as qinghaosu, is isolated from the leaves of the Chinese herb sweet wormwood *Artemisia annua* L and is the only natural antimalarial agent since the discovery of quinine (272). Historical accounts report the antipyretic properties of artemisinin from Chinese scripture dating back over 2000 years with the first accounts of the specific effect of malarial fever documented in the 16th century (128, 228, 238, 570, 574). In 1972, Chinese scientists characterised the structure of artemisinin as a sesquiterpene lactone with an internal peroxide linkage and within 10 years the drug was introduced into clinical practice (128, 238, 520). The semi-synthetic artemisinin derivatives dihydroartemisinin (DHA), artesunate, artemether and arteether (Fig. 1.7) were subsequently developed and introduced into clinical practice where they have become renowned as the most potent and rapidly acting of the antimalarial drugs available on the market today, with clearance of parasites noted within 48 h in most cases (212, 238, 521).

Although artemisinins are an exciting class of antimalarial drugs with their short elimination half-life, rapid onset of action and no major side-effects, a major drawback of these drugs is the high parasite recrudescence rates within 2 to 3 weeks of monotherapy (187). In view of this high recrudescence rate, it has been concluded that to prevent the formation of artemisinin resistant parasites,

artemisinins should only be used in combination therapies rather than in monotherapy (77, 521, 552, 570).

Figure 1.7. Chemical structure of 1. Artemisinin; and derivatives 2. Dihydroartemisinin (DHA); 3. Artemeter; 4. Arteether; 5. Artesunic acid (artesunate) and 6. Artelinate (570).
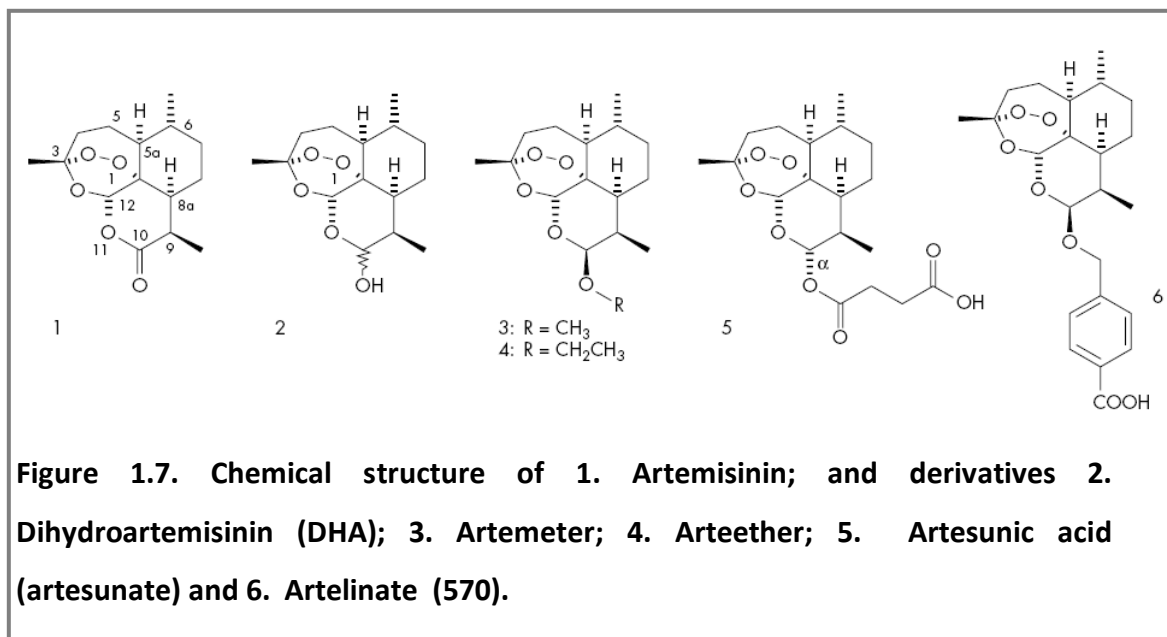
1.3.2.1 Mode of action

For decades after their discovery, the antimalarial action of artemisinin and its derivatives was based on their unusual chemical structure incorporating a peroxide bridge and was attributed to their chemical capability to generate free radicals (213, 570). It was thought that the endoperoxide bridge underwent ring-opening via protonation or formation of a complex with metal iron causing destructive, free-radical generation with the parasite altering the function of key parasite proteins, including membrane transporters (128, 152, 363). Early studies indicated that the modulation of oxidative stress may be important for antimalarial activity as those artemisinin compounds lacking the peroxide oxygen atom, were without activity (213, 363).

More recently an alternative mechanism of action for artemisinins was proposed which is based on the inhibition of the malaria parasite's calcium ATPase 6, sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) (152, 213, 570). SERCAs act to reduce cytosolic free calcium concentration by actively concentrating Ca^{2+} into membrane-bound stores with this activity is critical to cellular survival (213). Eckstein-Ledwig et al. (152) demonstrated that artemisinins inhibited the

SERCA ortholog of *P. falciparum* (PFATP6) with marked specificity, ultimately resulting in the death of the parasite (152, 263, 515). However, SERCA inhibition as a mechanism of action for artemisinins remains contentious (355, 569). Whilst recent publications have offered further possible mechanisms for artemisinin's antimalarial action (136, 532), the drugs' biological targets still remain ambiguous (355).

1.3.2.2 Antiparasitic activity

Artemisinin derivatives are active against all human malaria parasites, including multidrug-resistant *P. falciparum* and have a broad activity against the intraerythrocytic stages of the parasite, ranging from young ring-forms to schizonts (128, 212, 213, 238, 574). In falciparum malaria, it has been shown that artemisinin also kills gametocytes, including stage 4 gametocytes which are otherwise only sensitive to primaquine (213, 574). This gametocytocidal activity of artemisinin derivatives was also shown in clinical studies in Thailand which showed that artemisinin derivatives (artesunate and artemether) reduced the transmission potential of falciparum malaria (398).

1.3.2.3 Pharmacokinetics

Pharmacokinetic studies of artemisinins have been limited by difficulties of assays with several different techniques employed by various groups giving ranging accuracies (344, 570). Whilst high performance liquid chromatography (HPLC) with electrochemical detection was originally deemed the method of choice for measurement of artemisinin and its derivatives, with a sensitivity of 1–5 ng/mL (520, 521), more recently LC-MS methods have shown to have equal sensitivity with the additional quality of measurement of drug concentrations from small sample volumes (480, 524, 533). Although the pharmacokinetic parameters of artemisinin and its derivatives have been determined, unfortunately very few pharmacokinetic studies have focused on the variation of artemisinin profiles in different populations of patients, particularly children and pregnant women (570).

1.3.2.3.1   Artemisinin

After oral or rectal administration of artemisinin the peak plasma concentrations occur 3 h and 11 h later, respectively (344, 574). Artemisinin is a potent inducer of its own metabolism with an estimated elimination half life of 1-3 h (32, 128, 238).

1.3.2.3.2   Dihydroartemisinin (DHA)

DHA is the main active metabolite of the artemisinin derivatives, but can also be administered orally and rectally as a drug in its own right. DHA is rapidly absorbed following oral administration reaching peak plasma concentrations approximately 2.5 h after dosing. After rectal dosing, peak plasma concentrations occur approximately 4 h later. The elimination half-life of DHA is estimated to be 40-60 min and it is metabolised to an inactive glucuronide (128, 238, 347, 574).

1.3.2.3.3   Artesunate

Artesunate is rapidly absorbed after dosing and is almost entirely converted to DHA, the active metabolite through which the antimalarial activity of artesunate is determined (344). Artesunate peak plasma concentrations occur 0.2–1.6 h (53, 337, 345, 347), 1.4–3.1 h (255, 343) and 0.2–0.6 h (237) after oral, rectal and i.m. administration, respectively (344, 574). The elimination half-life of artesunate is estimated to be between 2-5 min before conversion to DHA (elimination half-life 40-60 minutes) (128, 238, 344).

1.3.2.3.4   Artemether

Artemether is the methyl ester of DHA and is metabolized to DHA. Artemether can be administered orally or intramuscularly with peak plasma concentrations occurring 2-3 h after oral dosing or approximately 6 hours after i.m. injection. However, following i.m. injection absorption is variable and slow and although the mean absorption time is approximately 6 h, peak plasma concentration can occur as long as 18 h after dosing (574). The elimination half-life of artemether ranges between 2-11 h (128, 238).

1.3.2.3.5   Artemotil (previously arteether)

There are limited data on the pharmacokinetics of artemotil, however, absorption is said to be slower and more erratic than the other artemisinin derivatives, with some patients having peak plasma concentrations more than 245 h after administration (574). A limited study in healthy volunteers suggested that the absorption half-life of artemotil varied from 0.6 to 1.7 h with a long elimination half-life of 25-72 h (249).

1.3.2.4 Clinical applications

Artemisinin derivatives can be used for the treatment of both uncomplicated and severe malaria in both adults and children (570). Since their introduction into the clinical setting as a chemotherapeutic agent artemisinins have been used extensively, predominantly in combination therapies, with impressive results (27-29, 31, 165, 180, 209, 217, 227, 230, 244, 256, 310, 521, 522, 571). The most rapidly acting of all antimalarials, artemisinins are effective against all *Plasmodium* species and are active by parenteral, oral or rectal administration (77, 552, 570). To date, there has been no significant toxicity reported in humans (218, 318, 552, 570).

1.3.2.5 Adverse Effects

In the clinical setting artemisinin and its derivatives have been found to be safe and remarkably well tolerated. However, occasional reports of mild gastrointestinal disturbances, dizziness, tinnitus, reticulocytopenia, neutropenia, elevated liver enzymes and electrocardiographic abnormalities including bradycardia and prolongation of the QT interval have been reported (238, 520, 574) .

Animal toxicology studies have reported neurotoxicity, particularly when using very high doses of i.m. artemotil and artemether, however, these results have not been substantiated in humans (218, 574). In the animal studies, administration of high doses of artemether and artemotil resulted in selective damage to the brain stem centres involved predominantly in auditory processing and vestibular reflexes (68, 350, 436, 506). However, although neurotoxic and cardiotoxic effects have been observed in experimental animals, these all occurred after high dosing regimens

and therefore the probability of adverse effects in humans using the clinical dosing regimens is minimal (42).

Animal studies have also provided evidence of morphological abnormalities and embryo death after artemisinin derivatives were administered during early pregnancy (582). A comprehensive review by Clark (104), on the embryotoxic effects of artemisinin derivatives in animal models, suggested that malformations of the cardiovascular and skeletal systems occurred when embryolethal doses were administered in the early gestational period, but not in late gestation. Furthermore, such adverse effects were reported in the absence of maternal toxicity (104). The effect of artemisinin and its derivatives have not been thoroughly evaluated in the first trimester of pregnancy in Humans (534). Therefore, the WHO recommends that use of artemisinin derivatives during the first trimester of pregnancy should be avoided until further investigation has taken place (582).

1.3.2.6 Resistance

It was generally regarded that through the tight control of artemisinin therapies, and having their use limited to combination therapies, resistance towards this highly efficacious drug could be prevented (149, 551, 563). In 2000, a report was published which described the reduced parasite clearance noted in 4 patients in Sierra Leone after treatment with artesunate (431). Whilst the authors concluded that this was evidence that resistance towards artesunate was developing on the African continent, no other non-responsive or confirmed cases of parasite resistance were published and it was still generally acknowledged that there was no resistance towards artemisinin drugs (31, 550). However, further cases of artesunate resistance in *P. falciparum* infection were published in 2009 (143), where it was clearly demonstrated that there was a prolongation of parasite clearance time after artesunate therapy in clinical cases in Western Cambodia compared to north-western Thailand (143). The described prolonged period to parasite clearance could not be explained by drug pharmacokinetics or other host factors and was therefore concluded to be artesunate resistance (143).

1.3.3 Piperaquine

Piperaquine (PQ) is a bisquinoline antimalarial which was first synthesized in the 1960s by both the Shanghi Pharmaceutical Research Institute in China and Rhone Poulenc in France (92, 127). During the next 20 years PQ was used extensively as both a prophylactic agent as well as for malarial chemotherapy in China and Indochina. Once the Chinese National Malaria Control Programme recommended PQ over CQ for the treatment of malaria, over 140 million adult treatment doses were distributed (127, 217). However, with the emergence of PQ-resistant *P. falciparum* strains as well as the emergence of the artemisinin derivative drugs, the use of PQ in the clinical setting declined (127).

In the quest for the development of new antimalarial combination therapies, PQ was 'rediscovered' by Chinese scientists as a suitable drug partner for combination with an artemisinin drug (127).   PQ was found to be a useful partner drug for artemisinin derivatives as early clinical studies demonstrated that PQ had good tolerance, mild side-effects, high-level prophylactic efficacy for 3 weeks after a single oral administration and rapid blood schizontocidal action against *P. falciparum* (46). Subsequently, several artemisinin combination therapies including PQ as the partner drug have emerged in the clinical setting, with results to date demonstrating good tolerability and efficacy (10, 27, 29, 139, 178, 189, 209, 217, 252, 253, 305, 456, 480).

1.3.3.1 Mode of action

PQ is a member of the 4-aminoquinoline class of antimalarial drugs and contains the 7-chloro-4-aminoquinoline structure found in every member of this drug class (127) (Fig. 1.8). As a result, it is likely that PQ has a similar mode of action as other 4-aminoquinolines such as CQ. Evidence suggests that the mode of action of PQ is the inhibition of the haem-digestion pathway in the parasite food vacuole (127, 356). It is suggested that PQ accumulated in the malaria parasite by iron trapping, resulting in high drug concentrations in the food vacuole, which leads to an inhibition in the formation of the haemozoin polymer (356).

Figure 1.8. Chemical structure of piperaquine phosphate (127)
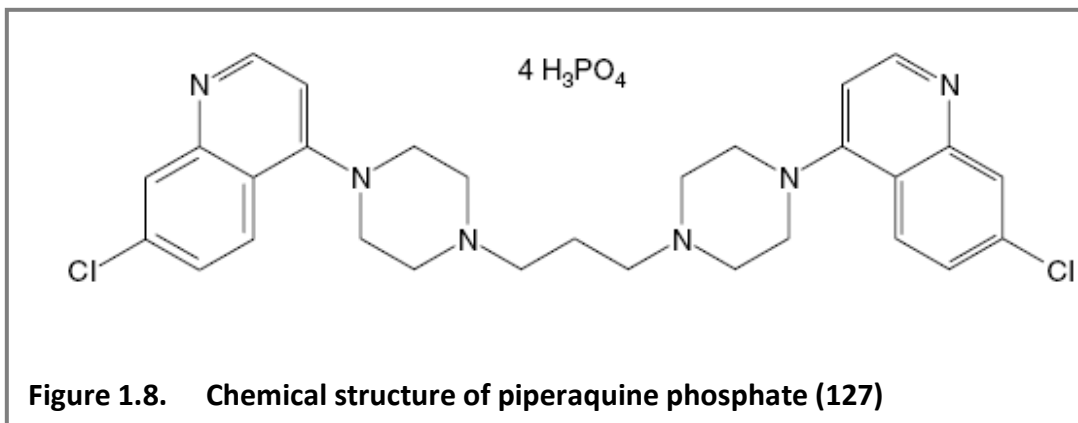
1.3.3.2 Antiparasitic activity

The parasite maturation stage predominantly affected by PQ administration is the trophozoite, as this is the only asexual stage which contains a functional food vacuole (91, 127). Microscopic studies on the effect of PQ on the structure of erythrocytic stages of *P. berghei* ANKA, of which all asexual parasite stages are observed within the peripheral circulation, supports the proposed mechanism of action of PQ as after treatment the ultrastructure of trophozoites are predominantly affected whilst ring-form, schizonts and gametocytes showed few morphological changes (93, 94). Changes to the structure of trophozoites was first identified as the progressive swelling of the food vacuole membrane with eventual autophagocytosis of the parasite, 12-24 h after drug administration (93). It is therefore highly likely that the target of PQ efficacy is the food vacuole of the trophozoite (90, 93).

1.3.3.3 Pharmacokinetics

Despite use as an antimalarial agent in clinical practice since the early 1960s, pharmacokinetic data of PQ in humans has only recently become available (10, 232, 286, 425, 443, 480). The first clinical study describing the pharmacokinetics of PQ was published by Hung et al, (232) who described the population pharmacokinetics of PQ in Cambodian children and adults with uncomplicated *P. falciparum* and *P. vivax* malaria. All patients received the artemisinin combination therapy Artekin 2® (each tablet containing 40 mg of DHA and 320 mg of PQ phosphate (PQP)). It was

shown that PQ had a slow absorption and a long terminal elimination half-life ($t_{\frac{1}{2}}$) of 22.6 days (543 h) in adults and 13.5 days (324 h) in children (232).

With the increasing popularity of PQ as a partner drug to the artesmisinins for combination therapy, this initial report was rapidly followed by a succession of studies investigating the pharmacokinetics of PQ in healthy and malaria-infected populations, across a variety of ethnicities, age groups and infecting parasite species (254, 286, 425, 480). Despite a general consensus that PQ has an extremely long elimination half-life, designating a consistent value to this parameter has proven to be a contentious issue (127, 483). The accuracy of the published and generally accepted half-life of 23 days (231) was challenged by Tarning et al. (483) who suggested that the previously published value was likely to be an underestimation as a result of insufficient assay sensitivity, short duration of sampling and oversimplified fitting of two-compartment models. In their study, the duration of sampling was increased from 35 days (previous published report (231)) to 93 days (483). As a result of the extended period of monitoring of plasma PQ concentrations, the terminal elimination half-life was suggested to be in the order of 36 days although if based on the last measurements, could be as long as 80 days (483). However, since these early pharmacokinetic data, further studies in healthy volunteers have reported PQ half-life in the range of 11.7 to 20.3 days after a single dose (286, 425, 443), and as 12.5 days after multiple doses (286). In patients with uncomplicated malaria the mean elimination half life ($t_{\frac{1}{2}}$) was suggested to range between 13.5 and 28 days (232, 254, 480). The aforementioned studies demonstrate the issues involved in accurately measuring and defining pharmacokinetic parameters for long half-life drugs, such as PQ. Reported half-lives appear to be strongly dependent on sample numbers, assay sensitivity and the duration of follow-up after drug administration. Such issues are best illustrated in Fig. 1.9 (483), which shows the relationship between follow-up duration and assay sensitivity and the potential problems associated with the estimation of terminal elimination half-life for drugs, like PQ, that have multiphasic elimination kinetics and a long terminal half-life.

Figure 1.9 **Pharmacokinetic elimination of piperaquine**. The diagram illustrates the different estimates of the terminal elimination half-life as a function of follow-up duration and assay sensitivity (483)
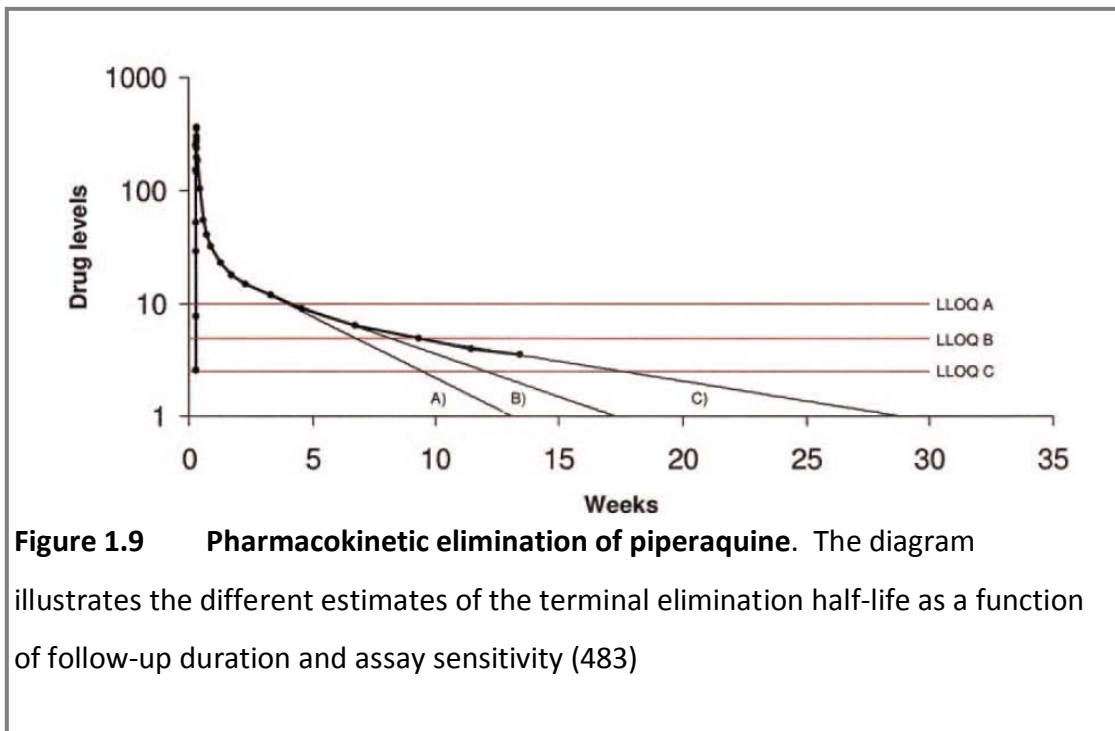
1.3.3.4 Clinical Efficacy

Published preclinical studies, although limited, have concluded that PQ is a relatively safe and well tolerated drug, with a good toxicity profile (50, 92, 127). It is effective against all *Plasmodium* strains, including those strains of *P. falciparum* which are resistant to CQ, with few patients reporting adverse affects. Despite the rapid acquisition of parasite resistance towards PQ after extensive monotherapeutic use, the excellent tolerability, effectiveness, long half-life and low cost of PQ make it a promising partner drug for short-course antimalarial combination therapies (127).

Even with the successful implementation of the artesunate-mefloquine and artemether-lumefantrine combinations in malaria endemic countries, the quest for a more desirable Artemisinin Combination Therapy (ACT), with a partner drug that fulfils more of the WHO recommendations, continues. Whilst searching for new partner drugs for ACTs, PQ underwent a renaissance and as a result a number of ACTs including PQ as the partner drug have been successfully formulated and implemented in the clinical setting (Table 1.4)(456). The first PQ combination therapy combined the artemisinin derivative DHA with PQP in a co-formulation with

Table 1.4   Combination therapies including PQP currently used in the clinical setting. *Abbreviations: Dihydroartemisinin (DHA); Artemisinin (AR); Piperaquine phosphate (PQP); Trimethoprim (TMP); Primaquine (PRIM); days (d); hours (h).*

Commercial name	DHA (mg)	AR (mg)	PQP (mg)	TMP (mg)	PM (mg)	Dosing schedule (adult)	Cure rate	Ref
CV-8®	32	0	320	90	5	2 tablets at t = 0, 24 and 48 h	28 d: 94%	(178)
Artecom®	32	0	320	90	0	2 tablets at t = 0, 6, 24 and 48 h	28 d: 96.9% 56 d: 97.4%	(560) (217)
Duo-Cotexcin®	40	0	320	0	0	3 tablets at t = 0 and 24 h, 2 tablets at t = 48 h	28 d : 97.8%	(595)
						2.1 mg/kg/day DHA 17.1 mg/kg/day PQP t = 0, 24 and 48 h	42 d: 98%	(589)
Artekin®	40	0	320	0	0	2 tablets at t = 0, 6-8, 24 and 48 h	28 d: 92.3–100% 56 d: 98.7% 63 d: 97.5%	(27, 139, 253) (217) (244)
						1.6 mg/kg/day DHA 12.8 mg/kg/day PQP t = 0, 8, 24 and 48 h	63 d: 100% 63 d: 96.1%	(29) (27)
						1.6 mg/kg/day DHA 12.8 mg/kg/day PQP t = 0, 24 and 48 h	28 d: 95.2–99% 63 d: 99.4%	(252, 477) (29)
						2.1 mg/kg/day DHA 16.8 mg/kg/day PQP t = 0, 24 and 48 h	28 d: 99% 42 d: 100% 63 d: 98.4%	(456) (305) (189)
						2.25 mg/kg/day DHA 18 mg/kg/day PQP t = 0, 24 and 48 h	42 d: 87–89.9% 63 d: 92.2%	(209, 397) (413)
Artequick®	0	80	400	0	4	3.2 mg/kg/day AR 16 mg/kg/day PQP 0.16 mg/kg/day PRIM t = 0, 24 and 48 h	28 d: 98.5%	(265, 476)

trimethoprim and primaquine called CV-8® (127, 178). This combination was implemented as national policy for first-line treatment of uncomplicated malaria in Vietnam in 2000, with a 3-day regimen giving 28-day cure rates of 96%, a similar outcome to the combination atovaquone-proguanil (Malarone) (178). After slight modification of the original CV-8® formulation, Artecom® was registered in both Vietnam and China, however, its use in the clinical practice appears to have been limited (127, 217). The most recent PQ formulations Artekin® (PQ-DHA) and Artequick® (PQ-artemisinin) have been more successfully implemented into the clinical setting, particularly in Southeast Asia, with both drugs being shown to be highly efficacious (27, 29, 139, 189, 209, 217, 244, 252, 253, 265, 305, 336, 397, 413, 456, 476, 477, 589, 595).

Benefits of DHA-PQ over artemether-lumefantrine were that DHA-PQ offered better post-treatment prophylactic effect, a significantly lower risk of recurrent parasitaemia after treatment, simple dosing schedule, more consistent intestinal absorption, relatively low cost, a lower risk of gametocytaemia after therapy and better haemoglobin recovery (336, 589). Several advantages that DHA-PQ has over artesunate-mefloquine are that the treatment course is cheaper (approximately US$1.50 per adult treatment), better tolerated (217) and available as a co-formulation which means the treatment course is easier to take and should result in an increase in adherence thus limiting the chance of the emergence of resistant parasites to either the DHA or PQ (456).

Studies comparing the combinations artesunate-mefloquine and DHA-PQ have demonstrated that both treatment regimens were highly efficacious and well tolerated treatments (27, 29, 217, 253, 305, 456) with reported cure rates of 95 to 99% with 3-day dosage regimens in uncomplicated falciparum malaria (27, 139, 217, 252, 456, 477). Therefore, with the successful implementation of PQ based ACTs in the clinical setting, it is important to further understand the relationship between PQ plasma concentrations and toxicity or efficacy (253) as well as determining the therapeutic index for PQ, which, remains poorly defined.

1.3.3.5 Adverse effects

Overall, studies have shown that when taken in monotherapy or as an ACT, PQ is a well tolerated drug with few patients reporting adverse effects (127, 139, 178, 217). Minor complains that have been reported include mild headache, dizziness, nausea, abdominal pain and vomiting   (27, 29, 92, 127, 139, 209, 217, 232, 456, 477) although these symptoms are often difficult to distinguish from the symptoms resulting from the malaria infection. However, in a healthy volunteer study it was found that mild nausea, abdominal pains, dizziness and fatigue occurred transiently after PQ administration (443) suggesting that the mild adverse effects noted in clinical studies were in fact due to either a combination of symptoms of malaria and drug administration or simply due to PQ administration.

To date there are limited data available relating to the safety of PQ use during pregnancy, lactation or in children younger than 12 months of age (127, 139, 232, 252, 253, 413).

1.3.3.6 Resistance

After the extensive and unregulated use of PQ as a monotherapy for antimalarial prophylaxis and chemotherapy in China, PQ resistance soon emerged with $IC_{50}s$ significantly increasing in areas of southern China where PQ use was widespread (127). As a result, the use of PQ for either prophylaxis or chemotherapy was stopped and its use has only recently resumed, albeit in combination therapies under much stricter regulation.

There are conflicting views in the literature to whether any cross-resistance between PQ and other antimalarial drugs (particularly other 4-aminoquinolines and artemisinins) exist (89, 90, 127, 279, 280, 356). In two PQ resistant mouse strains, *P. berghei* ANKA and K173 PR strains, cross-resistance was found between PQ, hydroxy-PQ, artesunate, artemisinin and mefloquine (279, 280). However, studies looking at the cross-resistance of PQ in CQ-sensitive and CQ-resistant isolates of *P. falciparum* from Cameroon suggested that with $IC_{50}s$ of <100 nmol/L there was only a slight correlation between the two drugs therefore minimizing the risk of *in vitro*

cross-resistance (46). More recently *in vitro* studies observing the interactions between PQ, DHA, mefloquine, quinine, CQ, pyronaridine and naphthoquine demonstrated that PQ had no interaction with DHA, CQ, pyronaridine and naphthoquine but antagonism with mefloquine (126). This suggests that there should be little clinical interaction of PQ with any of the drugs studies except mefloquine which, according to these results should be limited (126).

1.3.4 Chloroquine

The antimalarial CQ (originally named Reochin) was discovered in 1934 by Hans Andersag at Bayer I.G. Farbenindustrie A.G. laboratories in Germany (39, 105, 111). However, it wasn't until during World War II that British and U.S. scientists recognized CQ as an effective and safe antimalarial drug (39, 341). Therefore, CQ became the first-line treatment for uncomplicated and severe malaria as well as the principal drug for malaria prophylaxis (105). In the late 1950s the first cases of CQ-resistant *P. falciparum* were documented in Thailand and Columbia which was soon after followed by the spread of CQ-resistant strains worldwide (39, 63, 326).

Despite the extensive spread of CQ-resistant strains of *P. falciparum* and more recently the emergence of chloroquine-resistant *P. vivax* strains in New Guinea and Indonesia (38, 40, 110, 412), CQ remains by far the most widely used antimalarial drug worldwide (450, 540, 562).

1.3.4.1 Mode of action

Whilst the precise mechanism of action of CQ is not fully elucidated, it is generally considered that the antimalarial action of CQ is attributed to the drug's accumulation within the haem-rich lysosomes of the food vacuole of malaria parasites. This accumulation results in an increase in the intralysosomal pH of the parasite which directly inhibits the lysosomal hydrolysis of haemoglobin. As a consequence, toxic metabolites accumulate within the parasites leading to the arrested growth of the parasite (219, 297, 356, 409, 562).

1.3.4.2 Antiparasitic action

CQ only acts on those stages of the parasite life cycle that can actively digest haemoglobin within the erythrocyte, thus trophozoite stage parasites (540). This has been confirmed by ultrasturctural studies which have shown that the parasite's food vacuole is the target of the activity of CQ (424, 540).

1.3.4.3 Pharmacokinetics

CQ has a long terminal elimination half-life ranging from 3 h to 1500 h (490) although most recent studies suggest a longer terminal elimination half-life of 1–2 months (148, 264, 504, 540, 562). In healthy patients and those with uncomplicated malaria, oral chloroquine is rapidly absorbed with peak plasma concentrations of approximately 250 µg/L reached within 2 h of dosing. After i.m. or s.c. injection, absorption is so rapid that dangerously high peak plasma concentrations (500–3500 µg/L) may be reached within 5–20 min after administration of a 5 mg base/kg dose (540).

CQ is extensively bound to tissues, particularly the liver, connective tissue and pigmented tissues such as the skin and retina resulting in a very large total volume of distribution. CQ is also concentrated in erythrocytes, granulocytes and platelets with approximately 55–60% bound in plasma (148, 540). Following administration, the drug is rapidly dealkylated in the liver via cytochrome P450 enzymes into the pharmacologically active desethylchloroquine (DECQ) and bisdesethylchloroquine (148, 264).

1.3.4.4 Clinical efficacy

Whilst CQ-resistant *P. falciparum* is present worldwide, with high grade resistance in many areas, CQ remains the drug of choice for combating CQ-sensitive parasites in Africa (59, 551, 561). However, clinical failure after a course of CQ now exceeds 25% in much of Africa but as the drug is cheap and readily available, it remains the first-line therapy throughout much of Africa and parts of Southeast Asia (562).

In the clinical setting CQ is most commonly used for the oral treatment of uncomplicated malaria and is effective against all CQ-sensitive parasite species (111, 562). Recent efficacy studies of CQ in Burkina Faso, a region which has had high grade CQ-resistant *P. falciparum* since 1983, demonstrated that after administrating a standard course of CQ to children, parasitological and clinical failure rates were reported to be 27% and 10%, respectively (330). These results were surprising as clinical failure rates in the same region in 1988 were estimated to be closer to 70% (437). Therefore, this efficacy study suggested that since replacing CQ as a first-line drug, the incidence of CQ resistance has decreased. Such observations appear to correspond to a general trend seen across areas of Southeast Asia. Furthermore, data collected in 1994 in Gabon showed a decrease in CQ resistance isolates (385) and further data in 1996 (67) showed a significant increase in *P. falciparum* sensitivity to CQ. These data imply that if CQ monotherapy is replaced in a region for a period of time, there is the capacity for the parasites to become more sensitive to the drug. Therefore, CQ should not be disregarded as an antimalarial therapy and, with better understanding of the mechanisms of resistance, there is a potential for CQ to be restored to clinical utility (111, 183). For example, it has been suggested that sequential treatment with CQ and a glutathiamine-depleting drug, may increase the efficacy of CQ in the clinical setting (183). Furthermore, it has been demonstrated that combination of CQ with a sensitising drug, such as antihistamines or antidepressants, may increase the clinical efficacy of the drug particularly against CQ-resistant parasites (56). Studies reported that addition of the antihistaminic drugs cyproheptadine (377), azatadine (47), or chlorpheniramine (462) potentiated the activity of CQ against mild to moderately CQ-resistant parasites (56).

1.3.4.5 Adverse effects

CQ is generally well tolerated, however, when plasma concentrations exceed 250 µg/mL, unpleasant side-effects such as dizziness, headache, diplopia, disturbed visual accommodation, dysphagia, nausea and malaise may develop. Therefore, the adverse effects of CQ are usually limited to patients receiving intravenous infusions (364, 540). In Africans, Haitians and dark-skinned Asians pruritus of the palms, soles

and scalp may also be a problem and is particularly seen in cases of therapeutic noncompliance (364, 540, 562). Pruritus is suggested to occur in up to 70% of adult Africans taking CQ (364). Serious adverse effects such as hypotension, electrocardiograph abnormalities, leucopenia and aplastic anaemia are rare but can occur at high drug concentrations (562).

1.3.4.6 Resistance

The first case of CQ-resistant *P. falciparum* malaria was described in Colombia in 1960 and within a short period, had spread to most of the malaria endemic regions of the world (326, 461). So rapid was the progression of resistance that within 10 years of the first case, CQ-resistant *P. falciparum* had spread across the entire African continent (547). Today, CQ remains effective for *P. vivax, P. ovalae and P. malariae* infections worldwide and for falciparum malaria in restricted areas such as central America northwest of the Panama Canal, Haiti, the Dominican Republic and parts of the middle east (540). However, caution must be taken before treating any cases of malaria with CQ as reports of chloroquine-resistant *P. vivax* have also started to emerge (461, 540).

1.3.5 Antimalarial combination therapies

In light of the ever increasing pressure of the emergence of drug resistance, antimalarial combination therapies are now thought to be the way forward in the treatment of uncomplicated and severe malaria (581, 583{World Health Organization, 2010 #1255)}. The concept of combination therapy is based on the synergistic or additive potential of two or more drugs, which have independent modes of action and/or different biochemical targets, so as to improve therapeutic efficacy and delay the development of resistance to individual components of the combination (127, 581). In order to determine the merits of a proposed combination therapy for different epidemiological conditions, a set of criteria have been suggested by the WHO (581). The major criteria include the following:

- Therapeutic efficacy of the combination, irrespective of the efficacy of the individual components.
- Safety of the drugs in combination, especially amongst high risk groups.

- Potential for widespread use at all levels of heath care, including use for home management.

- Potential for consumer compliance.

- Cost effectiveness.

- Potential for the delay or prevention of development of resistance.

- Other factors: including product availability, production capacity and potential for wide-spread use at a sub-regional level.

In the clinical setting, a number of drug combinations are currently available and used successfully to treat uncomplicated and severe malaria. The most common are the ACTs (150, 335, 351, 551, 555, 563, 581).

1.3.5.1 Artemisinin combination therapy

In recent times ACTs have become increasingly popular in Southeast Asia for malaria chemotherapy and are expected to become more frequently used in tropical Africa (438, 581). Although ACT has been recommended by the WHO since 2001 as the 'ideal strategy' for malaria control, overall deployment has been slow. This is primarily related to their high relative cost, limited public awareness of the concept and advantages of combination therapy, limited knowledge on the safety of ACTs in pregnancy and other operational issues including inappropriate drug use, lack of suitable formulation and the imbalance between supply and demand (335, 562).

The advantages of ACT relate to the unique properties and mode of action of the artemisinin compound including; its rapid reduction of the parasite biomass, rapid resolution of clinical symptoms, effective action against multidrug-resistant *P. falciparum*, reduction of gametocyte carriage thus possibly reducing transmission of resistance, little documented resistance to artemisinin or its derivatives in most regions and few reported adverse clinical effects (335, 581). Ideally, in ACT the partner drug should have a half-life exceeding 4 days (eg. mefloquine or PQ) which both protects the artemisinin derivatives against resistance and offers an extended period of malaria prophylaxis whilst enhancing efficacy (46, 232, 252, 351, 581).

With the increasing emergence of drug-resistant *P. falciparum*, the use of ACTs in the clincal setting has also grown, resulting in the WHO recently recommending ACTs as the treatment of choice for uncomplicated malaria (253, 574, 581)}.   In order to provide efficacious antimalarial therapy, without the risk of drug resistance, the WHO has stipulated that when devising new ACTs the following factors must be considered   (128, 351):

- Components have different modes of action.

- No interactions.

- Short course regimens (3 days at most).

- At least one drug which clears asexual forms rapidly.

- At least one drug with a long half-life.

- Well tolerated, low toxicity.

- Broad spectrum of action (including against gametocytes).

- Co-formulation, if possible.

It is currently estimated that more than 60 malaria endemic countries are advocating the change of antimalarial policy to ACTs, however, there is still much debate over which are the most suitable combinations for first-line treatment of uncomplicated malaria (408). To date, artemether-lumefantrine (Coartem®) is the only co-formulated ACT which has international registration (28, 408). However, despite not being commercially available as a co-formulation, artesunate-mefloquine is a popular choice of ACT, particularly in Southeast Asia (28, 352, 457, 476, 477, 522, 560). Artesunate-mefloquine is used as first-line antimalarial therapy, as a 3-day regimen (artesunate 12 mg/kg and mefloquine 25 mg/kg), in many Southeast Asian countries, demonstrating excellent efficacy and tolerability (28, 352, 456, 457, 477, 522). Despite the efficacy of this combination, there are a number of drawbacks for relying solely on this treatment regimen, particularly when considering the stipulations made by the WHO for ACTs. These include:

1. The high price of each adult treatment of artesunate-mefloquine (approximately US$3 per treatment) means that most people in endemic

areas cannot afford the treatment and thus are treated with less effective alternatives (eg. CQ) (456)

2. Mefloquine has some common adverse effects, and although not serious, can result in poor adherence to the multiple dose therapy. Mefloquine can also be associated with neurological and neuropsychiatric side-effects, however, these are usually mild and are not long lasting (118, 125, 456)

3. To date, co-formulated mefloquine and artesunate are not widely available commercially. Hence, if patients experience adverse effects to mefloquine they can stop taking this drug but continue to take the artesunate which thereby compromises efficacy and risks parasite resistance (456, 457). In 2010, a co-formulation of mefloquine and artesunate (ASMQ) was registered for clinical trials and several trials in Africa and Latin America are currently in progress.

The co-formulation artemether-lumefantrine has proved to be highly effective and well tolerated in several studies from Africa and Southeast Asia (2, 29, 30, 160, 161, 329, 404, 492, 522, 589, 595). Like the artesunate-mefloquine combination, however, artemether-lumefantrine has several drawbacks. Disadvantages of this combination include; the need for twice-daily dosing, that each dose should be taken with a fat-rich meal or soya milk (30, 161) as well as the relatively high recurrence of parasitaemia within 28 days (317, 589).

Alternative ACTs, which are either currently available or in the process of undergoing clinical investigation, include:

1. DHA-PQP (Artekin®)
2. Artesunate plus sulfadoxine-pyrimethamine
3. Naphthoquine plus DHA
4. Artesunate plus amodiaquine
5. Artesunate plus pyronaridine

1.3.5.2 Non-artemisinin combination therapies

Before the introduction of the ACTs several successful non-artemisinin combination therapies were used in the clinical setting, specifically quinine-tetracycline (or doxycycline), sulfadoxine-pyrimethamine and most recently atovaquone-proguanil (Malarone™), amodiaquone-sulfadoxine-pyrimethamine and chlorproguanil-dapsone (LapDap) (128, 362). However, with each of these combinations a number of issues have hampered their continued application in the clinical setting, particularly relating to side-effects (quinine-tetracycline), compliance (each dosing strategy usually spans 7–10 days) and declining efficacy (high-grade resistance to sulfadoxine-purimethamine is common). Although atovaquone-proguanil is given as a short-course regimen it is one of the most expensive antimalarial therapies currently available and despite being only introduced in 2000, highly resistant cases have already been reported (128, 464).

Whilst the majority of comparative efficacy studies conducted between ACTs and non-ACTs demonstrate that ACTs have the highest cure rates (particularly when referring to treatment failure) and tolerability, ACTs are not always the best or most feasible option for malarial chemotherapy (262, 362). The high cost of artemisinin based therapies and their availability, particularly on the African continent remain major concerns (588). As a result clinicians in Africa are turning to the inexpensive and widely available non-artemisinin combination therapies, particularly amodiaquone plus sulfadoxine-pyrimethamine, which may still be a feasible treatment option in areas where sulfadoxine-pyrimethamine resistance is not endemic (314, 588).

The most commonly used non-artemisinin combination therapies available in the clinical setting, which still remain efficacious in many regions and are therefore a suitable option for ACT, if necessary, include the following (581):

1. Amodiaquine plus sulfadoxine-pyrimethamine
2. Atovaquone-proguanil (Malarone™)
3. Quinine plus tetracycline (or doxycycline)

1.4   AIMS OF THE THESIS

Although the rodent *P. berghei* model has been the primary animal model for in vivo screening of new antimalarial compounds, its application has been limited to efficacy assessment. The potential of this model to provide pharmacokinetic and additional pharmacodynamics data, has therefore not been fully explored. Whilst the overall aim of this thesis was to further investigate and develop the *P. berghei* murine malaria model as a tool for the assessment of the pharmacokinetic and pharmacodynamic properties of antimalarial drugs, the specific objectives included:

1. **To evaluate the efficacy of dihydroartemisinin (DHA) in an asplenic murine malaria model.**

    The pharmacodynamic properties of DHA were investigated through a single dose ranging study conducted in both *P. berghei* infected and uninfected asplenic and intact mice. Given published clinical findings, it was hypothesised that the efficacy of DHA would be compromised in the asplenic mice. (Chapter 3; Paper I)

2. **To obtain robust pharmacokinetic and pharmacodynamic data following the single dose administration of piperaquine (PQ) to mice**

    The pharmacokinetic and pharmacodynamic properties of PQ were investigated in *P. berghei* infected and uninfected mice after administration of single doses of PQ phosphate (PQP). Similarly to human pharmacokinetic studies it was hypothesised that in the murine malaria model, PQ would demonstrate a long elimination half-life, with little difference in drug plasma concentrations between *P. berghei* infected and uninfected mice. (Chapter 4; Paper II).

3. **To investigate drug efficacy, reinoculation outcomes, and parasite viability after administration of a single dose of PQ in the murine malaria model**

    Previous investigations (Chapter 4) demonstrated that high dose PQP had an extended antimalarial efficacy, with resolution of detectable

parasitaemia, although for the majority of the pharmacokinetic profile, PQ plasma concentrations were deemed to be at less than efficacious concentrations. Therefore, the aim of this study was to determine whether the extended antimalarial efficacy was a result of drug effects alone or a combination of drug efficacy and host factors. It was hypothesised that in combination with initial PQ efficacy, the host immune system played an important role in the resolution of infection. (Chapter 5; Paper III)

4. **To obtain robust pharmacokinetic data for chloroquine (CQ) and its major metabolite desethylchloroquine (DECQ) after single and multiple dose administration in the murine malaria model.**

Despite CQ being used extensively throughout the clinical setting, the pharmacokinetic parameters of this drug in preclinical models is not well defined. Furthermore, there are limited data on the pharmacokinetics of multiple dose CQ administration or single and /or multiple dose DECQ administration in mice. It was hypothesised that in the murine malaria model, CQ would have a long elimination half-life in both malaria-infected and uninfected mice. (Chapter 6; Paper IV).

CHAPTER TWO

METHODS

2.1   MATERIALS

2.1.1   Animals and animal welfare

2.1.1.1 Mice

2.1.1.1.1   Experimental mice

All experimental work was performed using 5-6 week old male outbred albino male Swiss Arc:Arc(S) mice which were obtained from the Animal Resource Centre (ARC; Murdoch, Australia). The ARC reports that the original breeding stock of Arc:Arc(S) mice was received from the Charles River Breeding Laboratories (Kingston, NY) in 1991 as a SPF CD1 animal stock (22). This particular mouse strain was then re-imported from the Charles River Laboratory in 2006 (22). Whilst genetically stable inbred mouse strains are often used in animal studies involving specific disease processes or immunological responses, outbred mouse strains are more commonly associated with drug development or efficacy studies (18, 122, 268, 378, 379, 381). The outbred Swiss mouse model was therefore used for all studies as it provided a robust and economical mouse model. Male mice were selected for use in these studies which eliminated the chance of variable biochemical parameters as result of fluctuating hormone levels which can be linked to females (268).

The experimental design of each animal investigation was based on a modified Rane *in vivo* screen system (Refer to 1.2.2.1.1.4). The number of animals used in each drug treatment group throughout the thesis investigations was selected based on a statistical power calculation. For the pharmacodynamic studies, it was determined that statistically significant results could be achieved in this biological system with at least 8 mice per treatment group (>25% difference in outcomes; power = 80%; α=0.05).

2.1.1.1.2   Mice for P. berghei passage

Inbred albino male BALB/cArc mice (ARC; 7-8 weeks of age) were used for the weekly passage of *P. berghei* infection. The BALB/c mice were originally sourced by

the ARC from the Animal Breeding Unit, University of Western Australia (Crawley, Australia) in 1981 (21). In order to obtain specific pathogen free status in this strain, the first litters were derived by caesarean (21). The genetic make-up of the BALB/c mice used in all investigations were the inbred albino Tyr^c, $Tyrp1^b$, A, $H-2^d$ strains (21). As the experimental procedures involving BALB/c mice required a stable infection for weekly passage, this inbred strain was chosen as it is the most established method of passage and it reduces immunological responses when transferring the infection between mice.

2.1.1.1.3   Sentinel mice

Male Swiss mice (ARC; 5-6 weeks of age) were used as sentinel mice for the animal holding room. At quarterly intervals, 6 mice (of similar age to experimental mice) were obtained and separated into 3 open-top cages (n=2 per cage) with the cages then distributed around the animal room (1 cage close to the entry door, 2 cages dispersed within the experimental cages). These mice received the same food, water and bedding as the experimental mice. Additionally, when changing dirty bedding in the experimental cages, a small amount of dirty bedding was randomly selected and placed in the sentinel cages. At the conclusion of the interval, blood was harvested from the mice and sent for laboratory testing (Murine Virus Monitoring Service, Gilles Plains, South Australia) against a panel of known murine viral pathogens, most importantly including Mouse Hepatitis Virus (MHV), Mouse Parvovirus (MPV), and Pneumonia Virus of Mice (PMV) (Full testing panel outlined in Appendix 1). For the duration of research undertaken for this thesis, the sentinel mice remained pathogen free.

2.1.1.2 Animal welfare

2.1.1.2.1   Ethical approval

All experimental protocols described in this thesis were approved by the Curtin University Animal Ethics Committee. All research was conducted in accordance with the NHMRC Code of Practice for the Care and Use of Animals for Scientific Purposes (342).

2.1.1.2.2 Housing facilities

Between 2005 and 2007, all animals were housed in the School of Pharmacy animal laboratory in an air-conditioned room set at $22^{o}C$ with a 12-h light/dark cycle. This animal laboratory was decommissioned at the end of 2007 and all animal research was moved to the Curtin University PC2/PC3 Animal Research Laboratories. As a result, the *P. berghei* inoculum had to be re-established in the new environment from frozen blood stocks. Within the new facility, animals were housed in a PC2 rated animal holding room, maintained at $22^{o}C$, with a 12-h light/dark cycle.

2.1.1.2.3 Cages

Non-porous, translucent plastic cages with stainless steel bar tops were used to house the mice. For experiments over short periods (1-2 weeks) small cages housed a maximum of 4 mice whilst large cages housed up to 12 mice. For experiments lasting more than 2 weeks, a maximum of 2 and 8 mice were housed in each small and large cage, respectively. Stainless steel nameplates were used to hold identification cards on the front of each cage. Plastic water bottles with stainless steel drinking teats were used for water dispensing.

