DEVELOPMENT OF A NOVEL, QUANTITATIVE ASSAY FOR DETERMINING THE RATE OF ACTIVITY OF ANTIMALARIAL DRUGS

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ABSTRACT

Malaria, caused by an intracellular *Plasmodium* parasite, remains a devastating disease, having claimed approximately 655000 lives worldwide in 2010. The Medicines for Malaria Venture suggests a ‘single-dose radical cure” as the ideal malaria treatment since rapid clearance of blood-stage parasites and symptom relief improves patient compliance and limits drug resistance. Thus, novel antimalarials should be rapid-acting and assessing their rate of activity is critical to drug discovery. Traditional evaluation of this rate by morphological assessments is flawed by highly subjective, operator-specific interpretations, mainly due to heterogeneous parasite morphology under routine culture conditions. This study aimed to develop an alternative, quantitative assay. Energy is vital for the growth and maintenance of all living organisms. Commercially available kits allow rapid quantification of the cell‘s energy currency, ATP. Therefore, quantification of parasite ATP shows potential for diagnosing abnormal parasite metabolism and the kinetics of drug action. In this study, a rapid protocol for detecting ATP in *Plasmodium falciparum* parasites using a luminescence-based kit was developed and optimised. Furthermore, luciferase-expressing transgenic parasites, in which luciferase activity is detected using a similar kit, were acquired. The utility of both methods for evaluating the rate of drug-induced stress was explored using antimalarials with varying modes of action and, presumably, rates of activity. Results showed that parasite ATP remained unchanged, increased or decreased during drug exposure. Morphological examinations by light microscopy and a Recovery assay, aided interpretation of the drug-induced changes in parasite ATP. These investigations suggested that unchanged parasite ATP levels reflect poor drug action, increased ATP levels indicate a stress response and partially compromised viability, while significantly reduced ATP reflects severely compromised viability. Concerning the Luciferase assay, parasite luciferase activity decreased during drug exposure, even in the presence of proteasome inhibitors. Changes in parasite ATP and luciferase activity occurred at rates which suggested that chloroquine is slow-acting, mefloquine has a moderate rate of activity and artemisinin is rapid-acting. These findings are compatible with the expected rates of activity of these established antimalarials. Hence, measurement of parasite ATP and/or luciferase activity may support assessments of parasite health and the kinetics of antimalarial action during drug discovery.

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LIST OF ABBREVIATIONS

ACT	Artemisinin-based combination therapy
APAD	3-Acetylpyridine adenine dinucleotide
ART	Artemisinin
ATP	Adenosine triphosphate
BC	Before Christ
BSA	Bovine serum albumin
CAM	Calmodulin
CMCM	Complete malaria culture medium
CQ	Chloroquine
CSIR	Council for Scientific and Industrial Research
CV	Coefficient of variation
DFMO	DL-α-difluoromethylornithine
DNA	Deoxyribonucleic acid
Dr.	Doctor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GR	Glutathione reductase
GRX	Glutaredoxin
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione-S-transferase
H^+	Hydrogen ion (proton)
HAP	Histoaspartic protease
HPLC	High-pressure liquid chromatography
hrp2	Histidine-rich protein 2
HSPs	Heat shock proteins
ID	Identification number
IRS	Indoor residual spraying
ITN	Insecticide-treated bednets
K^+	Potassium ion
LC	Lactacystin

Luc	Luciferase
LDH	Lactate dehydrogenase
MG	MG-132
MI	Michigan
MMV	Medicines for Malaria Venture
MQ	Mefloquine
Na^+	Sodium ion
NADH	Dihydronicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NBT	Nitro blue tetrazolium
Pb	*Plasmodium berghei*
PBS	Phosphate-buffered saline
PES	Phenazine ethosulphate
P. falciparum	*Plasmodium falciparum*
pLDH	Plasmodial lactate dehydrogenase
PM	Plasmepsin
Prof.	Professor
RBC	Red blood cell
RBM	Roll Back Malaria
RLU	Relative light units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RSA	Republic of South Africa
SD	Standard deviation
SERCA	Sarco-endoplasmic reticulum calcium ATPase
SOD	Superoxide dismutase
TR	Thioredoxin reductase
TRX	Thioredoxin
US	United States
USA	United States of America
WHO	World Health Organisation
WI	Wisconsin

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CHAPTER 1: GENERAL INTRODUCTION

1.1. MALARIA

Malaria is caused by an obligate intracellular protozoan parasite of the genus *Plasmodium* and transmitted by the female *Anopheles* mosquito (Francis *et al*., 1997). This potentially fatal parasitic disease targets several vertebrate hosts including humans, monkeys and birds (Cox, 2010). Humans have been victims of the disease since ancient times, with reports detailing signs and symptoms of malaria dating back to as early as 2700 BC (Cox, 2010).

The parasites responsible for the disease were discovered in 1880 by Charles Louis Alphonse Laveran, a French army surgeon, while working in Algeria (Cox, 2010). On the basis of reports that a pigment was observed in the spleens of malaria patients, Laveran used light microscopy to search for the pigment in fresh, unstained blood samples of malaria patients. His efforts were rewarded when he observed the pigment in leucocytes and in, or on, erythrocytes. Upon closer examination, Laveran noticed that the parasite took on several different forms including crescents, and motile and motionless spherical forms that contained pigment. He described a course of events that started with clear spots which grew, obtained pigment and filled the erythrocyte before rupturing at a point which coincided with fever, a well-known symptom of malaria. Laveran‘s description of events, though based solely on light microscopy without the use of stains or an oil immersion lens, is still compatible with the currently accepted description of the intra-erythrocytic development of the malaria parasite. After carefully examining the blood of 200 patients, Laveran noted that the crescent-shaped bodies were present in all malaria-infected individuals but not in uninfected patients. Despite the leading theory at the time which suggested that malaria was caused by a bacterium, Laveran realised that the erythrocytic bodies which he had discovered were in fact protozoan parasites which he named *Oscillaria malariae*, and proposed were the cause of malaria.

Laveran‘s theory initially received much scepticism, with leading malariologists and microbiologists not convinced that what Laveran was describing was anything more than disintegrating erythrocytes (Cox, 2010). However, he persevered and by 1884, had convinced leading Italian malariologists that the causative agent of malaria was a protozoan and not a bacterium (Cox, 2010). The development of novel staining techniques and the