All cage components were autoclaved at $121^{o}C$ for 15 min on a hard good cycle, using an accredited autoclave in the School of Pharmacy and/or Curtin University Animal Research Laboratories.

2.1.1.2.4 Bedding

ALPHA-dri bedding (Shepherd Specialty Papers; Able Scientific, Perth, Western Australia) was autoclaved at $121^{o}C$ for 15 min on a hard goods cycle, using an accredited autoclave. Cellulose bedding was selected for use as it ensured low dust accumulation and ingestion of bedding by the mice would not interfere with animal biochemistry parameters, which can be seen when using wood chips as a bedding source (251). A number of autoclaved tissues were also placed in each cage for use as nesting material.

2.1.1.2.5   Acidified water

Acidified water (pH2.5) was prepared by adding 400 µL of 32% w/v HCl to 1 L glass bottles of de-ionised water.   Before use, the prepared water solution was autoclaved for 20 min at 121°C on a fluid cycle in an accredited autoclave.   The water was acidified in order to prevent bacterial contamination whilst water was either in storage or in the cage water bottles (439, 469).

2.1.1.2.6   Rodent food

Rodent food cubes, purchased from Glen Forrest Stock Feeders (Perth, Western Australia), were used for animal feed.   The food was autoclaved on a hard goods cycle for 20 min at 121°C in an accredited autoclave before distribution to the animals.

2.1.1.2.7   Environmental enrichment

Cardboard tubes and small cardboard boxes were autoclaved at 121°C for 15 min on a hard goods cycle, with a selection placed in each cage to provide stimulation. The goal of adding environmental enrichment to the cages was to decrease fighting, particularly in the large cages, as part of established animal welfare recommendations (342).

2.1.2   Parasites

The rodent specific parasites, *P. berghei* ANKA parasites were used for all experimental work.   Originally sourced from the Australian Army Malaria Research Institute (Enoggera, Queensland; acquired by Prof K Ilett and Dr K Batty in 1995), the parasites were well established in the laboratory through weekly passage of infection through BALB/c mice.   The *P. berghei* ANKA strain was selected as it induced high mortality in mice, thus providing a good model to estimate antimalarial drug efficacy in reducing parasitaemia, and is sensitive to all currently used antimalarial drugs (379). Occasionally, volumes of infected blood were prepared as stock samples and stored at -80°C, should a new infection line require development at a later date.

2.1.3　Antimalarial drugs

Antimalarial drugs selected for evaluation included DHA, PQ and CQ. DHA (MW=284.3) was obtained from Dafra Pharma N.V. Turnhout, Belgium. PQ phosphate (PQP; MW=927.5) was sourced from Yick-Vic Chemicals and Pharmaceuticals, Kowloon, Hong Kong whilst CQ diphosphate salt (MW= 515.9) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.1.4　Sodium pentobarbitone

Pentobarbitone injection is commercially available as Lethobarb® (300 mg/mL; 500 mL) which is 5-10 times the required concentration (murine doses were 3-4 mg) and was unsuitable for the present studies. Therefore, for the present studies, all sodium pentobarbitone for injection (sodium pentobarbitone at 30 mg/mL, propylene glycol at 40% [vol/vol], and ethanol at 10% [vol/vol] in water [pH 9.5]) was prepared in house by a registered pharmacist (Dr K Batty). After preparation the sodium pentobarbitone solution was stored in 10 mL volumes in sterile glass bottles in a cool, dark environment. At time of use the solution was diluted 50:50 with 0.9% NaCl for injection.

2.1.5　Reagents and chemicals

May-Grunwald Giemsa stain was obtained from the Department of Microbiology, Royal Perth Hospital, Western Australia. All general laboratory chemicals and solvents were of analytical grade (Sigma-Aldrich Chemical Co., Milwaukee, WI; BDH Laboratory Supplies, Poole, England; and Merck Pty. Limited, Kilsth, Victoria, Australia).

2.2　METHODS

2.2.1　Mouse manipulations

2.2.1.1 Animal identification

As each animal cage contained at least 4 animals, and all results were mouse specific, it was desirable that each individual could be identified. This was accomplished by marking the topside of the base of each tail with a coloured permanent marker. The markings were monitored closely and renewed over the

period of the study should they have faded over a period of time or were rubbed/licked off, particularly after nesting during each night.

Whilst there are a number of techniques that are commonly used for animal identification, including the use of either tattoos or punctures in the ear, the experiments used in these studies were short term and did not require such invasive identification methods.

2.2.1.2 Restraint for manipulation

In order to gain greater control of the mouse for i.p. injection, correct restraint had to take place to reduce the possibility of injury during manipulation. The mouse was first removed from the cage, by lifting it at the base of the tail, and then placed on the cage lid. The tail was held firmly and using the right hand, the tail pulled back gently, causing the mouse to pull in the opposite direction and clasp the cage bars with all four feet (Fig. 2.1; A). Using a quick firm action, the thumb and forefinger of the left hand grasped the scruff of the neck, near the base of the head whilst the tail was still held by the right hand (Fig. 2.1; B). With the tail held in the right hand, and the scruff in the left, the mouse was lifted and laid against the left palm. The tail was then tucked under the third and fourth finger of the left hand resulting in the manual restraint of the animal (Fig. 2.1; C). This position allowed adequate access to the abdominal region for i.p. injection and ensure little movement from the mouse, thus preventing injury during injection processes.

Figure 2.1 Manual restrain of a mouse. **A:** The mouse is removed from the cage by lifting the base of the tail and placed on the cage lid and gently pulled backwards. **B**: The scruff of the mouse's neck is grasped with the thumb and forefingers whilst the right hand still holds the tail. **C**: The tail is tucked under the little finger of the left hand whilst the mouse's back lies across the palm of the hand, manually restrained.

2.2.1.3 Tail bleeding procedures

Peripheral blood smears were prepared using a single drop of blood collected from one of the veins of the mouse tail. The mouse was initially removed from the cage by picking it up at the base of the tail and drawing it backwards into a bleeding cone by the tail. The mouse tail was held firmly between the fore and middle fingers at the base of the cone to limit mouse movement, whilst the thumb and little finger held the top of the tail to prevent movement. A small prick was then made in one of the tail veins using a 26Gx½ inch needle which resulted in the formation of a single droplet of blood. The blood was collected in a 15 µL heparinised capillary tube and further blood flow from the vein was stemmed by placing a tissue over the needle prick and gently applying pressure until a clot formed. The mouse was then returned to its cage.

2.2.1.4 Intraperitoneal injection

Intraperitoneal injections were made into the caudal left abdominal quadrant. The mouse was restrained manually (Section 2.2.1.2) and the head and body tilted downwards. A 26Gx½ inch needle, with the bevel facing upwards to prevent tissue tearing, was inserted firmly through the skin past the abdominal wall and the syringe contents deposited into the abdominal cavity. During insertion, care was taken to note the change in tissue densities as incorrect positioning of the needle

may result in the penetration of an abdominal organ and/or slow or erratic absorption of the injected compound (469).

2.2.1.5 Cardiac puncture

The mouse was manually restrained (Section 2.2.1.2) and injected i.p. with 200 µL of diluted (15 mg/mL) sodium pentobarbitone and then rested to allow the anaesthetic to take effect. After 5 to 10 min mouse reflexes were tested to see if the anaesthetic had taken control. Foot reflexes were tested by squeezing the back foot between the thumb and middle finger. If the mouse was still conscious, this would result in a kicking reflex. To test tail reflexes, the base of the tail was pressed firmly with the thumb. If the mouse squeaked or flinched then anaesthetic control had not been obtained and the mouse was further rested. If no response was observed in either reflex test, cardiac puncture procedures could continue.

The mouse abdominal midpoint was determined and using surgical scissors, a small incision was made by lifting the skin and cutting a small hole through the fur and skin layers. From this point a midline vertical incision was made to the chin.   Four further incisions were made, two from the primary incision radiating out to the left and right side of the abdominal cavity, and two incisions running left and right of the vertical incision at the mouse diaphragm to each forelimb, respectively (Fig. 2.2; A). Using forceps, the sternum was lifted exposing the chest cavity and diaphragm. Using a sharp blade, a hole was made in the diaphragm resulting in the collapse of the lungs. The ribs were parted by excising the sternum, exposing the heart and collapsed lungs (Fig. 2.2; B). Whilst still raising the sternum with forceps, a 1 mL syringe with a 26Gx½ inch needle was used to harvest blood from the left cardiac ventricle (Fig. 2.2; C). The vacuum within the syringe, combined with the pressure of the beating heart, resulted in the syringe filling with blood.

A male mouse with an approximate weight of 30g has a total blood volume in the range of 3-5 mL. However, for the purposes of this project, only 1-1.5 mL of blood was collected, preferentially in a single draw. Once drawn, the blood was placed

into a paediatric citrate anticoagulant tube (Citrate 0.105M, 1 mL, BD Vacutainer) and mixed thoroughly.

Figure 2.2   Schematic demonstrating the procedures of internal cardiac puncture.   A: A primary inscision was made at the abdominal mid-point, immediately above the external genetalia, and a vertical inscision was made to the mouse chin. 4 radiating cuts were made along the primary vertical incision towards each limb. **B:** The skin was pealed back revealing the abdominal organs. To gain access to the heart the diaphragm was first punctured to collapse the lungs, then the sternum excised, revealing the cardiothorassic organs underneath. **C**: A 26Gx½ inch needle, connected to a 1 mL syringe, was placed in the left ventrical and the maximum volume of blood removed. Organs observed in the cardiothorassic cavity included 1. Thyroids; 2. Esophagus; 3. Trachea; 4. Thymus; 5. Heart; 6. Lungs. *The original image, sourced from (366), was modified for explanatory purposes.*

2.2.1.6 Euthanasia

Pre-determined end points of >40% parasitaemia, >10% reduction in mouse body weight in less than 24 h, animal distress or the termination of the experimental protocol were selected and firmly adhered too in all studies.

When euthanasia was required the mouse was manually restrained (Section 2.2.1.2) then injected with 200 µL of 15 mg/mL sodium pentobarbitone injection by i.p.

administration (dose=100 mg/kg). A further 100-200 µL of sodium pentobarbitone injection was administered 3-5 min later if the mouse had not expired. Variable volumes of sodium pentobarbitone were administered to the mice dependant of age, body weight, health status and sex, all which may influence the sensitivity to both the desired and undesired effects of anesthetics (469).

2.2.2 Parasite preparations

2.2.2.1 Establishment of parasitaemia

For the purpose of these investigations the murine malaria model was initiated from cryopreserved stock parasites. Two BALB/c mice were initially infected with 200 µL of thawed cryopreserved *P. berghei* ANKA parasites by i.p. injection (Section 2.2.1.3). Mice were maintained and monitored daily until a significant level of parasitaemia had been established (>30% parasitaemia). At this time the total blood volume was collected via cardiac puncture (Section 2.2.1.5). After determining an accurate blood parasitaemia, the blood was diluted to a standard inoculum of 10^7 parasitised erythrocytes per 100 µL. The standard inoculum (100 µL) was passaged into two new BALB/c recipients to maintain constant passage of the infection.

2.2.2.2 Parasite inoculation

To determine parasite density within a blood sample, total red blood cell and parasite counts were performed. The total number of red blood cells was determined using a cell count performed in a Neubauer chamber (Section 2.2.2.3) while parasitaemia was estimated by counting the number of parasites in tail blood films taken before mouse euthanasia (Section 2.2.1.6). The volume of blood required for an inioculum of 10^7 parasites was determined using the following calculation.

$$\text{Volume of blood} = \left(\frac{\text{Total RBCC}}{0.004*} \right) \times 100 \times \left(\frac{\% \text{ Parasites}}{100} \right)$$

* For these series of investigations a standard value of 0.004 was used, which took into account the counted area of the Neubauer chamber (10 squares = 0.4mm^2), the chamber depth (0.1mm) and the dilution factor (1:100).

Once calculated, the volume of blood was drawn from the anticoagulant tube into a positive displacement pipette and then diluted in the appropriate volume (1 mL blood volume) of citrate phosphate dextrose buffer (Appendix 2) and mixed thoroughly on a vortex. When infecting, 100 µL of the standard inoculum was given to each mouse by i.p. injection.

2.2.2.3 Neubauer cell counting chamber (haemacytometer)

The Neubauer cell counting chamber has a glass-etched grid on its base to allow the number of cells to be accurately determined. To determine red blood cell numbers, a blood suspension was prepared by diluting 10 µL of blood in 990 µL of formaldehyde in trisodium citrate (Appendix 2). The blood/diluent preparation was vortexed then 10 µL was pipetted into the chamber and left for 5 minutes to allow the erythrocytes to settle. Under x40 light microscopy, red blood cell numbers were counted in 10 of the inner 25 grid squares (Fig. 2.3).
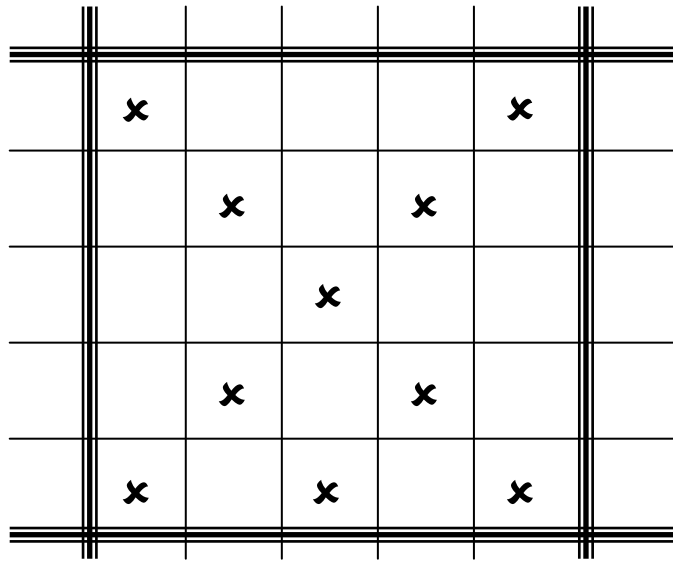
Figure 2.3 **Neubauer cell counting chamber**. After loading 10 µL of diluted blood suspension into the chamber, erythrocyte numbers were counted in 10 of the 25 squares within the specified area (as shown by ✘). Using this cell total we are able to calculate the total number of erythrocytes per µL of blood.

2.2.2.4 Preparation and staining of blood films

Peripheral blood films were prepared using blood collected from one of the tail veins from each mouse (Section 2.2.1.3). To prepare the blood film a single droplet of heparinised blood was placed at the base of a clean, labelled glass microscope slide. A spreader slide was then drawn back into the blood droplet causing the droplet to spread across the edge of the spreader slide. Using a smooth, uninterrupted movement, the spreader slide was moved away from the droplet of blood forming a thin blood smear. An ideal blood smear should have an area of thickly stacked red blood cells at the base of the slide, and an area in which red blood cells are spread in a single uniform layer.

All thin blood films prepared in these investigations were stained using May-Grunwald Giemsa, a commonly used stain for the improved visualisation of malaria parasites. Thin blood smears were fixed in absolute methanol for 30 sec. The slides

were then air dried (approximately 2 min) before placing them on the HemaTek staining machine loaded with a 10% May-Grunwald Giemsa preparation (Department of Microbiology, Royal Perth Hospital, Perth, Australia). Whilst passing through the staining machine each film was exposed to stain for 4 min, buffer solution for 3 min and a wash solution for 2 min. The machine then dried the stained films by blowing air against the slide for 2 min. Once dried, the slides were cover slipped using DePex as the mounting medium.

2.2.2.5 Determination of parasitaemia

Prepared blood films were stained, air dried, cover slipped with DePex then left to air dry for 1 to 2 days. Once dried these films were observed under light microscopy using a x100 oil immersion objective. Within the eyepiece of the microscope, a 1cm x 1cm, 100 square graticule had been placed, allowing more accurate cell counting within each field of view (FOV).

To determine blood parasitaemia two types of cell counting took place. After selecting a section of the film that showed a uniform distribution of red blood cells, all red blood cells, including parasitised cells, were counted within the 100 graticule squares. The total number of parasitised erythrocytes was then counted within the same area. The lens was then moved upwards 1 field of view with a similar procedure undertaken. This process was followed for the number of fields of view required (30 and 100 fields of view for parasitaemias <0.05% and >0.5%, respectively). Using the values obtained for total number of red blood cells and parasitised red blood cells in 100 squares, parasitaemia was then calculated using the formula below. An accelerated method of counting was also employed when >30 fields of view were required to be counted. At this time, the number of erythrocytes was counted in only ¼ of each FOV and multiplied by 4 to determine the total number of cells per FOV. However, this accelerated method could only be used when the film being counted had an even distribution of red blood cells.

$$\% \text{ Parasitaemia} = \left(\frac{\text{PRBC}}{\text{RBCC}} \right) \times 100$$

2.2.2.6 Parasite staging
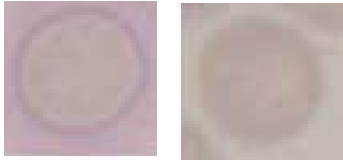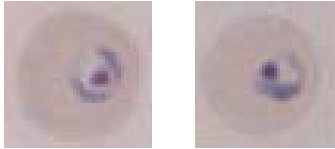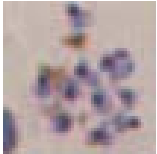
Studies in asplenic mice required stage-specific parasite counts (Chapter 3). Parasites were separated into the 4 different asexual maturation stages; ring-form, early trophozoite, late trophozoite and schizonts, using a pre-prepared identification chart (Table 2.1). In order to numerically tally the different stages, a haematology differential counter was used which allowed the operator to tally each parasite stage separately, whilst calculating the total number of parasites counted. All parasite staging was performed using x100 oil immersion light microscopy. All microscopy was performed by a single operator to maintain continuity and reproducibility of results.

2.2.3   Statistical analysis

All statistical analysis of the counting data was performed using the statistical computer packages SigmaStat® 2004 (SPSS Inc., Chicago) obtained from Hearne Scientific Software Pty, Melbourne and Microsoft Excel for Windows (Microsoft® Windows 2000 Professional). From raw data the mean, standard deviation, 95% confidence interval and linear interpolation were determined. Student's t-test and one way analysis of variance (ANOVA) testing was also performed using the SigmaStat® program. A P value of <0.05 was deemed significant for all statistical analysis.

All statistical data was plotted on a series of linear and semi-logarithmic graphs using the statistical program SigmaPlot® 2004 (SPSS Inc., Chicago) obtained from Hearne Scientific Software Pty, Melbourne.

Table 2.1 **Asexual *P. berghei* parasite stages.** Examples of parasite morphology used for the identification and staging of *P. berghei* maturation stages *(Gibbons, unpublished; based on (241)).*

Maturation stage	Morphology	Characteristics
Uninfected erythrocytes		Erythrocyte containing no intracellular parasite.
Ring-form		Substantial nucleus with a thin chromatin band and large vacuolated area.
Early trophozoite		Regular shape, vacuole beginning to retreat due to haematin pigment.
Late trophozoite		Regular or irregular in shape. Vacuole half to completely filled with haematin pigment.
Schizont		Irregular shape with multiple nuclei due to forming merozoites. 4-16 nuclei.
Meroizoites release by schizont rupture		Erythorycte's integrity destroyed as mature merozoites are released into circulation

CHAPTER THREE

PHARMACODYNAMIC STUDY OF DIHYDROARTEMISININ

IN AN ASPLENIC MURINE MALARIA MODEL

3.1 INTRODUCTION

3.1.1 Role of the spleen

3.1.1.1 Function of the Spleen

The central role of the spleen, the largest lymphatic organ of the human body, is the selective clearance of erythrocytes, microbes and other foreign particles from the blood (76, 97, 158, 313, 428). However, the highly organised architecture of the spleen, consisting of lymphoid follicles (white pulp) and intervening sinusoids (red pulp) (Fig. 3.1), extends splenic functions to also include phagocytosis, immunological reactivity, haematopoiesis and platelet storage (544). The distinct cellular organisation of both the red and white pulp lends itself towards their specific functions which are phagocytic activity and immune activity, respectively (303, 517, 518).

The success of the red pulp as a filtering organ is due to its unique vasculature, where numerous filtration beds, consisting of a three dimensional network of branched fibroblastic contractile reticular cells, are interposed between the end of an artery and the beginning of a vein (71, 428, 544) (Fig. 3.2). After filtering through the red pulp, where the cells are in intimate contact with immunologic effector cells, the blood re-enters the venous lumen by passing through inter-endothelial slits (IES) in the splenic sinuses. The IES measure 1 to 3 µm and in order to pass from the cords of Billroth to the venous lumen, cells must be deformable with those unable to change shape remaining trapped in the cords of Billroth (428, 544). Due to the contractility of their actin-myosin filamentous cytoskeleton, leukocytes are able to easily pass through the IES (303, 426). Reticulocytes are somewhat less deformable than mature erythrocytes and therefore will usually spend 1 to 2 days in the cords of Billroth before they are sufficiently mature to resume circulation (518).
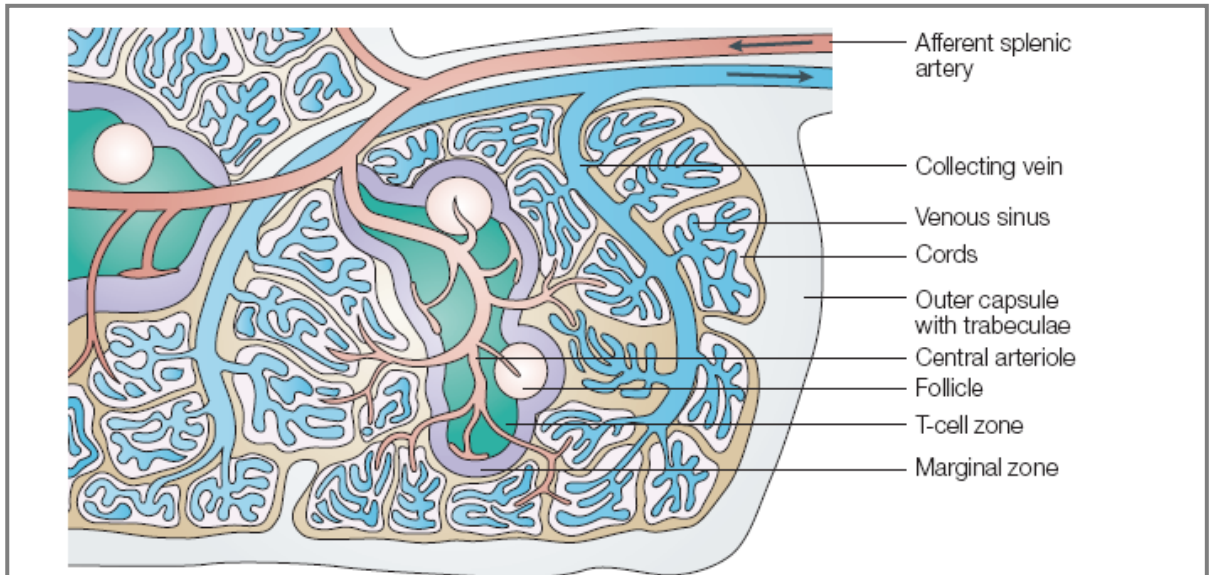
Figure 3.1   Schema of the spleen. The afferent splenic artery branches into central arterioles, which are sheathed by white-pulp areas; these white pulp areas consist of the T-cell zone (also known as the periarteriolar lymphoid sheath, PALS), arterioles and B-cell follicles. The arterioles end in cords in the red pulp, from where the blood runs into venous sinuses, which collect into the efferent splenic vein. The larger arteries and veins run together in connective-tissue trabeculae, which are continuous with the capsule that surrounds the spleen. *[Schematic and figure description as presented in (313)]*
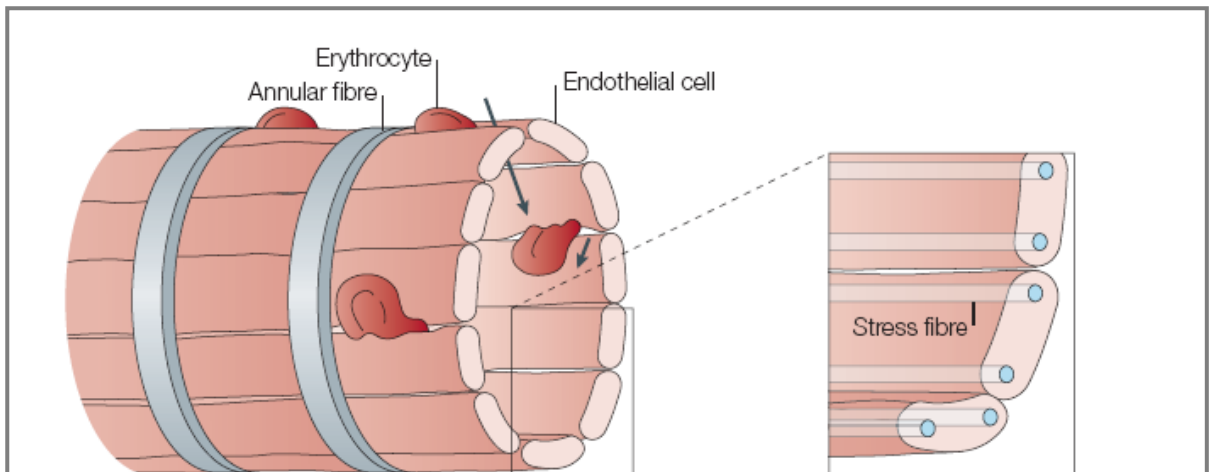
Figure 3.2   Venous sinuses in the red pulp of the spleen. Schema of a venous sinus located in the cords of the red pulp. Blood from the cords collects in the sinuses (shown by arrows). The venous sinuses consist of a lining of endothelial cells that are positioned in parallel and connected by stress fibres to annular fibres. Contractility of the stress fibres allows the formation of slits between the endothelial cells, thereby regulating the passage of blood and blood cells from the red-pulp cords into the sinuses and back into the venous system. Because the red-pulp cords contain a large number of macrophages, ageing erythrocytes that are no longer able to pass through the slits are phagocytosed. *[Schematic and figure description as presented in (313)]*

The immune activity of the white pulp may be attributed to its architecture of clusters of T and B lymphocytes, mononuclear phagocytes and reticular cells. All cell types have an active role in the regulation of immune function and host defence through antibody and cytotoxic cell production (84, 441). When foreign bodies stimulate antigen presenting cells (including monocytes, macrophages or dendritic cells) in the marginal zone of the spleen, adaptive immune responses are most often initiated. After receiving an inflammatory signal from an antigen presenting cell, either from the pathogen themselves or from components of the innate immune system responding to infection, the antigen presenting cells migrate deep into the white pulp and activate naïve and memory T cells which are clustered around the periarterial lymphatic sheath (97, 290, 497). The T lymphocytes are then responsible for the generation of an immunological defence against the foreign organism (428).

3.1.1.1.1 Removal mechanisms of intraerythrocytic bodies

To maintain a healthy population of circulating erythrocytes, the spleen removes those with reduced deformability, IgG sensitisation and those containing intraerythrocytic particulate material by three distinct processes (312, 428, 517).

(1) Phagocytic mechanisms due to IgG sensitization of erythrocytes (428, 544).

(2) Trapping of deformable erythrocytes in the cords of Billroth (97).

(3) Removal of intracellular particles through splenic pitting mechanisms (20, 289, 312, 346, 428).

3.1.1.1.1.1 Splenic pitting

The complex architecture of the cords of Billroth results in a reduced blood flow through the red pulp which creates a hypoxic, hypoglycaemic environment, that is lethal to aged erythrocytes or those with damaged membranes (544). The reduced blood flow through the cords is also important for phagocytosis as it prolongs the exposure of erythrocytes to the splenic macrophages, increasing the time for detection of surface antibodies or intraerythrocytic inclusions. Once detected by a macrophage, it is thought that attachment between the erythrocyte and

macrophage occurs and the inclusion/antibody is removed along with their cell's outer membrane, in a process known as splenic pitting (312, 428, 544). Although this is the most accepted theory, another pitting mechanism has been suggested in which pitting occurs whilst the cell is attempting to traverse the IES. In this mechanism, erythrocytes containing intracellular bodies attempt to cross the IES, however, they become stuck with the healthy portion of the cell through the IES and the intracellular body on the cord side. It is at this stage that the body is 'pitted' from the cell by splenic macrophages or other phagocytic cells (290, 426). Either way, it is believed that the erythrocyte, from which the inclusion was removed, is then returned to the circulation and continues its normal circulatory functions albeit possessing a slightly more fragile membrane. The most commonly removed intracellular bodies in this process include Pappenheimer bodies, Howell Jolly bodies, Heinz bodies and mature or damaged malarial parasites (290).

3.1.1.1.1.2 Immune response

Although the precise protective mechanisms of the spleen are not completely elucidated, as the body's largest lymphoid organ it is a major site of antibody production (428, 453). Extensive research has provided valuable evidence that in both humans and animals the spleen clears erythrocytes and foreign matter, through both rheological and immunological means (453). However, the precise processes involved in the production of antibodies towards malaria parasites is yet to be conclusively identified (289, 497). The most accepted theory is that in the removal of erythrocytes with particulate material, the immunological process of opsonisation occurs, this being the alteration of target cells to aid in more effective and efficient removal. This involves the binding of IgG antibodies to the surface of an erythrocyte containing particulate material. When opsonisation occurs, the cell is detected by splenic macrophages and circulating neutrophils and is removed by phagocytosis (97, 123, 194, 221, 240).

3.1.2   Removal of malaria parasites

As the majority of asexual *Plasmodium* parasites dwell within erythrocytes and reticulocytes, the spleen plays a central role in host defence. This occurs through

the elimination of parasites by recognition of altered erythrocyte deformability after parasite invasion, IgG sensitization of erythrocytes as due to the insertion of foreign antigens into erythrocyte membranes, modulating parasite antigen expression as well as cellular and humoral immune responses of the host (221).

Recent studies in humans suggest that during both acute and chronic malaria infections the splenic structure, cellular composition and function may change, which appears to be of increasing importance in the stimulation of both rheological and immunological functions of the spleen (137, 289, 290, 518). During the course of a malaria infection, increased numbers of parasite-specific antibody forming cells have been detected in the spleen.   It was observed that the total population of T-cells (which are native to the white pulp) increased, resulting in a heightened rate of immune reactivity against the parasites. It was also recorded that there was a marked increase in the number and function of splenic macrophages, cytokines produced by CD4+ T cells and other lymphocytes present within the red pulp (20). These findings suggest that there was an interrelationship between the observed stimulation and activation of splenic components and the presence of increased parasitic load within the host circulation (20).

3.1.2.1 Splenic pitting mechanisms

Studies using *P. falciparum* suggest that the elimination of mature parasites from the peripheral circulation involves two spleen dependent processes, with the primary process very similar to the sequestration mechanisms used to protect parasites against removal.   The primary process involves the adherence of erythrocytes containing mature stage parasites (parasitised red blood cells; PRBCs) to capillary and post-capillary venular endothelium through interactions between receptor molecules on the endothelium and parasite ligands found on the surface of the infected erythrocyte (20, 97, 347). The parasite ligand ring-infected erythrocyte surface antigen (RESA) is associated with the dense granules of the merozoite which is deposited in the erythrocytic membrane during cell invasion and is therefore present from an early stage of parasite maturation (290). Late stage parasites, such as trophozoites and schizonts, insert PfEMP1 neoantigens into the erythrocyte

exterior membrane. Both of these parasite antigens act in parasitic antigenicity and as ligands mediating the attachment of the cell to venule endothelium (290). Adherence of PRBCs to the endothelium stimulates a secondary process leading to their removal from the peripheral blood through splenic clearance mechanisms after the detection of the endothelium-ligand interactions (20, 129). Further evidence supporting these mechanisms was obtained in human studies using radio-labelled infected erythrocytes which demonstrated that the spleen removes both infected erythrocytes and those sensitized by IgG via the two spleen dependent mechanisms (96).

Studies in the 1960s suggested that the spleen could remove intraerythrocytic parasites leaving the host cell intact through splenic pitting, however, parasite clearance in human malaria was previously considered to be through the obligatory destruction of the parasitised erythrocyte (96). The theory of splenic pitting was further demonstrated by Chotivanich et al. (96) who used immunofluorescent staining with antibodies against *P. falciparum* ring-infected erythrocyte surface antigen (RESA) to show an increase in the number of circulating erythrocytes that detected positive to RESA (showing parasitisation) but did not contain an intracellular parasite (290). These results clarified that splenic pitting was the main route of removal of circulating parasites after antimalarial therapy, which also explains why after drug treatment in some patients with hyperparasitaemia, the parasite count falls dramatically but the red blood cell count remains stable or only slightly depressed (20, 96, 321).

Despite the observed stability in red blood cell counts after treatment with antimalarials, severe anaemia is one of the most lethal complications, particularly in children, with *P. falciparum* infection (133, 399, 528). However, it is recognised that the anaemia of malaria is multifactorial (316). Various studies describe potential mechanisms for malarial related anaemia including increased surface IgG (528), deficiencies in complement regulatory proteins CR1 and CD55 potentially leading to complement mediated lysis (468, 528), increased red blood cell susceptibility to phagocytosis (3, 399, 528) and insufficient erythropoesis (87) to name a few.

Although the precise mechanism of malaria related anaemia is unknown, it is widely acknowledged that loss of parasitised erythrocytes does not account for the degree of anaemia seen in many patients (316, 399). In fact, a population based study demonstrated that whilst 18% of patients (750 of 4,007) in Thailand presented with anaemia, the loss of parasitised red blood cells only accounted for <10% of the overall blood cell loss (399).

3.1.2.2 Immune response

In malaria infections the protective immune response stimulated in the spleen may be demonstrated by observation of an increased susceptibility and severity of infection observed in splenectomized animals (154, 156, 401). Immunity provided by the spleen appears to be directed mainly against intraerythrocytic parasites. Recent studies have demonstrated that splenectomy does not affect host susceptibility to sporozoite-induced infections or the development of the subsequent exoerythrocytic stages of the parasite (453). The mechanisms of immunological protection against malaria infections are not well understood, but it is suggested that the spleen is the major site of production of protective antibodies, immune defence and the production of antiparasitic antibodies during malarial infections (60, 70).

While the natural resolution of a malaria infection is highly dependent on the rheological removal mechanisms of the host, the presence and activity of antimalarial antibodies is also thought to be of major importance (518). There is evidence that parasitised erythrocytes, particularly those that harbour multiple parasite stages, bind a specific antibody on the infected erythrocyte membrane. The antimalarial antibody, an IgG isotype, results in the increased recognition of the infected erythrocytes to cytotoxic T cells and phagocytic macrophages, leading to the removal of the parasite through phagocytic processes (240). Phagocytosis of parasite debris and parasitised erythrocytes has also been demonstrated in antigen-presenting cells, including macrophages, monocytes, and dendritic cells, in the marginal zone of the spleen which may also stimulate adaptive immune responses (453). Evidence from animal models also shows the importance of the host's

defence against malaria infections. Antigen presentation and recognition during the initial infection occurs primarily in the splenic macrophages with the spleen continuing to play an important role in the immune responses of the host until cure or death (62, 303, 426). In experimental investigations performed by Hunter and colleagues (233), the process of opsonisation and subsequent phagocytosis was observed *in vitro* using a model of *P. berghei* schizont-infected erythrocytes and rat macrophages. It was suggested that the findings in the experimental procedure could be correlated with processes observed in the clearance and organ sequestration of parasitised cells *in vivo* (124).

3.1.2.3 Parasite defence mechanisms to splenic clearance

In order to both maintain and increase parasite biomass in the infected host, *P. falciparum* parasites have developed defence mechanisms to prolong the survival time of the parasite within the human host by allowing the parasite to mature and reach schizogony (97). Referred to as parasite sequestration, mature *P. falciparum* parasites insert neoantigens into the surface of the infected erythrocyte which enables them to avoid passage through the splenic cords. Of the four established human malaria parasites, *P. falciparum* is the only species which has defended itself from splenic clearance by sequestration, and as a result is the parasite species responsible for the majority of severe malaria deaths (221).

Throughout its asexual blood stage cycle the *P. falciparum* parasite decreases the possibility of detection through maintenance of erythrocyte membrane integrity or as it matures, insertion of neoantigens into the erythrocyte membrane (129). The immature ring-form parasite, present in the first 6–8 h of the blood stage cycle, has a small, soft morphology that does not perturb the erythrocyte membrane configuration or express parasite antigens on the erythrocytic membrane. Consequently, the invaded erythrocyte maintains its integrity and avoids detection or recognition by the splenic macrophages (129, 221). As the parasite matures, the young flexible form develops into a larger more rigid parasite that is more likely to cause damage to the host cell function and membrane integrity. These changes increase the likelihood of recognition by the host's sensitive defence mechanisms.

To compensate for its increased susceptibility, the parasite inserts specific neoantigens (including *P. falciparum* RESA and PfEMP1) into the host erythrocyte cell membrane forming electron-dense protrusions on the surface of the host erythrocytes (129).

Through interaction of the specific parasite ligand and endothelial receptors, the parasitised erythrocytes have the ability to cytoadhere to the vascular endothelium, particularly in organs such as the brain, heart and kidney (20, 97, 346). Consequently, the parasitised erythrocytes anchored to the epithelium do not pass through the splenic filtration system, thereby reducing the likelihood of their detection and removal from the host. Such deep vascular sequestration is thought to be a major factor in the beginning of vital organ dysfunction and may also contribute to the rapid development of anaemia in the host (97). As a result of sequestration in *P. falciparum* infections, only ring-form parasites may be observed in the peripheral blood (320). If damage occurs to the malarial parasites during their life cycle, usually due to antimalarial therapy, the parasites' host defence mechanisms will be affected leading to their rapid detection by splenic macrophages and roving phagocytic cells resulting in their subsequent removal (320).

3.1.3   Effect of Splenectomy

As the spleen is a soft encapsulated organ it remains relatively unprotected within the abdominal cavity. Consequently, the spleen is the most common intra-abdominal organ to be injured, often requiring its removal in an attempt to stem internal bleeding (62). Whilst low velocity blunt trauma, most often due to domestic violence, assault or automobile accidents is the primary cause for splenectomy, splenic removal may also be required in cases of splenomegaly or cellular damage as a result of infection, inflammation or invasion by cancer cells (36, 62, 289, 517, 542).

Although the filtration and immune responses of the spleen are important in the regulation of homeostasis, the life expectancy of a person may not be impaired

after splenectomy (331). Currently, the only documented consequences of splenectomy are related to immune function and a decrease in immunity (69, 195, 290). Surgical removal of the spleen has been shown to result in a reduced clearance of particulate antigens, be they intracellular (malaria) or extracellular (bacteria); diminished response to new antigens, particularly polysaccharides; impaired phagocytosis of unopsonized and opsonized bacteria and cells; and decreased levels of tuftsin and poperdin (246, 447, 517). Although lymphoid organs involved in immune defences remain (eg. lymph nodes), the response time to invading bacterial or pathogenic bodies is delayed. This may result in a splenectomized patient having a far more severe and sometimes life-threatening infection, which before splenectomy may have been quickly resolved (97, 240). Blood filtration, once a major function of the spleen, is compensated for by the liver where Kupffer cells (perisinusoidal macrophages) undertake the majority of phagocytic activity. After splenectomy, it may be histologically observed that the number of circulating macrophages and Kupffer cells increase as a splenic compensatory mechanism. Although splenectomy results in the prolonged circulation of many foreign bodies or damaged cells, these inferior bodies will eventually be removed through phagocytosis within the liver (97).

3.1.3.1 The effect of splenectomy and malaria infection

During malaria infections the absence of a functioning spleen results in reduced immunological and rheological mechanisms necessary for parasite removal, with high parasite counts observed in asplenic patients up to several months after drug therapy (97, 290, 497). Morphological studies, observing the circulating parasite population, suggested that the majority of parasites remaining in the circulation were (in most cases) no longer considered viable, thereby reducing the risk of further infections with these parasites (97). These studies were based on microscopic comparisons of drug affected parasites and those not exposed to any drug. The results showed an identifiable change in parasite shape, density and structure. It was found that the shapes of the persistently circulating drug affected parasites in asplenic patients were similar to those of parasites detected in an *in vitro* cell culture exposed to the antimalarial artesunate. The morphological

changes in the parasites included a shrunken cytoplasm, rapidly expanding vacuoles and pyknotic nuclei, which were highly suggestive of a degenerative process directly resulting from the effects of the antimalarial drugs on the active parasites. It was concluded that although the parasites were present, they were no longer viable (97).

It has also been shown that during malaria infections in monkeys (particularly *P. knowlesi* (138, 221)*, P. falciparum* (45, 123, 221) *and P. fragile* (5, 34, 138, 240)) the spleen is essential for maintaining the expression of parasite antigens on the surface of the infected erythrocytes, including those cytoadherent ligands that are essential for parasite sequestration (449). These studies have demonstrated that the parasite antigens are not present on the erythrocytic membranes in splenectomized animals and as a result the infected cells from splenectomized animals are unable to sequester *in vivo* and do not bind to endothelium or melanoma cells *in vitro*. However, the ability to cytoadhere and express surface antigens is restored when these parasites are passaged into intact hosts (449). A number of clinical cases also correlate with the animal findings as in these malaria-infected splenectomized patients all intraerythrocytic developmental stages were observed in the peripheral blood smears suggesting that the mature parasites are not sequestering (5, 34, 138, 240, 290). This may imply that the parasite has the capacity to determine whether or not it is required to activate its defence mechanisms dependant on the state of splenic clearance. Although this possibility has not been investigated further it could be an important observation, especially when considering new drug developments, as a lack of sequestration may result in a decreased mortality from cerebral malaria.

3.1.3.2 Asplenic model of malaria infection

In experimental and naturally occurring malaria models, splenectomy has been found to have an adverse effect on the host's defence against infection, in that normally non-lethal infections become lethal and *P. falciparum* infections are always a life-threatening disease (401). The splenectomized host is also abnormally

prone to other health issues including meningitis, septicaemia and pneumococcal infections (401).

In studies performed in a splenectomized murine model, hosts with innate resistance to infection became susceptible, transient infections became lethal and there was a loss of immunity (290). Consequently, Eling (155) concluded that the spleen should be considered essential for the resolution of an acute malaria infection or, at least for the induction phase of immunity, to both the avirulent and virulent malaria parasites. This conclusion was more recently challenged based on several experimental studies performed in Saimiri monkeys and clinical cases (401, 449, 542) where it was suggested that the spleen is not essential for the resolution of malaria infections. In a primate experimental model Pye et al. (401) used a total of 31 monkeys, of which 6 were splenectomized. Each monkey was infected with an inoculum of 10^7 parasites of the Indochina 1/CDC malaria strain of *P. falciparum*. Results demonstrated that the splenectomized monkeys had higher peak parasitaemias and longer duration of detectable parasitaemia (about 20 days) compared to intact monkeys, which had parasite duration of 11–14 days. In the splenectomized population several of the animals required treatment with mefloquine when their parasitaemia exceeded 10% of the total erythrocyte count. Mefloquine treatment was not required for any of the intact monkey population, to aid in parasite resolution (290). The researchers reported an interesting and unexpected observation in the splenectomized monkey population in that a number of the animals resolved their infection without antimalarial drug intervention. This indicated that the initiation of effective antimalarial immune responses were independent of the spleen (62, 124, 290). The observations made by Pye et al. (1994) were further supported by studies conducted in patients with *P. falciparum* infections (124). In the human studies the humoral and cellular immune responses to blood-stage antigens in splenectomized patients were similar to those individuals with spleens. Thus, the spleen may not be essential for the processes leading to parasite clearance in partially immune, splenectomized patients (449, 542). It may therefore be suggested that the involvement of the spleen in the resolution of malaria infection is important but not essential.

To date only a limited number of case studies describing the pharmacodynamic response to antimalarial therapies in the asplenic patient have been referenced (34, 62, 138, 194, 240, 290, 432, 449, 497). However, despite similar antimalarial therapies being used, the parasitological responses described after antimalarial therapy are inconsistent and often contradictory. For example, Thu et al. (497) described an asplenic patient who presented with a moderately severe malaria infection. Despite intensive antimalarial therapy over a period of 18 days, including the use of oral mefloquine (1 dose), oral artemisinin (6 doses), i.v. artesunate (2 doses), i.v. quinine (9 doses) and oral quinine (12 doses), a rapid elimination of parasites was not observed until 13 days after the initiation of antimalarial therapy, with eventual resolution of infection 18 days after the initiation of therapy (in a patient with a functional spleen expected resolution of infection would be no more than 7 to 10 days with the use of only one of these antimalarial drugs) (497). The conclusion of this clinical case, as with other cases (138), was that the prolonged parasitaemia was a direct result of the patient's asplenic status. In contrast, further clinical cases have demonstrated the clearance of asexual parasitaemia and parasitological recovery from infection after a normal duration of antimalarial therapy (34, 194). In fact, using similar antimalarial regimens, Bach et al. (34) determined in 33 splenectomized individuals that although asplenic status resulted in an increased susceptibility to malaria infection, it did not contribute to the clinical outcome of infection (34). The variability of clinical outcomes described in splenectomized patients could potentially be the result of a number of factors including; prior immunity to the infecting parasite species, infection with drug resistant parasites, selected antimalarial therapy, other pre-existing health problems (including haemoglobinopathies), concurrent bacterial or viral infections and/or the initial level of parasitaemia.

Therefore, although it is acknowledged that comparison of human and animal responses to a particular disease process should proceed with caution, there are several explanations for the parasitological responses in animal species and human infections to differ so significantly. Firstly, the apparently contradictory outcomes described in the range of case studies in splenectomized patients are compounded

by the small number of patients in each report. The largest cohort of splenectomized patients studied for parasitological response was 33 patients with the majority of conclusions drawn from single case studies, from a wide range of clinical manifestations and different patient ethnicities (34, 194, 290, 432, 497). By contrast, animal models are generally more consistent. Unlike the clinical setting, most aspects of infection and antimalarial therapy within animal models are tightly controlled to ensure consistency and reproducibility of results. For example, within an animal laboratory the researcher is able to guarantee infection with a single parasite species, a standardised infecting inoculum, a similar level of parasitaemia at the time of drug administration, constant environmental influences, controlled diet and perhaps most importantly, maintenance of a pathogen-free environment to eliminate the possibility of concurrent infections that may alter the course of antimalarial therapy. Animal models also have the capacity to involve large numbers within each cohort, which is often not feasible in the clinical setting, particularly when studying a specific population.

Secondly, in clinical case studies where delayed parasite removal is noted in splenectomized patients (497), the parasitaemia observed in the peripheral circulation after antimalarial therapy may include dead or drug affected (non-viable) parasites that are still present as a result of the patient's asplenic status (96, 97). Case studies generally fail to indicate whether such a factor was taken into account when determining the peripheral parasitaemia, and even if considered, differentiating the microscopic changes to parasite structure when affected by antimalarial drugs is only reliable if undertaken by an experienced microscopist. Therefore, it is possible that in the case studies where a delayed parasitaemia was observed, the majority of parasites were killed by early antimalarial therapy but their removal from circulation was delayed. If this were the case, parasitological and clinical recovery in the splenectomized patients could have occurred significantly earlier than indicated in the report and if so, the results in these case studies could be similar to results described in animal models. It is also possible that in the absence of a spleen, the compensatory clearance mechanisms of the

liver, observed in simian and rodent models are not as significant, or rapidly achieved, in the human host.

3.1.3.3 Antimalarial treatment strategies

Compared to normal individuals, asplenic people living in malaria-endemic areas are more commonly parasitaemic, and have a delayed clearance of parasites after treatment (542). In addition, an asplenic status can result in a malaria infection being more severe or even fatal (241). Ideally, splenectomized individuals living in endemic areas should take lifelong antimalarial prophylaxis and be immunized against pneumococcal infections, but this is often difficult and prohibitively expensive (87, 388, 543). If the person lives in the tropics, but in an area of low transmission (such as the highlands), malaria prophylaxis must be taken when they plan to travel into an endemic zone (489).

3.1.4   Study Aims

The principal aim of this study was to evaluate the effects of DHA in asplenic and intact mice infected with *P. berghei*. Histopathological examination of key organs was also performed to investigate the role of the liver, and other key organs, in the removal of parasites after DHA treatment in this murine model.

3.2   METHODS

3.2.1   Materials

3.2.1.1 Mice

This study was approved by the Curtin University Animal Experimentation Ethics Committee. Male Swiss mice (5 to 6 weeks of age; average weight 29.5 ± 3.3 g) were obtained from the ARC for all experimental work. Male BALB/c mice (7 to 8 weeks of age; ARC) were used for weekly passage of malaria parasites. All animal handling and housing procedures were performed as outlined in Sections 2.1.1 and 2.2.1.

3.2.1.1.1   Splenectomy procedures

All splenectomy procedures were performed by registered veterinarians at the ARC on 3-5 week old mice. After surgery all mice were observed for a period of 2 weeks at the ARC laboratories before delivery to the Curtin University Animal Holding Facilities.

3.2.1.2 Parasites

P. berghei ANKA parasites were maintained by continuous weekly blood passage in BALB/c mice (Section 2.2.2.1). A standard inoculum of 10^7 parasitised erythrocytes per 100 µL was prepared by dilution of blood harvested from infected BALB/c mice (>30% parasitaemia) in citrate-phosphate-dextrose solution (Section 2.2.2.2) and administered by i.p. injection to infect the experimental mice (Section 2.2.1.4).

3.2.1.3 Drug preparation

DHA (Dafra Pharma N.V., Belgium) was used for all antimalarial therapy experimental procedures. All drug doses were prepared by dissolving the DHA powder in a solution of 60% dimethyl sulfoxide (DMSO) in polysorbate 80 (Tween 80). In order to ensure all DHA powder was completely dissolved and evenly distributed, the drug solution was sonicated for 1-2 min to break down any large particles and aid in dissolution.   When completely dissolved, the drug solution was drawn into 1 mL syringes for administration using a 26Gx½ inch needle.

3.2.2 Study Design

3.2.2.1 Single dose pharmacodynamic study

As clinical case reports show a delayed clearance in peripheral parasitaemia in asplenic patients, the basis of this investigation was to determine if a single dose of DHA would lead to the successful clearance of parasitaemia in asplenic mice. The aim of this study was therefore to compare the pharmacodynamic response of subtherapeutic doses of DHA in malaria-infected intact and asplenic mice.

Asplenic (n=32) and intact (n=32) mice were inoculated with 10^7 *P. berghei* parasites. Groups of asplenic (n=8) and intact (n=8) mice were given DHA

approximately 56 h after inoculation (anticipated parasitaemia 2-5%, confirmed by thin blood film examination) at doses of 0, 10, 30 and 100 mg/kg (Fig. 3.3).

1. NAIVE MICE (n=32)

2.

3. | 0 mg/kg (n=8) | 10 mg/kg (n=8) | 30 mg/kg (n=8) | 100 mg/kg (n=8) |
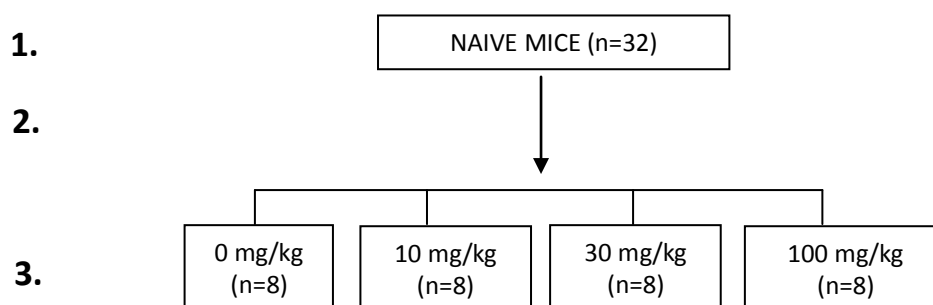
Figure 3.3   Schematic simplifying the experimental protocol for the single dose pharmacodynamic study. 1. Naive asplenic (n=32) and intact (n=32) mice were inoculated with 10^7 *P. berghei* parasites; **2.** Mice were left for a period of 56 h at which time the peripheral parasitaemia was expected to reach 2-5%; **3.** Mice were broken into 4 groups of n=8 mice. Each group of mice received a single dose of DHA (0, 10, 30 or 100 mg/kg) by i.p. injection. Parasitaemia was then monitored through the preparation of regular peripheral blood smears.

To monitor peripheral parasitaemia, tail vein bleeds were performed every 4 h for the first 52 h after drug administration, then twice daily until the time of euthanasia (>40% parasitaemia). Peripheral blood smears were prepared after each tail vein bleed. Once air dried, each smear was stained with May Grunwald Giemsa then observed under 100x oil immersion microscopy.   In addition to determining total parasitaemia, parasites were classified into the four erythrocytic maturation stages (Fig. 3.4; ring-form, early trophozoite, late trophozoite and schizont) according to guidelines from Leiden University Medical Centre (24).

A   B   C   D
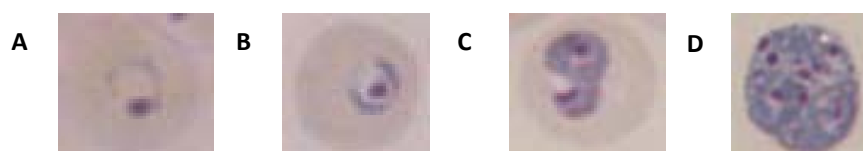
Figure 3.4   Maturation stages of *P. berghei* parasites during the asexual erythrocytic cycle. (A) ring-form; (B) early trophozoite; (C) late trophozoite; (D) schizont.

3.2.2.3 Histopathological study

Although *P. berghei* parasites are known to not sequester in the Swiss mouse model, mature stage parasites cause disruption to the host erythrocyte surface membrane causing it to become rigid and unable to easily transverse the microcirculations of organs, particularly in cases of high parasite density (97, 158). Furthermore, it would be expected that those organs that perform a blood filtration function would demonstrate a higher presence and density of parasites. As the spleen is the primary organ for the filtration of blood, with selective removal of infected cells, it would be expected that this organ would demonstrate the greatest presence of parasites throughout the stages of *P. berghei* infection. However, as the asplenic host does not have the capacity to remove the parasites through splenic 'pitting' or phagocytic mechanisms, it would be presumed that either another organ may perform this function or there would be a lack of parasite removal after drug therapy. Therefore, the purpose of this investigation was to determine the parasite presence and distribution of *P. berghei* parasites, throughout the course of malaria infection, in treated and untreated intact and asplenic mice. After consideration of the function and microcirculation properties of each of the major organs, the liver, spleen, lung and kidney were all selected for observation.

To observe the distribution of parasites throughout different stages of *P. berghei* infection, time-points were selected to reflect situations of light, moderate and heavy infections as well as key time-points such as the time of drug administration in drug treated mice. Two series of mice were investigated in parallel. The first series of mice (Group A; n=24) consisted of untreated intact and asplenic mice where groups (n=4) were euthanized and organs harvested at 0, 36, 56, 76, 96 and 168 h after inoculation. A second series (Group B; n=12) of intact and asplenic mice were given a single i.p. dose of 30 mg/kg DHA 56 h after inoculation and groups (n=4) were euthanized 76, 96 and 168 h after inoculation for organ examination (Fig. 3.5).

1. NAIVE MICE (n=36)

2. GROUP A (n=24)   GROUP B (n=12)

3.
- 0 h (n=4)
- 36 h (n=4)
- 56 h (n=4)
- 76 h (n=4)
- 96 h (n=4)
- 168 h (n=4)

**4.**

5.
- 76 h (n=4)
- 96 h (n=4)
- 168 h (n=4)
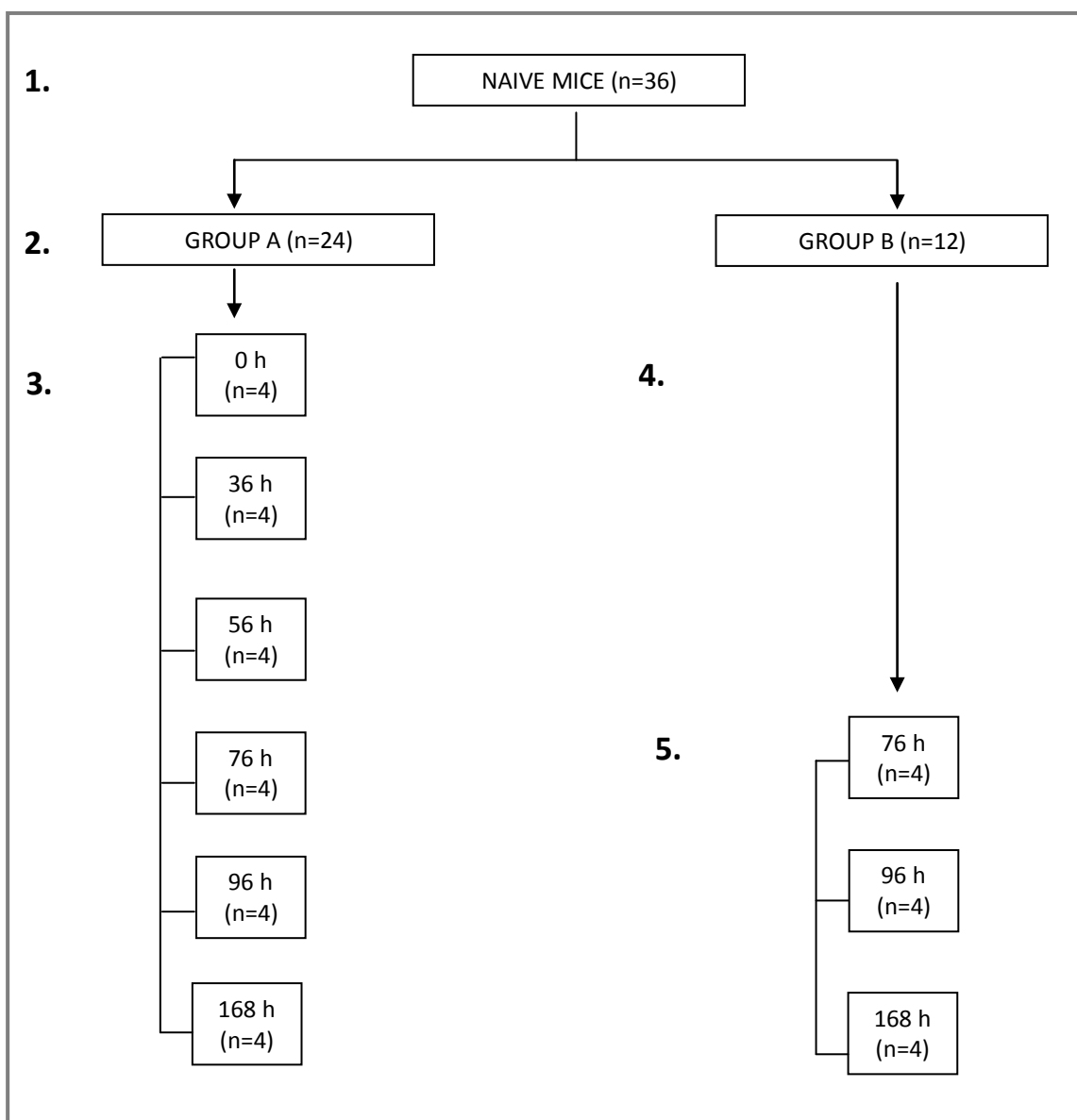
Figure 3.5   Schematic simplifying the experimental protocol for the histopathology study.   **1.**   Naive asplenic (n=36) and intact (n=36) mice were separated into 2 groups, Group A (untreated mice; n=24) and Group B (DHA treated mice; n=12);   **2.** All mice in Group A and Group B were inoculated with 10^7 *P. berghei* parasites; **3.** Group A mice were broken into 6 groups of n=4 mice which were euthanized and specific organs harvested at 0, 36, 56, 76, 96 and 168 h after parasite inoculation; **4.**   Group B mice were left for a period of 56 h (peripheral parasitaemia 2-5%) at which time each mouse received a single dose of 30 mg/kg DHA;   **5.**   Group B mice were broken into 3 groups of n=4 mice which were euthanized and specific organs harvested at 76, 96 and 168 h after parasite inoculation (20, 40 and 112 h after DHA treatment).

At the designated time of organ harvesting each mouse was euthanased by a single i.p. dose of sodium pentobarbitone (Section 2.2.1.6), which was carefully injected as to not perturb or damage any abdominal organs. A slightly higher dose of sodium pentobarbitone was used in this investigation as it is normal practice to harvest the blood volume of the mouse via cardiac puncture and it is desirable to anesthetise the mouse, but not interfere with cardiac function. However, in this experiment, no blood was harvested from these mice before organ harvest (as to preserve the natural distribution of parasites within each of the organs) so a higher dose of sodium pentobarbitone was used to ensure prompt cessation of cardiac and respiratory function. Once it was determined that the respiration of the mouse had ceased, organ harvesting was performed.

To remove the organs from the mouse without causing unnecessary damage, the abdominal cavity was opened as previously described for performing internal cardiac punctures (Section 2.2.1.5).   Using sterile surgical equipment, each organ was removed in its entirety, washed in 0.9% NaCl solution to remove external contaminants, weighed and then observed macroscopically. The organ was then placed in a vial containing a solution of 10% formalin in 0.9% NaCl for fixation. Due to their small size, the spleen, heart and kidney were able to be fixed in their entirety, however, in order to ensure complete and adequate fixation a single lobe of liver (left lobe) was selected for fixation. As the malaria infection progressed, splenomegaly was seen to occur and in many cases only half of the spleen was placed into fixative to ensure adequate fixation.

When preparing organs for histological fixation it is common practice to perfuse the organs with fixative before they are removed from the animal, to minimise the alteration of cell structures as a result of post-mortem effects (426). However, for this study perfusion was deemed unsuitable because forcing the fixative into the organ would disrupt the natural distribution of parasites. Therefore each organ was promptly processed and placed in fixative.

3.2.2.3.1 Histology

After removal from the body, each organ remained in fixative until histological processing took place (a maximum of 6 months after harvesting). For histological preparation the organs were dehydrated in increasing concentrations of alcohol (70 to 100%) and finally embedded in paraffin blocks. The histological preparation took place using a Shandon Hypercenter XP enclosed tissue processor (Thermo Fisher Scientific, Scoresby, Victoria, Australia). Each organ section was removed from its fixation vial and placed in a cassette which was then loaded into the processing racks. When all samples were loading into the processing racks, these racks were inserted into the processor and the cycle initiated. The purpose of the processing cycle was to remove all of the fixative from the tissues through dehydration and replacing it with molten paraffin. This was achieved by taking the samples through cycles of increasing concentrations of ethanol, then xylene and finally molten paraffin. The schedule followed during this process was as outlined in Table 3.1.

Table 3.1   Cycle Schedule for the Shandon Hypercentre XP Enclosed Tissue Processor.

SOLUTION	TIME
70% Ethanol	2 h
90% Ethanol	2 h
100% Ethanol	1 h
100% Ethanol	2 h
100% Ethanol	2 h
Xylene	2 h
Xylene	2 h
Paraffin wax	2 h
Paraffin wax vac	2 h
Paraffin wax vac	30 min
Paraffin wax vac	30 min

Once tissue processing was completed the samples were ready for embedding into paraffin blocks. Embedding took place using the Tissue embedding Centre (Electron Microscopy Services, Hatfield, PA, USA). Solid paraffin was placed in the thermal and dispensing consoles and the temperature gauges set to 65°C to ensure a smooth consistency. The embedding plate was cleaned to remove all foreign contaminants and the cold plate switched on. When all solid paraffin was melted in the thermal and dispensing consoles the tissue cassettes containing processed organ samples were removed from the tissue processor and placed into the wax bath. Plastic paraffin embedding cassettes (25 mm diameter) were used to set the organ sections. The organ sections were embedded using the following process:

1. A plastic embedding cassette was labelled using a 2B pencil and placed on the cold plate facedown and molten paraffin dispensed into the cassette to a height of 2–3 mm.

2. The organ section was placed into the molten paraffin with the cutting surface placed facing down.

3. Liquid paraffin was then slowly dispensed into the cassette as to completely cover the organ section and fill the plastic cassette.

4. Once filled the cassette was left to set on the embedding plate.

This process was followed until all sections were embedded in cassettes and the paraffin set to solid wax. Each cassette was then stacked in cardboard storage boxes and placed in a cool dark room until time of cutting.

Tissue sectioning took place using a Leica rotary microtome (Leica Microsystems, Gladesville, NSW, Australia) with tissue sections floated on a Leica temperature controlled waterbath (Leica Microsystems, Gladesville, NSW, Australia). Blocks were mounted on the microtome and roughly trimmed at 10 μm sections until a good clean tissue face was achieved. Once trimmed, the trimming blade was replaced with a cutting blade, which was thoroughly cleaned and inspected for nicks which could tear the delicate tissues. Before sectioning, the steel cutting plate was chilled using ice cubes to ensure the thin paraffin sections would not stick to the plate. Each block was then sectioned in serial 4 μm slices which consisted of 10 to

15 slices of tissue. The tissue ribbons were gently picked up using steel forceps and floated in a sectioning water bath set at 30°C. The cut ribbon was floated on the heated distilled water until the tissue sections had re-expanded to their original shape and size at which time each section was separated and mounted on a clean glass slide. All mounted sections were air dried overnight.

After 12 h of air drying, to ensure section stability on the slide, the sections were prepared for staining in Haematoxylin and Eosin. The prepared slides were first placed in a drying oven set at 60°C for 45 h to melt excess paraffin wax surrounding the tissue sections. The sections were then ready for deparaffinization and staining following the procedure outlined in Table 3.2.

Table 3.2   Haematoxylin and Eosin staining procedure (Note: Times were variable depending on organ section requiring staining)

SOLUTION	TIME
Xylene	1 min
100% ethanol	1 min
95% ethanol	1 min
80% ethanol	1 min
Deionised water	rinse (> 30 sec)
Haematoxylin	5 min
Deionised water	rinse
Scott's tap water	5-10 dips
0.3% Acid ethanol	5-10 dips
Deionised water	rinse
Eosin	3-5 min
80% ethanol	30 sec
95% ethanol	30 sec
100% ethanol	30 sec
Xylene	> 30 sec

Once stained in Haematoxylin and Eosin the tissue sections were left to air dry then cover-slipped using DePex as the mounting medium. The slides were then placed on a flat surface for 24 h to allow the mounting medium to dry at which time the slides were stored in designated slide storage trays. Organ sections were examined under oil immersion light microscopy for parasite visualization and evaluation of tissue morphology.

3.2.2.3.2   Haemozoin quantification

Several methods have been described for the detection of haemozoin in blood and tissues, including microscopy, spectrophotometry, fluorescence and chemiluminescence (215, 271, 328, 419, 470, 511). However, although microscopic techniques have been further advanced in more recent years, it remains a useful tool only for the identification of pigment rather than as a quantitative measure (271, 339, 419, 470). The use of magnetic field separation, spectrophotometry, fluorescence and chemiluminescence are all more sensitive quantitative measures.

For the purpose of this study an alternative quantification method had to be developed to determine haemozoin density in liver sections as the only tissue samples that were available were the stained histology samples. Therefore, a microscopic method was developed based on that used for the determination of peripheral parasitaemia in blood films. Liver sections from 4 mice per dose group were observed under 100x oil immersion light microscopy. A high resolution digital image was taken for 10 consecutive fields of view from each liver section (Olympus DP70 digital imaging system, Olympus DP70 controller version 2.1.1.183 and DP manager version 2.1.1.163; Olympus Australia Pty Ltd, Mt Waverley, VIC Australia). A calibrated scale (200 µm x 150 µm) was superimposed on the digital image and the numbers of haemozoin deposits (regardless of size) were counted within the defined area. This process was repeated for each of the 10 fields of view with the total count used to estimate haemozoin density (haemozoin deposits per mm^2).

It is acknowledged that whilst the proposed method does not quantify the mass of haemozoin within the liver tissue it does identify the number of haemozoin deposits

thus allowing comparison between liver sections and varying time-points from the asplenic and intact mouse populations.

3.2.2.3.3   Blood pathology

Haematological changes, which are the most common complications of malaria infections, play a major role in the pathogenicity of infections (463). These changes can involve all blood components and include anaemia, white blood cell changes as a result of cytokine induction, thrombopathy and possible coagulopathies (312, 463, 543).   It is also acknowledged that during malaria infection the increased destruction of red blood cells, as well as mechanical destruction to soft organ tissues by the rigid parasites, can contribute to selective changes in biochemistry (182, 258). The liver in particular is an organ which is directly affected by malaria parasites and as a result the release of liver enzymes into blood circulation is often seen to occur as parasitaemia increases (258).

The purpose of this study was to investigate the progressive changes in haematology, liver biochemistry and electrolyte levels from groups of asplenic and intact mice that were either untreated or received a single dose of 30 mg/kg DHA 56 h after parasite inoculation. At each designated time point (0, 36, 56, 76, 96 and 168 h after inoculation) blood was collected by internal cardiac puncture from each infected Swiss mouse (n=6 asplenic and intact mice at each time point). After harvesting, the blood from 3 mice at each time point was placed into EDTA anticoagulant for haematological testing, whilst blood from the remaining 3 mice was placed in lithium heparin tubes for biochemistry.

EDTA samples were processed daily on a Cell-Dyn3200 (Abbott Diagnostics, Illinois, USA) to determine full blood parameters as anaemia and thrombocytopaenia are recognised features of malaria infection and these specific tests are routinely used to monitor disease progression (382). As mouse blood cells are distinctly smaller than human cells, the samples were run under the Veterinary mode using a Mouse parameter setting. As cell separation and counting occurs due to pre-calibrated

MCV values, the use of this package ensured that red blood cells were counted as such, rather than being counted as large platelets in the human mode.

For biochemistry analysis, heparinised blood was immediately centrifuged for 10 min at 3000 g (Biofuge Primo, Heraeus Instruments/ Kendro Instruments Australia Pty Ltd, Lane Cove, NSW, Australia) and the separated plasma was stored in micro-centrifuge tubes and refrigerated at 4°C until analysed at the conclusion of the study. The plasma was separated from the red blood cell pellet to eliminate the possibility of enzyme leaching from the pellet into the plasma which would falsely elevate enzyme levels. The plasma samples were processed in a single batch using a Synchron LX® 20 PRO (Beckman Coulter Australia Pty Ltd., Gladesville, NSW, Australia) for liver function tests (bilirubin, alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (AFP)) as indicators of liver damage associated with malaria infection. The same procedure was performed for mice treated with DHA (56 h after inoculation) using blood that was harvested at 76, 96 and 168 h after inoculation. Both haematology and biochemistry results from intact and asplenic mice were compared to determine differences in disease pathology between the two groups.

3.3   RESULTS

3.3.1   Single Dose Pharmacodynamic Study

3.3.1.1 DHA pharmacodynamics

Single dose DHA produced a prompt decline in parasitaemia, reaching a nadir 24 h after drug administration (Fig. 3.6). The nadir was 2.8, 4.4 and 6.0 fold below starting parasitaemia in asplenic mice and 2.7, 5.1 and 6.9 fold in intact mice at doses of 10 mg/kg, 30 mg/kg and 100 mg/kg, respectively. Parasite recrudescence was observed in all groups of mice. To determine if there was a significant difference in the rate of parasite recovery, the time to reach a 5% parasitaemia after dosing ($t_{5\%}$) was determined by linear interpolation. The value of 5% was selected as the recrudescence comparison time point, as this both reflected the maximum parasitaemia at time of dosing, and a previous study had determined that
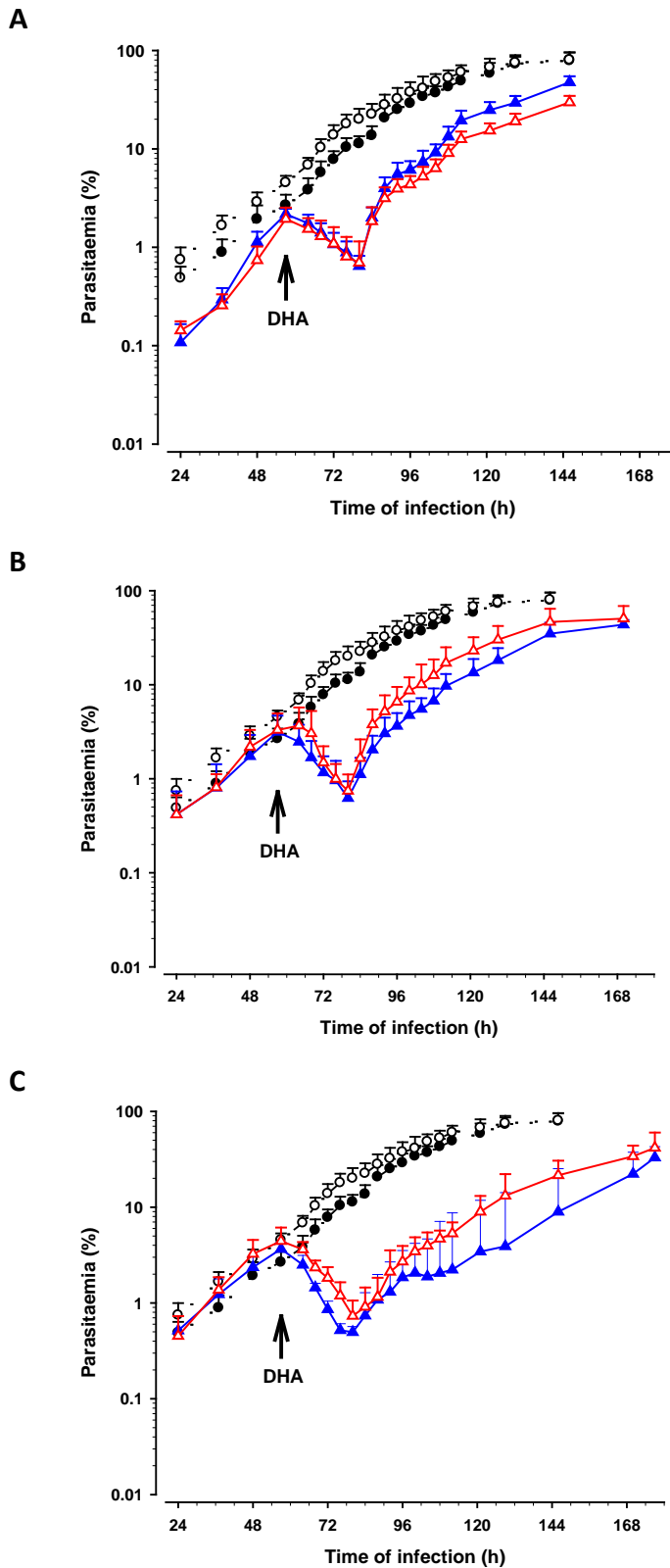
Figure 3.6. Effect of dihydroartemisinin on parasitaemia in intact and asplenic mice. Data are shown as mean total parasitaemia (% of infected erythrocytes) + SD versus time in relation to the dose of DHA. Mice were inoculated with 10^7 parasitised erythrocytes 56 h prior to DHA administration [A: 10 mg/kg DHA, B: 30 mg/kg DHA, C: 100 mg/kg DHA; ● untreated intact mice, O untreated asplenic mice, ▲ treated intact mice, △ treated asplenic mice; n=8 per group].

a recrudescent parasitaemia reaching 5% in mice would likely lead to a fatal outcome, whilst no significant changes would occur in the erythrocyte count (179).

The mean $\pm$ SD $t_{5\%}$ was 39 $\pm$ 5 h, 34 $\pm$ 8 h and 48 $\pm$ 5 h (P=0.003; ANOVA) in asplenic mice, and 32 $\pm$ 3 h, 43 $\pm$ 7 h and 62 $\pm$ 24 h (P=0.004; ANOVA) in intact mice at 10 mg/kg, 30 mg/kg and 100 mg/kg, respectively. There was a significant difference in the $t_{5\%}$ between asplenic and intact mice at each dose studied (P=0.004, P=0.044 and P=0.012 for 10 mg/kg, 30 mg/kg and 100 mg/kg, respectively; t-test).

3.3.1.2 Parasite Staging

No significant difference in staging profiles was observed between asplenic and intact mice, both untreated and DHA-treated (Fig. 3.7). Late trophozoites were the predominant stage at each time point. In untreated mice, early trophozoites and ring-forms comprised the remainder of the total observed parasitaemia up to 2-3 days post-dose (4-5 days post infection; Fig. 3.7, panels A and B). Schizonts became more prominent than the ring-forms as the infection advanced beyond a total parasitaemia of at least 10%. All erythrocytic stages were affected by the administration of DHA, with nadir at 20-28 h (Fig. 3.7, panels C-F), clearly demonstrating that DHA was broadly effective in this *in vivo* model. This finding is consistent with other published findings which have shown that artemisinin derivatives are effective on all stages of the *Plasmodium* parasite (238).
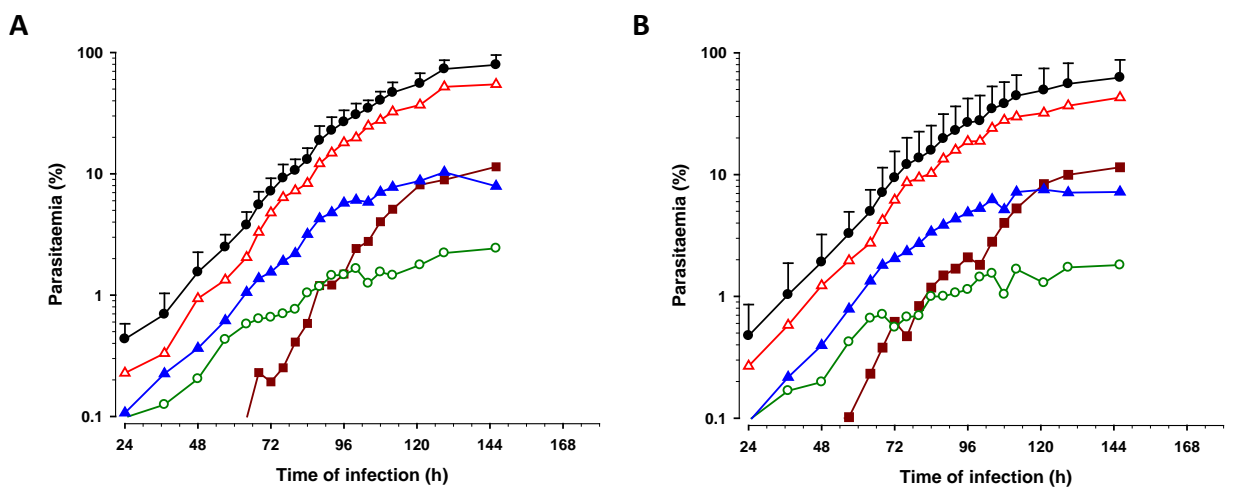
Figure 3.7 Parasite stage differentiation at varying doses of dihydroartemisinin. Data are shown as mean parasitaemia (% of infected erythrocytes) versus time in relation to dose (error bars excluded for clarity; n=8 per group). [A: untreated intact mice, B: untreated asplenic mice]
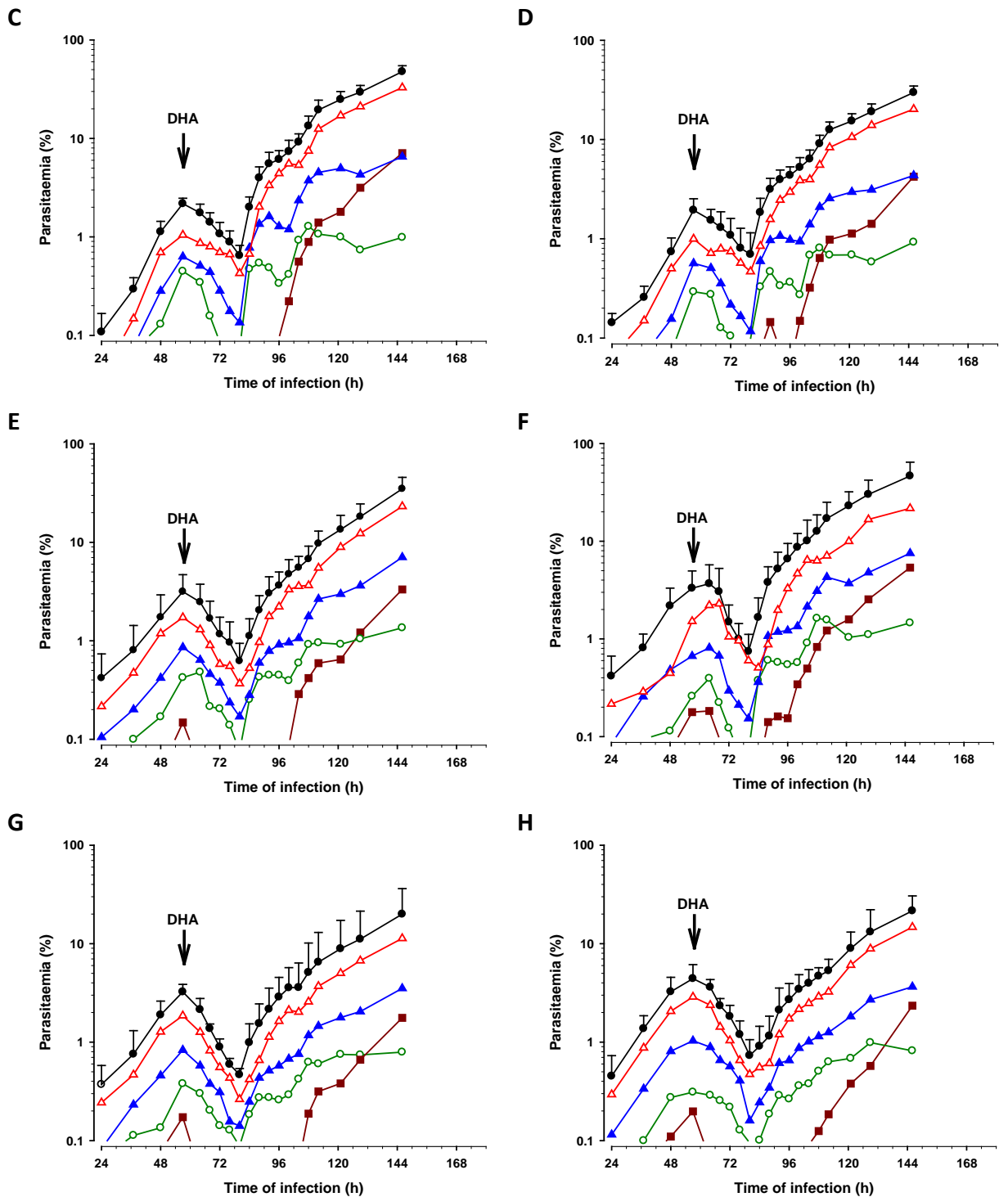
Figure 3.7 Parasite stage differentiation at varying doses of dihydroartemisinin. Data are shown as mean parasitaemia (% of infected erythrocytes) versus time in relation to dose (error bars excluded for clarity; n=8 per group). [C: 10 mg/kg DHA treated intact mice, D: 10 mg/kg DHA treated asplenic mice, E: 30 mg/kg DHA treated intact mice, F: 30 mg/kg DHA treated asplenic mice, G: 100 mg/kg DHA treated intact mice, H: 100 mg/kg DHA treated asplenic mice; ● mean total parasitaemia, ○ ring-forms, ▲ early trophozoites, △ late trophozoites, ■ schizonts].

3.3.2 Histopathological Study

3.3.2.1 Histology

In order to observe pathological changes in tissue sections, as well as the presence of malarial parasites and haemozoin deposits, histological sections of spleen, liver, lung and kidney sections were observed and morphology compared at 36 h (Fig. 3.8), 56 h (Fig. 3.9), 76 h (Fig. 3.10), 96 h (Fig. 3.11) and 168 h (Fig. 3.12) after parasite inoculation. Furthermore, organ sections were compared from mice that received DHA 56 h after parasite inoculation. For comparative purposes, these organ sections were also taken 76 h (Fig. 3.13), 96 h (Fig. 3.14) and 168 h (Fig. 3.15) after parasite inoculation (20, 40 and 112 h after DHA administration).

Liver and lung weights increased as the infection progressed and were significantly greater than uninfected mice at 168 h after inoculation in both asplenic and intact mice (Table 3.3). A similar outcome was observed in DHA-treated asplenic and intact mice (Table 3.3). By contrast, the kidney weight was not affected by malaria infection. The mean proportionate weight of the spleen was significantly greater in the infected mice, compared to the uninfected animals, from 56 h of infection (the time of DHA administration) in treated and untreated mice (Table 3.3).

As anticipated in the intact mice in the study, the spleen increased in size and weight as the infection progressed. Along with haemozoin accumulation, organ histology showed an increase in the cellularity of both red and white pulp and the progressive loss of a defined marginal zone. These expected histological changes were associated with increasing parasite burden (15, 210). The increase in red pulp cellularity is reported to occur as the phagocytic and erythropoietic activity of the spleen is enhanced to replace infected erythrocytes with a healthy population of cells (210). White pulp expansion occurs as the immune function of the spleen is stimulated by the presence of parasites resulting in both lymphocyte and antibody production (15). In asplenic mice, careful examination of the abdominal cavity occurred post mortem to check for any remnants of splenic tissues which may account for the increased parasite clearance. No evidence of residual spleen was found in any of the asplenic mice.

Figure 3.8 Light microscopy of Haematoxylin and Eosin stained mouse organ sections (x40) 36 h after parasite inoculation. Sections are from the spleen of infected mice and liver, lung and kidney sections are from both intact and asplenic mice 36 h after inoculation with *P. berghei* parasites. Arrows demonstrate the presence of haemozoin deposits (↑), parasitised erythrocytes (↑) and liver Kupffer cells (↑).

Figure 3.9 Light microscopy of Haematoxylin and Eosin stained mouse organ sections (x40) 56 h after parasite inoculation. Sections are from the spleen of infected mice and liver, lung and kidney sections are from both intact and asplenic mice 56 h after inoculation with *P. berghei* parasites. Arrows demonstrate the presence of haemozoin deposits (↑), parasitised erythrocytes (↑) and liver Kupffer cells (↑).
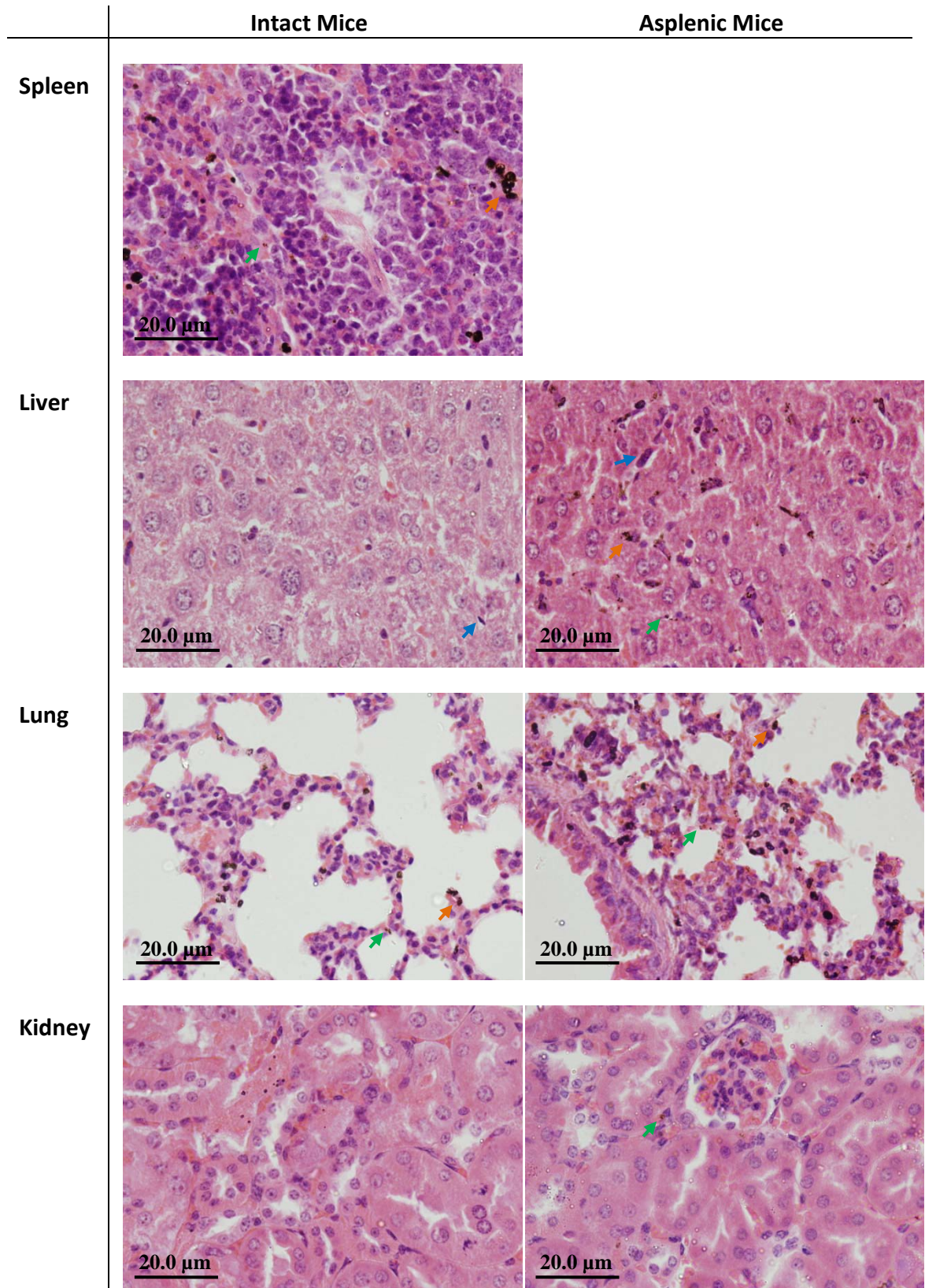
Figure 3.10 Light microscopy of Haematoxylin and Eosin stained mouse organ sections (x40) 76 h after inoculation. Sections are from the spleen of infected mice and liver, lung and kidney sections are from both intact and asplenic mice 76 h after inoculation with *P. berghei* parasites. Arrows demonstrate the presence of haemozoin deposits (↑), parasitised erythrocytes (↑) and liver Kupffer cells (↑).
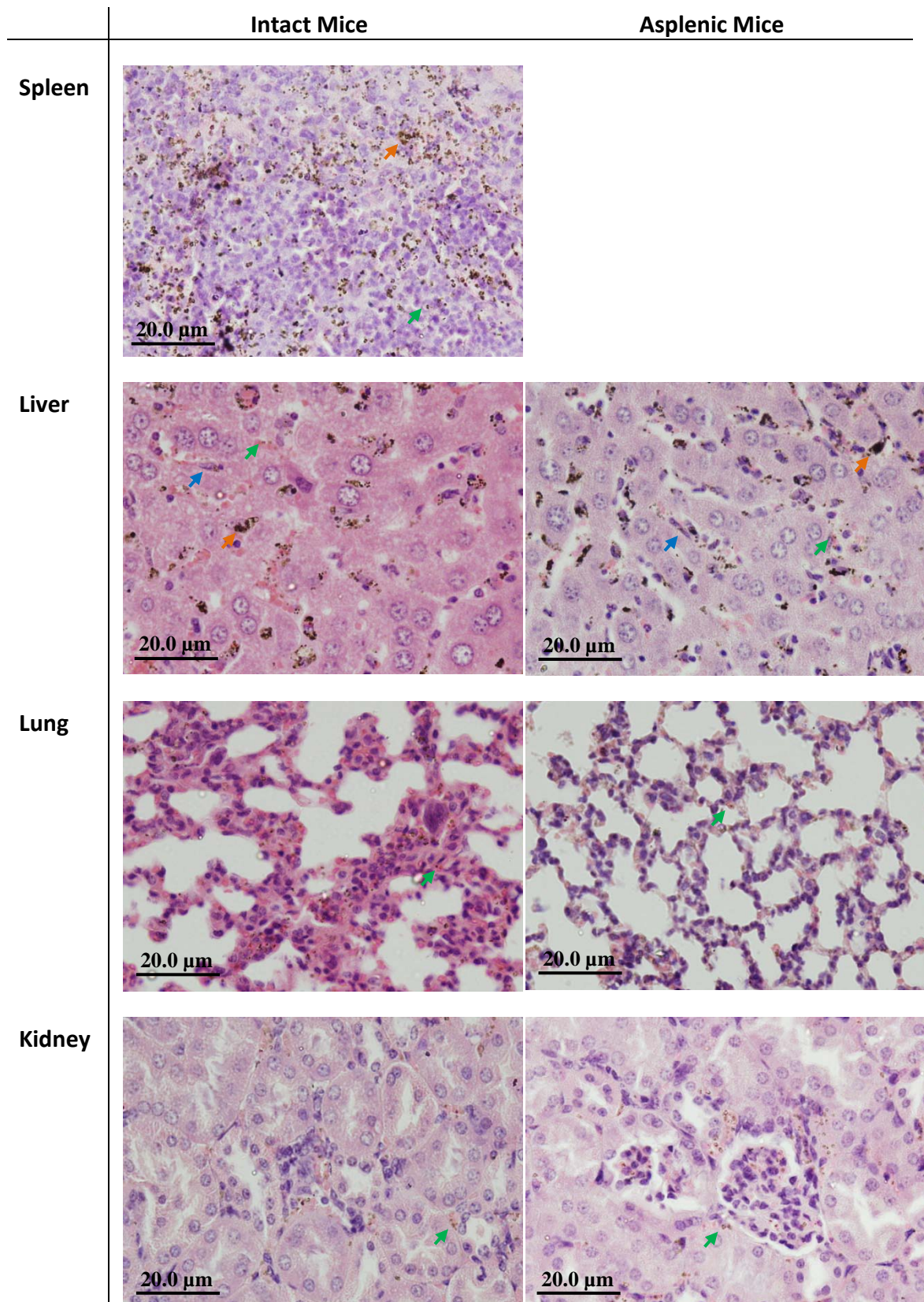
Figure 3.11. Light microscopy of Haematoxylin and Eosin stained mouse organ sections (x40) 96 h after parasite inoculation. Sections are from the spleen of infected mice and liver, lung and kidney sections are from both intact and asplenic mice 96 h after inoculation with *P. berghei* parasites. Arrows demonstrate the presence of haemozoin deposits (⬆), parasitised erythrocytes (⬆) and liver Kupffer cells (⬆).

	Intact Mice	Asplenic Mice
Spleen		
Liver		
Lung		
Kidney		
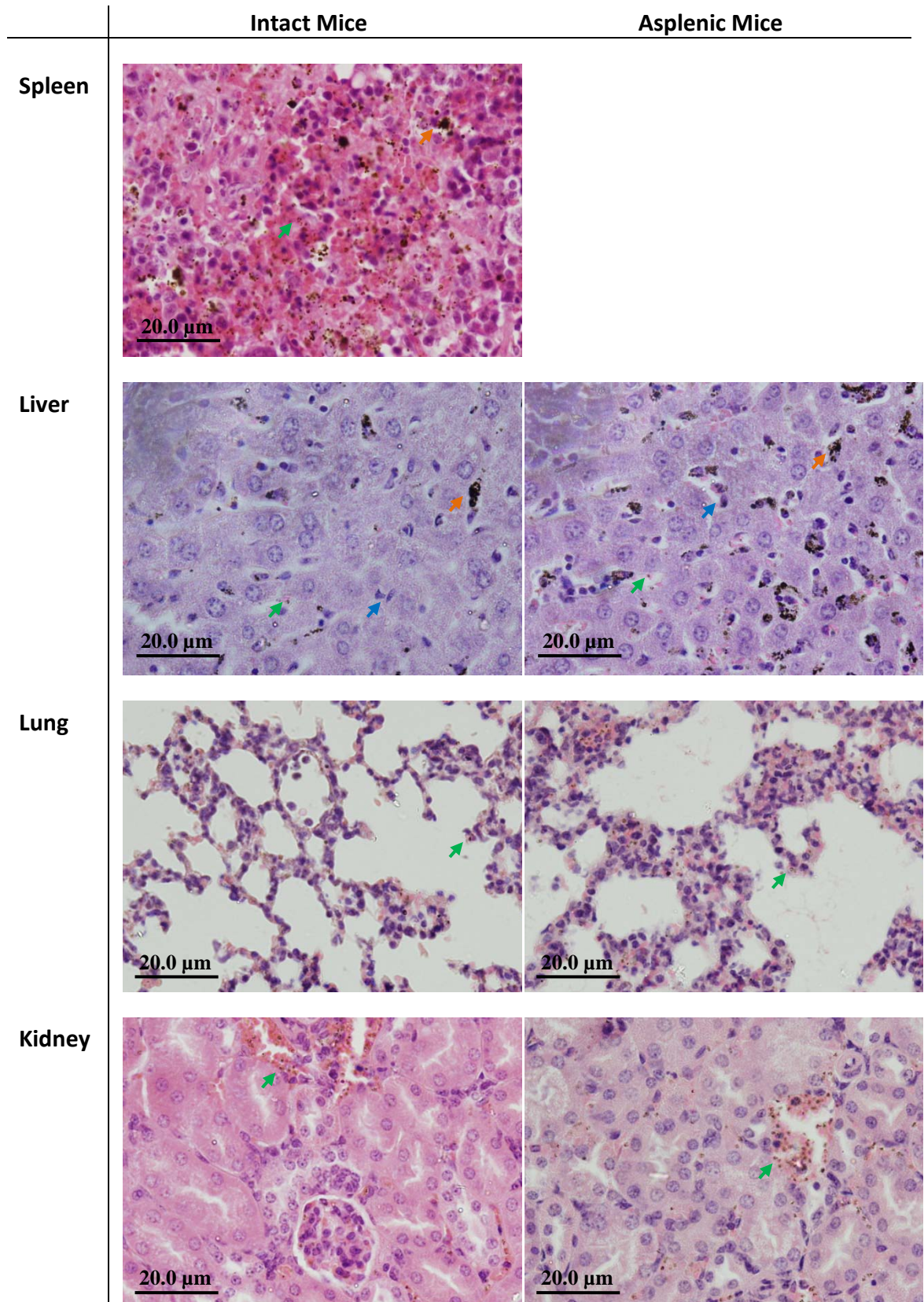
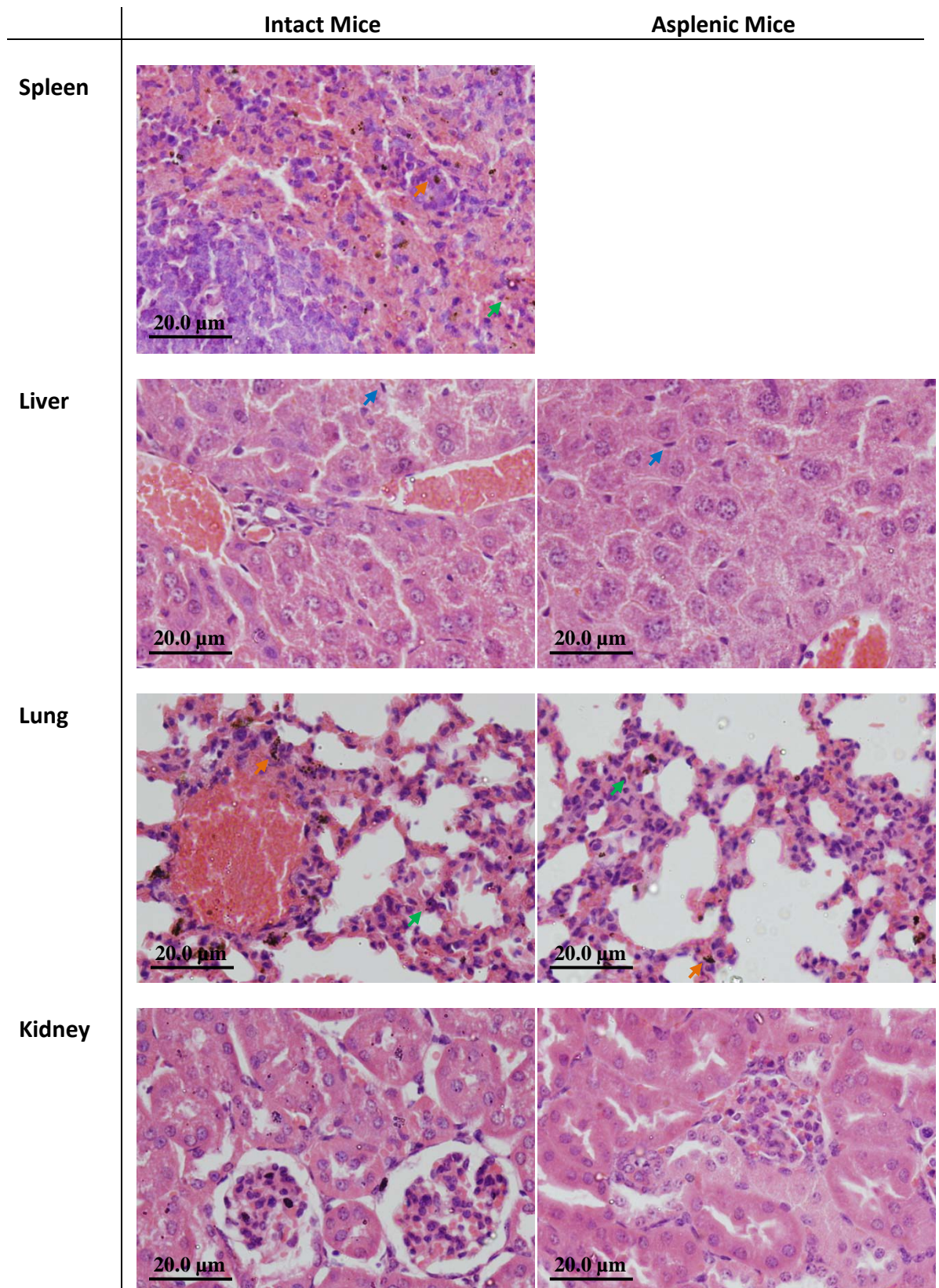**Figure 3.12 Light microscopy of Haematoxylin & Eosin stained mouse organ sections (x40) 168 h after parasite inoculation**. Sections are from the spleen of infected mice and liver, lung and kidney sections are from both intact and asplenic mice 168 h after inoculation with *P. berghei* parasites. Arrows demonstrate the presence of haemozoin deposits (⬆), parasitised erythrocytes (⬆) and liver Kupffer cells (⬆).
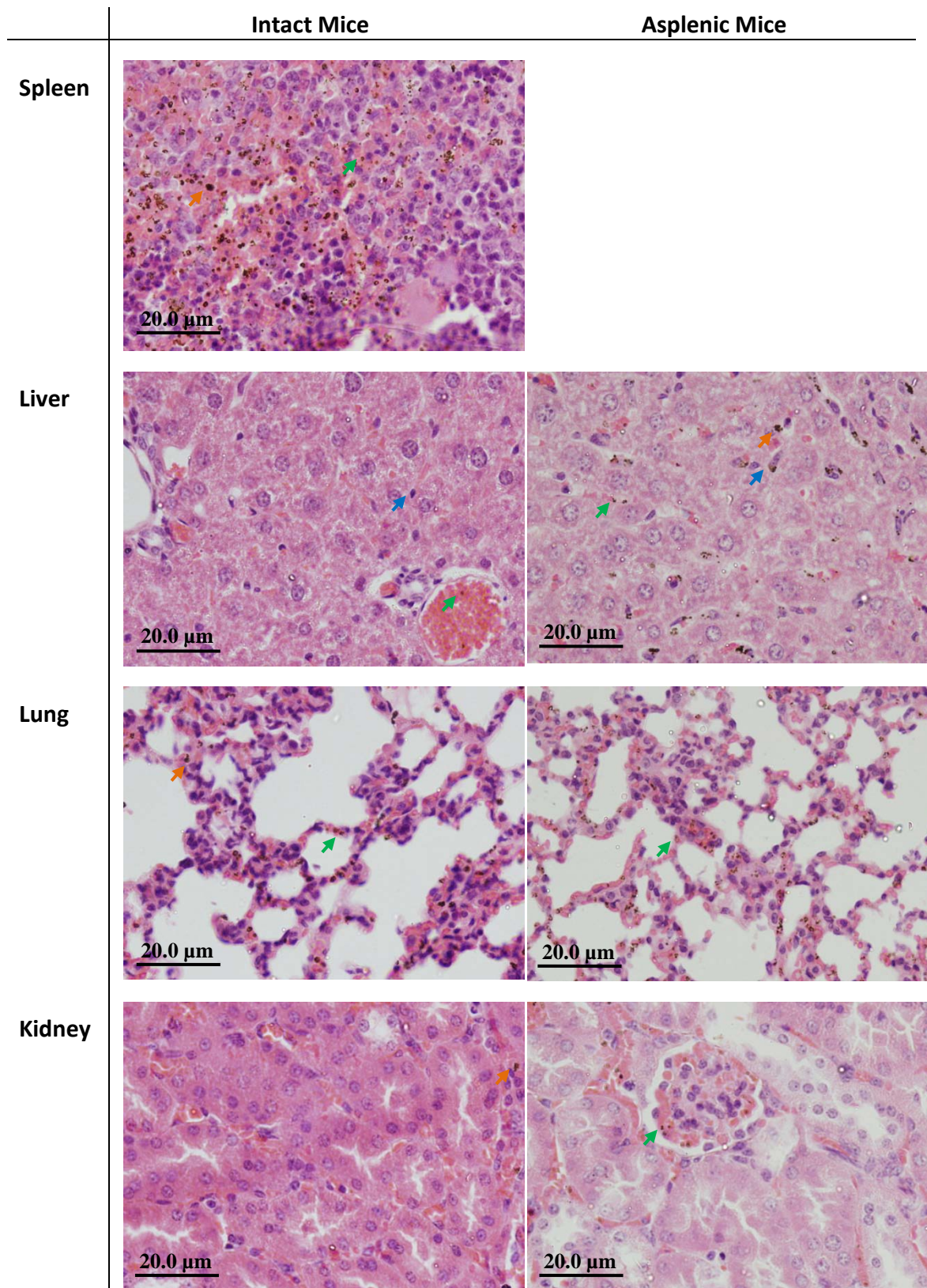
Figure 3.13 Light microscopy of Haematoxylin and Eosin stained mouse organ sections from DHA treated mice (x40) 76 h after parasite inoculation. Mice were inoculated with P. berghei parasites then at 56 h of infection were treated with 100 mg/kg DHA. Sections are from the spleen of infected mice and liver, lung and kidney sections are from both intact and asplenic mice 76 h after inoculation with *P. berghei* parasites. Arrows demonstrate the presence of haemozoin deposits (↑), parasitised erythrocytes (↑) and liver Kupffer cells (↑).

	Intact Mice	Asplenic Mice
Spleen		
Liver		
Lung		
Kidney		
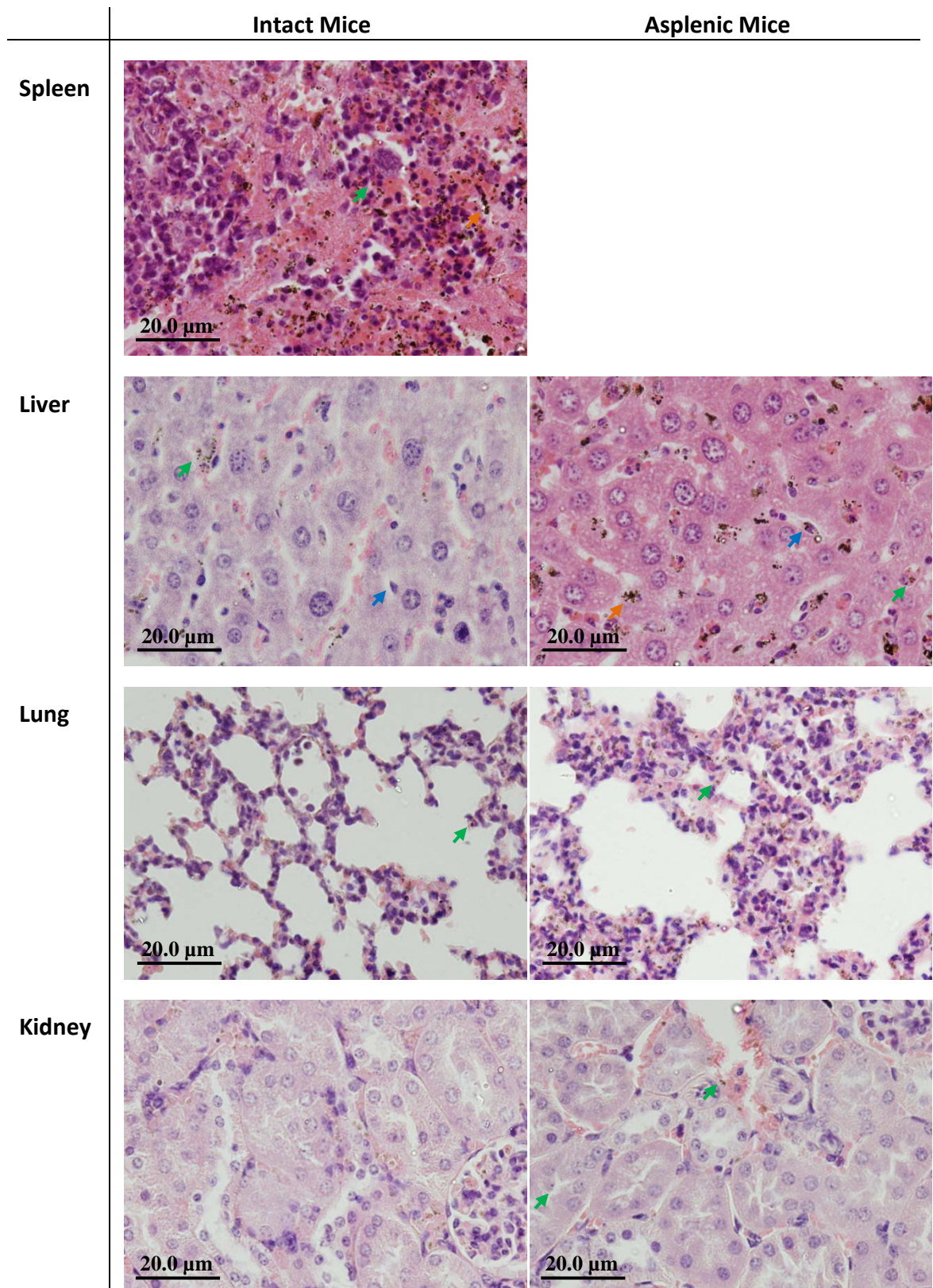
Figure 3.14 Light microscopy of Haematoxylin and Eosin stained mouse organ sections from DHA treated mice (x40) 96 h after parasite inoculation. Mice were inoculated with *P. berghei* parasites then at 56 h of infection were treated with 100 mg/kg DHA. Sections are from the spleen of infected mice and liver, lung and kidney sections are from both intact and asplenic mice 96 h after inoculation with *P. berghei* parasites. Arrows demonstrate the presence of haemozoin deposits (↑), parasitised erythrocytes (↑) and liver Kupffer cells (↑).
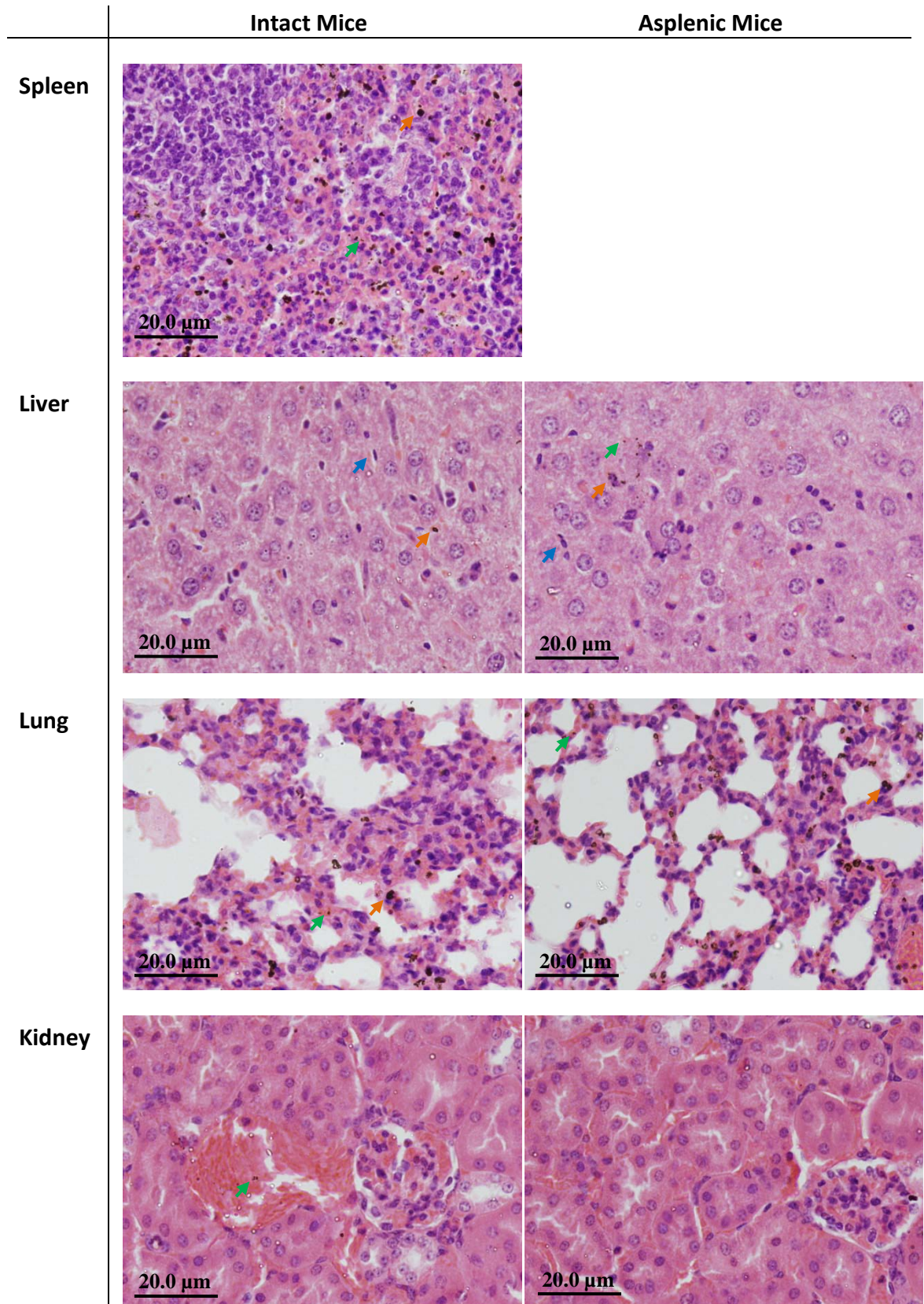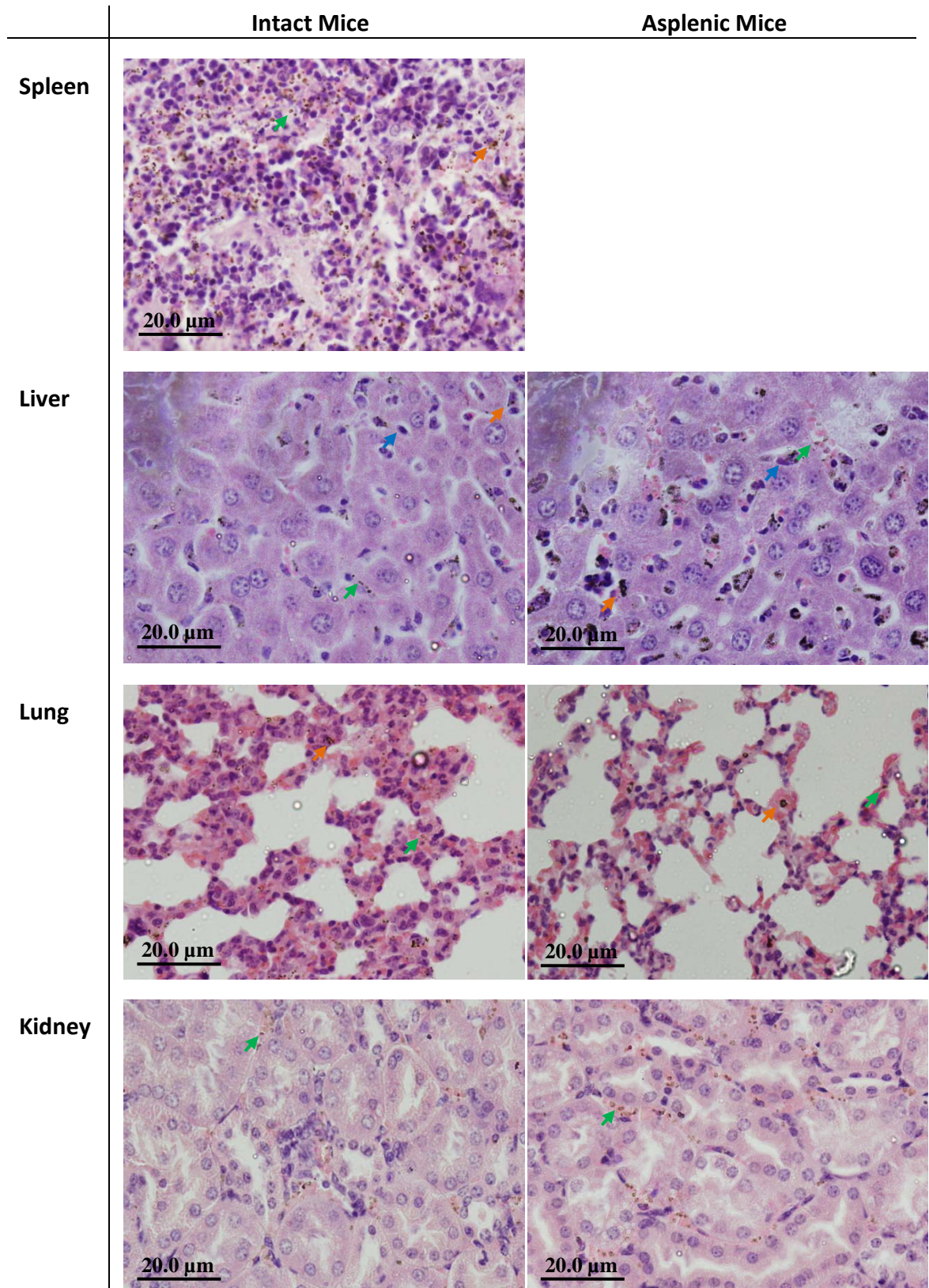
Figure 3.15 Light microscopy of Haematoxylin and Eosin stained mouse organ sections from DHA treated mice (x40) 168 h after parasite inoculation. Mice were inoculated with *P. berghei* parasites then at 56 h of infection were treated with 100 mg/kg DHA. Sections are from the spleen of infected mice and liver, lung and kidney sections are from both intact and asplenic mice 168 h after inoculation with *P. berghei* parasites. Arrows demonstrate the presence of haemozoin deposits (↑), parasitised erythrocytes (↑) and liver Kupffer cells (↑).

Sections of liver and spleen showed that after 56 h of infection, both organs were accumulating parasitised erythrocytes (Fig. 3.9–Fig. 3.15). This was demonstrated by the presence of haemozoin within the splenic cords, an increased number of phagocytic cells in the spleen and liver and Kupffer cell hyperplasia within the liver. In asplenic mice, parasites were observed in the liver as early as 36 h after inoculation (Fig. 3.8) and Kupffer cell hyperplasia became prominent from 56 h of infection (Fig. 3.9). Comparison of liver sections from asplenic and intact mice indicated a greater density of parasites in asplenic mice throughout the infection (Fig. 3.9). An interesting observation made in liver sections taken from asplenic mice was that from 56 h of infection clusters of basophilic staining cells were randomly present within the liver lobules (Fig. 3.16). The clusters of cells could be observed in all asplenic liver sections in increasing size (approximately 30-50 cells) and numbers as the infection progressed. These basophilic staining clusters are not a unique observation as they have been previously described when looking at the increased antibody production within liver tissue during malaria infection in splenectomized rats (Fig. 3.16) (545). Liver sections from intact mice did not demonstrate the presence of the basophilic clusters until after 96 h of infection, and only in sporadic numbers.

Overall, haemozoin density in the liver was significantly greater in asplenic mice, compared to intact mice, and the haemozoin density was greater in untreated mice than DHA treated mice at the same time post-inoculation (Table 3.3). These data provide an indication of total parasite presence in the liver, but may not reflect natural parasite accumulation/extraction by the liver if haemozoin transport between tissues has occurred (179).

The lungs showed parasite presence from 36 h of infection (Fig. 3.8 to Fig. 3.12) with evidence of haemorrhage observed by both gross and histological examination after 76 h of infection. Increasing numbers of macrophages and lymphocytes were observed as the infection progressed in both intact and asplenic mice. The kidneys were generally unremarkable and did not show parasite presence in the tubules until 168 h of infection in both intact and asplenic mice (Fig. 3.11).
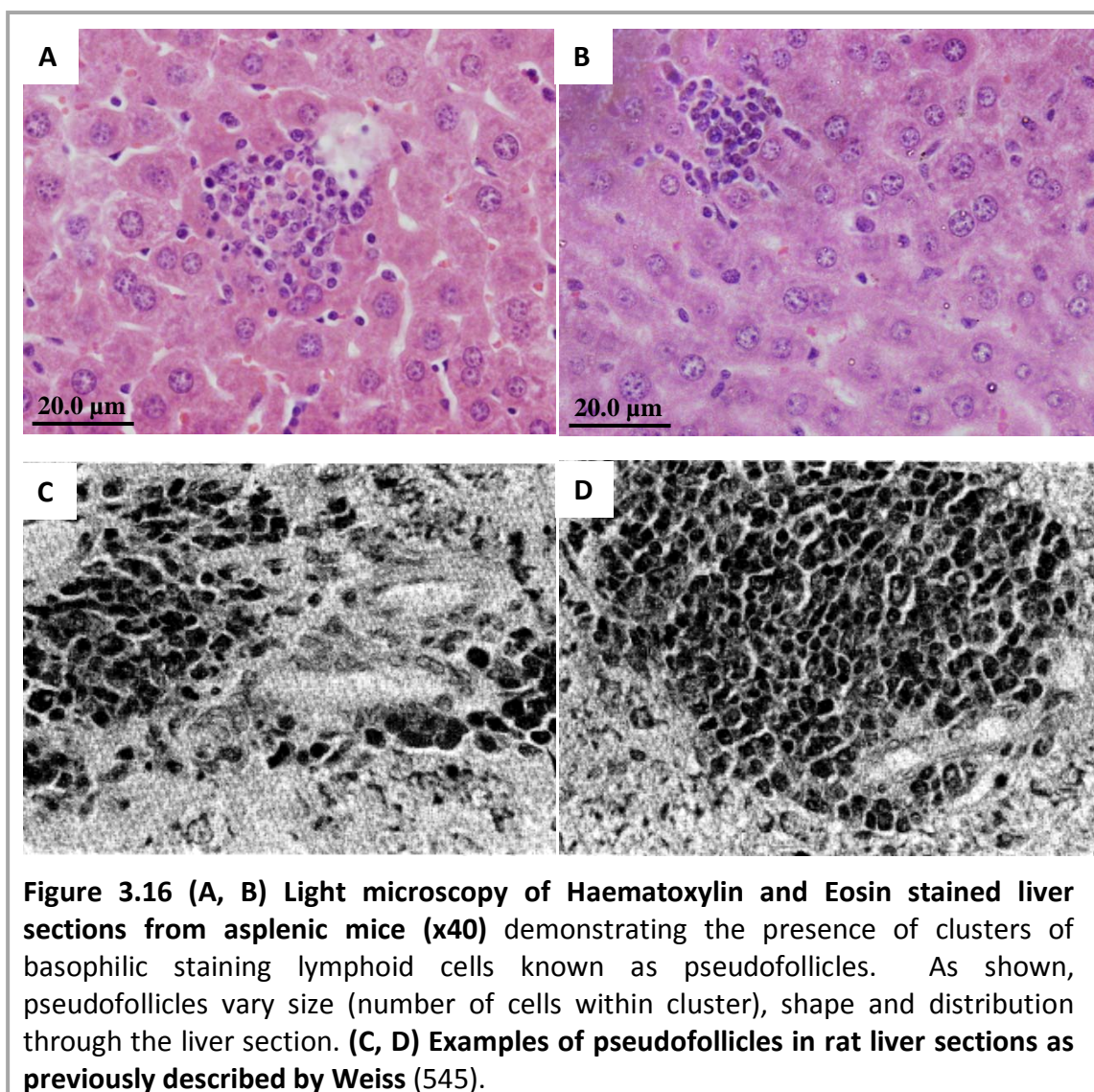
Figure 3.16 (A, B) Light microscopy of Haematoxylin and Eosin stained liver sections from asplenic mice (x40) demonstrating the presence of clusters of basophilic staining lymphoid cells known as pseudofollicles. As shown, pseudofollicles vary size (number of cells within cluster), shape and distribution through the liver section. **(C, D) Examples of pseudofollicles in rat liver sections as previously described by Weiss** (545).

Table 3.3   **Haemozoin deposits per mm^2 of liver from mice inoculated with *P. berghei* parasites**. Treated mice were given DHA 56 h after inoculation. Data are mean ± SD.

	Untreated		DHA Treated	
	Intact (n=4)	Asplenic (n=4)	Intact (n=4)	Asplenic (n=4)
Time after inoculation				
0 h	0	0	-	-
36 h	0	5 ± 4[a]	-	-
56 h	34 ± 10	45 ± 13	-	-
76 h	190 ± 92	532 ± 67[a]	64 ± 34[b]	123 ± 78[b]
96 h	215 ± 145	765 ± 117[a]	127 ± 53	317 ± 87[ab]
168 h	1684 ± 242	1776 ± 107	301 ± 137[b]	809 ± 85[ab]

[a] $P<0.05$ for comparison between intact and asplenic mice at designated time point (t-test); [b] $P<0.05$ for comparison between untreated and DHA-treated mice at designated time point (t-test)

The kidneys and lungs were also examined, with particular consideration of parasite trapping in the microvasculature. No significant pathological change was observed in kidney sections in our study (up to 168 h post-inoculation), suggesting that the kidneys did not have a significant role in the removal of parasitised erythrocytes from circulation. However, there was a significant increase in the mass of the lungs as the infection progressed (Table 3.4). Based on autopsy examination and organ histology, this was apparently due to oedema and haemozoin deposition in the microcirculation of both asplenic and intact mice. Histological examination of lung tissue demonstrated parasite presence from 36 h of infection with evidence of haemorrhage observed by both gross and histological examination after 76 h of infection. Increasing number of macrophages and lymphocytes were observed as infection progressed in both intact and asplenic mice.

3.3.2.2 Pathology

3.3.2.2.1 Haematology

During the course of infection, red blood cell indices progressively changed in both intact and asplenic mice, as would be expected. Although total red blood cell count (RBCC) and haematocrit (HCT) remained stable in both cohorts of mice for the first 76 h of infection, at 96 h, when parasitaemia exceeded 10%, both values steadily declined with significantly decreased values observed at 168 h in both asplenic and intact mice when compared to the RBCC and HCT indices at baseline (Table 3.6). After treatment with DHA no statistically significant difference in RBCC was observed in intact mice throughout the malaria infection, however, a significantly different HCT value was observed at 168 h (Table 3.7). In treated asplenic mice both the RBCC and HCT were significantly different at 168 h compared to both the starting indices as well as when compared to intact mice at the same time point (Table 3.7).

Haemoglobin concentrations declined in both asplenic and intact untreated mice from 36 h after inoculation but this decrease was not statistically significant until 168 h after inoculation (Table 3.6). A similar pattern was observed in asplenic,

Table 3.4 Organ weights as a proportion of total body weight (%TBW) from mice after inoculation with *P. berghei* parasites. Treated mice were given DHA 56 h after inoculation and blood was harvested 20, 40 and 112 h after dosing (76, 96 and 168 h after inoculation). Data are mean ± SD.

Time after inoculation	Parasitaemia (%) Intact (n=10)	Parasitaemia (%) Asplenic (n=10)	Liver (%TBW) Intact (n=10)	Liver (%TBW) Asplenic (n=10)	Lung (%TBW) Intact (n=10)	Lung (%TBW) Asplenic (n=10)	Kidney (%TBW) Intact (n=10)	Kidney (%TBW) Asplenic (n=10)	Spleen (%TBW) Intact (n=10)
Untreated mice									
0 h	0	0	5.5 ± 0.9	5.8 ± 0.1	0.57 ± 0.06	0.60 ± 0.05	0.86 ± 0.12	0.88 ± 0.1	0.31 ± 0.05
36 h	0.41 ± 0.1	0.39 ± 0.2	5.2 ± 0.1	4.9 ± 0.1	0.51 ± 0.14	0.45 ± 0.15[b]	0.71 ± 0.09[b]	0.63 ± 0.12[b]	0.33 ± 0.12
56 h	2.3 ± 0.7	3.2 ± 1.4	5.9 ± 0.1	5.8 ± 0.1	0.74 ± 0.06[b]	0.71 ± 0.08[b]	0.85 ± 0.08	0.81 ± 0.08	0.58 ± 0.07[b]
76 h	9.3 ± 3.4	15.2 ± 9.3	6.5 ± 0.2	6.2 ± 0.1	0.64 ± 0.05	0.72 ± 0.14[b]	0.86 ± 0.07	0.86 ± 0.1	0.62 ± 0.11[b]
96 h	27.1 ± 12.0	28.2 ± 17.1	6.6 ± 0.1	6.6 ± 0.1	0.61 ± 0.07	0.68 ± 0.06[a]	0.83 ± 0.13[a]	0.89 ± 0.05[a]	0.81 ± 0.13[b]
168 h	68.1 ± 17.3	66.3 ± 20.0	7.5 ± 0.1[b]	7.0 ± 0.1[b]	0.84 ± 0.10[b]	0.75 ± 0.07[ab]	0.97 ± 0.14	0.90 ± 0.13	1.0 ± 0.3[b]
DHA treated mice (56 h after inoculation)									
76 h	0.10 ± 0.01	1.3 ± 0.5[a]	6.0 ± 0.1	5.9 ± 0.7	0.62 ± 0.05	0.66 ± 0.07	0.87 ± 0.11	0.90 ± 0.14	0.66 ± 0.17[b]
96 h	1.5 ± 0.4	2.5 ± 0.8	6.5 ± 0.1	6.1 ± 0.6	0.65 ±0.08[b]	0.65 ± 0.08	0.88 ± 0.14	0.71 ± 0.12	0.64 ± 0.13[b]
168 h	14.6 ± 6.1	30.1 ± 2.5[a]	7.1 ± 0.1[b]	7.3 ± 0.5[b]	0.96 ± 0.08[b]	0.74 ± 0.06[ab]	0.91 ± 0.12	0.90 ± 0.11	0.86 ± 0.24[b]

[a] P<0.05 for comparison between intact and asplenic mice at designated time-points (t-test); [b] Parameter significantly different to uninfected animals (0 h group, P<0.05, ANOVA; not applicable to parasitaemia).

treated mice, but not in intact mice treated with DHA where haemoglobin concentration remained stable after treatment with DHA (Table 3.7).

Mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) values remained stable for the entire malaria infection in both asplenic and intact mice, although in asplenic mice both the MCV and MCH were increased at 168 h. However, these increases were not significantly different compared to baseline values (Table 3.6). However, in treated intact mice, the MCV was significantly lower compared to treated asplenic mice at both 96 and 168 h. The MCH in treated intact mice remained stable for the course of infection, however, in treated asplenic mice the MCH was significantly decreased compared to baseline values (Table 3.7).

In untreated asplenic mice the red blood cell distribution width (RBDW) was significantly increased compared with intact mice at the same time-points of 56, 76, 96 and 168 h post-inoculation. At 168 h the RBCDW in asplenic mice was also significantly increased compared to the RBCDW in asplenic mice at 0 h (Table 3.6). The RBCDW value for untreated intact mice was only significantly increased at 168 h when compared to baseline values (Table 3.6). After treatment with DHA, the RBCDW in both intact and asplenic mice remained consistent through all examined time-points (Table 3.7).

Although an initial decrease in white blood cell count (WBCC) was seen at 36 h in both intact and asplenic mice, the general trend was an increase in white blood cells during the course of infection in both treated and untreated mice (Table 3.6 and Table 3.7). However, a statistically significant difference in parameters was only observed at 168 h. The total WBCC was also differentiated at each time point with absolute counts for granulocytes, lymphocytes and monocytes generally all increasing as expected (Table 3.6). After treatment with DHA, the WBCC and absolute differential counts for all three white blood cell populations, in both intact and asplenic mice, increased during the course of infection (Table 3.7).

Declining platelet counts were seen in both treated and untreated intact mice with a statistically significant decrease seen after 36 h of infection in untreated mice (Table

3.6). In the asplenic mice, platelet counts fluctuated in both treated and untreated mice but were not significantly different to the uninfected animals at any of the sample times (Tables 3.6 and 3.7).

3.3.2.2.2 Biochemistry

ALP and ALT concentrations showed no significant difference throughout the infection. Bilirubin concentrations increased during the course of infection but were not significantly greater than uninfected mice until 168 h after inoculation in all groups of mice (Table 3.8).

The mean AST concentration was significantly higher in all infected mice than in uninfected mice (Table 3.8). In the asplenic groups a significant difference in AST concentrations was seen when comparing untreated infected and uninfected mice (Table 3.8). After treatment with DHA there was a statistically significant increase in AST concentrations in infected intact mice compared to uninfected intact mice, however, there was no significant difference in AST concentrations between DHA treated asplenic and uninfected asplenic mice (Table 3.9). AST concentrations were higher in the untreated asplenic mice than in intact mice (statistically significant 56 and 76 h after inoculation; Table 3.8).

Table 3.6 Haematology parameters from mice after inoculation with *P. berghei* parasites. Data are mean ± SD.

| | Time after inoculation | | | | | | | | | | | |
| | 0 h | | 36 h | | 56 h | | 76 h | | 96 h | | 168 h | |
	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)
Parasitaemia (%)	0	0	0.46 ± 0.11	0.35 ± 0.19	1.6 ± 0.3	3.0 ± 0.1	5.1 ± 0.4	5.6 ± 1.2	12.6 ± 6.5	14.3 ± 0.9	51.1 ± 1.2	54 ± 7
RBCC (x10^{12}/L)	8.60 ± 0.02	8.10 ± 0.78	9.39 ± 0.64	8.44 ± 0.45	8.41 ± 0.58	8.02 ± 0.84	8.67 ± 0.69	8.36 ± 1.7	7.42 ± 0.43	7.86 ± 1.1	4.88 ± 0.81[b]	3.69 ± 1.05[b]
Hb (g/L)	131 ± 4	127 ± 10	139 ± 8	131 ± 4	126 ± 8	123 ± 12	128 ± 11	130 ± 20	117 ± 5	122 ± 11	87 ± 20[b]	71 ± 20[b]
HCT	0.43 ± 0.01	0.42 ± 0.03	0.45 ± 0.02	0.42 ± 0.01	0.41 ± 0.02	0.40 ± 0.03	0.43 ± 0.04	0.44 ± 0.07	0.38 ± 0.00	0.40 ± 0.04	0.25 ± 0.06[b]	0.21 ± 0.06[b]
MCV (fL)	50 ± 1	52 ± 1	48 ± 1	50 ± 1	44 ± 9	50 ± 2	50 ± 1	52 ± 2	50 ± 1	51 ± 2	52 ± 1	55 ± 1[a]
MCH (pg)	15.2 ± 0.5	15.6 ± 0.4	14.5 ± 0.2	14.7 ± 0.1	14.5 ± 0.7	15.0 ± 0.7	18.9 ± 0.8	15.5 ± 1.1	14.6 ± 0.53	15.5 ± 1.3	12.2 ± 0.9	19.3 ± 0.31
MCHC (g/L)	304 ± 3	303 ± 1	301 ± 2	296 ± 7	301 ± 7	301 ± 3	284 ± 3	297 ± 10	294 ± 8	305 ± 11	358 ± 30[b]	349 ± 11[b]
RBCDW	10.0 ± 0.3	11.4 ± 1.2	9.2 ± 0.2	10.4 ± 0.8	9.5 ± 0.3	11.4 ± 0.7[a]	10.2 ± 0.2	13.7 ± 1.1[a]	10.1 ± 0.32	12.1 ± 0.42[a]	12.2 ± 0.94[b]	14.7 ± 0.44[ab]
WBCC (x10^9/L)	1.4 ± 0.6	1.5 ± 0.7	0.54 ± 0.1	0.54 ± 0.09	2.32 ± 1.67	3.5 ± 2.1	1.8 ± 0.5	2.6 ± 0.6	1.8 ± 0.46	3.9 ± 2.4	4.9 ± 0.8[ab]	3.1 ± 0.3[b]
Gran (x10^9/L)	0.32 ± 0.46	0.56 ± 0.10	0.13 ± 0.05	0.20 ± 0.11	0.43 ± 0.14	2.04 ± 1.46	0.23 ± 0.31	0.34 ± 0.27	0.39 ± 0.08	0.5 ± 0.4	1.06 ± 0.88	0.35 ± 0.3
Lymph (x10^9/L)	0.97 ± 0.27	0.87 ± 0.24	0.19 ± 0.02	0.28 ± 0.0[a]	0.74 ± 0.11	1.24 ± 0.41	1.43 ± 0.67	1.88 ± 0.18	1.27 ± 0.17	1.33 ± 1.7	2.86 ± 1.71	2.62 ± 0.22[b]
Mono (x10^9/L)	0.07 ± 0.08	0.10 ± 0.09	0.05 ± 0.00	0.07 ± 0.01	0.12 ± 0.04	0.26 ± 0.22	0.13 ± 0.15	0.33 ± 0.23	0.19 ± 0.18	0.04 ± 0.03	0.96 ± 0.72[b]	0.16 ± 0.19
% Gran	18 ± 21	34 ± 11	35 ± 10	36 ± 13	25 ± 16	55 ± 9	13 ± 18	13 ± 8	22 ± 2	15 ± 13	23 ± 18	11 ± 8
% Lymph	79 ± 24	61 ± 14	52 ± 9	52 ± 10	44 ± 27	38 ± 11	80 ± 27	76 ± 11	74 ± 18	60 ± 24	58 ± 30	84 ± 14
% Mono	4 ± 3	6 ± 3	14 ± 1	12 ± 4	6 ± 2	7 ± 2	7 ± 9	12 ± 8	10 ± 8	25 ± 24	19 ± 13	5 ± 5
Platelets (x10^9/L)	1022 ± 92	1079 ± 25	802 ± 10[b]	828 ± 7	845 ± 74[b]	1173 ± 654	591 ± 81[b]	888 ± 117[a]	668 ± 40[b]	807 ± 160	463 ± 45[b]	1097 ± 76

[a] P<0.05 for comparison between intact and asplenic mice at designated time-points (t-test); [b] Parameter significantly different to uninfected animals (0 h group, P<0.05, ANOVA; not applicable to parasitaemia). Abbreviations – RBCC: Red blood cell count; Hb: Haemoglobin; HCT: haematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; RBCDW: Red blood cell distribution width; WBCC: White blood cell count; Gran: Granulocytes; Lymph: Lymphocytes; Mono: Monocytes).

Table 3.7 Haematology parameters from mice that were treated with DHA 56 hours after inoculation with *P. berghei* parasites. Blood was harvested 20, 40 and 112 h after dosing (76, 98 and 168 h after inoculation). Data are mean ± SD.

	Time after inoculation							
	0 h		76 h		96 h		168 h	
	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)
Parasitaemia (%)	0	0	0.45 ± 0.4	0.92 ± 0.5[a]	3.8 ± 2.2	7.5 ± 3.9[a]	29.2 ± 13	38 ± 9.1
RBCC (x10^{12}/L)	8.60 ± 0.02	8.10 ± 0.78	8.27 ± 0.40	7.67 ± 0.93	8.09 ± 0.55	7.64 ± 0.07	7.68 ± 0.38	5.82 ± 0.32[ab]
Hb (g/L)	131 ± 4	127 ± 10	124 ± 4	118 ± 10	121 ± 7	119 ± 4	121 ± 4	97 ± 6[a]
HCT	0.43 ± 0.01	0.42 ± 0.03	0.42 ± 0.02	0.39 ± 0.03	0.39 ± 0.03	0.38 ± 0.02	0.37 ± 0.01[b]	0.28 ± 0.02[ab]
MCV (fL)	50 ± 1	52 ± 1	50 ± 1	51 ± 3	48 ± 1[b]	50 ± 3	48 ± 1[b]	49 ± 1
MCH (pg)	15.2 ± 0.5	15.6 ± 0.4	15.0 ± 1.2	14.7 ± 0.5	14.4 ± 0.4	15.2 ± 1.2	15.3 ± 1.1	14.6 ± 0.9[b]
MCHC (g/L)	304 ± 3	303 ± 1	291 ± 9	292 ± 4	298 ± 7	305 ± 11	317 ± 18	300 ± 13
RBCDW	10.0 ± 0.3	11.4 ± 1.2	10 ± 1	11 ± 1	10 ± 1	12 ± 1	10 ± 1	12 ± 1
WBCC (x10^9/L)	1.4 ± 0.6	1.5 ± 0.7	2.1 ± 0.7	2.7 ± 1.1	2.3 ± 7	2.3 ± 0.9	4.8 ± 2.1	6.0 ± 0.9[b]
Gran (x10^9/L)	0.32 ± 0.46	0.56 ± 0.10	0.67 ± 0.42	1.03 ± 0.86	0.55 ± 0.33	0.16 ± 0.15	2.60 ± 1.49[b]	2.51 ± 0.7
Lymph (x10^9/L)	0.97 ± 0.27	0.87 ± 0.24	1.16 ± 0.10	1.34 ± 0.16	1.24 ± 0.24	2.02 ± 0.97	1.34 ± 0.64	2.52 ± 1.42
Mono (x10^9/L)	0.07 ± 0.08	0.10 ± 0.09	0.30 ± 0.24	0.32 ± 0.22	0.47 ± 0.46	0.08 ± 0.01	0.83 ± 0.62	0.94 ± 1.2
% Gran	18 ± 21	34 ± 11	30 ± 9	34 ± 17	23 ± 8	8 ± 8	52 ± 14	42 ± 5
% Lymph	79 ± 24	61 ± 14	58 ± 15	55 ± 18	59 ± 21	88 ± 8	33 ± 21	41 ± 17
% Mono	4 ± 3	6 ± 3	13 ± 6	11 ± 7	18 ± 14	4 ± 2	15 ± 8	17 ± 23
Platelets (x10^9/L)	1022 ± 92	1079 ± 25	784 ± 114	865 ± 141	582 ± 23[b]	935 ± 92[a]	227 ± 217[b]	1266 ± 306[a]

[a] P<0.05 for comparison between intact and asplenic mice at designated time-points (t-test); [b] Parameter significantly different to uninfected animals (0 h group, P<0.05, ANOVA; not applicable to parasitaemia). Abbreviations – RBCC: Red blood cell count; Hb: Haemoglobin; HCT: haematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; RBCDW: Red blood cell distribution width; WBCC: White blood cell count; Gran: Granulocytes; Lymph: Lymphocytes; Mono: Monocytes).

Table 3.8 Biochemistry parameters from mice after inoculation with *P. berghei* parasites. Data are mean ± SD.

	Time after inoculation											
	0 h		36 h		56 h		76 h		96 h		168 h	
	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)
Parasitaemia (%)	0	0	0.42 ± 0.2	0.63 ± 0.8[a]	2.9 ± 0.7	4.7 ± 0.8[a]	12.3 ± 1.0	22 ± 4[a]	37.2 ± 5.9	41.2 ± 12.8	79.4 ± 6.1	82.1 ± 3.7
Total Bilirubin (µmol/L)	10 ± 1[a]	7 ± 1	12 ± 2	13 ± 2[b]	14 ± 6	14 ± 7	9 ± 2	9 ± 0[b]	11 ± 2	12 ± 1[b]	21 ± 2[b]	20 ± 4[b]
ALP (IU/L)	57 ± 22	41 ± 13	47 ± 11	45 ± 25	34 ± 3[a]	54 ± 10	39 ± 2	53 ± 10	49 ± 12	47 ± 7	36 ± 8	49 ± 8
ALT (IU/L)	36 ± 12	38 ± 10	73 ± 34	44 ± 10	48 ± 3	59 ± 17	46 ± 9	63 ± 16	48 ± 12	54 ± 15	30 ± 8[a]	66 ± 1
AST (IU/L)	107 ± 19	85 ± 13	134 ± 25	142 ± 18[b]	148 ± 7[ab]	155 ± 53[b]	134 ± 10[a]	161 ± 4[b]	140 ± 9	152 ± 16[b]	165 ± 39	176 ± 19[b]
Total Protein (g/L)	43 ± 2	41 ± 3	43 ± 1	47 ± 1	43 ± 1	43 ± 3.5	40 ± 2	40 ± 1	45 ± 3	44 ± 3	46 ± 2	42 ± 2
Albumin (g/L)	10 ± 1	9 ± 1	10 ± 1	11 ± 2	8 ± 0	10 ± 1	10 ± 1	10 ± 1	11 ± 0	10 ± 1	11 ± 1	11 ± 1

[a] P<0.05 for comparison between intact and asplenic mice at designated time-points (t-test); [b] Parameter significantly different to uninfected animals (0 h group, P<0.05, ANOVA; not applicable to parasitaemia).   Abbreviations – ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate transaminase).

Table 3.9 Biochemistry parameters from mice that were treated with DHA 56 hours after inoculation with *P. berghei* parasites. Blood was harvested 20, 40 and 112 h after dosing (76, 98 and 168 h after inoculation). Data are mean ± SD.

	Time after inoculation							
	0 h		76 h		96 h		168 h	
	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)	Intact (n=3)	Asplenic (n=3)
Parasitaemia (%)	0	0	1.0 ± 0.2	1.1 ± 0.4	4.1 ± 2.0	11.3 ± 2.1[a]	33.0 ± 17.4	42.4 ± 8.2
Total Bilirubin (µmol/L)	10 ± 1	6.5 ± 0.7	11 ± 2	12 ± 5	13 ± 6	11 ± 3	20 ± 4[b]	17 ± 3[b]
ALP (IU/L)	57 ± 22	41 ± 13	43 ± 10	45 ± 13	50 ± 6	45 ± 11	39 ± 13	30 ± 3
ALT (IU/L)	36 ± 12	38 ± 10	75 ± 15	71 ± 16	76 ± 12[a]	42 ± 7	133 ± 52	69 ± 16
AST (IU/L)	107 ± 19	85 ± 13	342 ± 43[b]	230 ± 72	266 ± 40[ab]	177 ± 31	351 ± 60[ab]	197 ± 12
Total Protein (g/L)	43 ± 2	41 ± 3	41 ± 6	44 ± 11	47 ± 1	41 ± 4	42 ± 2	41 ± 3
Albumin (g/L)	10.3 ± 0.6	9.0 ± 0.6	10 ± 0	10 ± 1	11 ± 0	11 ± 1	9 ± 1	10 ± 1

[a] P<0.05 for comparison between intact and asplenic mice at designated time-points (t-test); [b] Parameter significantly different to uninfected animals (0 h group, P<0.05, ANOVA; not applicable to parasitaemia). Abbreviations – ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate transaminase).

3.4 DISCUSSION

This series of investigations demonstrates that:

1. DHA has potent antimalarial efficacy in asplenic and intact mice infected with *P. berghei* malaria.

2. DHA appears to be effective against most erythrocytic stages of the malaria parasite.

3. The liver is able to remove parasites from the circulation, thus compensating for asplenic status in malaria infection (in the murine malaria model).

The single dose-ranging pharmacodynamic study verified that the therapeutic effect of DHA extends well beyond the duration of detectable drug concentration. Previous studies have identified that DHA has an extremely short elimination half-life of 12–15 min in mice (48, 554). Therefore, it would be expected that the drug is effectively eliminated from the blood circulation within a few hours. However, parasite nadir occurred at 24 h after a single dose of DHA, regardless of drug dose, followed by parasite recrudescence (Fig. 3.6). These results indicate that DHA is rapidly taken into the red blood cell where it exerts a more prolonged antimalarial effect against the intraerythrocytic parasite. This speculation correlates with the observed low plasma DHA concentrations described in the literature (48).

In contrast to case reports of malaria in asplenic patients (34, 290, 497), this murine malaria model showed that single dose administration of DHA (10, 30 and 100 mg/kg) to asplenic and intact mice resulted in similar parasitaemia-time profiles. The only significant difference between the pharmacodynamic profiles of asplenic and intact mice was during parasite recrudescence, where after high dose DHA administration the rate of recrudescence was faster in the asplenic group ($t_{5\%}$ 48 h v 62 h) (Fig. 3.6). Whilst previous murine studies have shown that splenectomized and intact mice infected with *P. yoelii* did not differ in their course of infection (434), and that the effects of splenectomy on the progression of malaria infection can be mouse strain dependant (156), it was expected that in the current study a significantly delayed and/or less effective parasite clearance would be observed in the asplenic mice following drug administration. The results obtained were

therefore surprising.   Although elucidating the precise mechanisms of parasite clearance was beyond the scope of the present investigation, further investigations were persued into the pathology and histology of blood and organ systems of the mouse in an attempt to provide an alternative explanation for the unexpected pharmacodynamic findings.

Through the examination and differentiation of the asexual erythrocytic stage parasites, it was determined that DHA appears to be efficacious against all of the asexual erythrocytic stages (Fig. 3.7).   This finding is consistent with *in vitro* data, showing that artemisinin compounds are effective against most of the *P. falciparum* erythrocytic life cycle (451, 554), and indications of stage-specific activity of artemisinin drugs *in vivo* (554). The apparent broad spectrum stage efficacy of DHA is presumably a result of the novel mechanism of action of the artemisinin derivatives. Although other mechanisms of action have been proposed, interaction of the SERCA orthologue should be considered given that DHA is shown to be effective against all stages of the parasite, including ring-forms that lack haemozoin (34, 290, 497).

It is recognized that differentiating between dead and live parasites in the blood circulation and organs is difficult, although differences in parasite morphology have been noted after treatment with antimalarial drugs, particularly artemisinin derivatives (435). It is possible that ineffective single doses would result in a mix of both dead and live parasites in circulation, making it difficult to quantify the viable parasite burden. However, due to the rapid clearance (short half-life) of DHA (156), drug concentrations beyond the nadir parasitaemia would be negligible. Hence, in the absence of drug pressure, the majority of parasites detected would be live and viable, as dead parasites would likely have been rapidly removed from the circulation by phagocytic mechanisms. In the case of splenectomy, with a reduced parasite clearance capacity, a combination of both live and dead parasites could be expected in the circulation. However, as clearly shown in the asplenic model (Fig. 3.6) parasite clearance was rapid in both intact and asplenic mice. Therefore, it is presumed that the parasitaemia results reported in this thesis are reflective of the

true number of viable parasites in the peripheral circulation at each time point due to both the successful clearance of parasites by both asplenic and intact mice, but also due to the experience of the microscopist (candidate) at differentiating viable parasites and those affected by DHA administration.

In this study, malaria infection caused an increase in liver mass and rigidity, as well as changes in organ histology. This was attributed to haemozoin deposition and parasitised erythrocyte accumulation in the organ, predominantly through Kupffer cell and macrophage phagocytosis, as well as accumulation of the rigid parasitised erythrocytes in the liver sinusoids. The observed Kupffer cell hyperplasia and hypertrophy also would have contributed to the increased mass of the organ (276). Liver integrity was compromised during the infection, in both asplenic and intact mice, as reflected by increased levels of bilirubin, ALT and AST (Table 3.8 and 3.9) and as previously reported (35). These changes have been attributed to the malaria infection, as liver toxicity is unlikely to be caused by DHA (238), although the possibility of changes in liver enzyme concentrations due to the injection vehicle cannot be excluded. Comparison of asplenic and intact mice revealed little difference in liver mass and biochemistry, but a notable contrast was observed in the liver histology. Beyond 76 h post inoculation, at a parasite density in excess of 5%, liver sections from asplenic mice showed occasional clusters of basophilic staining cells of varying sizes (30-50 cells per cluster; Fig. 3.16), which were consistent with cells of lymphoid origin closely resembling areas of white pulp of the spleen. These clusters were previously described in an asplenic murine malaria model where they were identified as 'pseudofollicles' (545). It is thought that pseudofollicules are cellular infiltrates of macrophages and lymphocytes of T and B cell origin, that act as secondary, ectopic, splenic germinal centres in asplenic mice (545). This is consistent with a report suggesting that in the absence of a spleen, processing of malaria antigens, previously regulated by the spleen, becomes a role of the liver (435). These observations have so far only been described in the murine model which may suggest why mice have the capacity to survive at much higher parasitaemia than humans, although such intensive liver histology is not realistic in malaria-infected patients. In all other compared organs (kidney, lungs) there was

little difference in the tissue histology between the asplenic and intact mouse groups.

Haematology data were broadly consistent with expectations (543). In the advanced malaria infection, haemoglobin levels decreased as the parasitaemia exceeded 20% in both asplenic and intact mice regardless of drug treatment. This was most likely related to well recognized mechanisms such as increased cell haemolysis as a result of schizont rupture and erythropoietic depression (87). Furthermore, these observations are consistent with human (34) and murine (142) reports showing that haemoglobin concentrations were similar in both asplenic and control groups.

In contrast to the haemoglobin data, there was a significant difference between the intact and asplenic mice in relation to the platelet counts. In the intact mice (treated and untreated), the platelet count decreased as the infection progressed, a common feature of malaria infection. However, in asplenic mice the platelet count was relatively stable throughout the infection in both treated and untreated groups. Although the mechanism of thrombocytopenia during malaria infection is yet to be elucidated, in mice infected with *P. berghei* it is thought to be a result of reduced platelet life span, platelet adherence to vasculature and immune mechanisms (188, 190, 388). The relatively stable platelet count observed in the asplenic mice was not expected, as in clinical cases, thrombocytopenia occurs in asplenic patients.

Investigation into plausible explanations for the observed differential pathology revealed a number of possibilities:

a) A Type 1 error as a result of an analytical error due to spurious automated platelet determinations could result in a falsely high platelet count as observed in the asplenic group. As the automated cell counter differentiates between red blood cells and platelets as a function of size, any circulating red blood cells fragments (as a result of erythrocyte rupture during merozoite release) would erroneously be counted as platelets. However, microscopic examination of a representative selection of blood films determined that the increased platelet count of asplenic mice was not a

result of increased numbers of schistocytes/red blood cell fragments or other microcytic cells that would create a false positive. Furthermore, examination of blood films from intact mice with thrombocytopenia rules out the presence of platelet clumps which may have resulted in a falsely decreased platelet count. Therefore, it is presumed that the observed platelet differences are most probably not a result of a Type 1 error.

b) As a recognised function of the spleen is as a platelet reservoir, containing up to 1/3 of the total circulating numbers, it would be expected that platelet counts would be substantially higher after splenectomy due to the lack of platelet sequestration. However, although this may explain why higher numbers of platelets are present in circulation it does not account for the lack of thrombocytopenia seen in asplenic mice during infection.

c) The antigenic nature of malaria infections could result in the production of antibodies that bind to platelets during malaria infection resulting in their sequestration into vascular endothelium. Alternatively, expression of malarial antigens to the surface of thrombocytes may result in splenic clearance due to its hyperactivity.

In conclusion, although DHA had similar overall efficacy in asplenic and intact mice, this study showed that asplenic mice had a reduced capacity for parasite clearance. Although the current findings in asplenic mice did not reflect observations in recent clinical case reports, there is a relative paucity of human studies for detailed comparison of the results from rodent models. The current study has shown an enhanced role of the liver in clearing parasites in asplenic mice following DHA treatment. However, a detailed investigation of the mechanisms of clearing parasites in asplenic mice was not feasible in the present study. Overall, this asplenic murine malaria model could be useful in studying the splenic function in parasite clearance of new antimalarial compounds. This study also demonstrates the potential value of the model for investigations of disease pathology.

CHAPTER FOUR

PIPERAQUINE PHARMACOKINETIC AND PHARMACODYNAMIC STUDY

4.1   INTRODUCTION
4.1.1   Physicochemical properties

PQ is commercially available as either a base ($C_{29}H_{32}Cl_2N_6$; 4,4`-(1,3-propaneiyldi-4,1-piperazinediyl)bis[7-chloro]quinoline; Molecular weight: 535.51 ), or as the water soluble salt, piperaquine phosphate (PQP; $C_{29}H_{32}Cl_2N_6.4H_3PO_4$; 1,3-bis[1-(7-chloro-4`-quinolyl)-4`-piperazinyl] phosphate; Molecular weight: 927.48) (Fig 4.1) (253). PQ base is a highly lipophilic basic molecule with a melting point of 212-213°C and poor solubility in water (283, 482), whilst PQP is slightly soluble in acidic solutions (482). Plasma protein binding has also been estimated to be at least 97% (231, 232).

Figure 4.1   **Chemical structure of piperaquine phosphate (PQP)** (253)
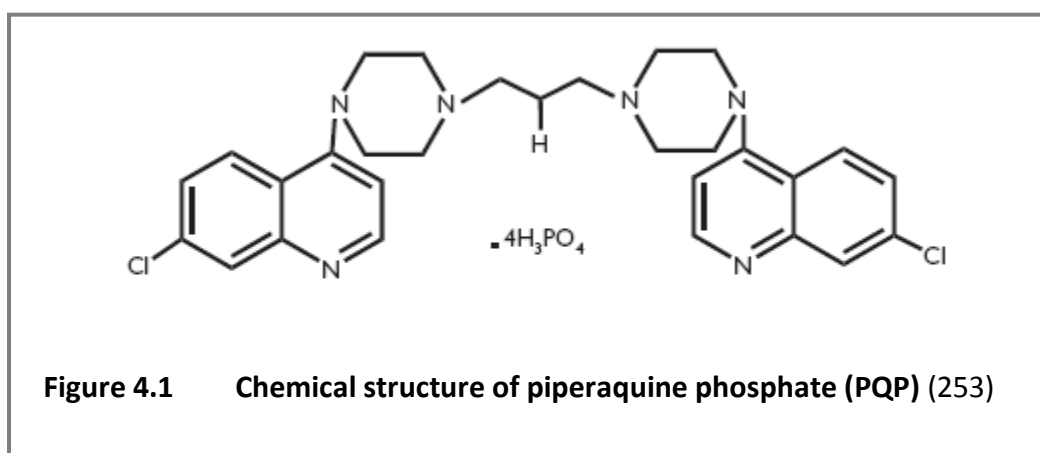
PQ has been shown to concentrate moderately in red blood cells, with a mean erythrocyte: plasma ratio of 1.5:1 at 46% haematocrit over the plasma concentration range 50-500 µg/L (232).   It has also been shown that PQ concentrations in EDTA and heparinised blood are equivalent, whilst serum concentrations prepared from the same blood are approximately 58% higher (127, 282). This is most likely because the clotting process in the anticoagulant tubes releases drug which are concentrated in leucocytes or platelets (55, 282).

4.1.2 Piperaquine as an antimalarial

In recent years, the increased incidence and spread of multidrug-resistant *P. falciparum* malaria has meant that new antimalarial drugs or drug combinations are essential. Whilst developing new antimalarial combination therapies, PQ was "rediscovered" by Chinese scientists as a suitable drug partner, particularly for combination with the artemisinin derivative drugs (127). To date, two fixed-dose combination therapies including PQ and the artemisinin derivatives DHA (Artekin®)(27, 61, 94, 127, 139, 252, 253, 477) and artemisinin (Artequick®) (389, 476) have emerged in the clinical setting with all clinical results to date showing excellent tolerability and efficacy (27, 29, 139, 209, 217, 252-254, 305, 413, 456). PQ is listed in the Chinese Pharmacopeia and commercially available in China, however, it is not yet available in the western world as either single dose or fixed-dose combination therapies (127, 231, 284, 285, 425, 485).

4.1.3 Animal studies

Although PQ has been used in monotherapy for many years in China and as an emerging candidate for ACTs, there is little preclinical data available in western literature on the pharmacokinetic and pharmacodynamic profiles of PQ (127). Reports are limited to several animal studies published in Chinese literature in the early 1980s, focusing on PQ toxicology and PQ monotherapy (127, 484). Therefore, we rely on more recent animal investigations (484), as well as PQ review articles (127), for information about the pharmacokinetics and pharmacodynamics of PQ and possible implications for dosing regimens in the clinical setting. Furthermore, no preclinical studies of the combination DHA and PQ were found in a detailed literature search.

Chinese pharmacodynamic studies, comparing the efficacy of PQP and PQ base in mice infected with CQ-sensitive or CQ-resistant strains of *P. berghei,* found that PQ and PQP have different potencies in prophylactic and therapeutic roles (127). Qu et al. (402) reported that the oral doses of PQ and PQP required to suppress infections were significantly different, with PQP apparently more effective at lower doses than PQ base (87 ± 4 mg/kg and 65 ± 3 mg of base/kg for PQ base and PQP, respectively;

P<0.01) (402). These results were further supported by Zhu et al. (594) who demonstrated that PQP was more potent than PQ base in their model of murine *P. berghei* malaria at doses of 50 and 200 mg base/kg (127, 594). However, it is thought that the difference in efficacy seen between PQ and PQP are more likely to be a result of the differences in solubility of the two forms (127). Further studies using the CQ-sensitive and CQ-resistant murine models demonstrated that there may be a low level of cross resistance between PQ and CQ, an observation that corresponded to later observations in the clinical setting (127, 402).

The pharmacokinetic parameters of PQ have only recently been elucidated in both animal models and clinical studies, despite PQ being used as an antimalarial agent for over 20 years (127, 232, 425). In an early pharmacokinetic study in mice, ^{14}C labelled PQP was used to determine the accumulation and absorption patterns of PQ in mice (95). The study suggested that PQ had a rapid absorption from the gut, with a high systemic availability of 80-90%, and preferentially accumulated in the liver, kidney and spleen (95, 127). Using this method it was also determined that PQ had a half-life of 9 days (95, 127). However, the validity of these results is unclear as the method used to measure the absorption and half-life of PQ is neither sensitive nor specific. Chen et al. (95) measured the total ^{14}C, hence it is unclear if there were any radiolabelled metabolites also present at the time of measurement, which would contribute to the total ^{14}C measurements made at each time point (95, 127). Therefore the results obtained may not reflect the true fate of PQ in the murine model. The pharmacokinetic parameters of PQ in dogs were also described in a toxicology study by Sheng et al. (440) where the half-life was reported as 9.4 days. However, the accuracy of the reported half-life is unclear as details on how the value was calculated were not described (127). As a result it is important to further investigate the pharmacokinetics of PQ in preclinical models to address issues such as the erratic absorption of PQ (425), possible enterohepatic recirculation (232, 443), parenteral administration, extreme volume of distribution (232, 425), or long half-life (483, 484).

A recent study of PQ pharmacokinetics in the rat examined pharmacokinetic parameters of PQ after either a single oral dose or i.v. administration (484). It was found that the half-life for PQ after a low dose (13 mg PQP/kg) was 34 ± 19 h and 23 ± 8 h following intravenous and oral administration, respectively. After high (26 mg PQP/kg) dose PQP, a similar half-life of 38 ± 16 h and 25 ± 3 h was obtained for both intravenous and oral administration (484). However, in this study blood samples were only collected for 80 h after administration, which does not take into account the possibility that PQ could remain in the system for longer than 3.5 days. Despite the short duration of sampling, the authors concluded that as the pharmacokinetic properties and metabolism of PQ was similar in the rat to that found in humans, the rat is a suitable species for PQ preclinical studies (484).

4.1.4 Study aims

In order to address the paucity of preclinical pharmacokinetic, efficacy, and safety data that are normally required by regulatory authorities and are essential for future research, the aim of this study was to obtain robust pharmacokinetic and pharmacodynamic data following the administration of single doses of PQ in the *P. berghei* murine malaria model. To achieve this aim, the study comprised three arms: (i) the determination of single dose pharmacodynamic profiles in mice infected with *P. berghei* and given 10, 30 or 90 mg/kg PQP at 2 to 5% starting parasitaemia. Furthermore, at 60 days after drug administration, the 90 mg/kg PQP group was reinoculated with *P. berghei* parasites to test for acquired immunity; (ii) the determination of PQ pharmacokinetic parameters in healthy and malaria-infected mice after administration of 90 mg/kg PQP; (iii) investigation of the combination efficacy of 10 mg/kg PQP and 30 mg/kg DHA.

4.2   METHODS

4.2.1 Materials

4.2.1.1 Mice

This study was approved by the Curtin University Animal Experimentation Ethics Committee. Male Swiss mice (5 to 7 weeks of age; average weight 28.9 ± 3.8 g) were obtained from the ARC for all experimental work. Male BALB/c mice (7 to 8

weeks of age; ARC) were used for weekly passage of malaria parasites. All animal handling and housing procedures were performed as outlined in Sections 2.1.1 and 2.2.1.

4.2.1.2 Parasites

Plasmodium berghei ANKA parasites were maintained by continuous weekly blood passage in BALB/c mice (Section 2.2.2.1). A standard inoculum of 10^7 parasitised erythrocytes per 100 μL was prepared by dilution of blood harvested from infected BALB/c mice in citrate-phosphate-dextrose solution (Section 2.2.2.2) and administered by i.p. injection to infect the experimental mice (Section 2.2.1.4).

4.2.1.3 Drug Treatment

PQP was available as a finely crushed powder. As PQP is only slightly soluble in water (231), a uniform suspension had to be prepared for i.p. administration. To prepare the suspension, an accurately weighed mass of PQP was placed in a mortar and pestle and several drops (3-5 depending on drug mass) of ethanol added to wet the drug. An accurately measured volume of drug vehicle (Polysorbate 80: glycerol 80:20% [vol:vol]; drug suspension formulated by Andrzejewski, C. BPharm (Hons), Curtin University, 2004) was then added to the drug and well mixed using the mortar and pestle. Care was taken during mixing to prevent the formation of bubbles in the suspension.

When administering the doses to experimental mice, 1 mL of suspension was drawn from the stock suspension with careful mixing taking place between draws to ensure an even distribution of drug throughout the polysorbate 80:glycerol suspension. Due to the high viscosity of the suspension, a 23G needle was required for drug administration.

4.2.2 Study design

4.2.2.1 Single dose pharmacodynamic study

4.2.2.1.1 Dosing

For the single dose pharmacodynamic study, 3 doses of PQ were chosen for investigation, 300 µg, 900 µg and 2,700 µg PQP (approximately 10, 30 and 90 mg/kg PQP for 30 g mice; concentration of the PQP suspension was variable and a standard 100 µL volume of suspension was administered to the mice). These doses were chosen for investigation as a previous study on the preventive and therapeutic effects of PQ in mice infected with *P. berghei* had demonstrated that the median preventative dose of PQ after oral administration was 33.6 mg/kg (402). Based on this premise, the efficacy of PQP was tested in the *P. berghei* murine malaria model using three logarithmically distributed doses. Considering the observations of Qu et al. (402), it was anticipated that the 3 chosen doses (10, 30 and 90 mg/kg PQP) would demonstrate ineffective, partially effective and highly effective dosing, respectively.   These doses were run in parallel with an untreated control group which received only drug vehicle (0 mg/kg PQP) at the time of dosing.

4.2.2.1.2 Drug administration

Male Swiss mice (n=50) were infected with a standard 10^7 *P. berghei* parasitised erythrocytes inoculum by i.p. injection. The mice were then divided into 4 treatment groups consisting of n=8 mice in the untreated control group and n=14 mice in each of the PQP dose groups. Sixty-four hours after parasite inoculation, when the peripheral parasitaemia had reached a level of 3–5% (confirmed by thin blood film microscopy) each mouse received a single i.p. dose of 0, 10, 30 or 90 mg/kg PQP, depending on their selected treatment group.

After drug administration, the parasitaemia in each mouse was monitored through the preparation of peripheral tail vein blood smears which were subsequently stained in May-Grunwald Giemsa and then examined under 100x oil immersion light microscopy.   In order to characterise the pharmacodynamic response to PQP administration with rich data, tail vein bleeds were performed 3 times a day for the first 5 days after treatment, twice daily for the next 2 weeks and then daily until the

time of euthanasia (>40% parasitaemia, >10% reduction in mouse body weight in less than 24 h or termination of the experimental protocol). Mice were euthanased with sodium pentobarbitone injection (50-100 mg/kg i.p.).

4.2.2.1.2   Parasite re-inoculation at 60 days post PQP-administration

Preliminary pilot studies (Andrzejewski, C. BPharm (Hons), Curtin University, 2004) demonstrated that after dosing with 90 mg/kg PQP, all mice survived to the experimental end point (56 days) with many mice maintaining persistently low parasitaemias. Therefore, one objective of this investigation was to determine if these mice had acquired immunity towards the *P. berghei* parasites as a result of the persistent subclinical levels of infection.

All mice that had initially received a single 90 mg/kg dose of PQP in the single dose pharmacodynamic study were re-inoculated with a second 10^7 *P. berghei* parasitised erythrocyte i.p. inoculum 60 days after drug administration. Furthermore, three control arms were run in parallel with the re-inoculated group consisting of uninfected age matched mice that received either (a) drug vehicle (n=4), (b) 90 mg/kg PQP (n=8) or (c) remained untreated (n=4) on day 0. These groups were required to control for age and/or PQ concentrations, 60 days after drug administration. All control groups were subsequently inoculated with 10^7 *P. berghei* parasitised erythrocytes 60 days after dosing. Parasitaemia was monitored by daily peripheral blood films as previously described.

4.2.2.2   Pharmacokinetic Study

4.2.2.2.1   Drug administration and blood sampling

The pharmacokinetic parameters of PQ were determined in both healthy and malaria-infected mice. To determine the pharmacokinetics of PQ in healthy mice, 55 male Swiss mice (6 weeks old) received a single i.p. dose of 2,700 μg PQ phosphate (PQP; 100 μL of suspension; approximately 90 mg/kg). This PQP dose was selected as pharmacodynamic time-density profiles suggest that 90 mg/kg PQP had prolonged antimalarial efficacy and ensured lengthy survival for malaria-infected mice. It was therefore desirable to ascertain whether the observed

antimalarial efficacy was a result of drug concentrations, immunological mechanisms or a combination of the two. Mice were divided into 11 groups of n=5 mice, with each group representing a collection time point.

Blood was harvested from each group by cardiac puncture at 0, 2.5 and 8 h, and 1, 2, 4, 7, 10, 14, 18 and 25 days after drug administration. Each mouse blood sample was placed into a lithium heparin anticoagulant tube (Vacutainer®; Becton-Dickinson, NJ, U.S.A), centrifuged at 10,000 g for 5 min with the plasma then separated and stored at -80°C until HPLC analysis.

A second pharmacokinetic study was conducted using malaria-infected mice. Male Swiss mice (n=100) were inoculated with 10^7 *P. berghei* parasitised erythrocytes by i.p. injection and then were dosed, 64 h after inoculation, with a single i.p. dose of 2,700 μg PQP (100 μL of suspension; approximately 90 mg/kg). Blood was harvested from groups of n=5 mice at 0, 2, 4, 6, 12, 16 h and 1, 1.25, 2, 2.3, 3, 4, 7, 9, 15, 22, 30, 40 and 52 days after drug administration. All blood samples were prepared for HPLC analysis as described previously for the healthy mice.

4.2.2.2.2   Pharmacokinetic analysis
4.2.2.2.2.1 Preparation of stock solution and standard curve for HPLC assay
All HPLC analyses were performed by Dr Madhu Page-Sharp (Research Fellow, Curtin University / UWA).

Stock solutions of PQP (1 mg/mL PQ base in water) and the internal standard CQ phosphate (1 mg/mL CQ base in water) were prepared and stored in the dark at 4°C. When stored in these conditions the stock solutions were deemed stable for up to 50 days (231). Working stock solutions were regularly prepared from the stock solutions. For each analytical batch, a 5-point linear calibration curve was prepared by spiking blank human plasma with appropriate volumes of the working standards. Quality Control (QC) samples (5 μg/L and 50 μg/L) were also included in each analysis. Human plasma was used for all quality control samples as this was readily available in large volumes.

4.2.2.2.2.2 Piperaquine assay

Mouse plasma samples were assayed for PQ based on the method published by Hung et al. (231) with several minor modifications made as a result of the limited sample volume. Briefly, plasma samples (0.5 mL) were spiked with internal standard (200 ng CQ), then alkalinized with 0.1 mL of 1M NaOH. The samples were extracted by manual shaking (10 min) with 8 mL of hexane:isoamyl alcohol (90:10). After centrifugation at 1,500 g for 10 min, the supernatant (7 mL) was back extracted into 0.1 mL of 0.05M HCl by manually shaking for 5 min, followed by centrifugation at 1,500 g for 10 min. The organic layer was aspirated to waste whilst the HCl layer was transferred to a round-bottomed borosilicate glass tube which was then centrifuged at 1500 g for 20 min. The HCl extract was then transferred to an HPLC sample vial and a 100 µL aliquot was injected onto the HPLC.

Separations were achieved on a Waters Xterra^{TM} RP$_{18}$ (3.5 µm, 4.6 x 100 mm) column attached to a Waters Symmetry^{TM} C$_{18}$ (5 µm, 3.9 x 20 mm) guard column. The mobile phase consisted of acetonitrile (7% v/v) in water containing sodium chloride (0.1% w/v), trifluroacetic acid (0.025% v/v) and triethylamine (0.008% v/v). The mobile phase was pumped at 1.2 mL/min and the UV absorbance of analytes was detected at 340 nm.

The intra-day relative standard deviations of PQ were 10.8, 8.2, and 9.4% at 5, 200 and 1000 µg/L, respectively (n=5). Inter-day relative standard deviations were 11.6, 4.4 and 6.7% at 5, 200 and 1000 µg/L, respectively (n=25). The limit of quantification and limit of detection were 1.5 µg/L and 0.7 µg/L, respectively.

All samples were assayed within the frozen storage stability limits for PQ, which had previously been established as 12 months (Hung, T-Y, B Med Sci Thesis, University of Western Australia, 2003).

4.2.2.3 PQ and DHA combination study

The combination study compared the pharmacodynamic responses between groups of mice that received either single doses of PQP, DHA or PQP + DHA. Male Swiss

mice (5-6 weeks; n=50) were inoculated with 10^7 *P. berghei* parasitised erythrocytes by i.p. injection, then divided into a control group (n=8) and three treatment groups (Groups A, B and C; n=14 per group). Sixty-four h after inoculation Group A mice received 10 mg/kg PQP, Group B mice received 30 mg/kg DHA and Group C mice received successive doses of 10 mg/kg PQP and 30 mg/kg DHA, by i.p. injection. Parasitaemia was monitored by examination of peripheral blood smears.

4.2.3   Pharmacodynamic analysis

Peripheral blood films were stained with May-Grunwald Giemsa using a Hema-Tek staining machine within an hour of preparation with all films cover-slipped (DePex mounting medium) and stored until time of examination (Sections 2.2.2.4 and 2.2.2.5). To minimise counting variability, which is inevitable when multiple microscopists determine parasitaemia within a single study, all peripheral blood smears were examined and parasitaemia determined by the author. Parasitaemia was determined by counting either 30 or 100 fields of view for >0.5% and <0.5% parasitised erythrocytes, respectively. This procedure ensured an acceptable standard error of 22% at 0.1% parasitaemia (277) and a limit of detection in the order of 0.002% parasitaemia.

4.2.4   Pharmacokinetic analysis

For pharmacokinetic modelling, measured plasma PQ concentrations were normalised to a dose of 90 mg/kg PQP (52 mg/kg PQ base), according to the weight of each mouse at the time of dosing. Consistent with the principles of destructive testing (37, 593), the mean normalised plasma PQ concentration for each group of mice was used to estimate the pharmacokinetic parameters of PQ. Pharmacokinetic analysis was performed using Kinetica™ Version 4.2 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Non-compartmental analysis of the plasma drug concentration-time data was used to estimate area under the curve (AUC; log-linear trapezoidal method), terminal elimination half-life ($t_{1/2}$), apparent clearance (CL/F) and apparent volume of distribution (V/F) (429). A two-compartment model was fitted to the data to estimate pharmacokinetic descriptors for the observed biphasic elimination ($t_{1/2\alpha}$ and $t_{1/2\beta}$; weighting = $1/y^2$).

4.3 RESULTS

4.3.1 Single dose pharmacodynamic study

Administration of single dose PQP (10, 30 or 90 mg/kg) resulted in a decline in parasitaemia at all doses tested (Fig. 4.2). The nadir parasitaemia in the 10 mg/kg group occurred 36 h after dosing and was 12.8 ± 3.1 fold below the pre-dose parasitaemia. Parasite nadir was immediately followed by parasite recrudescence and a rapid rise in parasitaemia until time of euthanasia. The median survival time was 10 days (range 5 to 12 days).

After a single dose of 30 mg/kg PQP, the parasitaemia immediately declined and fell below the limit of detection (0.002%) 1.8 days after dosing. However, a detectable parasitaemia was observed briefly, 2.5 days after dosing, but otherwise remained undetectable until 7-8 days after dosing. At this time, recrudescence was observed and the parasitaemia continued to rise for a further 2-3 days (Fig. 4.2). From 10-18 days, the mean parasitaemia remained stable (0.5-2.5%) and then decreased to approximately 0.1% from days 22-30. Beyond day 30, the mean parasitaemia slowly increased until mice required euthanasia. The median survival time for the 30 mg/kg group was 54 days (range 8–59 days). Within this 30 mg/kg group, 4 mice (30%) showed a steady increase in parasitaemia after recrudescence and required euthanasia after a median of 22 days (mean parasitaemia 11%).

In the 90 mg/kg group, the mean parasitaemia declined rapidly and was undetectable by 36 h after dosing. Recrudescence occurred after 7-8 days in all mice, with a mean peak parasitaemia of 1.8 ± 1.6% observed 16 days after dosing. The parasitaemia declined and generally remained below 0.1% until the experimental end-point, 60 days after dosing. All mice were active, alert and had stable body weight throughout the course of the study.
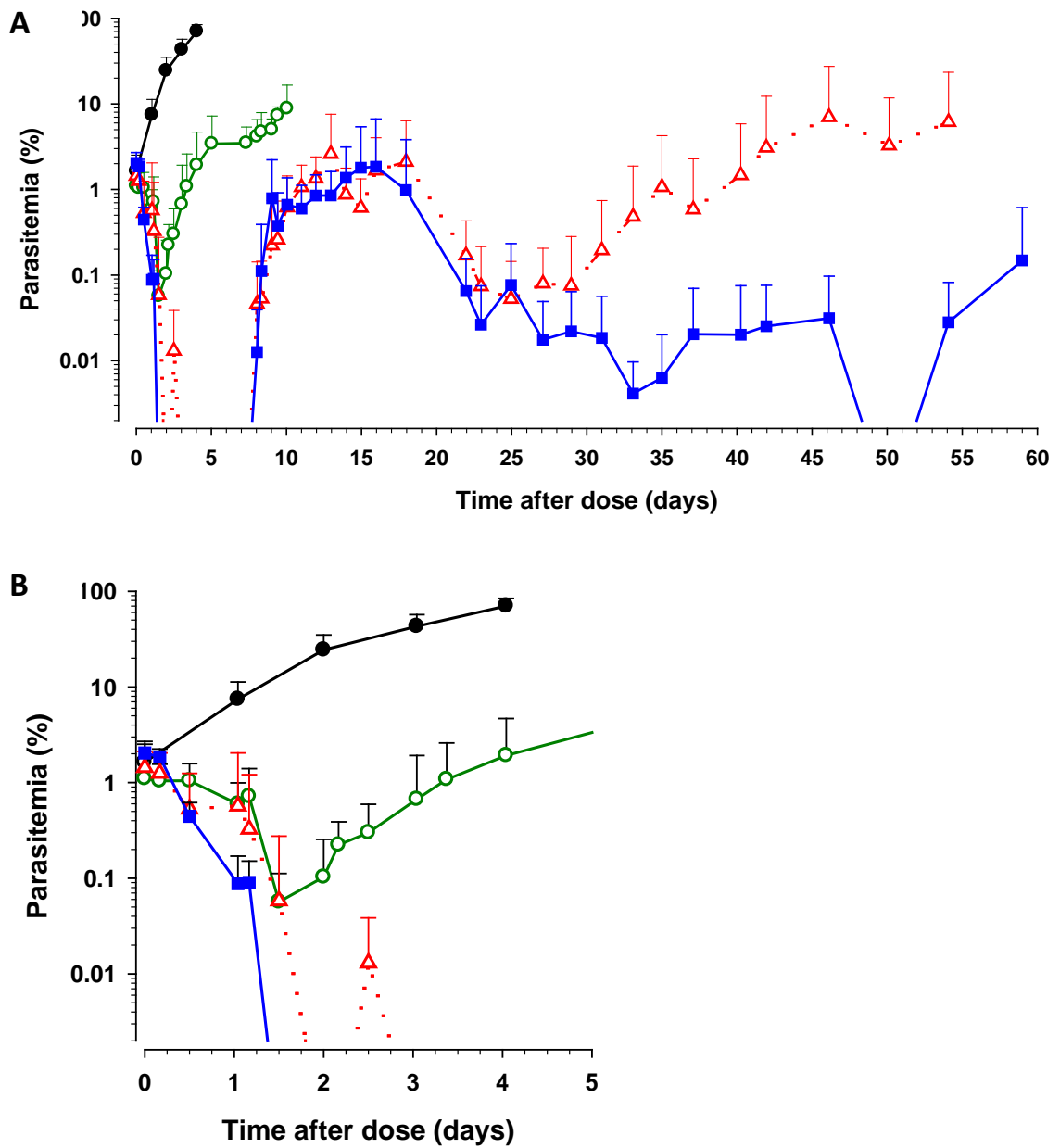
Figure 4.2 Parasitaemia-time profile in Swiss mice following a single i.p. dose of PQP administered 64 h after inoculation with 10^7 *P. berghei* parasitised erythrocytes. Data are shown as total parasitaemia (% of infected erythrocytes ± SD), commencing from the time of PQP administration. Control (n=8; ●——); 10 mg/kg (n=14; ○——); 30 mg/kg (n=13; △– – –); 90 mg/kg (n=14; ■——). Panel B shows an expanded view for the first 5 days after drug administration.

4.3.2 Parasite re-inoculation at 60 days

All 3 groups of control mice, which were either untreated or received vehicle or 90 mg/kg PQP on Day 0 and then inoculated with 10^7 *P. berghei* parasites on day 60, demonstrated similar parasite responses. After inoculation all groups showed rapidly increasing parasitaemias with euthanasia required 4 days after infection as peripheral parasitaemias reached >10% (Fig. 4.3). Mice that had previously been inoculated and received 90 mg/kg PQP on Day 0, and were re-inoculated on Day 60, remained asymptomatic for two weeks (pre-determined end-point) with only low-level parasitaemias observed during this period of time (generally <1%) (Fig. 4.3).

4.3.3 Pharmacokinetic Study

HPLC chromatograms are shown in Fig 4.4. The CQ internal standard eluted at 1.4 min whilst PQ eluted at 4.5 min (Fig. 4.4; B). The chromatogram confirmed that CQ was a suitable internal standard as there was adequate separation between the two peaks, thus a reduced probability of the peaks interfering with one another. The blank plasma was also shown to be free of endogenous substances (Fig. 4.4; A). All analysed mouse plasma samples showed two drug peaks (CQ and PQ) and no indication of drug metabolites or other interfering substances (Fig. 4.4; C). Mouse plasma sample 22 (Fig. 4.4; C), taken from a healthy mouse 12 hours after drug administration, had a PQ concentration of 136 µg/L.

Pharmacokinetic data from both healthy and malaria-infected mice are summarized in Table 4.1 and presented in Fig. 4.5. The plasma PQ concentration-time profiles and pharmacokinetic descriptors were similar in healthy and malaria-infected mice.
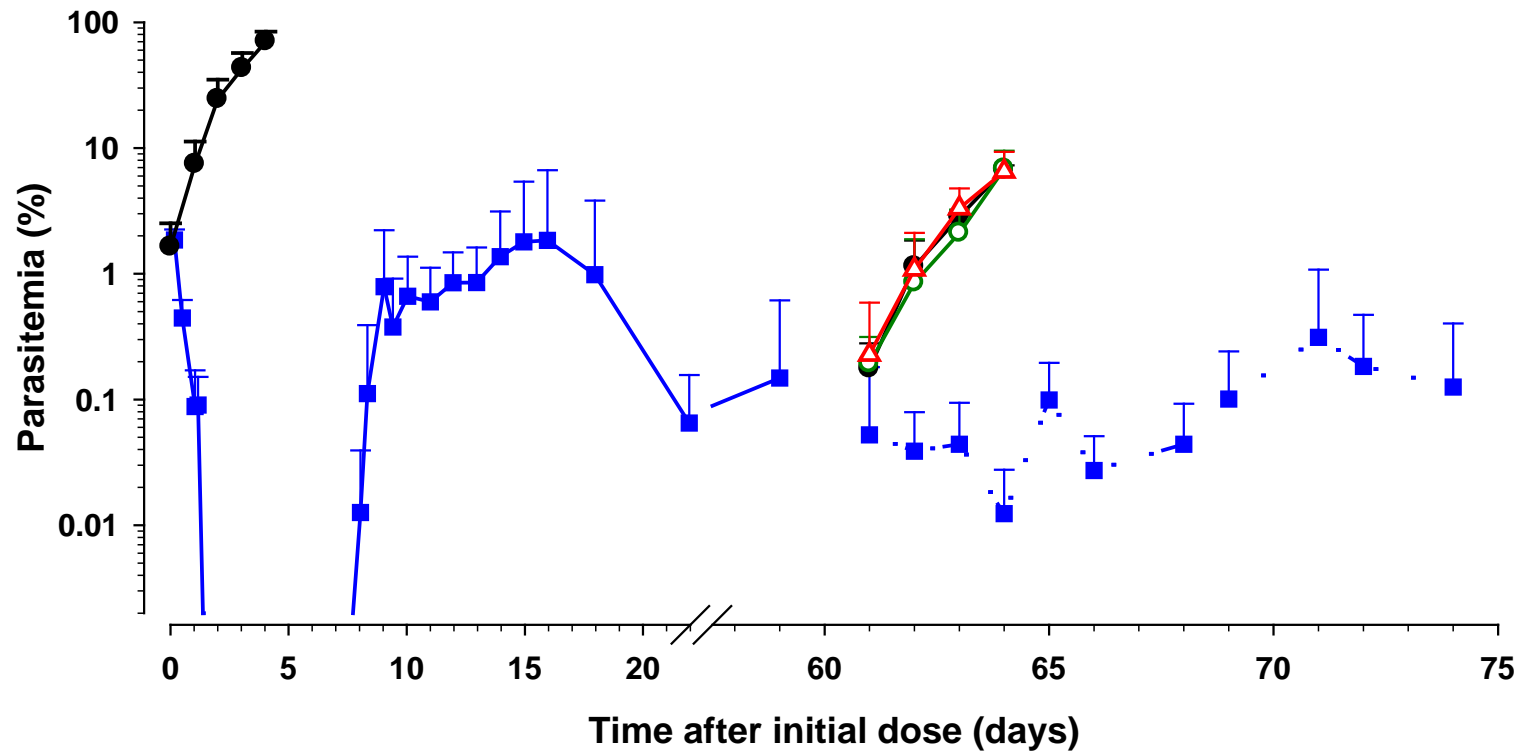
Figure 4.3   Parasitaemia-time profile in Swiss mice following a single dose of PQP (90 mg/kg; n=14; ■ ——) at time zero, and also following subsequent re-inoculation with 10^7 *P. berghei* parasitised erythrocytes at 60 days. Data are shown as total parasitaemia (% of infected erythrocytes ± SD). Three control groups studied at the 60 day point comprised uninfected Swiss mice that were age-matched and inoculated for the first time after 60 days: Untreated at day 0 (n=4; ● ——); Vehicle at day 0 (n=4; ○ ——); PQP 90 mg/kg at day 0 (n=4; △ ——).
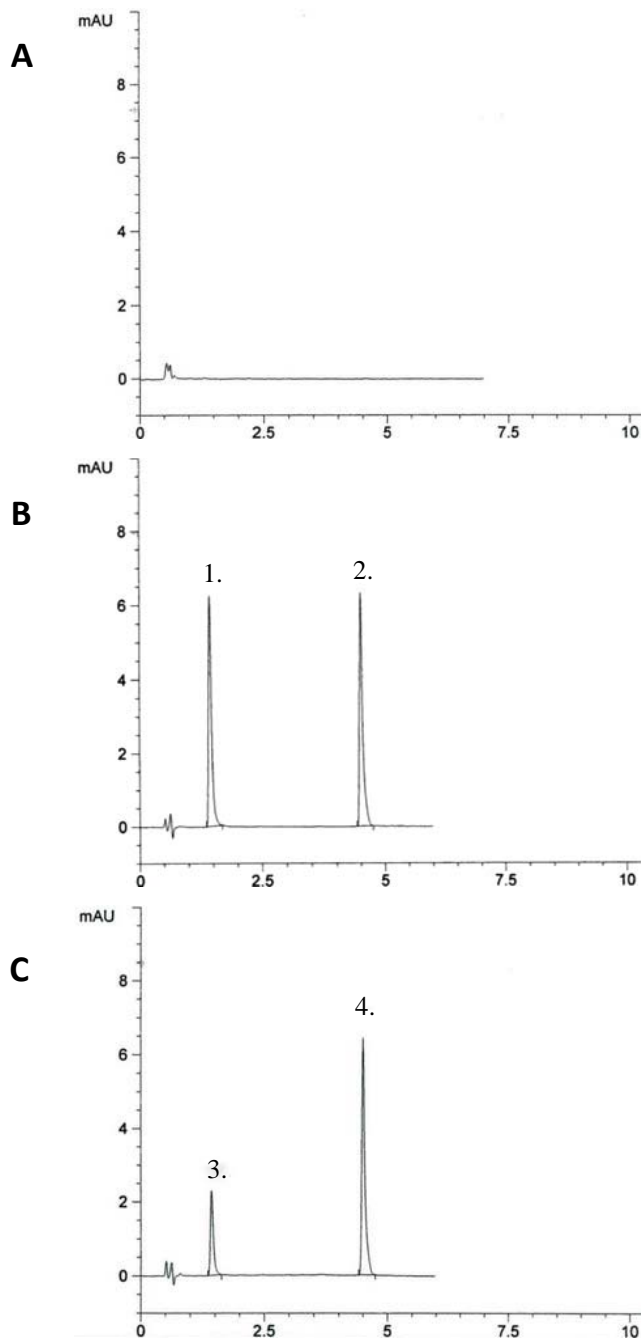
Figure 4.4   High performance liquid chromatography chromatograms for analysis of piperaquine concentrations in mouse plasma samples.   Chromatograms demonstrate results for **A**: Blank human plasma; **B**: Drug Standards; **C**: Mouse plasma sample analysis.   Peaks identified were **1.** PQ internal standard (1.4 min); **2.** CQ standard (200ng; 4.5 min); **3.** PQ in mouse plasma sample 22 (136 µg/L) and **4.** CQ internal standard.
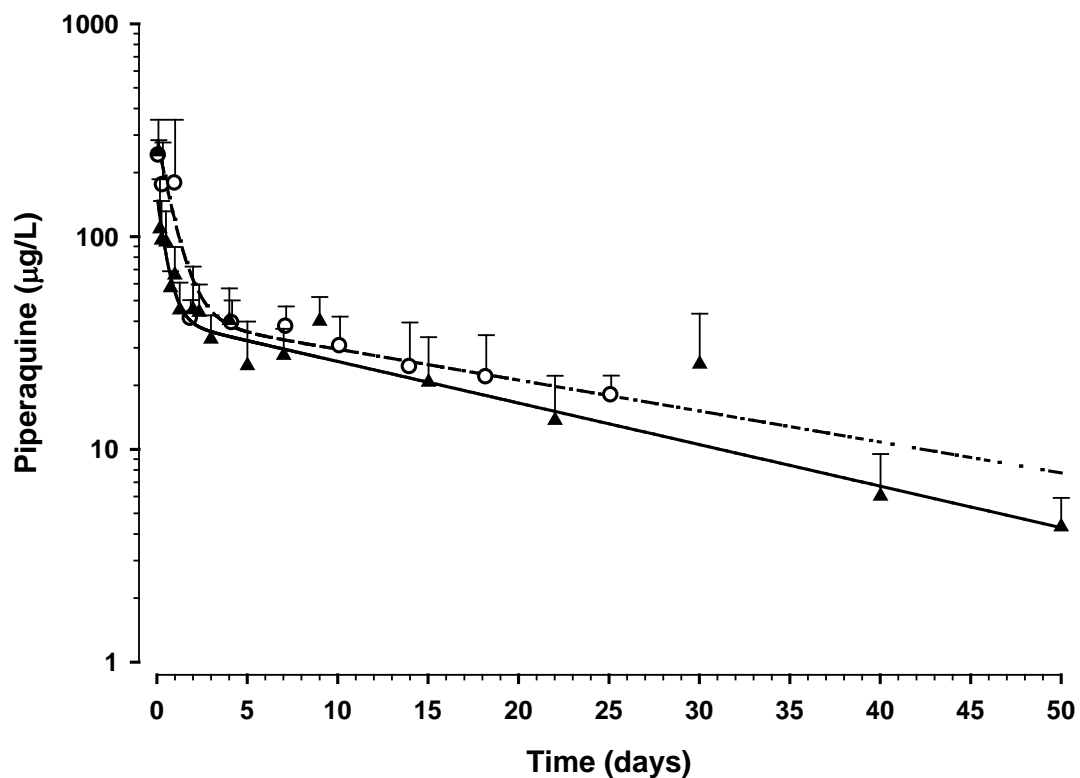
Figure 4.5 **Concentration-time profile of PQ in mice given approximately 90 mg/kg i.p. PQP (normalized for pharmacokinetic analysis, Section 4.2.3).** Data are given as mean ± SD plasma PQ concentration (n=5) in healthy (○ — —) and malaria-infected (▲ ——) mice. The lines represent the best fit of a two-compartment model to the respective data sets (extrapolated beyond the last data point for healthy mice (25 days) to facilitate comparisons).

Table 4.1 **Pharmacokinetic parameters for PQ following i.p. (2,700 µg; 90 mg/kg) administration in healthy and malaria-infected male Swiss mice.** Pharmacokinetic parameters were determined using both non-compartmental analysis ($t_{1/2}$, AUC, CL/F and V/F) and from a two-compartment model ($t_{1/2\alpha}$ and $t_{1/2\beta}$).

	Uninfected mice (n=50)	*P. berghei* infected mice (n=100)
$t_{1/2}$ (days)	17.8	16.1
$t_{1/2\alpha}$ (days)	0.59	0.35
$t_{1/2\beta}$ (days)	20.7	15.4
AUC (mg.h/L)	33.53	27.34
CL/F (L/h/kg)	1.55	1.9
V/F (L/kg)	956	1059

4.3.4 PQ and DHA combination study

The effects of single dose PQP (10 mg/kg), DHA (30 mg/kg) and a combined dose of PQP + DHA (10 mg/kg PQP + 30 mg/kg DHA) are summarized in Figure 4.6 (data normalized for clarity) The starting parasitaemia was 4.5 ± 1.1% for control mice (n=8), 4.6 ± 1.1% for DHA (n=14), 1.5 ± 0.6% for PQP (n=14; P<0.001 compared to other groups; ANOVA) and 3.7 ± 1.5% for PQP + DHA combination (n=14). Because of the differences in the starting parasitaemias between the groups, it was decided to normalize the parasitaemias (expressing parasitaemia as a proportion of the initial parasitaemia) to facilitate comparison between treatment groups, particularly when determining the time to, and level of, parasite nadir.

Parasite nadir occurred at 24, 28 and 36 h after administration of DHA alone, PQP+DHA and PQP alone, respectively. Comparison of parasite nadirs for the DHA, PQP and PQP + DHA treatment groups showed a 11.9 ± 4.8, 12.8 ± 3.1 and 22.4 ± 11.8 fold decrease in parasitaemia from the time of drug administration (P=0.007; PQP + DHA compared to both DHA and PQP alone; ANOVA).
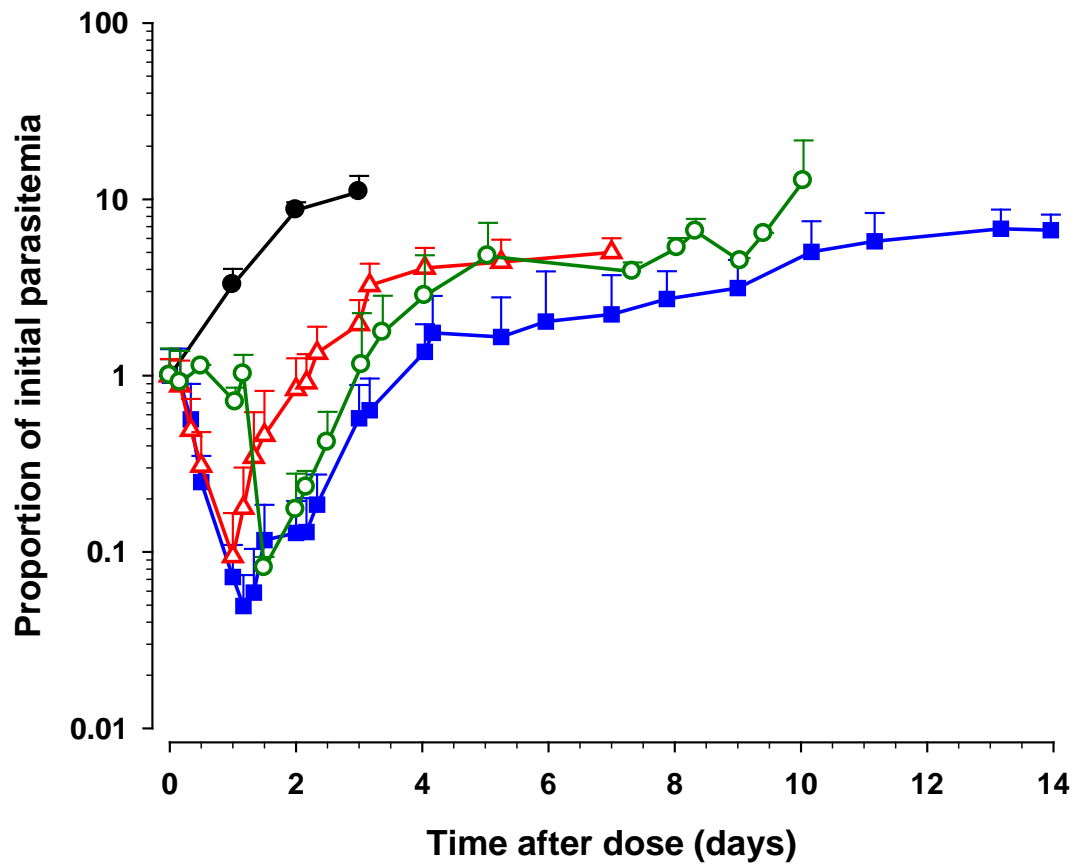
Figure 4.6 **Parasitaemia-time profiles in mice as a proportion of parasitaemia at the time of dosing (mean ± SD).** Mice were given vehicle i.p. (n=8; ● ——), 10 mg/kg PQP i.p. (n=14; ○ ——), 30 mg/kg dihydroartemisinin i.p. (n=14; △ ——), 10 mg/kg PQP plus 30 mg/kg DHA (n=14; ■ ——).

4.4 DISCUSSION

Using a modified murine efficacy model (Rane) this novel and detailed investigation of PQ demonstrated a potent, long lasting antimalarial efficacy in mice infected with *P. berghei* parasites. Furthermore, the combination of PQP and DHA led to enhanced efficacy, compared to either drug alone, with a slightly faster decline in parasitaemia and a significantly lower nadir, suggesting an additive effect.

The pharmacokinetic properties of PQ have recently been well established in humans with the highly lipid-soluble drug having a long elimination half-life of approximately 23 days (19-28 days) in adults and 14 days (10-18 days) in children (10, 203, 232, 253, 254, 286, 425, 443, 480, 484). Meanwhile, pharmacokinetic parameters of PQ in a murine model are scant with a single Chinese report (95) which suggested a half-life of 9 days in mice using ^{14}C-labelled PQ phosphate. The current study demonstrated that PQ has a long terminal elimination half-life. Malaria-infected and healthy mice showed similar pharmacokinetic profiles with biphasic elimination and a terminal half-life of 17.8 and 16.1 days, respectively. Although the half-life determined in this study is almost double that previously reported, the method used to determine plasma PQ concentration (231) is more robust than the ^{14}C-labelled PQP method used by Chen et al. (95), which has been described as problematic (127).

The single dose pharmacodynamic study showed that PQ has a relatively prompt initial effect in decreasing the parasitaemia with an ED_{50} that appears to be in the order of 30 mg/kg. An ED_{50} within this dosing range was presumed because after receiving a single i.p. dose of 30 mg/kg PQP, the drug appeared to be relatively ineffective for 1/3 (n=4) of mice in this cohort (n=13) whilst the remaining 2/3 of mice showed long lasting antimalarial efficacy. By comparison, an *in vivo* study of PQ and PQP in a CQ-sensitive strain of *P. berghei* determined that the ED_{50} of PQ and PQP (base equivalent) was 5.0 ± 0.2 mg/kg and 4.5 ± 0.1 mg/kg, respectively (127, 402).

A single dose of 90 mg/kg PQP was seen to be highly effective for the clearance of parasitaemia in all mice. Initially the parasitaemia fell below the limit of detection for approximately one week, at which time peripheral parasitaemia again became evident. However, instead of a terminal recrudescence, all mice maintained subclinical levels of

infection until the experimental endpoint, 60 days after the initial dose of PQ. At this time all mice were re-challenged, resulting in an apparent resistance to the inoculum with no notable changes in parasitaemia observed. The observed response is highly suggestive of the mice having a degree of immunity towards the *P. berghei* parasites. In order to consider the mechanisms responsible for the current observations, further investigation into the effect of host age, host immunological status, parasite viability and potential drug resistance was conducted in the next phase of PQ studies, which are reported in Chapter 5.

Linking the parasitaemia-time profiles for the 90 mg/kg PQ dose group (Figs. 4.2; A and B), to the corresponding pharmacokinetic data (Fig. 4.4) indicates that plasma PQ concentrations fell from a mean of 250 μg/L, 2 h after the dose, to 45 μg/L approximately 36 h after the dose, at which time the parasitaemia was below the limit of detection (0.002%). During the period of undetectable parasitaemia, 2-7 days after drug administration, the plasma PQ concentration was approximately 20-50 μg/L. In most mice, PQ concentrations >10 μg/L persisted for at least 30 days and the extrapolated mean plasma PQ concentration at 60 days after dosing (the time of the re-inoculation experiment) was 3 μg/L in malaria-infected mice and 5.5 μg/L in control mice. At these low drug concentrations it was presumed that the antimalarial efficacy of PQ was insufficient to control or prevent relapsed parasitaemia.

Although the introduction of the potent artemisinin derivatives into the clinical setting has proved to be highly successful in the treatment of uncomplicated malaria, their characteristics as short acting drugs with high recrudescence rates (in short-term dosing regimens) means that it is imperative that they be combined with a second antimalarial drug for clinical use (127, 584). It has been recommended that the partner antimalarial should be a long-acting drug with a half-life that extends at least two parasite life-cycles (>4 days), have good tolerability, be low cost and have limited pre-existing drug resistance (127, 232). In this context, PQ makes a good partner drug for the artemisinin derivatives as it has a median half-life that is 3 to 6 times greater than that recommended and rates well on tolerability, cost and shows limited resistance (127, 232).

The purpose of the combination study was to demonstrate the initial response of PQ both in direct comparison and in combination with DHA. After a single dose of PQ the parasitaemia was seen to remain stable for 12 h before any decline was observed. In comparison, those mice receiving a single dose of DHA demonstrated an immediate decline in parasitaemia followed by a rapid rate of parasite recovery as shown previously (Chapter 3 and (179)). Parasite nadir was also seen to differ between the two single dose administrations occurring 24 and 36 h after dosing for PQ and DHA, respectively. The combined dose resulted in a rapid decline in parasitaemia which was consistent with the single dose DHA profile, although the parasite nadir was lower and later than the single dose curve. This therefore demonstrates the influence of PQ in the combination dose. Those mice receiving the combined dose also demonstrated a slower rate of parasite recovery and a longer survival time in comparison to the single dose administrations. This suggests that although single dose PQ is an effective antimalarial therapy, the rate of parasite decline and overall efficacy is enhanced when given in combination with DHA.

The current data suggests an additive interaction between the combined PQ and DHA dose, however, the results do not provide enough evidence to dismiss the possibility of synergism. This is consistent with published data in that previous animal models have demonstrated additive efficacy between DHA and PQ (27, 512), whilst a recent *in vitro* study conducted by Davis et al. (2006) demonstrated that DHA showed either no interaction or was mildly antagonistic when combined with PQ (126).

Despite the persistence of low concentrations of PQ post-treatment, re-inoculation with *P. berghei* parasites shows that the low concentrations of drug in circulation may not necessarily impede the development of an infection. The long elimination half-life of PQ is a predisposing factor for the potential emergence of any resistance, which was evident when PQ was used as a monotherapy in China in the 1970s (127, 209, 217, 425). However, when used in combination with artemisinin derivatives, which have a parasiticidal effect that both accelerates therapeutic responses and reduces parasite biomass, the extended efficacy of PQ is thought to prevent any potential recrudescence. This would thereby eliminate residual parasites thus reducing the number of surviving mutant parasites which in turn prevents any resistance towards

either drug (46, 209, 252, 477). Clinical studies have actually suggested that the extended post-treatment prophylaxis of PQ may reduce both relapse and reinfection for a period of 4-6 weeks after treatment (27, 209, 408). As a result, patients remain asymptomatic for a longer period, which increases the time for haematological recovery, resulting in the risk of anaemia been halved, as well as a reduced gametocyte carriage rate (209, 408). Therefore, it is thought that although the post-treatment prophylaxis provided by PQ in this combination therapy will not prevent subsequent relapses, this drug combination has the capability of delaying the potential of relapse (408).

Results obtained in this series of investigations have determined that in this *P. berghei* malaria model PQ gave effective antimalarial efficacy after single dose administration, enhanced antimalarial efficacy when combined with DHA and an extended period of post-treatment prophylaxis. However, the results also raised a number of questions relating to whether the demonstrated extended antimalarial efficacy was due primarily to PQ or if the animal had acquired immunity towards the infecting species of parasites. In order to address these questions, further investigation into parasite viability, drug efficacy, drug resistance and immunological influences was performed (Chapter 5).

CHAPTER FIVE

PIPERAQUINE EFFICACY STUDY

5.1   INTRODUCTION

5.1.1   Clinical efficacy of long half-life drugs

Clinical studies involving the use of contemporary ACT strategies, where PQ has been incorporated as a partner to either DHA (27, 29, 127, 139, 209, 217, 244, 252, 253, 305, 413, 456, 595) or artemisinin (476), demonstrate high efficacy and good tolerability for the treatment of both *P. falciparum* (27, 29, 139, 217, 252, 305) and *P. vivax* (209, 232, 253, 336) infections.   The success of these ACT strategies, both in the resolution of parasitaemia and the prevention of drug resistance, has been attributed to PQ having a long terminal half-life in humans of 12-28 days (232, 286, 480, 481), fulfilling the requirement for a partner drug that is a long-acting schizontocide with a half-life exceeding 4 days (or two asexual parasite life-cycles) (232, 351).

However, the long half-life of PQ also raises concerns about adverse effects and drug resistance (209, 232, 336, 386).   Traditionally, drugs with a long terminal elimination half-life have been sought as partner drugs in combination therapies, as the use of a single or small number of doses in treatment regimens ensures therapeutic compliance. Also, if used for prophylaxis, treatment is only required on a once weekly basis (558).   It is well established that drugs with a long elimination half-life are potentially vulnerable to the development of resistance as parasites are inevitably exposed to suboptimal/ subtherapeutic drug concentrations for extended periods of times when these drugs are used as monotherapy (541, 558).   This phenomenon has been described by White (550), who demonstrated how slowly eliminated antimalarials such as CQ or PQ present a lengthy opportunity for the selection of resistance among sensitive parasites (MIC A; Fig. 5.1).   However, once resistance has become established (MIC B), blood concentrations are no longer inhibitory and the terminal elimination phase is no longer a factor in parasite clearance (Fig. 5.1; (550)).
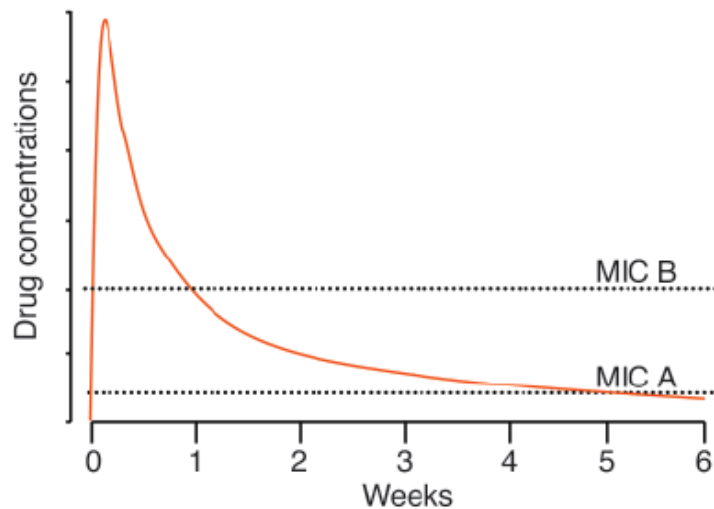
Figure 5.1   Demonstration of prolonged duration of exposure to subtherapeutic concentrations of drug and the subsequent acquisition of drug resistance.   After the use of an antimalarial drug with a long terminal elimination half-life, such as chloroquine or piperaquine, sensitive parasites (MIC A) are exposed for prolonged periods of time to sub-therapeutic blood concentrations of the drug in monotherapy facilitating the opportunity for the acquisition of parasite resistance towards the antimalarial drug in use.   Once parasites have gained resistance towards the antimalarial drug (MIC B), the long terminal half-life effect of the drug is no longer effective and blood concentrations are no longer inhibitory. *Diagram from White, 2004 (550).*

5.1.2   Acquired immunity

In 1980, Bruce-Chwatt (72) wrote "Malaria immunity may be defined as the state of resistance to infection brought about by all those processes which are involved in destroying the plasmodia or by limiting their multiplication. Acquired immunity may be either active or passive. Active immunity is an enhancement of the defence mechanism of the host as a result of a previous encounter with a pathogen. Passive immunity is conferred by the prenatal or postnatal transfer of protective substances from mother to child by the injection of such substances".

Whilst the principal features of naturally acquired immunity have been defined, there is currently little known about the underlying mechanisms behind its activation and maintenance (25, 140, 144, 229, 321, 369). The development of clinical and parasitological immunity to malaria is evident by the ability of the host to control the parasite density and hence the progression of the infection (144, 159). Although achievable after a single infection, acquired immunity is seen to be primarily directed towards the erythrocytic stage with induction of adequate protective immunity usually requiring repeated infections (144, 159, 592). Immunity is species specific (102, 245, 591) but not necessarily strain specific (65, 66, 245). Furthermore, acquired defence mechanisms often have been shown to lose their protective effect soon after the exposure to infection is interrupted, with the host becoming susceptible to new clinical infections of malaria (140). The introduction of asexual erythrocytic stage immunity has been demonstrated in humans, monkeys, birds and mice (167, 168, 223, 226, 308, 444, 460, 474, 479).

5.1.3 Relationships between antimalarial therapy and immunity

Despite the desire to eliminate parasites from infected patients as quickly as possible, it must also be considered that when treating patients living in endemic countries it may be preferable for the infection to clear through both a combination of active antimalarial therapy and the patient's immunological response mechanisms (157, 304, 478). This was demonstrated in a study by Enevold et al. (157) where it was observed that a child's naturally acquired immunity enhanced the clinical efficacy of drug therapy in the clearance of *P. falciparum* infections. These results suggested that recovery from malaria may depend on drug efficacy, parasite drug-resistance as well as a complex interaction with host factors such as acquired immunity and innate resistance (157). Should a patient have had prior opportunity to acquire immune responses towards a strain of *Plasmodium*, the combination of both the natural immune response and drug therapy will strongly influence the rates of recrudescence following treatment, the perseverance of subclinical infection which normally would promote drug resistance, and the transmission of future progeny of infection (117, 304).

Immunity to re-inoculation has also been shown in mice from which parasites were eradicated by use of a curative drug (64). When this group of mice was challenged with a second infection (after treatment with drug), the majority of animals survived in comparison to those mice which remained untreated, which succumbed to the primary infection (64). Further studies combining the use of treatment and natural immunity for the control of infection, demonstrated that only mice that received sub-curative treatments, and thus had parasites present for a prolonged period during the primary course of infection, could induce a level of protective immunity (64, 113-115, 287).

5.1.4   PQ resistance in murine models

The development of PQ-resistant strains of the murine malaria *P. berghei* were first described by Li et al. (279, 280). Through increasing drug pressure over a period of 5 months, two PQ-resistant *P. berghei* strains *(P. berghei* ANKA *and P. berghei* K 173), were developed using *in vitro* methods (127, 279, 280). Although these two *in vitro* PQ-resistant strains were then utilised to test for cross resistance between PQ and a range of antimalarial drugs, it was observed that once drug pressure subsided the parasites returned to their sensitive phenotype (279, 280).

More recently, an *in vivo* model of PQ-resistant *P. berghei* ANKA was described in which resistance was selected through continuous drug pressure over a period of 18 months (259). However, in contrast to earlier models this line of PQ-resistant malaria demonstrated stable selection of PQ-resistance, which was defined by the maintenance of the resistant phenotype when drug selection pressure was removed, for at least 10 serial passages (177, 259).

5.1.5   Study aims

Dose ranging studies of sub-therapeutic doses of PQP in the *P. berghei* murine malaria model demonstrated that after high dose (90 mg/kg) PQP a chronic *P. berghei* infection developed that extended to the experimental end-point of 60 days (Chapter 4; see Fig. 5.2). Furthermore, when all mice were re-infected with a second 10^7 *P. berghei* parasite inoculum there was no change in the course of infection, with the subclinical chronic infection remaining stable. The previous study had also shown that

25 days after 90 mg/kg PQP, the subclinical parasitaemia was in the order of 1% infected erythrocytes and the plasma PQ concentration was approximately 14 μg/L (Section 4.3.3). By comparison, at 40 and 60 days the subclinical parasitaemia was stable at approximately 0.1% and the plasma PQ concentrations (3 to 7 μg/L) were considered unlikely to have an antimalarial effect. As the previous study did not extend beyond 60 days, the present study was designed to clarify the duration of antimalarial efficacy.

Therefore, the aims of the present study were to investigate drug efficacy, re-inoculation outcomes and parasite viability after a single dose of PQP in the *P. berghei* murine malaria model. The significance of determining all of these factors was to provide a greater understanding of how single dose administration of a long half-life drug such as PQ, influence parasite pharmacodynamics, mouse immunological responses and parasite response to continual exposure to suboptimal drug concentrations. This could clarify the influence of PQ on both parasite and host responses. Furthermore, detailed preclinical pharmacodynamic data for PQ, alone or in combination with artemisinin drugs will complement clinical studies, especially when there is interest in the therapeutic impact of persistent low PQ concentrations.

5.2   METHODS

5.2.1   Pharmacodynamic efficacy of 90 mg/kg PQP

In order to investigate whether the observed prolonged subclinical infection was a result of the antimalarial effect of PQ alone or a combination of factors, three distinct groups of mice were studied simultaneously. The purpose of this study design was to determine the effects of (i) animal age at time of inoculation, (ii) prolonged presence of PQP and (iii) acquired immunity at pre-determined time-points (25, 40, 60, 90 and 130 days after PQP administration) along the pharmacodynamic curve.

5.2.1.1 Selection of investigation time-points

After careful consideration of the pharmacodynamic profile of 90 mg/kg PQP in the *P. berghei* murine model, as demonstrated in the previous chapter, investigation time-points of 25, 40, 60, 90 and 130 days after drug administration were selected. These time-points reflected times of significant change in the drug pharmacodynamics (Fig.

5.2). After drug administration the parasitaemia was seen to rapidly decline, falling to undetectable levels by 36 h, with parasite recrudescence observed at 7 days after PQP. The parasitaemia was seen to plateau, and then steadily decline about 20-30 days after drug administration, with a persistent parasitaemia in the range of approximately 0.01-0.1% until day 60.

Therefore, the time-points selected for this investigation reflect a period of declining parasitaemia (25 days), low level persistent parasitaemia (40 and 60 days), and approximately one and two months after the experimental end point of the previous study (Fig. 5.2).
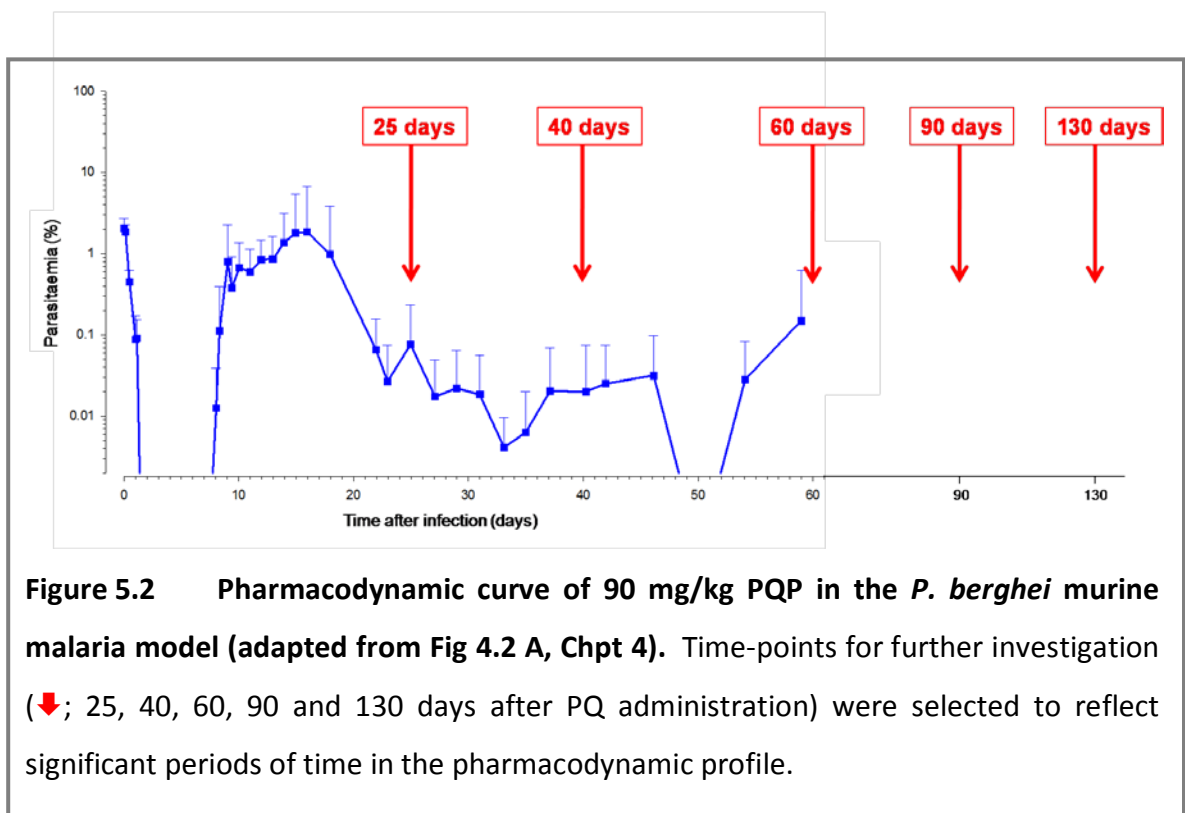
Figure 5.2   **Pharmacodynamic curve of 90 mg/kg PQP in the _P. berghei_ murine malaria model (adapted from Fig 4.2 A, Chpt 4).** Time-points for further investigation (⬇; 25, 40, 60, 90 and 130 days after PQ administration) were selected to reflect significant periods of time in the pharmacodynamic profile.

5.2.1.2 Experimental protocol

a) **Group A (Age control)** – Previous studies have identified that the age of a rodent, at the time of inoculation with _plasmodium_ parasites, influences the course of infection (4, 596). In particular, it was demonstrated in rats that when young (4 weeks) and mature (8 weeks) animals were infected with similar inoculums of _P. berghei_ parasites, the young animals were more susceptible to infection and had a higher mortality rate, than the older rats (4). It was

therefore desirable in the present study to determine whether there were any age related effects on time of inoculation, in the murine *P. berghei* model.

Uninfected 5-6 week old male Swiss mice (n=20) were given a single administration of drug vehicle at Day 0. At each of the pre-determined time-points, groups (n=4) of mice were inoculated with a standardised inoculum of 10^7 *P. berghei* parasites by i.p. injection (Fig. 5.3). The mouse age at each inoculation time point was therefore, 9-10 weeks, 11-12 weeks, 14-15 weeks, 18-19 weeks and 24-25 weeks at 25, 40, 60, 90 and 130 days, respectively. Parasitaemia was monitored through the preparation and examination of twice daily blood films. Mice were euthanased when parasitaemia exceeded 40%.

b) **Group B (PQ control)** – The purpose of the second control group was to determine the antimalarial efficacy of PQ by establishing whether the PQ concentration at each designated time point was sufficient to suppress or resolve parasitaemia after inoculation.

Uninfected 5-6 wk old male Swiss mice (n=30) received a single administration of 90 mg/kg PQP on Day 0 (Fig. 5.3). At each pre-determined time point (25, 40, 60, 90 or 130 days after drug administration) groups of mice (n=6) were injected with a standardised inoculum of 10^7 *P. berghei* parasites. Parasitaemia was monitored through the preparation and examination of twice daily blood films.

c) **Group C** – The purpose of the primary study group was to determine whether those mice that maintained a persistently low parasitaemia after drug treatment had acquired a degree of immunity to this strain of *P. berghei*. In order to determine whether this had occurred, *P. berghei* infected and PQP-treated mice were exposed to a second inoculum of *P. berghei* parasites at each designated time-point (25, 40, 60, 90 and 130 days after drug administration). It was expected that if any parasite suppression was observed or if a new infection failed to develop, which could not be explained by animal

age or drug influences (control arms), this would suggest that a degree of immunity to the infecting strain of parasites had been acquired.

The efficacy group consisted of 5-6 week old male Swiss mice (n=50) that were initially inoculated with a standardised inoculum of 10^7 *P. berghei* parasites. When parasitaemia had reached a level of approximately 3-5% (64 h after inoculation), each infected mouse received a single dose of 90 mg/kg PQP by i.p. injection (Fig. 5.3). At each designated investigation time point, groups of mice (n=6) were re-inoculated with a standardised inoculum of 10^7 *P. berghei* parasites (from the same initial parasite stock as the initial infection; refer to 5.2.2) by i.p. injection. Furthermore, at each experimental time point a second sub-group of mice (n=4) was used for parasite viability passage (Refer to 5.2.2). Parasitaemia was monitored through preparation and examination of twice daily blood films until time of euthanasia.

5.2.1.3 Pharmacodynamic profile

In order to determine the pharmacodynamic time-density profile for this series of investigations, the parasitaemia was monitored through the preparation and examination of twice daily blood films from all mice. As sub-groups of mice were used for further investigations at each designated time-point (25, 40, 60, 90 and 130 days after PQP administration) the numbers of mice used to construct the pharmacodynamic profile decreased accordingly throughout the experimental period. Therefore, for the first 25 days the parasitaemia curve reflects the mean parasitaemia for n=50 mice from Group C, between 26 and 40 days n=40 mice, between 41 and 60 days reflects the mean parasitaemia for n=30 mice, between 61 and 90 days is the mean parasitaemia for n=20 mice and between 91 and 130 days reflects the parasitaemia of n=10 mice. The mean parasitaemia for the entire Group C mice has been reported.

In a complementary arm of the Group C investigations, a group of age-matched mice were simultaneously infected with 10^7 *P. berghei* parasitised erythrocytes but remained untreated, thereby acting as a control for the parasite inoculation (Fig. 5.3).
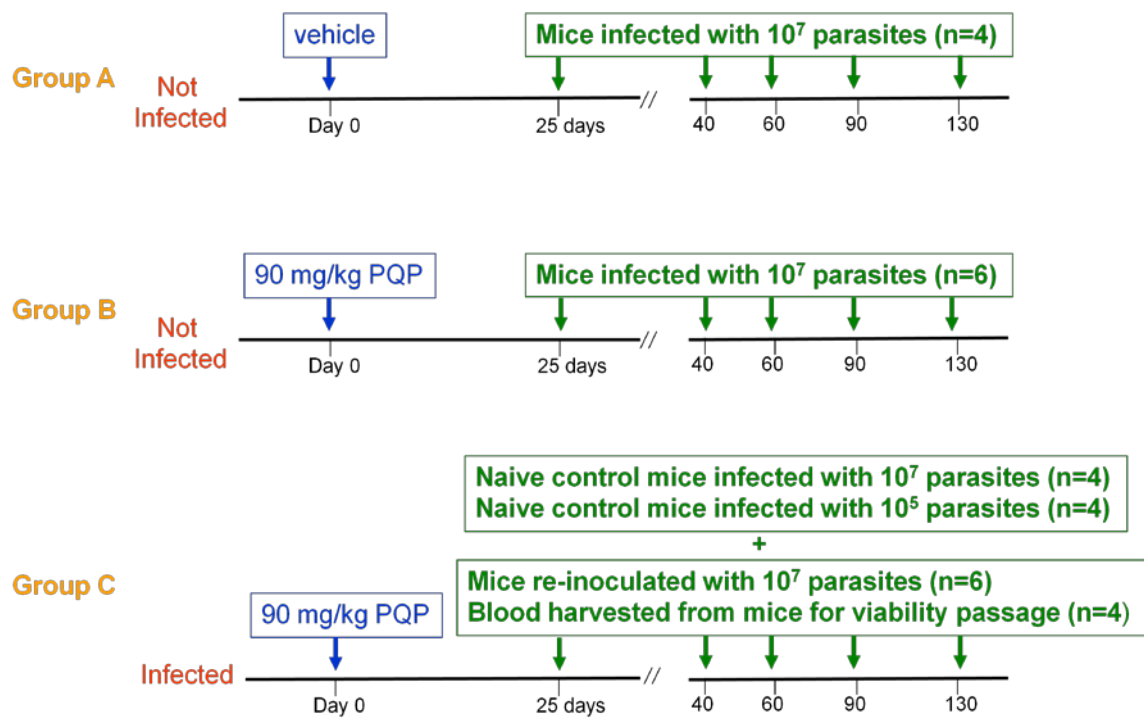
Figure 5.3 **Schematic describing the experimental protocol for each of the 3 arms investigated in this study.** Group A consisted of uninfected mice that received drug vehicle on Day 0 with groups of mice infected at each experimental time point to determine if mouse age at time of infection affected the course of infection. Group B consisted of uninfected mice that received PQP on Day 0 with groups of mice inoculated with parasites at each time-point. This control group was run to determine whether 90 mg/kg PQP was efficacious against infection at each experimental time point. Group C consisted of infected mice that were treated with PQP on Day 0. At each experimental time point a group of mice was re-inoculated with parasites to determine if the experimental mice had acquired immunity towards the infection whilst blood was harvested from a second sub-group of mice for passage into naive hosts to test for parasite viability. Furthermore, two separate control arms that consisted of groups of naive mice were inoculated with either a 10^5 or 10^7 *P. berghei* inoculum at each time point to demonstrate a normal time-density profile with inoculums of this size.

5.2.2   Parasite viability

This arm of the study was designed to evaluate the viability of parasites from previously infected, treated mice with subclinical parasitaemia. Although parasites were present in the peripheral circulation, albeit at low levels, it was not known if these parasites were dead or drug affected and therefore unable to produce progeny, or whether drug or immune mechanisms were able to suppress the parasitaemia by preventing replication. This was investigated by removing blood (containing parasites) from the host, washing the red blood cells to remove any drug or antibodies and then passaging the washed cells into uninfected, naive mice.

Included in this component was an evaluation of *P. berghei* resistance to PQ. It has been well documented than when used in monotherapy, parasites have had the propensity to develop resistance mechanisms against PQ.   Furthermore, several publications have demonstrated the acquisition of resistance to PQ in rodents, although this is predominantly obtained through repeated exposure to drug pressure (259, 402).   However, it was of interest in the present series to observe whether there was any difference in the pharmacodynamic response of 90 mg/kg PQP when administered to mice that had been inoculated with parasites from treated (Group C) hosts with subclinical infections.

5.2.2.1 Experimental design

At each designated time point (25, 40, 60, 90 and 130 days) blood was harvested from a sub-group of Group C mice (n=4) via cardiac puncture. The blood was centrifuged at 3,000 g for 5 min and the plasma was separated, measured and stored at -80°C for later PQ analysis. The red blood cells were washed 3 times in 0.9% w/v NaCl (centrifuged each time for 5 min at 3,000 g), with care taken to remove all of the washing saline and buffy coat, in which sensitised leukocytes or free antibody may be present. The packed red blood cells were then re-suspended in the same volume of 0.9% w/v NaCl as the plasma that was originally removed from the packed erythrocytes. A blood smear was prepared using the red blood cell suspension to estimate the parasitaemia for the donor sample.

Passage of the red blood cell suspension to naïve recipient mice was dependent upon the level of parasitaemia. In general, the parasitaemia was <1% and recipient mice were inoculated with a 1:5 dilution of the original suspension (200 µL i.p. injection). Where the parasitaemia was >1%, the parasite suspension was diluted to provide a standard inoculum of 10^5 *P. berghei* parasites in 100 µL. Each of the four donor inoculums was passaged into a group of naïve male mice (5 weeks old; n=5) resulting in a total group size of 20 for each time point (Fig. 5.4). Simultaneously, a group of naive untreated age-matched mice (n=4) were inoculated with a 10^5 *P. berghei* inoculum to act as a control arm for the viability study. Five days after inoculation, three of the mice from each group of recipients were treated with a single 90 mg/kg i.p. dose of PQP and the remaining two mice per group remained untreated (Fig.5.4). Hence, at each time point both the parasite viability and the effect of PQ were evaluated. Parasitaemia was monitored by twice daily peripheral blood films.

5.2.3   Pharmacokinetic parameters

The plasma PQ concentration was determined in both malaria-infected (n=4) and uninfected age-matched control mice (n=2) at each time point (25, 40, 60, 90 and 130 days). Blood was harvested by cardiac puncture, centrifuged at 3,000 *g* for 5 min and the plasma was separated and stored at -80°C until analysed using a validated HPLC assay with limits of quantification and detection of 1.6 µg/L and 0.7 µg/L, respectively (231, 254). The mean plasma PQ concentration was compared with data from the previous pharmacokinetic study (Chapter 4; Section 4.3.3).

1.

2.

3.

4.

| Drug vehicle | 90 mg/kg PQP |
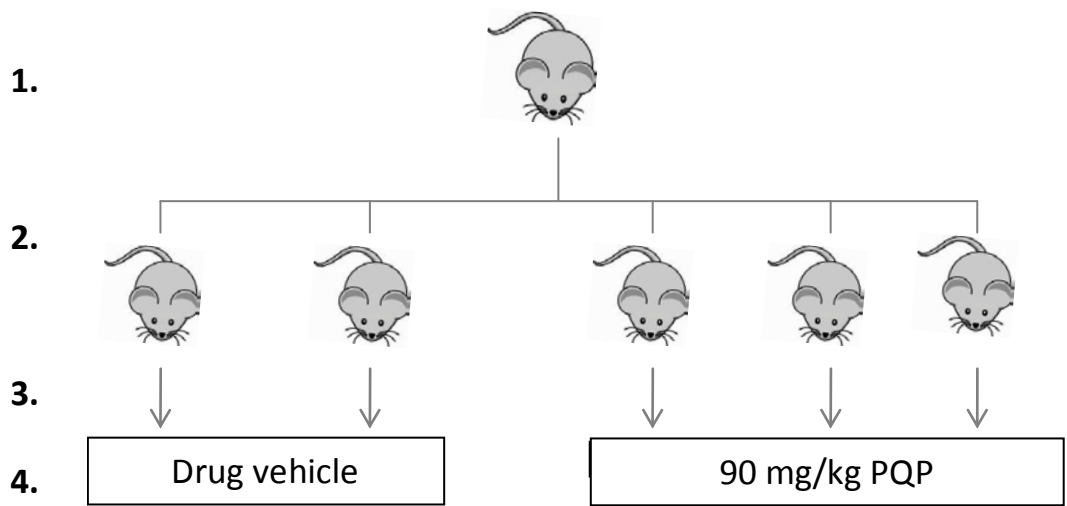
Figure 5.4 **Schematic simplifying the experimental protocol for testing parasite viability and drug response.** Groups of mice (n=4), initially infected then treated with 90 mg/kg PQP at Day 0 (Group C), had blood harvested at each experimental time point for testing of parasite viability and drug response. **1.** At each experimental time point, blood from each mouse was harvested via cardiac puncture, washed to remove antibodies or residual plasma PQ concentrations, then prepared for inoculation to recipient mice; **2.** The prepared blood was injected into 5 naive mice by i.p. injection; **3.** Mice were returned to cages with peripheral blood films prepared twice daily to monitor parasitaemia; **4.** Five days after inoculation with donor blood, 3 out of the 5 mice received a single i.p. dose of 90 mg/kg PQP whilst the remaining two mice received drug vehicle. Parasitaemia was monitored in all mice through preparation of twice daily blood films until time of euthanasia (parasitaemia > 40% or at experimental end-point, 25 days after inoculation)

5.3   RESULTS

5.3.1   PQ pharmacodynamics.

Administration of a single dose of 90 mg/kg PQP resulted in a rapid decline in parasitaemia which fell below the limit of detection (0.002%) by 36 h after dosing (Fig. 5.5). In all mice, recrudescence occurred 7-8 days after drug administration, with a mean peak parasitaemia of 1.32% ± 0.56% at 13 days. The parasitaemia remained relatively stable for approximately 8 days and declined to a mean of 0.03% by Day 25 (Fig. 5.5). Thereafter the mean parasitaemia remained below 0.015% until 80 days after dosing, beyond which there were no detectable parasites in any mice up to and including the experimental endpoint of 130 days after initial dosing. These findings were consistent with the previous study (Chapter 4).

5.3.1.1 Group A

Group A control mice that were initially uninfected and untreated then inoculated at each pre-determined time (25, 40, 60, 90 or 130 days), showed similar parasite density-time profiles to those in previous studies (Chapters 3, 4 and (49, 467)). This was characterised by rapidly rising parasitaemias until the time of euthanasia (6-8 days after inoculation; peripheral parasitaemia >40%; Fig. 5.6). Hence, in this model, mouse age (between 5 and 25 weeks) did not influence the course of *P. berghei* infection.

5.3.1.2 Group B

Group B uninfected control mice, that received 90 mg/kg PQP on Day 0 and were inoculated for the first time at each experimental time-point, demonstrated a modest suppressive effect against the malaria parasites in the 25 day cohort only (Fig. 5.7). This cohort of mice had a mean parasitaemia approximately 10 fold lower than controls and a median survival time of 15 days (Fig. 5.7). By contrast, no apparent parasite suppression was seen at any other time-point, with median survival times of 8, 8, 8 and 7 days for the 40, 60, 90 and 130 day cohorts, respectively. In the latter groups, parasite density-time profiles and survival times were comparable to those for Group A control mice that received only drug vehicle. Therefore, it may be suggested that 90 mg/kg PQP was mildly suppressive until approximately 25 days after drug administration.
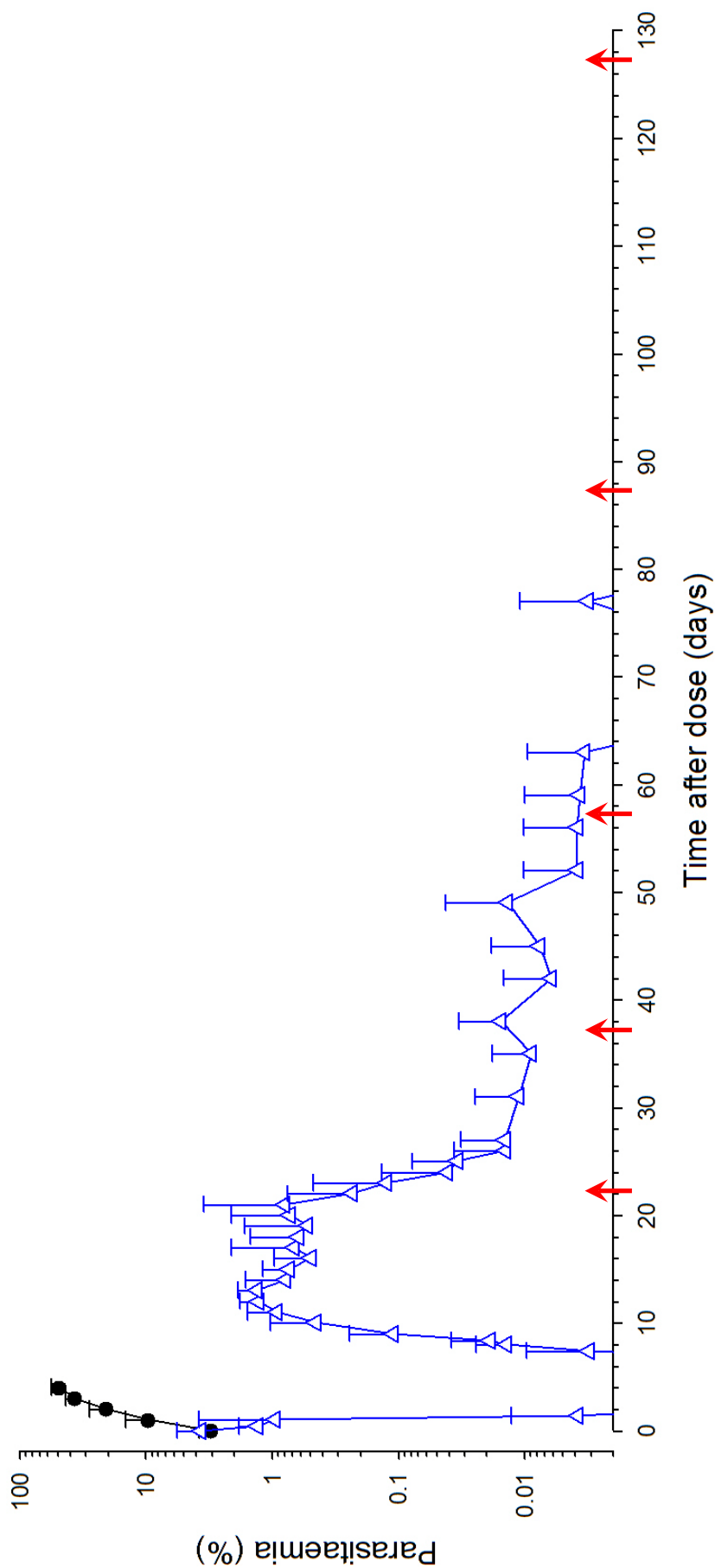
Figure 5.5   Parasitaemia-time profile in Swiss mice following the administration of a single dose of 90 mg/kg PQP administered 64 h after inoculation with 10^7 *P. berghei*-parsitised erythrocytes. Data are shown as total parasitaemia (mean percentage or erythrocytes infected ± SD), commencing from the time of PQP administration. Symbols: ●, control (n=4); △,90 mg/kg (n=60 initially, less 10 mice following each of the pre-determined study points at 25, 40, 60 and 90 days (indicated by ↑)

5.3.1.3 Group C

Data for Group C mice that were previously inoculated and received 90 mg/kg PQP on Day 0, are shown in Figure 5.8. The cohort that was re-inoculated on day 25 maintained a low, subclinical parasitaemia (<0.015%) with all mice remaining asymptomatic for 4 weeks (pre-determined endpoint of re-inoculation study; Fig. 5.8). A similar outcome was seen at 40, 60 and 90 days, with all mice remaining asymptomatic and peak parasitaemias of 0.03%, 0.3% and 0.9%, respectively. In contrast, mice re-inoculated 130 days after initial drug administration (which had been aparasitaemic for >50 days) showed a rapidly increasing parasitaemia, with a median survival time of 10 days (Fig. 5.8).

The naive control group, which was run in parallel with the re-inoculation study, confirmed that the standard inoculum of 10^7 parasitised erythrocytes injected into the experimental mice would lead to predictable outcomes of a rapidly increasing parasitaemia and lethal outcome within one week (as seen in previous chapters). Therefore, this control arm adequately established that the parasite inoculum prepared for re-inoculation purposes was consistent with expected pharmacodynamic responses obtained in naive mice.
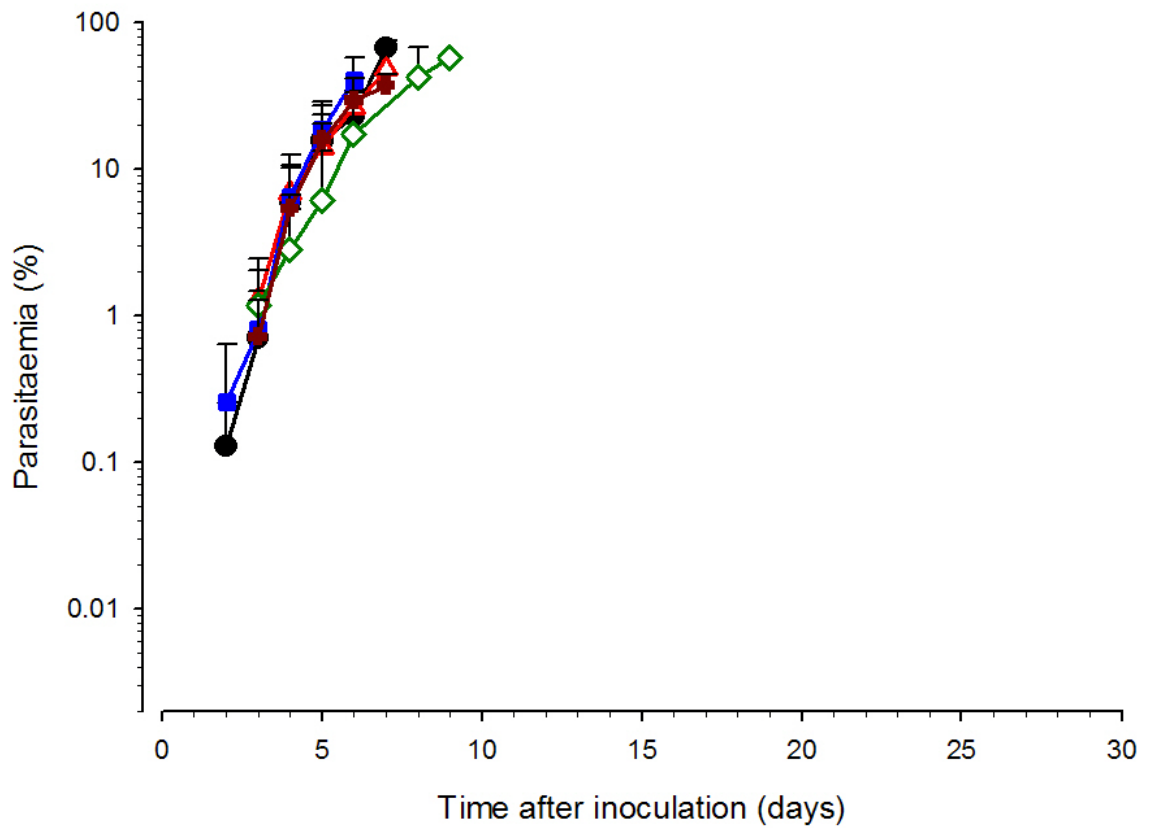
Figure 5.6　　　**Parasitaemia-time profiles from the parasite re-inoculation arm of the study.** Untreated uninfected age-matched Group A control mice (n=4 at each time point) that were inoculated with 10^7 *P. berghei* parasitised erythrocytes at pre-determined times (●, 25 days; △,40 days; ■, 60 days; ◇, 90 days; ✚, 130 days). Data are shown as total parasitaemia (mean percentage of erythrocytes infected ± SD), commencing from the time of inoculation. (see also text, sections 5.2.1.2a and 5.3.1.1).
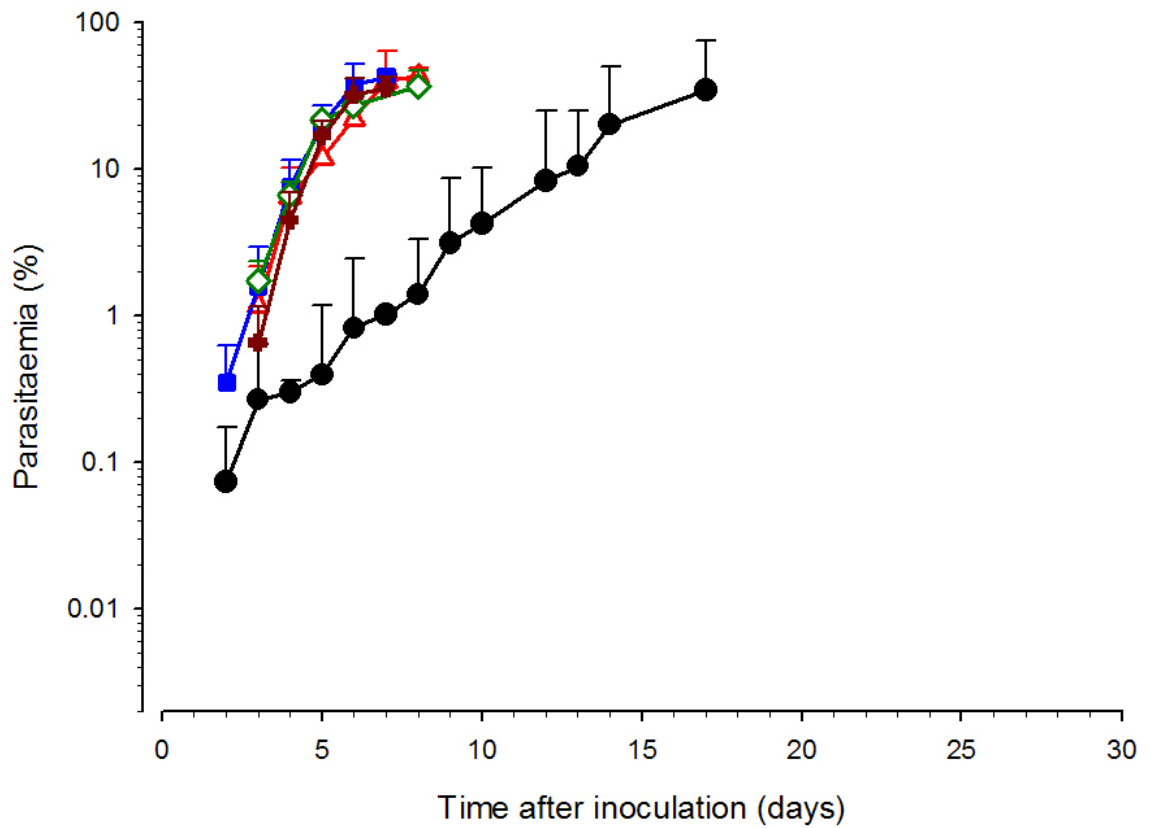
Figure 5.7 **Parasitaemia-time profiles from the parasite re-inoculation arm of the study.** Drug efficacy was assessed through the use of age-matched uninfected (Group B) mice that received a single dose of 90 mg/kg PQP at day 0 and later infected with 10^7 *P. berghei* parasitised erythrocytes at each pre-determined time point (n=6 at each time-point: ●, 25 days; △,40 days; ■, 60 days; ◇, 90 days; ✚, 130 days). Data are shown as total parasitaemia (mean percentage of erythrocytes infected ± SD), commencing from the time of inoculation. (see also text, sections 5.2.1.2b and 5.3.1.2).
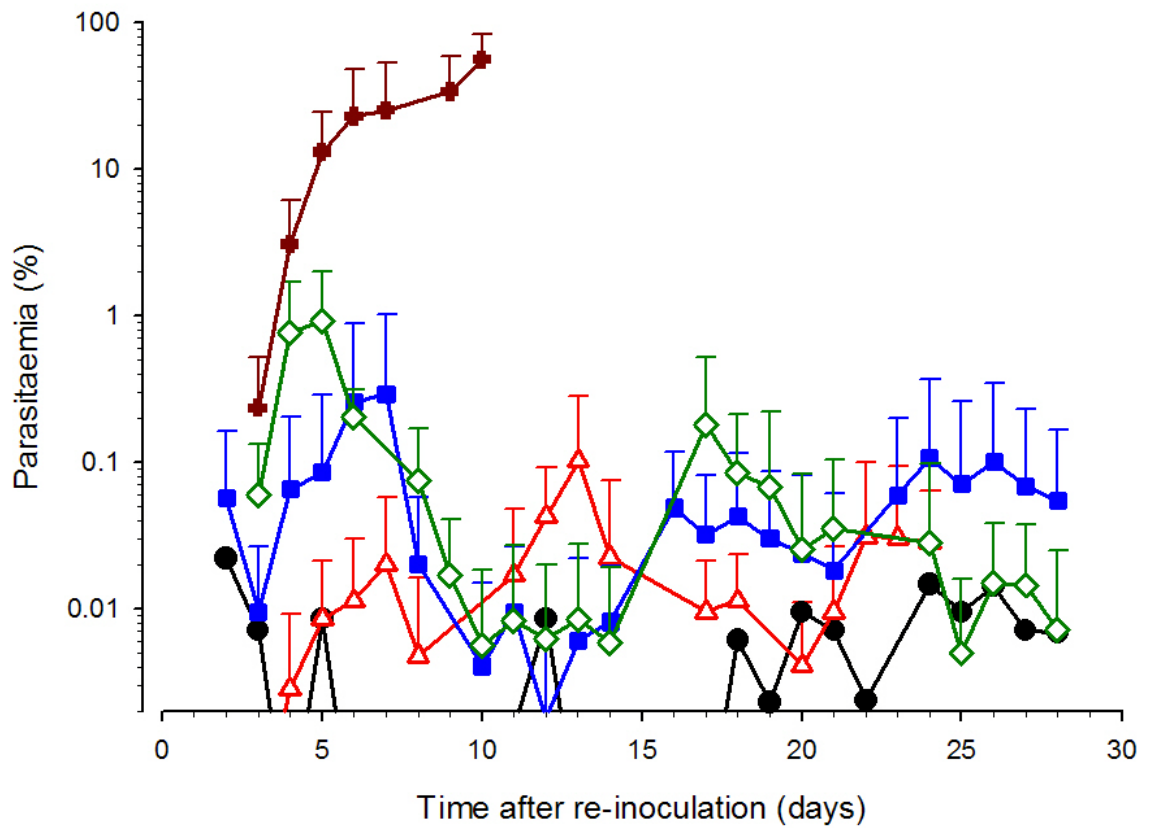
Figure 5.8 **Parasitaemia-time profiles from the parasite re-inoculation arm of the study.** Infected mice treated with 90 mg/kg PQP (Group C) at day 0 were re-inoculated with a second inoculum of 10^7 *P. berghei* parasitised erythrocytes at each study point (n=6 at each pre-determined time point: ●, 25 days; △, 40 days; ■, 60 days; ◇, 90 days; ✚, 130 days. Data are shown as total parasitaemia (mean percentage of erythrocytes infected ± SD), commencing from the time of inoculation. (see also text, sections 5.2.1.2c and 5.3.1.3).

5.3.3 Parasite viability and drug resistance study.

Blood harvested from Group C mice at the 25 day time-point (mean parasitaemia 0.03 ± 0.04% at time of harvesting) was shown to contain viable parasites, as all naïve mice inoculated with the donor red blood cell suspensions developed infections. Mice receiving drug vehicle five days after inoculation progressed to experimental endpoints (high parasitaemia) with a median survival of 19 days (Fig. 5.9). Following administration of 90 mg/kg PQP five days after inoculation, parasitaemia in the treated mice was not markedly different to that for the vehicle group, and the median survival was also 19 days. A similar outcome was observed in the 40-day mice (Fig. 5.10) with the data from both the 25 day and 40 day groups indicating that the parasites were resistant to PQ.

Blood harvested from the Group C mice at 60 days after drug treatment (mean parasitaemia 0.004 ± 0.006%) showed that viable parasites were present in only two of the donor mice, with the blood from two of the donor mice not producing a detectable parasitaemia in any of the naïve mice inoculated. In the 10 naïve recipient mice that developed an infection from the red blood cell suspension, mice that received drug vehicle five days after inoculation had a median survival of 10 days (n=4; Fig. 5.11). Administration of PQP to the remaining mice (five days after inoculation) resulted in a prompt decline in parasitaemia and recrudescence in all recipients (n=6; Fig. 5.11). Recipients from one donor (n=3) had a progressive infection that reached 10% parasitaemia within 25 days. However, the three recipients from the other donor showed a response to PQ that was consistent with successful treatment.

The naive control arm, which was run in parallel with each viability experiment, demonstrated the expected pharmacodynamic response for mice inoculated with a 10^5 *P. berghei* parasitised erythrocytes, consistent with Gibbons et al. 2007 (179). Furthermore, this control arm demonstrated that the 90 mg/kg PQP dose was efficacious, as all naive mice treated with the drug preparation exhibited the expected pharmacodynamic response. Therefore, this control arm adequately established that there were no inconsistencies with parasite inoculum or drug preparation.

Figure 5.9 **Parasitaemia-time profiles at 25 days.** Parasitaemia-time profiles in naïve Swiss mice (n=5) after inoculation with red blood cell suspensions harvested from previously inoculated and PQP-treated mice (n=4) at 25 days after initial drug administration. Five days after inoculation, three out of five mice from each donor group received a single i.p. dose of 90 mg/kg PQP. Inoculations from the four PQP-treated mice that were harvested at Day 25 produced a viable infection in all 20 recipient mice. The untreated mice (●; n=8) and PQP-treated mice (○; n=12) had similar parasite density-time profiles, apart from Days 7 and 8 (2-3 days after PQ administration), and the median survival time was 19 days in both groups. Data are shown as total parasitaemia (mean percentage of parasitised erythrocytes infected ± SD), commencing from the time of red blood cell passage.
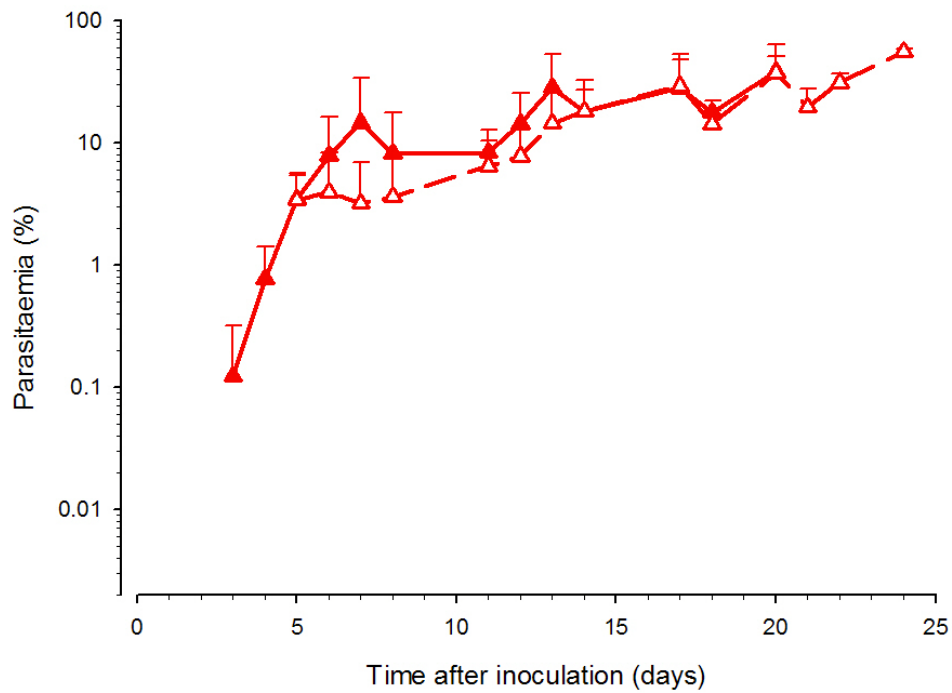
Figure 5.10 **Parasitaemia-time profiles at 40 days.** Parasitaemia-time profiles in naïve Swiss mice (n=5) after inoculation with red blood cell suspensions harvested from previously inoculated and PQP-treated mice (n=4) at 40 days after initial drug administration. Five days after inoculation, three out of five mice from each donor group received a single i.p. dose of 90 mg/kg PQP. Inoculations from the Day 40 mice produced viable infections in all recipient mice (▲ untreated, n=8; △ treated, n=12) and a similar profile to the Day 25 mice. Data are shown as total parasitaemia (mean percentage of parasitised erythrocytes infected ± SD), commencing from the time of red blood cell passage.
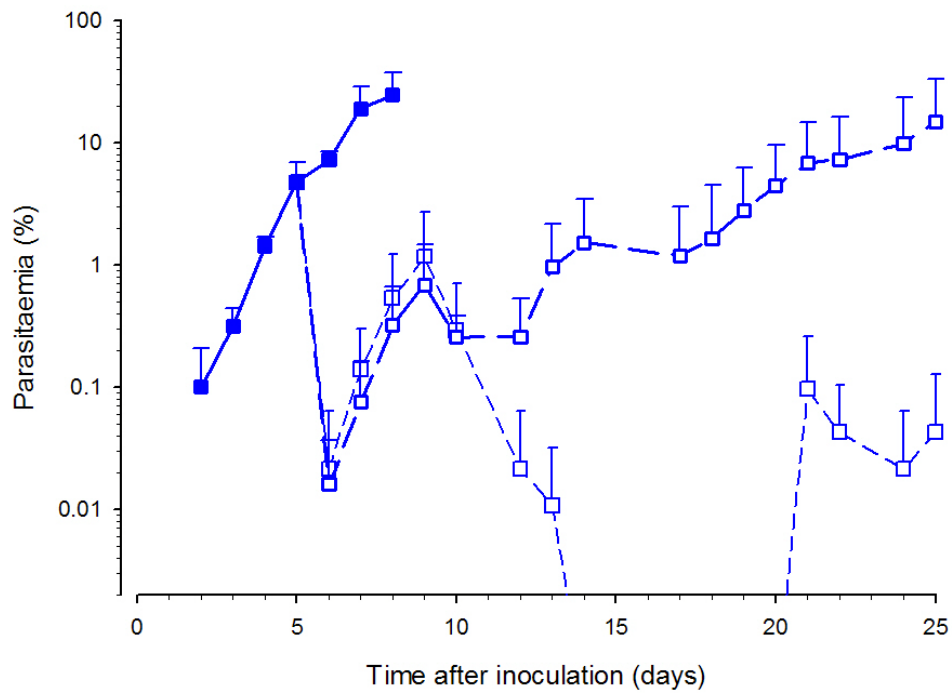
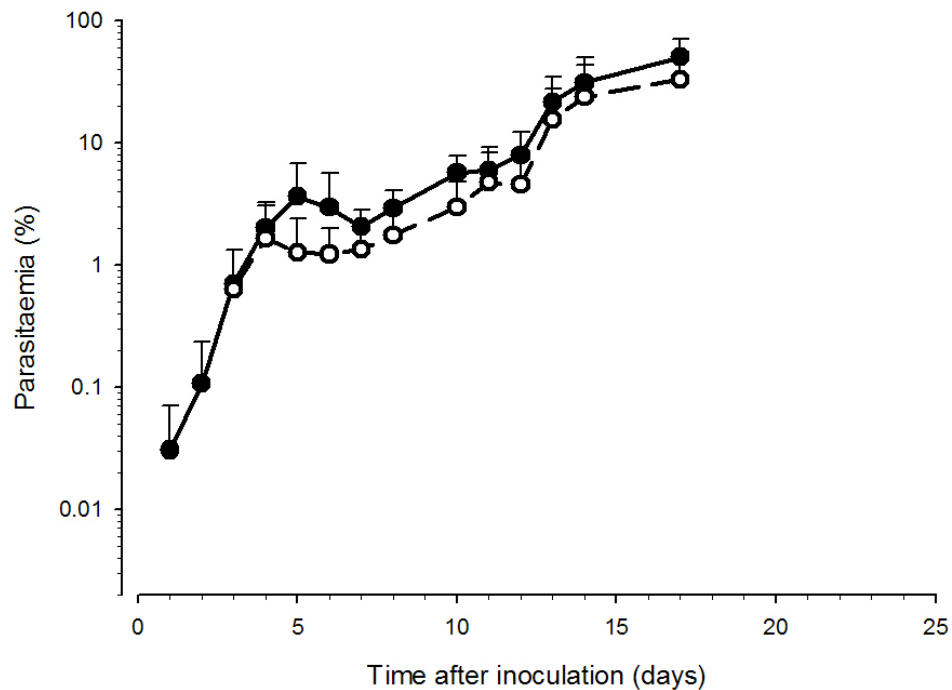**Figure 5.11** **Parasitaemia time-profiles at 60 days.** Parasitaemia-time profiles in naïve Swiss mice (n=5) after inoculation with red blood cell suspensions harvested from previously inoculated and PQP-treated mice (n=4) at 60 days after initial drug administration. Five days after inoculation, three out of five mice from each donor group received a single i.p. dose of 90 mg/kg PQP. In the Day 60 group, viable infections were produced from only two of the four donor mice. Untreated recipient mice (■; n=4) had a median survival of 10 days. Treated recipient mice (□; n=12) showed different profiles, with the cohort from one donor mouse having a progressive infection (   ) and the other cohort having a strong response to PQP treatment (– – –). Data are shown as total parasitaemia (mean percentage of parasitised erythrocytes infected ± SD), commencing from the time of red blood cell passage.

In the 90 day cohort mice, recipients from only one donor mouse developed a detectable infection (Fig. 5.12). All other recipients, from the other three donors, remained parasite free throughout the 30 day monitoring period. In mice with a positive infection, the lag period for the infection to develop suggested a low parasite count in the red blood cell suspension (inoculum). Furthermore, in these recipients PQ administration had little effect on the developing parasitaemia as the median mouse survival time (20 days) was similar to the mice given vehicle.

In the final subset (130 days), none of the recipient mice developed an infection during the 30 day monitoring period. This indicated that no viable parasites were present in Group C mice four months after inoculating and subsequent treatment with PQP.

5.3.4 PQ concentrations

The mean plasma PQ concentrations from malaria-infected mice (n=4) at each time point were 15 ± 8, 11 ± 1 and 2 ± 2 µg/L for 25, 40 and 60 days after PQP administration, respectively. Plasma PQ concentrations at 90 and 130 days were undetectable (Fig. 5.13). The plasma PQ concentrations in the present study were found to be comparable to the mean plasma concentration-time profile obtained in the previous chapter (Fig. 4.5, Chapter 4) Plasma PQ concentrations in uninfected control mice were 13.3 µg/L at 25 days (n=1), 4.9 µg/L at 40 days (n=1), 1.4 and 1.6 µg/L at 60 days (n=2), 1.9 µg/L and undetectable at 90 days (n=2) and undetectable 130 days (n=2) after PQP administration.
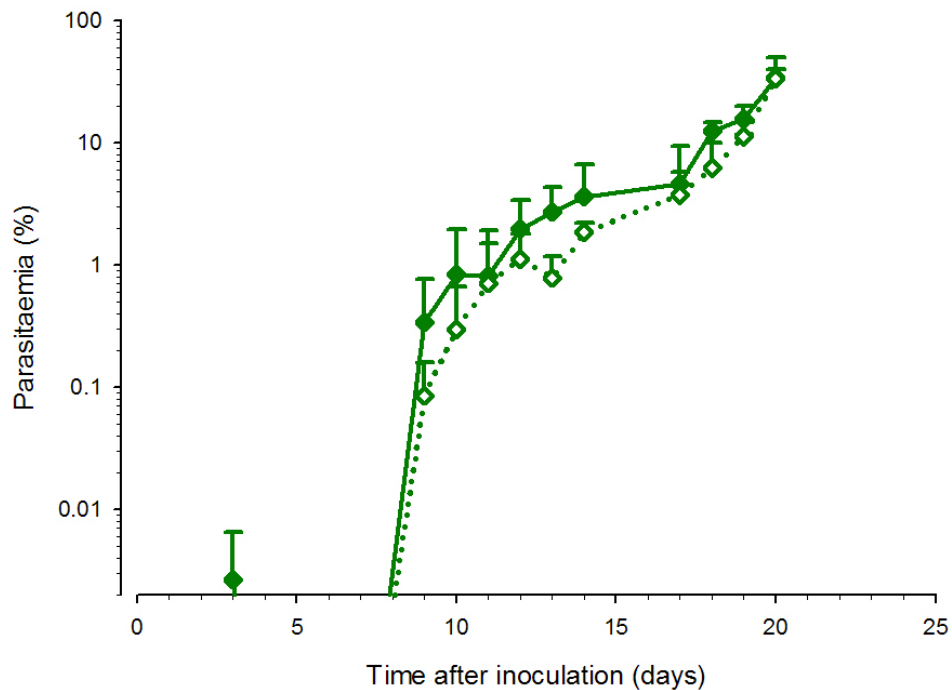
Figure 5.12   Parasitaemia-time profiles at 90 days. Parasitaemia-time profiles in naïve Swiss mice (n=5) after inoculation with red blood cell suspensions harvested from previously inoculated and PQP-treated mice (n=4) at 90 days after initial drug administration. Five days after inoculation, three out of five mice from each donor group received a single i.p. dose of 90 mg/kg PQP. In the Day 90 group, a viable infection was found in recipients from only one donor mouse and, although development of the infection was delayed, the parasite density-time profile of the untreated mice (◆; n=2) was similar to the treated mice (◇; n=3). Data are shown as total parasitaemia (mean percentage of parasitised erythrocytes infected ± SD), commencing from the time of red blood cell passage.
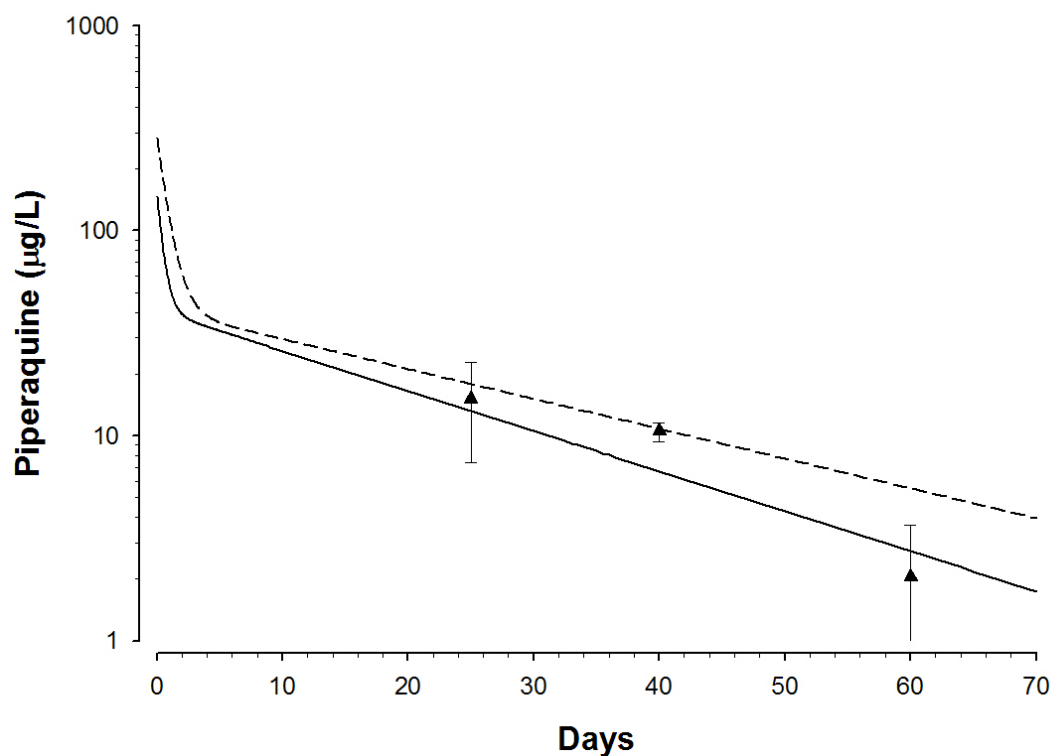
Figure 5.13   Plasma PQ concentrations in mice given PQP at approximately 90 mg/kg i.p. (normalized for pharmacokinetic analysis). Data are given as mean ± SD plasma PQ concentration for malaria-infected mice at 25, 40 and 60 days after treatment with PQP (▲; n=4). The lines show the mean concentration-time profile (two-compartment model) for healthy (- - -) and malaria-infected mice (——) as shown in Fig. 4.5, Chapter 4 (pg 166).

5.4 DISCUSSION

The purpose of the current investigation was to perform a series of viability and re-inoculation experiments to determine whether the previously observed, chronically low parasitaemia in PQ treated, *P. berghei* infected mice was a result of drug pressure, immune response or potentially a combination of both. Whilst specific immunological testing involving the use of monoclonal antibodies and/or labelled CD4+ and CD8+ T-cells would have added further support to the investigation, it was beyond the scope and resources of this study. Therefore, the conclusions from this study are based on the pharmacokinetic data and observed pharmacodynamic responses.

Pharmacodynamic data obtained for Group A mice demonstrated that when inoculated at each of the experimental time-points, the previously uninfected, untreated mice developed lethal infections. This suggests that in this *P. berghei* model, mouse age at the time of inoculation does not influence the course of infection. A similar finding has been previously reported where it was shown that age had no influence on the progression or lethality of *P. berghei* infections in mice (192, 387). By contrast, adult rats (>12 weeks) have an increased ability to clear *P. berghei* parasites and decreased mortality compared to young rats (4 weeks), albeit there was no significant difference in the initial progression of infection between the studied groups (4, 192, 387). This would therefore suggest that the differences in observed disease progression, in the young and adult rat groups, was due to an age-associated deregulation of immune responses to primary infection (4). By comparison, in the present studies, all experimental mice were obtained at an initial age of 5-6 weeks and by the time of inoculation would all be considered mature. Hence, it may be concluded that in these studies 'mouse age' at the time of inoculation may be excluded as a factor in improving survival.

The duration of therapeutic effect after single dose 90 mg/kg PQP, was evaluated in Group B mice after their inoculation with *P. berghei* parasites. The pharmacodynamic profiles clearly demonstrated that plasma PQ concentrations were only mildly efficacious at approximately 25 days after drug administration,

with mild parasite suppression being observed (Fig. 5.7). Analysis of plasma concentrations at each time point correlated with the pharmacodynamic observations. At 25 days, when modest parasite suppression was observed, the mean plasma PQ concentration was 15 ± 8 µg/L. Plasma concentrations at subsequent time-points (< 10 µg/L) correlated to a lack of parasite suppression. Therefore, it was concluded that in this murine malaria model the contribution of residual PQ concentrations <20 µg/L was low, whilst PQ concentrations <10 µg/L were ineffective. Although direct comparisons to human studies are not plausible, these findings are consistent with a recent report suggesting that plasma PQ concentrations >30 µg/L are required for successful treatment in clinical malaria (397).

Persistence of residual drug concentrations is a concern in the context of the selection of drug resistant parasites to long half-life partner drugs in ACTs, such as PQ. The most significant influence on the efficacy of antimalarial therapy is the parasite's ability to acquire and/or maintain drug resistance. General characteristics of the drug are perhaps the most important determinants in parasite resistance, particularly in regard to drugs with a long half-life such as mefloquine or PQ (548, 566). However, exposure to persistent subtherapeutic concentrations that inhibit, rather than clear, parasitaemia may exert substantial residual selection pressure (541, 548, 558, 566). Another issue to consider is the maintenance of adequate drug concentrations over a long period of time being important for the clearance of parasites within an infected individual. Subtherapeutic concentrations may eliminate the most susceptible parasites but not clear more resilient forms. As a result, eventually the necessary therapeutic dose required for therapy may increase beyond the maximum tolerated, thus drug resistance would emerge (566). Based on the results in this study, it is feasible that re-emergence of PQ resistant strains of *P. falciparum*, which in the 1980s led to the abandonment of PQ as an antimalarial agent (127, 386, 541), could occur as a result of residual PQ concentrations following ACT in endemic areas. However, to date, there are no clinical reports of resistance to PQ as a result of PQ-based ACT, which may reflect the high success rates associated with this regimen (27, 29, 139, 252, 253, 336, 413,

456, 477). That is, by curing the infection (parasite elimination) there is no residual biomass exposed to prolonged, low drug concentration.

In the current study, when parasites from previously treated mice were passaged into a naive host, the viable parasites appeared to not be affected by PQP, with parasite growth continuing at a constant rate after drug administration. Whilst inadequate drug formulation was considered, the corresponding control arm (naive mice inoculated with a fresh *P. berghei* inoculum and similarly treated with 90 mg/kg PQP) demonstrated the expected drug efficacy post drug administration. Therefore, the parasites' lack of response to a second 90 mg/kg PQP exposure appeared to be more indicative of drug resistance. By comparison, selected PQ resistance in a *P. berghei* ANKA murine malaria model after drug pressure was recently reported (259). Initially the ED_{90} for PQ was determined to be 3.52 mg/kg, however, after continued drug pressure over >27 passages, resistance was obtained which was reflected by an altered ED_{90} of 68.86 mg/kg (259). Furthermore, if drug pressure was removed it was observed that parasites slowly returned to being sensitive towards the drug. These results therefore suggest that it is plausible for *P. berghei* parasites to become resistant to PQ, however, it has previously not been described after a single prolonged exposure. Therefore, although the current parasite responses to repeated drug administration do suggest attainment of at least partial resistance, the small sample size and study design precludes definitive conclusions. Further studies, using a larger cohort of mice and appropriate immunological investigations, could provide more information on the mechanisms of resistance to PQ.

Parasite re-inoculation at 25, 40, 60 and 90 days after PQP administration in Group C mice, failed to establish a lethal infection with all mean parasitaemias remaining below 1%. However, when re-inoculated at 130 days after PQP, all mice developed a lethal infection. Given that plasma PQ concentrations were shown to be ineffective at curbing parasitaemia at any of these time-points, the results are indicative of an immune response being mounted towards the infection. Acquired resistance is considered to be the predominant factor in the development of

chronic *P. berghei* infections in mice (19, 114, 115). Murine *P. berghei* studies, using Atabrine or Manzamine A as treatment therapy, have independently demonstrated the expression of chronic *P. berghei* infections, which resulted in either fatal relapses (as late as 100 days after treatment) or spontaneous cure (19, 115). Furthermore, mice that exhibited chronic infections were shown to survive for extended periods of time after re-infection with a second *P. berghei* inoculum (115). These results, in conjunction with those obtained in the present study; suggest that if drug therapy has the ability to reduce the initial parasite burden to subclinical levels, the mouse immune system has the capacity to limit parasitaemia to low density chronic infections and a subsequent acquired partial immunity towards infection (114, 115, 278).

Investigations into murine host responses to infection have demonstrated that the immune response mounted towards malaria infection has the capacity to eradicate parasites after further challenge, at least within an interval of 4 to 6 weeks (332, 333). Once infection was resolved and parasites were no longer present in circulation, the titre of humoral protective factor was seen to wane markedly within 2 to 3 weeks of recovery from the *P. berghei* infection and as a result mice were once again susceptible to infection (332, 333). These observations further support the theory that if drug treatment has the ability to reduce parasitaemia to a critical, but as yet unknown, parasite density for a period of time, the mouse immune system has the ability to clear remaining parasites from the infected animal. This would then protect the mouse from further infection from the same parasites.

The extended period of the present study ensured that the full scope of drug efficacy, parasite viability and host response components could be evaluated. It was found that the mean parasitaemia beyond 60 days was very low (<0.01% where detectable) and, possibly due to the low parasite densities, the inoculation success was 50% and 25% at 60 and 90 days, respectively. Treatment with PQ also showed a variable response, with some infected recipients in the 60 day group showing evidence of drug resistance and others showing susceptibility to drug (although immediate drug effects were less than the standard response in control mice). Re-

inoculation of experimental mice with *P. berghei* parasites also failed to establish lethal infections at these time-points, suggesting persistence of immunity at a low parasitaemia and in mice with recently cleared infection.

The peripheral parasitaemia remained undetectable from 80 days after the original treatment with PQP, indicating that infections had resolved or the residual parasite biomass was very low (<0.001%; approximately 160,000 parasites in comparison to a starting biomass of 500 million parasites). At the final time point of our study, passaging of blood into control mice failed to establish an infection and, in contrast to earlier times, the second *P. berghei* inoculum led to a typical, lethal infection. These results suggest that any immunity or immunological memory was absent, a finding that is also consistent with the view that maintenance of immunological memory requires continuous exposure to the parasites (220, 414).

The present study may have been limited by the relatively small number of donor mice used to demonstrate parasite viability at each studied time-point. At the 25 and 40 day time-points, where parasite responses were consistent, the small sample size was not a conflicting issue. However, as time progressed the variability of parasite viability increased and by 60 days only 50% of the donor parasite inoculums from Group C mice proved to be viable upon re-inoculation. It is possible that a more accurate outcome could have been achieved if the number of donor mice was increased at each time-point.

In conclusion, the present study has demonstrated that the *P. berghei* murine malaria treatment model can be a valuable preclinical conceptual tool for investigation of the pharmacodynamic effects of antimalarial drugs such as PQ. It was found that PQ has a substantial antimalarial effect in this model, which appears sufficient for a host immunological response to be established. The study also suggests that residual PQ concentrations could lead to the development of PQ-resistant parasites if initial ACT was not curative and/or a new infection arose during the early post-treatment period.

CHAPTER SIX

INVESTIGATION OF THE PHARMACOKINETICS AND PHARMACODYNAMICS OF CHLOROQUINE IN MICE

6.1 INTRODUCTION

Since the discovery and development of chloroquine in the 1030s and its clinical application in the 1940s, CQ is still an important antimalarial drug today, particularly for the treatment of *P. vivax* malaria (REF). The clinical pharmacokenitics of CQ and its principal metabolites have been extensively studied in a variety of ethnicities, following various routes of administration, and in healthy subjects and malaria patients (6, 9, 74, 100, 135, 146-148, 153, 170-173, 199, 201, 254, 264, 270, 273, 338, 357, 359, 364, 410, 420, 490, 491, 504, 513, 514, 529, 549, 553, 559, 562). However, despite the intensive research into this drug and its metabolites in numerous populations, the pharmacokinetic parameters of the drug remain highly variable, if not inconclusive. For example, the terminal elimination half-life of CQ, reported from clinical studies, ranges from 3 to 1512 h (74, 146-148, 169-173, 199, 201, 264, 273, 357, 410, 491, 504, 513, 514, 540, 562). This significant variability may be attributed to different analytical techniques, whether plasma, serum or whole blood was used for analysis, or even incorrectly assuming that the tissue distribution phase was the elimination half-life. However, as drug administration and dosing regimens are often intimately linked with the reported terminal elimination half-life, it is concerning that there is a wide range of values for CQ, a potentially toxic drug, with a narrow therapeutic index (131). This point alone supports the strategy of acquiring basic pharmacokinetic information from preclinical models so that suitable and safe dosing regimens can be determined and implemented into the clinical setting.

6.1.1 Animal investigations

6.1.1.1 Pharmacodynamic efficacy

The pharmacodynamic efficacy of CQ has been previously investigated in a preclinical murine study observing the influence of initial parasite load and starting day of drug administration on the outcome of treatment, using both the Peter's 4 day test as well as treating post inoculation (239). An outbred mouse model inoculated with *P. berghei* NK61 parasites was used for all investigations. The results suggested that a complex relationship existed between the parasite load and efficacy of CQ (239). For example, mice dosed for four consecutive days with 20 mg/kg CQ before inoculation with 10^8 *P. berghei* parasites showed an undetectable parasitaemia until day 8 post-infection, with parasites observed from day 10 and eventual death by day 22 of infection (239). Conversely, all mice inoculated with $\leq$ 10^7 *P. berghei* parasites showed no presence of parasitaemia for the monitored period. In experiments where mice were dosed post inoculation it was demonstrated that in mice inoculated with 10^3 *P. berghei* parasites, if parasitaemia at the time of treatment was $\leq 0.1\%$, all infections resolved with no parasite recrudescence observed up to the experimental endpoint. By comparison, if peripheral parasitaemia was $\geq 0.1\%$ at time of treatment, 20 mg/kg CQ was shown to decrease the parasitaemia to undetectable levels until 7 days after treatment, however, parasite recrudescence soon followed and all mice died with progressively increasing parasitaemia by day 24 after CQ treatment (239). The authors concluded that in this *in vivo* antimalarial "drug-assay" model, several factors such as initial parasite load and the starting time of treatment influenced the drug response in the host (239).

This study may be relevant for other preclinical models, as it suggests the possibility of a threshold parasitaemia which could directly correlate to the treatment outcome. Furthermore, in the context of dose regimen considerations, this study indicates that at higher parasite burdens either a higher dose of CQ is required to resolve the infection or, if CQ toxicity prevents a higher dose being administered, a different antimalarial treatment strategy is required. In regards to murine treatment models, where it is important to observe the entire pharmacodynamic

response of the trial antimalarial, it highlights the importance of using parasite inocula > 10^7 *P. berghei* parasites. Further investigation into the influence of initial parasite load could provide useful preliminary data for the construction of comprehensive mathematical models. Such mathematical modelling could prove useful in the clinical setting where the selection of antimalarial therapy and corresponding treatment outcomes could be predicted given the initial blood parasitaemia.

Further efficacy data has been collated using murine malaria models including the determination of the minimum effective dose of CQ being 10 mg/kg (51, 239), and establishing that the acute lethal dose 50% (LD_{50}) of CQ in mice after i.p. injection was 79 mg/base/kg (range 68-78 mg/base/kg) (Table 6.1) (306).

Table 6.1   Parameters of toxicity of chloroquine (CQ) (306).   Data given as means ± standard error

**Original table modified to include murine data relevant to this chapter.

Route of administration	Acute LD_{50} mg/base/kg	Tolerated Dose mg/base/kg per day
	CQ	CQ
Intravenous	25 ± 2[†]	–
Intraperitoneal	79[†], 68–78[‡]	>40, >155[†,§]
Oral	387 ± 50[†], $1{,}000$[‡]	$400 = LD_{10}$[‡]

[†] Data from the files of the Sterling-Winthrop Research Institute

[‡] Data from Wiselogle, F.Y., 1946 (564)

[§] Five-day test

Murine models have also played an integral role in the investigation of the mechanism of action of CQ. Whilst a range of studies have determined that CQ affects *Plasmodium* parasites in several ways, including (i) interaction of CQ with parasite DNA (101, 108, 109, 202, 493), (ii) inhibition of parasite feeding by CQ

administration (372, 424), and (iii) accumulation of CQ in the acidic food vacuole of the parasite (11, 174, 175, 185, 211), perhaps the most significant results have been in the study of the effect of CQ on haem polymerisation (166, 295, 296, 375, 452). Morphological studies observing the effect of a single dose of 40 mg/kg CQ on *P. berghei* malaria showed a progressive aggregation of malarial pigment within the parasite (296). This effect was first observed within 30 min and was essentially complete within 4 h of drug administration. However, by 8 h after CQ there was a noticeable decline in the percentage of pigment-containing parasites and within 48 h very few parasites contained pigment (296). Further investigation using electron microscopy showed that the malaria pigment granules were accumulating within large vesicles that were limited by a single membrane. Within 1 h of CQ administration, these newly formed large vesicles also contained membrane bound inclusions of parasite cytoplasm and later, at 3–4 h, small vesicles contained unidentified material (296). These microscopy studies suggested that the formation of haematin pigment by the parasite may serve to concentrate the drug within the parasite, particularly in the digestive vesicles. It was further concluded that such an effect would account for the selective toxicity of CQ for the erythrocytic malarial parasites (296). This conclusion is further supported by murine studies which have demonstrated an accumulation or clumping of pigment granules in murine trophozoites (98, 375, 538). Furthermore, Peters demonstrated that a CQ-resistant line of *P. berghei* demonstrated significantly less pigment formation than a similarly treated CQ-sensitive *P. berghei strain*, which similarly suggests that the process of haemozoin formation is associated with the mechanism of action of CQ (375).

Similarly, *in vitro* studies have demonstrated the stage specific affinity of CQ along with an increased accumulation of pigment granules within the parasite after CQ administration (452, 471, 472, 587). In their investigation of the inhibition of haem polymerase by CQ, Slater and Cerami (452) used an *in vitro* model to identify specific aspects of the mechanism of action of CQ. The results obtained in this study closely correlated to those published in earlier murine studies (452) although it has been more recently suggested that in order for *in vitro* investigations to

reflect the true nature of haem polymerisation, specific preformed parasite haem polymers must be first added to the culture (166). Therefore, it may be suggested that both the *in vitro* and murine malaria preclinical models have contributed valuable, and correlating, information on the mechanisms of action of CQ.

6.1.2 CQ Pharmacokinetics

Preclinical pharmacokinetic studies in a variety of animal models have been reported, with all studies suggesting CQ has a long elimination half-life (8, 78, 396, 494). In healthy dogs after i.v. administration of 2 mg/kg CQ, the terminal elimination half life of CQ was estimated at 12.6 ± 1.84 days (8). Comparison of the pharmacokinetic parameters in both healthy and malaria-infected (*P. knowlesi*) Rhesus monkeys determined that the plasma CQ half-life was significantly increased in infected compared to healthy (P<0.05) (396). It was postulated that the significant difference in the elimination half-life of CQ in the infected monkeys could be due to hepatic involvement during malaria infection (396). This conclusion is further supported by an in vivo rat model which showed a decreased liver blood flow during malaria infection, therefore suggesting that the variations in hepatic metabolism could result in inconsistent CQ pharmacokinetics (148). Furthermore, investigations in the tissue distribution of CQ in monkeys and albino rats showed that CQ is extensively distributed, with the liver, spleen, kidney and lungs being the main repositories (7, 148, 306, 307).

Although murine malarias serve as preferable *in vivo* models for drug testing, the pharmacokinetic parameters of CQ in mice are limited. Three studies have been published on the pharmacokinetic paramters of CQ in mice (14, 78, 494). They have involved healthy mice administered s.c. 30 mg/kg CQ (494), and orally 100 mg/kg CQ (14), and in both healthy and malaria-infected mice administered s.c. either 5, 10 or 50 mg/kg CQ (78). However, all studies have limitations, especially incomplete pharmacokinetic analysis (78, 494). For example, in one study (78), pharmacokinetic analysis only included data up to 120 min post-dose and the reported values do not represent terminal elimination but instead indicate tissue distribution (78). Furthermore, no study takes into account the presence or

concentrations of CQ metabolites, such as desethylchloroquine (DECQ). All studies, however, produced interesting assumptions which, to date have been used as preclinical pharmacokinetic values for CQ. After measuring blood CQ concentrations at 0.5, 1, 2 and 4 h, Thompson et al. (494) suggested that the time of peak CQ concentration was 2 h after dosing. After observing the blood CQ concentration-time profile in healthy mice, Cambie et al. (78) estimated the distribution of CQ to be 40 min, which was quite similar to the distribution of CQ in humans using a double decay model. Furthermore, Cambie et al. reported that the rate of CQ elimination in malaria-infected mice is strongly influenced by the parasite load at the time of treatment. It was observed in mice with a heavy infection (21-25% parasitaemia) of *P. chabaudi*, at the time of CQ administration, that the V/F, AUC, $t_{\frac{1}{2}}$ and t_{max} of CQ were all significantly increased (78). However, as $t_{\frac{1}{2}\alpha}$ was not significantly different between mice with heavy infections and control mice, it was suggested that the increased t_{max} of CQ in heavily infected mice was due to an increase in $t_{\frac{1}{2}\beta}$. This observation was hypothesised to be a result of an increased capacity of the blood compartment to accumulate drug in those mice with a heavy load of parasites, thus the rate of drug efflux was smaller than the rate of influx (78). It was concluded that CQ was dynamically retained in infected erythrocytes and that the blood CQ concentration was considerably lower than that found in healthy controls or those with low level parasitaemia, thus accounting for the low drug elimination rate observed in heavy infection (78).

In order to address the paucity of pharmacokinetic data available for CQ in the murine malaria model the purpose of the present investigation was to produce robust pharmacokinetic data and corresponding pharmacodynamic data, for CQ in the *P. berghei* malaria model. Furthermore, as preclinical pharmacokinetic studies have been restricted to CQ alone, a further aim of the study was to investigate the pharmacokinetic profile for DECQ, the major metabolite of CQ. These pharmacokinetic data for CQ and DECQ in the *P. berghei* murine malaria model are expected to be a valuable guide for researchers using CQ in murine malaria studies.

6.2 METHODS

6.2.1 Pharmacodynamic evaluation of single and multiple doses of CQ

As the pharmacodynamic properties of CQ have been studied to some degree in murine malaria models (373, 374, 378, 381, 383), the focus of the present investigation was the pharmacokinetic properties of CQ, and the biologically active metabolite DECQ, after single and multiple dose administrations. In conjunction with this novel pharmacokinetic data, the pharmacodynamic responses would provide a unique evaluation of the effect of CQ in mice with established *P. berghei* infections.

A dose of 50 mg/kg CQ was selected for use in both the single and multiple dose studies. Previous pilot studies conducted in our laboratory (Jillian Stoney, BPharm (Hons) thesis (467)) evaluated single dose (10–50 mg/kg. Fig. 6.1A.) and multiple dose regimens (Fig. 6.1B.). Consideration of the results obtained in these studies, as well as toxicity data obtained for i.p. dose administration (LD_{50} after i.p. dosing in mice 79 mg/base/kg (306)) resulted in the selection of the 50 mg/kg dose, which after single dose administration, was expected to give a significant, yet subtherapeutic, antimalarial response.

For multiple dose administrations, 50 mg/kg CQ also was selected for pharmacokinetic analysis with 5 doses given at 24 h intervals. This dosing regimen was chosen because it had been observed in the pilot study (where doses were given 12 h apart; Fig. 6.1B.), that the antimalarial efficacy of CQ was extended when the treatment course was spread over more than one parasite erythrocytic cycle (24 h in *P. berghei*). Therefore, it was expected that although each individual dose would be subtherapeutic, the dosing regimen over a total of 4–5 parasite erythrocytic life cycles, could lead to parasitological cure.

6.2.1.1 Pharmacodynamic analysis

To monitor the pharmacodynamic effect of single and multiple doses of CQ on the parasitaemia of mice involved in the pharmacokinetic study, peripheral blood films were prepared and parasitaemia determined using two different subsets.
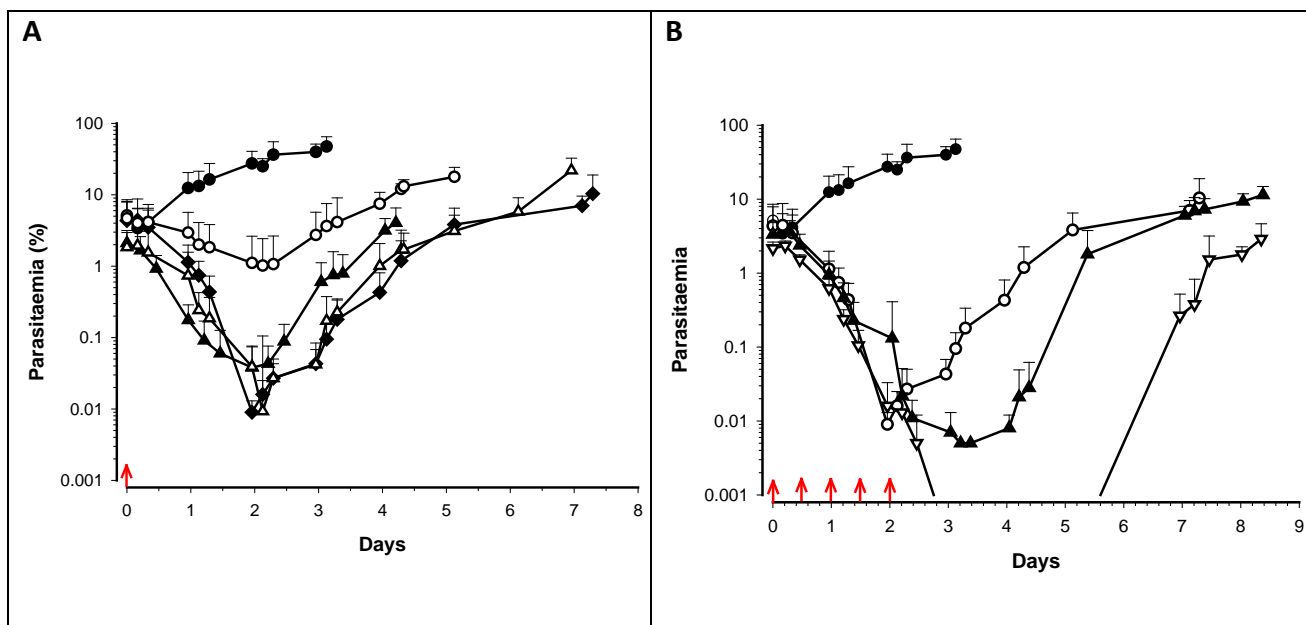
Figure 6.1A. Parasitaemia-time profile in Swiss mice following administration of single i.p. doses of CQ administered 65 h after inoculation with 10^7 *P. berghei-*parasitised erythrocytes ⇑). Data are shown as total parasitaemia (mean percentage of erythrocytes infected ± SD), commencing from the time of CQ administration. Symbols: ●, control (n=4); ○, 10 mg/kg CQ (n=9); ▲, 20 mg/kg (n=7); △, 30 mg/kg CQ (n=9); ◆, 50 mg/kg CQ (n=7). [Data from J. Stoney, BPharm (Hons) (467)].

Figure 6.1B. Parasitaemia-time profile in Swiss mice following administration of either a single i.p. dose of 50 mg/kg CQ (⇑ x 1), a three dose regimen of CQ (↑ x 3; 20, 20 and 10 mg/kg), or a five dose regimen of CQ (↑ x 5; 10, 10, 10, 10, and 10 mg/kg) with the first dose administered 65 h after inoculation with 10^7 *P. berghei-*parasitised erythrocytes, and subsequent doses administered ever 12 h. Data are shown as total parasitaemia (mean percentage of erythrocytes infected ± SD), commencing from time of initial CQ administration. Symbols: ●, control (n=4); ○, 50 mg/kg CQ (n=7); ▲, 3 dose regimen (n=8); ▽, 5 dose regimen (n=8). [Data from J. Stoney, BPharm (Hons) (467)].

Firstly, in both the single and multiple dose pharmacokinetic studies 10 mice (the mice grouped in the last 2 pharmacokinetic time-points i.e. 5 and 7 days for the single dose study and 21 and 30 days for the multiple dose analysis) were bled twice daily and parasitaemia determined. These data were used to construct comprehensive parasite density-time plots which represented the pharmacodynamic effect seen in each cohort of mice after CQ therapy.

In addition, peripheral blood films were prepared from every mouse immediately prior to dosing and at the time of blood collection and euthanasia. The mean parasitaemia for each group of mice was determined and these were plotted on the pharmacodynamic curve produced from the representative mice, signifying parasitaemia from the second subset.

All peripheral blood smears were prepared and stained, and parasitaemia quantified, as described in Chapter 2, Sections 2.2.1.3, 2.2.2.4 and 2.2.2.5.

6.2.2 Pharmacokinetics of CQ and DECQ in mice

6.2.2.1 Drug preparation for animal dosing

All drug solutions were prepared from a single source of CQ diphosphate salt which was obtained from Sigma-Aldrich (St. Louis, MO, USA).

To determine the required mass of CQ diphosphate salt to administer the required CQ dose, all mice were weighed prior to dosing. Whilst individual mouse weights were recorded (important for dose normalisation during pharmacokinetic modelling), the average mouse weight was used when calculating the mass of required CQ salt (MW base: 319.8; salt: 515.9) needed to administer a 50 mg/kg dose of CQ. As CQ diphosphate salt is readily soluble in water, the accurately weighed CQ diphosphate was dissolved in half the required volume of deionised water by vortex and, if necessary, sonification. After dissolution, the solution was made to volume and passed through a Millex®-HV 0.45 μm filter unit (Millipore S.A., Molsheim, France).

6.2.2.2 Single dose pharmacokinetic study

Pharmacokinetic parameters for CQ and DECQ were determined from 125 uninfected male Swiss mice (6-7 weeks old; mean weight 33.8 ± 2.8 g) and 125 malaria-infected mice (6-7 weeks old; mean weight 31.7 ± 2.9 g) which had been inoculated with 10^7 *P. berghei* parasitised erythrocytes 64 h prior to dosing. A standard 100 µL volume of drug solution was administered to each mouse i.p. as a single dose of 1,500 µg CQ.

To determine a robust pharmacokinetic profile of single dose 50 mg/kg CQ in mice, blood was harvested from groups of mice (n=5) at 0, 10, 15, 20, 30, 45, 60, 75, and 90 min, 2, 2.5, 3, 4, 5, 8, 12, 18, 24, 30, 36, 48 and 56 h, and 3, 4, 5, and 7 days after drug administration. All blood samples were collected by the author, and accurate records were taken for the time of drug administration and the time of blood harvest was calculated. For earlier time-points (10, 15, 20, 30, 45, 60, 75, and 90 min, 2, 2.5, 3, 4, and 5 h), the CQ drug solution was administered at 5 min intervals between each mouse within the group (n=5) to ensure that blood could be harvested at the required time-point.

At each pharmacokinetic data point, mice were anaesthetised with 50-100 mg/kg sodium pentobarbitone 5–10 min prior to blood collection. Blood was harvested by cardiac puncture in a single draw using a 1 mL syringe (Terumo) and 26Gx½ inch needle (Terumo) and placed into 1 mL lithium heparin tubes (Vacutainer®, Beckton-Dickinson, NJ, USA). The tubes were then mixed well by manually inverting the tube >10 times. At the completion of the time point, all five blood tubes were centrifuged at 3,000 *g* for 10 min and the plasma separated and stored at -80°C until analysed by HPLC (254). During separation care was taken to minimise the contamination of the plasma by constituents of the buffy coat (white blood cells and platelets).

A blood smear was prepared from each mouse blood sample at the time of harvesting to determine the peripheral parasitaemia.

6.2.2.3 Multiple dose pharmacokinetic study

The pharmacokinetic properties for CQ and DECQ were determined in a multiple dose study using a total of 125 uninfected male Swiss mice (6–7 weeks of age; mean weight 30.8 ± 3.1 g) and 125 malaria-infected male Swiss mice (6–7 weeks of age; mean weight 29.2 ± 3.1 g) that had previously been inoculated with 10^7 *P. berghei* parasitised erythrocytes, 64 h before initial drug administration. A total of five doses of 1,500 µg i.p. CQ were administered at 24 h intervals (as CQ phosphate solution in water; 100 µL injection volume). Each mouse was weighed prior to dose preparation on each day with the average mouse weight determined for dose calculations.

Blood was harvested for pharmacokinetic analysis from groups of mice (n=5) by cardiac puncture at 4, 8, 12, and 24 h after the first dose, 24 h after the 2nd, 3rd, and 4th dose, then 1, 2, 4, 6, 8, 12, and 18 h after the fifth dose and at 5, 5.25, 5.5, 6, 6.5, 7, 8, 10, 15, 21 and 30 days after commencement of the dosage regimen. All blood samples were harvested and processed as described in Section 6.2.2.2.

To monitor the pharmacodynamic effect of CQ on parasitaemia in mice used in the pharmacokinetic study, peripheral blood films were prepared and parasitaemia determined using two different subsets. Firstly, in both the single and multiple dose pharmacokinetic studies 10 mice (the mice grouped in the last 2 pharmacokinetic time-points; i.e. 5 and 7 days for the single dose study and 21 and 30 days for the multiple dose analysis) were bled twice daily and parasitaemia determined. The data were used to construct comprehensive parasite density-time plots which represented the pharmacodynamic effect seen in each cohort of mice after CQ therapy. In addition, peripheral blood films were prepared from every mouse immediately prior to dosing and at the time of blood collection and euthanasia. The mean parasitaemia for each group of mice was determined and these were plotted on the pharmacodynamic curve produced from the representative mice. This procedure was followed for both the single and multiple dose cohorts.

6.2.3   HPLC analysis of CQ and DECQ

All HPLC analysis was performed by the research group analyst, Dr Madhu Page-Sharp, according to an established method (254), as described below.

6.2.3.1 Materials

CQ diphosphate was obtained from Sigma-Aldrich (St. Louis, MO) and DECQ dioxalate was from Starks Associates (Buffalo, NY).   The internal standards amodiaquine (AQ) and PQ were purchased from Sigma (Stockholm, Sweden) and Yick-Vic Chemicals and Pharmaceuticals Ltd. (Hong Kong), respectively.   Acetonitrile was obtained from Merck (Damstadt, Germany).   All other solvents and chemicals were of HPLC or analytical grade.

6.2.3.2 Preparation of stock solution and standard curve

Stock solutions of CQ and DECQ were prepared separately, equivalent to 1 mg/mL base in water.   PQ was initially chosen for use as the internal standard, however, AQ was added as a second internal standard, should any interference occur with the primary standard.   For each analytical batch a 5-point linear calibration curve with a blank standard was prepared by spiking human plasma with appropriate volumes of working standards.   Quality control samples (5 µg/L and 50 µg/L) were included for each analysis.   All samples were assayed within the storage stability period of 6 months for CQ (unpublished data).

6.2.3.3 CQ and DECQ assay

CQ and DECQ were extracted based on a previously published method (254). Briefly, plasma standards and samples (500 µL) were spiked with internal standard (PQ; 200 ng and AQ; 200 ng) and mixed with 5 mL of *t*-butylmethyl ether and 200 µL of 5M sodium hydroxide and then manually shaken for 10 min.   All tubes were then centrifuged at 1,500 g for 10 min, with 4.5 mL of the organic phase then transferred into clean tubes and back extracted into 0.1 mL of 0.1 M HCl by shaking for 5 min. This procedure was then followed by centrifugation for 10 min at 1,500 g after which time the organic layer was aspirated to waste.   The HCl layer was transferred

to a round-bottomed borosilicate glass tube and re-centrifuged at 1,500 g for 20 min after which 60 µL aliquots were injected onto the HPLC column.

The HPLC system comprised of Hewlett Packard model 1100 with a gradient pump, autosampler and a variable wavelength UV detector (Agilent Technology, Waldbronn, Germany). Analysis of chromatograms was undertaken using Chemstation Software (Version 9, Agilent Technology, Waldbronn, Germany).

Separation was performed on a Gemini C_6-phenyl 110A (150 x 4.6 mm, 5 µm) column connected to a Gemini C_6-phenyl (4 x 3.0 mm) guard column (Phenomenex, Lane Cove, NSW, Australia) at 30°C. The mobile phase contained 0.05M KH_2PO_4 adjusted to pH 2.5 and 13% v/v acetonitrile. The mobile phase was pumped at 1 mL/min and analytes were detected by their UV absorbance at 343 nm. The approximate retention times for PQ, DECQ, CQ and AQ were 2.5, 4.2, 5.2 and 6.8 min, respectively (Fig. 6.2). Two small peaks were also observed at 6.0 and 7.5 min (either side of AQ internal control), however, their identities were not further investigated and remained unidentified.
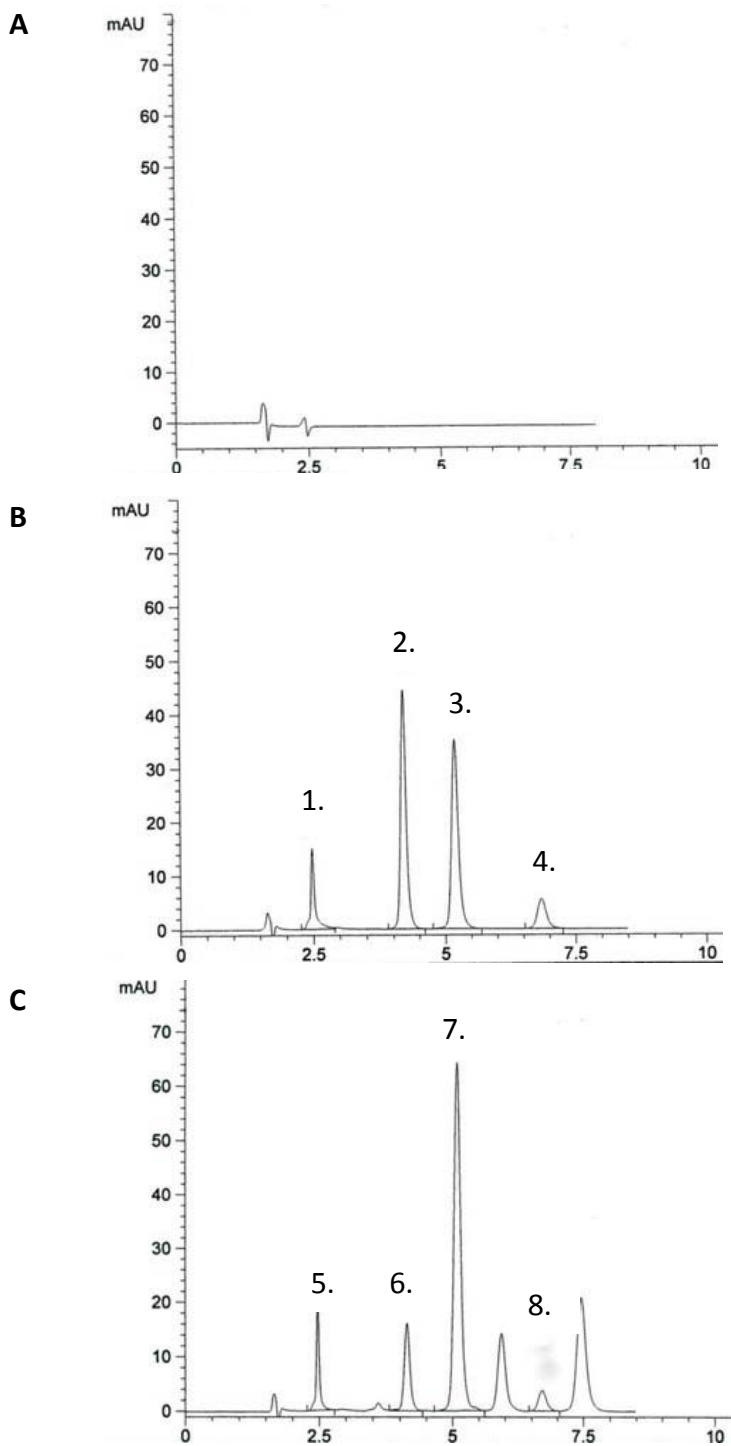
Figure 6.2   High performance liquid chromatography chromatograms for analysis of chloroquine and desethylchloroquine concentrations in mouse plasma samples. Chromatograms demonstrate results for **A**: Blank human plasma; **B**: Drug Standards; **C**: Mouse plasma sample analysis. Peaks identified were **1.** PQ internal standard (2.5 min); **2.** DECQ standard (200 ng; 4.2 min); **3.** CQ standard (200 ng; 5.2 min); **4.** AQ internal standard (6.8 min); **5.** PQ internal standard; **6.** DECQ in mouse plasma sample 20 (182 µg/L); **7.** CQ in mouse plasma sample 20 (925 µg/L); **8.** AQ internal standard.

The intra-day relative standard deviations (RSD's) for CQ were 7.8, 4.2 and 3.6% at 5 µg/L, 500 µg/L and 3,000 µg/L, respectively (n=5), while inter-day (RSDs) were 8.3, 5.8 and 5.2% at 5 µg/L, 500 µg/L and 3,000 µg/L, respectively (n=25). The intra-day relative standard deviations (RSD's) for DECQ were 6, 4.9 and 3.4% at 5 µg/L, 100 µg/L and 1,000 µg/L, respectively (n=5), while inter-day (RSDs) were 7.9, 6.3 and 5.4% at 5 µg/L, 100 µg/L and 1,000 µg/L, respectively (n=25). The limit of quantification and limit of detection was 1.2 µg/L and 0.6 µg/L for CQ and 1 µg/L and 0.5 µg/L for DECQ, respectively, with a signal to noise ratio of 3.

6.2.3.4 Pharmacokinetic analysis

For pharmacokinetic modelling, measured plasma concentrations were normalised to a dose of 50 mg/kg CQ, according to the weight of each mouse at the time of dosing. Consistent with the principles of destructive testing (37, 593), the mean normalised plasma concentration for each group of mice was used to estimate pharmacokinetic parameters. Pharmacokinetic analysis was performed using Kinetica^{TM} Version 4.4 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Non-compartmental analysis of the plasma concentration-time data was used to estimate AUC (log-linear trapezoidal method), $t_{\frac{1}{2}}$, CL/F and apparent V/F for the single dose data. A two-compartmental model was fitted to the data to estimate pharmacokinetic descriptors for the observed biphasic elimination of CQ ($t_{\frac{1}{2}\alpha}$ and $t_{\frac{1}{2}\beta}$; weighting = $1/y^2$). A two-compartment model (weighting = $1/y^2$) with first-order absorption was fitted to the DECQ plasma concentration-time data to estimate the formation rate constant (DECQ from CQ; k_F).

6.3   RESULTS

6.3.1   Pharmacodynamic evaluation of single and multiple doses of CQ

The complete (7-day) pharmacodynamic response to a single dose of 50 mg/kg CQ was determined in a representative group of 10 mice (final 2 groups for blood collection) and complemented by inclusion of the parasite density data for each sampling point in the pharmacokinetic arm of this study (Fig. 6.3). Administration of a 50 mg/kg i.p. dose of CQ resulted in a rapid decline in parasitaemia with parasite nadir reached 79 h after drug administration at a mean parasitaemia of 0.01 ±

0.02%. This represented a 406 fold decrease when compared to the starting parasitaemia. Parasite nadir was followed by an exponential rise (approximately 10 fold) in parasitaemia for > 3 days with all mice demonstrating high density parasitaemias at the experimental end-point (7 days).

In mice receiving a series of five 50 mg/kg doses of CQ administered 24 h apart, a prompt decline in mean parasitaemia was observed, falling below the limit of detection (0.002%) 54 h after the initial dose (Fig. 6.4). Parasites remained undetectable for a further 8 days until time of recrudescence, 10 days after the initial dose. From 14 to 21 days, the mean parasitaemia remained relatively stable (0.37 to 0.59%) and then steadily decreased until it again fell below the limit of detection, 24 days after the first dose of CQ. The parasitaemia remained undetectable until the experimental end-point, 30 days after the initial CQ dose. There was no statistically significant difference between parasitaemias determined from the representative groups of mice to that determined from each group of mice at time of euthanasia.
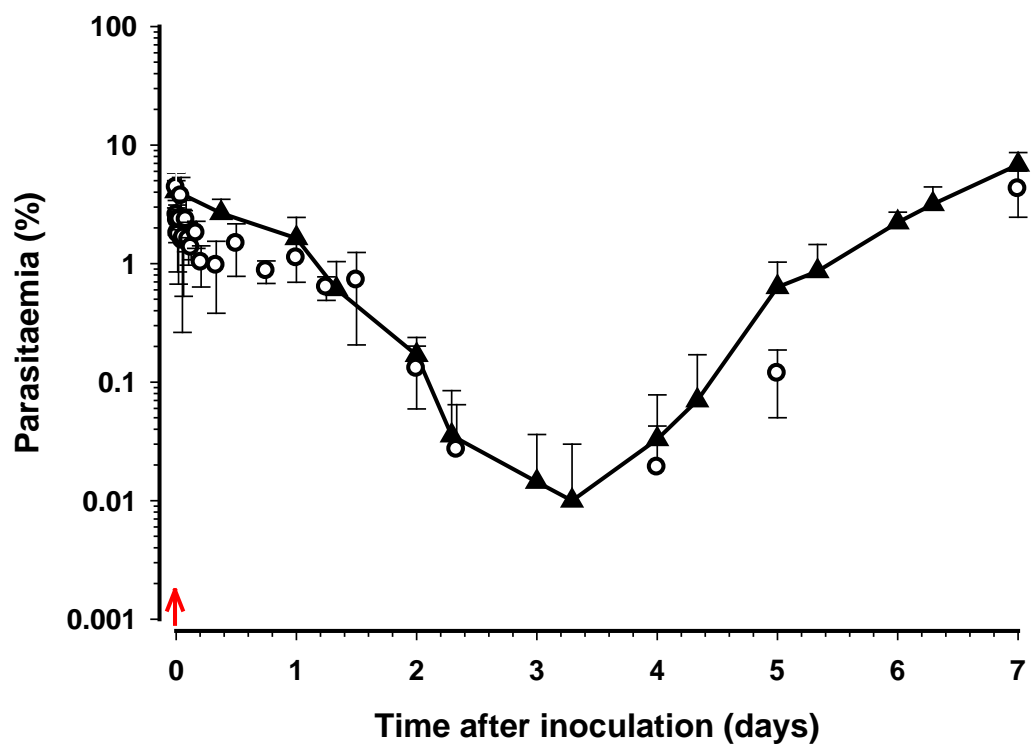
Figure 6.3. Parasitaemia-time profile in mice following administration of single i.p. doses of 50 mg/kg CQ, 64 h after inoculation with 10^7 *P. berghei*-parasitised erythrocytes. Data are shown as mean parasitaemia ± SD, from the time of drug administration (↑), in Swiss mice monitored for the duration of the study (▲, n=10) and from groups of mice whose blood was harvested at each pharmacokinetic time point (○, n=5 per group).
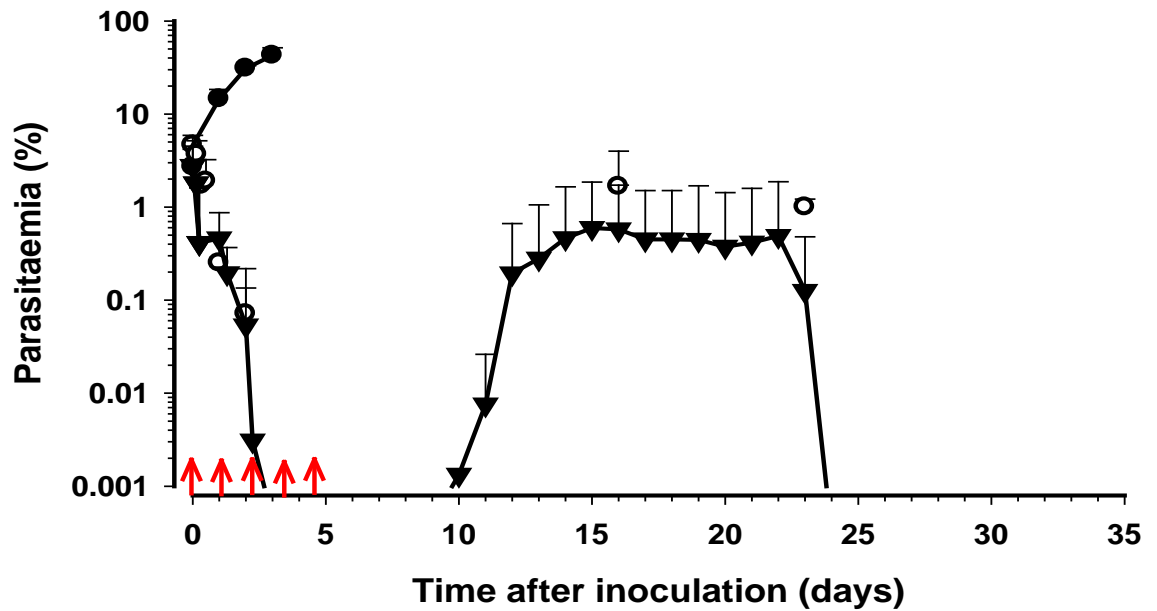
Figure 6.4. Parasitaemia-time profile in mice following administration of multiple doses of CQ (50 mg/kg CQ administered 65 h after inoculation with 10^7 *P. berghei*-parasitised erythrocytes, then single i.p. doses of 50 mg/kg CQ given every 24 hours for 4 days) ⇑). Data are shown as mean parasitaemia ± SD in both Swiss mice monitored for the duration of the study (▼, n=10) and from groups of mice whose blood was harvested at each pharmacokinetic time point (○ n=5 per group).

6.3.2 Pharmacokinetics of CQ and DECQ in mice

The plasma CQ and DECQ concentration-time profiles following single dose and multiple doses of CQ are shown in Figure 6.5 and Figure 6.6, respectively. The elimination C_{max}, t_{max}, $t_{1/2}$, CL/F and V/F of CQ were 1,708 µg/L, 20 min, 46.6 h, 9.9 L/h/kg and 667 L/kg, respectively, in healthy mice and 1,436 µg/L, 10 min, 99.3 h, 7.9 L/h/kg and 1,122 L/kg in malaria-infected mice (single dose data; non-compartmental analysis). The C_{max}, t_{max}, and $t_{1/2}$ of DECQ were 614 µg/L, 4 h, and 32.6 h, respecitively, in healthy mice and 345 µg/L, 2.5 h and 74.4 h in malaria-infected mice.

Based on the two-compartment model, $t_{1/2\alpha}$ and $t_{1/2\beta}$ were 3.3 and 53 h, respectively, in healthy mice and 4.7 and 163 h in malaria-infected mice (Fig. 6.5; Panel A; Table 6.1). CQ and DECQ data were incomplete (analytes not detected) after 7 days in the multiple dose study, hence detailed pharmacokinetic data could not be obtained from the two-compartmental model. Based on the control and malaria-infected data from the single and multiple dose studies, the mean rate of formation of DECQ from CQ (k_F) was 0.63 ± 0.55 h^{-1} and the formation half-life ($t_{1/2,Formation}$) was 1.7 ± 1.0 h. The mean $t_{1/2\alpha}$ from these four sets of data was 4.2 ± 0.7 h. The metabolic ratio of DECQ to CQ was estimated at 1.08 and 0.62 for healthy and malaria-infected mice, respectively.

Table 6.1   Pharmacokinetic parameters for CQ following i.p. (1,500 µg; 50 mg/kg) administration in healthy and malaria-infected male Swiss mice. Pharmacokinetic parameters were determined using both non-compartmental analysis ($t_{1/2}$, CL/F and V/F) and from a two-compartment model ($t_{1/2\alpha}$ and $t_{1/2\beta}$).

	Uninfected mice	*P. berghei* infected mice
$t_{1/2}$ (h)	46.6	99.3
$t_{1/2\alpha}$ (h)	3.3	4.7
$t_{1/2\beta}$ (h)	53	163
CL/F (L/h/kg)	9.9	7.9
V/F (L/kg)	667	1,122

Panel A
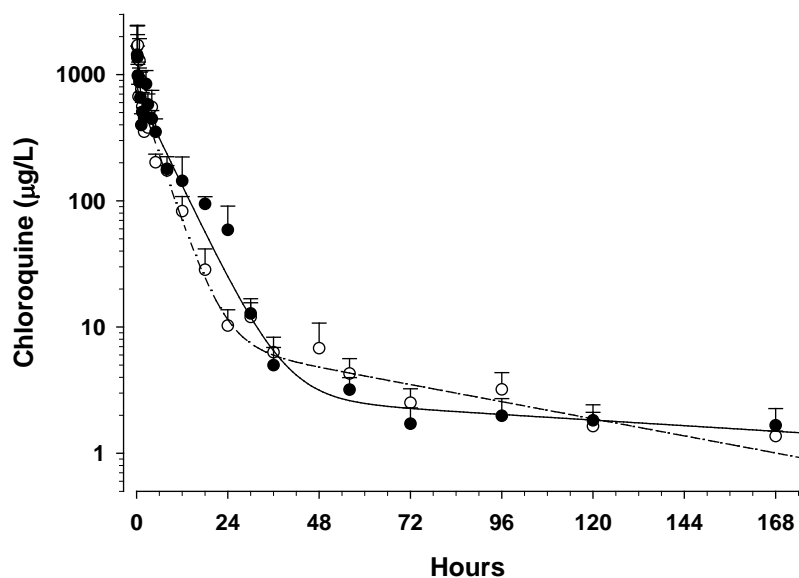
Panel B
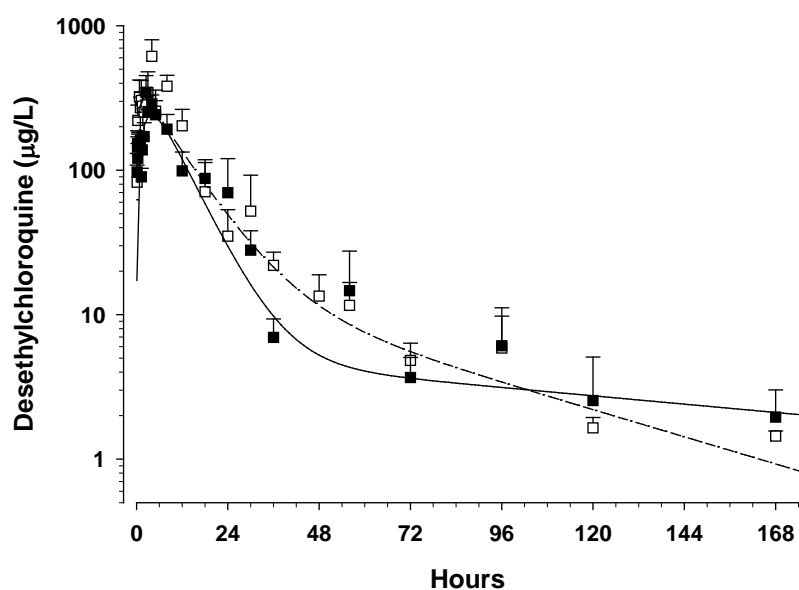
Figure 6.5   Concentration-time profile of CQ [Panel A] and DECQ [Panel B] in mice given a single dose of approximately 50 mg/kg i.p. CQ (as CQ phosphate; data normalised for pharmacokinetic analysis). Data are mean ± SD (n=5) plasma CQ concentration [Panel A] in healthy (○ ——) and malaria-infected (● —) mice and plasma DECQ concentration [Panel B] in healthy (□——) and malaria-infected (■—) mice. The lines represent the best fit of a two-compartment model to the respective data sets.

Panel A
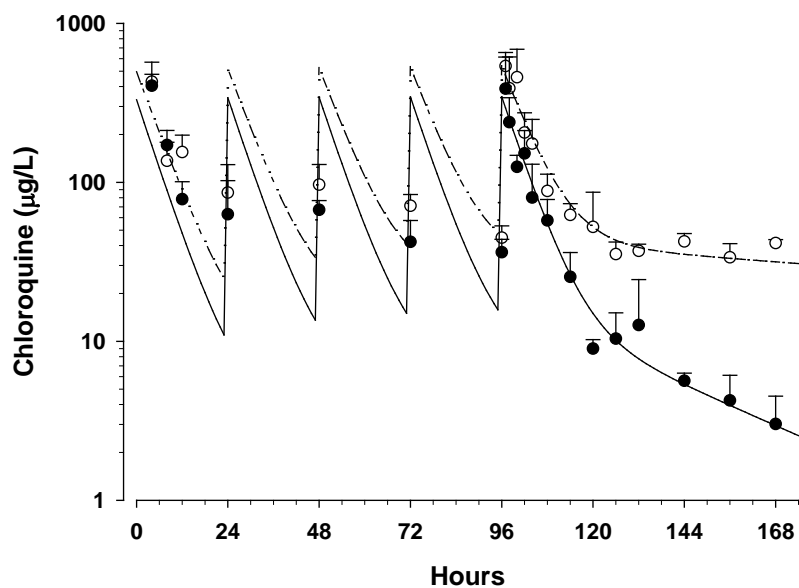
Panel B
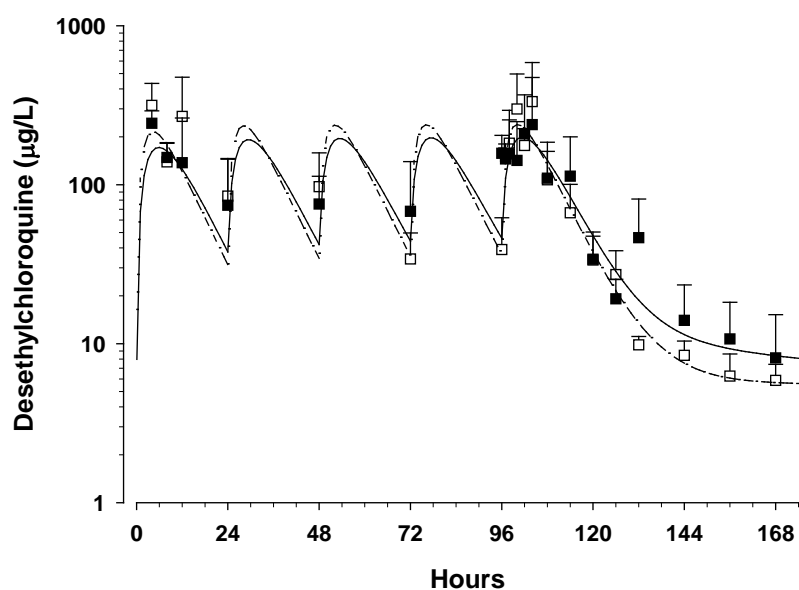
Figure 6.6 Concentration-time profile of CQ [Panel A] and DECQ [Panel B] in mice given five doses of approximately 50 mg/kg i.p. CQ (as CQ phosphate) at 24 h intervals. Data were normalised for pharmacokinetic analysis and are mean ± SD (n=5) plasma CQ concentration [Panel A] in healthy ($\bigcirc$ ——) and malaria-infected ($\bullet$ —) mice and plasma DECQ concentration [Panel B] in healthy ($\square$——) and malaria-infected ($\blacksquare$—) mice. The lines represent the best fit of a two-compartment model to the respective data sets.

6.4   DISCUSSION

The current study demonstrated that the *P. berghei* murine malaria treatment model is suitable for detailed preclinical investigation of antimalarial compounds, particularly intensive pharmacokinetic studies. Furthermore, this is the first investigation which comprehensively describes the pharmacokinetic properties of CQ and DECQ, in both healthy and *P. berghei* infected mice.   Furthermore, this study provides detailed pharmacodynamic data that demonstrate the rate of antimalarial response and provides an understanding of 'curative' effects of CQ in murine models.

To date, murine studies of CQ have not reported comprehensive pharmacokinetic data in either healthy nor malaria-infected mice, due to their short sampling periods (longest sampling period is 24 h) and/or small sample sizes (14, 78). The ability to compare results obtained from these studies is also hindered as Cambie et al. (78) determined CQ concentrations in whole blood after subcutaneous injection, whilst Ali et al. (14) established pharmacokinetic parameters in plasma after oral dosing. The purpose of the present study was therefore to improve the body of data by determining the pharmacokinetic parameters of CQ and DECQ in both healthy and *P. berghei* infected mice.

To determine the pharmacokinetic parameters of CQ and DECQ in this study it was decided to analyse the plasma drug concentrations, as a robust analytical method was already available within the laboratory and had been used in previous clinical studies (253, 254, 257).   However, CQ has a high blood: plasma ratio (>5:1) and is thought to be retained within infected erythrocytes hence plasma CQ concentrations are likely to be considerably lower than those found in healthy controls (78) and pharmacokinetic parameters will be matrix dependent (148, 264, 504). Despite this concern, a study by Frisk-Holmberg et al. (173) demonstrated that in healthy subjects there is a direct correlation between the plasma and whole blood CQ concentrations. Furthermore, whilst it is acknowledged that serum CQ concentrations are higher than corresponding plasma CQ concentrations, due to the release of platelet and leukocyte bound CQ during the clotting process, if

centrifuged correctly plasma CQ concentrations will reflt a comination of free and plasma protein bound CQ (148). It is therefore presumed that although parasitised erythrocytes would accumulate more of the drug than non-parasitised erythrocytes, a light infection (<5% parasitaemia) would not be expected to significantly change the CQ concentrations or pharmacokinetic properties (78). Conversely, it has been shown that heavy infections will significantly alter the pharmacokinetic profile of CQ; in particular the V, AUC and elimination half-life (78, 396). Considering these findings in relation to the present study, it is unlikely that the parasitaemia had significant effect on the plasma CQ concentration, particularly at the time of treatment when peripheral parasitaemia was <5%. However, during post-treatment recrudescence, towards the latter stage of sample collection, there may be some misrepresentation of plasma CQ concentrations as a result of the increasing parasitaemia. This was shown in a morphological study by Macomber et al. (295) in a *P. berghei* murine model, where 4 h following i.p. administration of 40 mg/kg CQ the erythrocyte CQ concentrations was approximately 100 times higher than corresponding plasma CQ concentration in infected mice, whilst in uninfected mice there was little accumulation of CQ into erythrocytes. Furthermore, CQ concentratiosn in erythrocytes progressively fell as the parasitaemia (295).

In the single dose pharmacokinetic study the pharmacokinetic profiles of healthy and *P. berghei* infected mice appeared to be quite similar. However, in the multiple dose study there appeared to be a significant difference in the plasma CQ concentrations from 120 h after starting CQ treatment (Fig. 6.6). The reasons for the variation in pharmacokinetic profiles, after the final 50 mg/kg CQ dose, are at this time unclear and it was beyond the scope of the study to explore this finding. However, accumulation of CQ in parasitised erythrocytes was considered unlikely because the pharmacodynamic profile demonstrates that the parasitaemia at this time was undetectable and the DECQ concentration-time profile was similar in malaria-infected and control mice.

A general limitation of all murine malaria studies is that direct extrapolation to human infections is not usually possible. However, murine malaria

pharmacokinetic/ pharmacodynamic models can be comparable to strategies used in the clinical setting (122). Therefore, such models could play an important role in the investigation of outcomes of antimalarial drug therapy both in single or combination therapies, in the development of new regimens as well as in the design of new drugs or drug combinations (122). Animal models also offer an opportunity for detailed investigations into the mechanisms of disease and the subsequent therapeutic response. For example, the current study demonstrates that although CQ has a rapid effect on eliminating parasite burden, single dose administrations are inadequate for resolving infection, even at higher doses (50 mg/kg CQ base; lethal dose suggested to range between 68-78 mg/kg base in mice (306)). Furthermore, investigation of a series of multiple dose treatment regimens showed the importance of selecting a suitable dosing regimen, as resolution of infection was only observed after repetitive high doses of CQ spread of several parasite life-cycles.

Pharmacodynamic data from the current multiple dose study demonstrated that in the *P. berghei* model, the immune system has a role in drug efficacy. Despite parasite recrudescence occurring 10 days after the initiation of therapy, a fatal parasitaemia was not observed in any mice with parasitaemias <0.5%. Furthermore, two weeks after parasite recrudescence was noted, the mean parasitaemia fell below the limit of detection (0.002%), with all mice appearing healthy and aparasitaemic for the remainder of the investigation. As the pharmacokinetic data demonstrated that plasma CQ concentrations were not detectable after 7 days, it may be concluded that the observed decline in parasitaemia, and eventual resolution of infection, was due to a combination of initial parasite reduction after drug administration and the stimulation of an immune response. A similar pharmacodynamic response was observed after high dose PQP (Chapters 4 and 5) at which time it was concluded that the resolution of infection was a result of both pharmacological and immunological mechanisms.

In conclusion, this study has shown that the *P. berghei* murine malaria model has the potential to be a valuable conceptual model for the comparison of single dose

and multiple dose therapies. Importantly, where immune and other regulatory mechanisms may be integral features, the murine model is a useful extension of more rapid, comparatively high-throughput *in vitro* studies. Despite WHO recommendations due to widespread CQ-resistant malaria (584), CQ monotherapy remains a popular choice of antimalarial for the prevention and treatment of uncomplicated malaria (111, 135, 513, 549, 556, 559, 562, 586). As a result, preclinical investigations into adequate treatment regimens (for single dose therapies) and acceptable combination therapies are of the utmost importance (357, 383, 522, 549). In light of this, the present study demonstrated the pharmacokinetic properties of CQ and its metabolite DECQ in both healthy and *P. berghei* infected mice, observations that are believed to significantly contribute to the preclinical knowledge of this drug, which to date has not been adequately investigated.

CHAPTER SEVEN

CONCLUSIONS

Murine malaria models have proved to be important in the preclinical evaluation of antimalarial compounds and their therapeutic regimens.   Furthermore, a well selected murine model can provide valuable insight on the pathology and immunology of malaria infection in the infected host. The Rane murine malaria model was initially proposed for the testing of antimalarial drug activity, using 'death' and 'survival' as descriptors of success. As a result, this model proved to be highly successful in the screening of potential antimalarial compounds. For the studies described in this thesis, it was thought that after minor modifications, the outcomes of the Rane model could be extended, thus allowing further investigation into descriptive pharmacokinetics, dose-ranging pharmacodynamics, organ histopathology and immunological interactions. Hence, this series of studies was designed to investigate the pharmacodynamic and pharmacokinetic relationship of a range of antimalarial drugs, both novel and established, in mice. This was deemed important, due to limited published (Western) literature wth detailed murine phamacodynamic-pharmacokinetic investigations of DHA, PQ and CQ. Whilst it is acknowledged that murine investigations are not readily comparable to the clinical setting, it is also suggested that the results obtained in these studies will prove to be valuable for more detailed preclinical research into antimalarial drug combinations and treatment regimens.

The research investigations presented in this thesis have demonstrated that the *P. berghei* murine malaria model provides a valuable conceptual model for the study of single dose, multiple dose and combination therapy pharmacodynamic and pharmacokinetic responses, which is clearly neither ethical nor feasible in the clinical setting. *In vitro* culture models also lack the immune and regulatory mechanisms of a living model and therefore the immunological responses observed in these studies, which proved to be important in the maintenance of subclinical

levels of infection, would not be able to be observed or considered in an *in vitro* model. Furthermore, pharmacokinetic data obtained from the present investigations have demonstrated that the *P. berghei* malaria model can provide robust evidence for 'concentration efficacy thresholds' with antimalarial drugs.

The study of DHA efficacy in intact and asplenic, healthy and *P. berghei* infected mice demonstrated that DHA had a potent antimalarial effect in both populations. Furthermore, an investigation into the stage specificity of DHA against *P. berghei* parasites showed that this antimalarial drug was effective against all of the erythrocytic stage *Plasmodium* parasites. Histopathological investigation of key organs (liver, kidney and lung) indicated an enhanced role of the liver in asplenic mice, which was a likely compensatory mechanism for the clearance of parasites in this population of mice.

Although PQ has been utilized as an antimalarial compound in the clinical setting since the early 1960s, to date there is little published data on the efficacy or pharmacokinetic-pharmacodynamic relationship of PQ in mice. The present detailed investigations therefore provided a valuable insight into this novel antimalarial drug. Single dose-ranging PQ studies demonstrated a potent, long lasting antimalarial effect in mice infected with *P. berghei* parasites. Furthermore, this antimalarial efficacy was shown to be enhanced when mice were treated with a combination of PQ and DHA. High dose PQP (90 mg/kg) administration resulted in a pharmacodynamic profile that suggested a period of post-treatment prophylaxis, which was further investigated to determine if this effect was due primarily to drug administration, the acquisition of immunity towards the infecting parasite strain or a combination of factors. A series of viability and re-inoculation experiments looking specifically at parasite viability, drug efficacy, drug resistance and immunological influences demonstrated that whilst PQ provided a substantial antimalarial effect, the period of post-treatment prophylaxis was predominantly a result of immunological mechanisms. Furthermore, the study suggested that residual PQ concentrations could lead to the development of PQ-resistant parasites.

The pharmacokinetic investigation of CQ in the *P. berghei* malaria model was performed because despite the extensive use of CQ as a comparator drug in preclinical efficacy investigations, there remains a paucity of murine pharmacokinetic data. This investigation found little difference, after single dose administration, between the pharmacokinetic profiles of healthy and *P. berghei* infected mice. However, after multiple dose administrations of CQ, a significant difference in the plasma CQ concentrations was observed. Corresponding pharmacodynamic data demonstrated that after multiple doses of CQ (5 x 50 mg/kg), parasitaemia was suppressed for a sufficient period of time in which an immunological response could be mounted towards infection.

The present series of investigations established that this *P. berghei* murine model is suitable for detailed preclinical investigations of antimalarial compounds, particularly intensive pharmacokinetic studies. Whilst the current studies appear to be the first to comprehensively describe the pharmacokinetic properties of PQ, CQ and DECQ, in both healthy and *P. berghei* infected mice, novel pharmacodynamic and immunological responses to the administration of DHA, PQ and CQ have also been demonstrated. It is therefore concluded that the work described in this thesis will be viewed as a valuable contribution to the preclinical investigation of antimalarial drugs.

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APPENDIX 1

MURINE SEROLOGY

Murine Virus Monitoring Service

101 Blacks Rad

Gilles Plan 5086

South Austalia

Telephone: +61 8 8261 1033

Fax: +61 8 8261 2280

SEROLOGY

'All samples will be tested by ELISA for antibody directed against: Cilia Associated Respiratory Bacillus (CAR), Murine Cytomegalovirus (CMV), E. Cuniculi (CUN), Ectromelia Virus (ECT), Hantaan Virus (HAN), Lymphocytic Choriomeningitis Virus (LCM), Mouse Adenovirus (MAD), Mouse Hepatitis Virus (MHV), Minute Virus of Mice & Mouse Parvovirus (combined test using recombinant antigen PARV – rNS1), Polyoma Virus (POL), Mycoplasma pulmonis (PUL), Pneumonia Virus of Mice (PVM), Reovirus Type 3 (REO), Epidemic Diarrhoea of Infant Mice (ROT), Sendai Virus (SEN), Theiler's Murine Encephalomyelitis Virus (TMV) and Clostridium piliformis (TYZ).'

All results will be reported as:

POS = Positive

NEG = Negative

EQU = Unconfirmed low level reaction

NS = Non-specific reaction

APPENDIX 2

REAGENTS AND BUFFERS

A2.1   Acetic Acid; 1 M

Acetic acid, glacial (17.43 M)	57.4 mL
Water	to 1 L

A2.2   Alsever's Solution

Dextrose (anhydrous)	20.5 g
Trisodium citrate ($Na_3C_6H_5O_7.2H_2O$)	8.0 g
Sodium chloride	4.2 g
Citric acid (monohydrate)	0.5 g
Water	to 1 L

A2.3   Citrate-Phosphate-Dextrose Solution; pH 6.9

Trisodium citrate ($Na_3C_6H_5O_7.2H_2O$	30.0 g
Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	0.16 g
Dextrose (anhydrous)	2.0 g
Water	to 1 L

A2.4   Formaldehyde-Citrate Solution

Formaldehyde solution (40% w/w CH_2O)	10 mL
Trisodium citrate solution (109 mM)	to 1 L

A2.5   Glycerol in Alsever's Solution

Glycerol	100 mL
Alsever's solution	to 1 L

* Sterilised by filtration (0.45 µm Millex ®-HA filter unit; Millipore, Bedford, MA)

A2.6 Phosphate Buffer

Potassium dihydrogen phosphate (KH_2PO_4)	2.72 g
Disodium hydrogen phosphate (Na_2HPO_4)	11.36 g
Water	to 1 L

A2.7 Sorenson's Phosphate Buffer (pH 7.0)

Potassium dihydrogen phosphate (KH_2PO_4)	3.75 g
Disodium hydrogen phosphate (Na_2HPO_4)	5.56 g
Water	to 1 L

A2.8 Trisodium Citrate Solution

Trisodium citrate ($Na_3C_6H_5O_7.2H_2O$)	32 g
Water	to 1 L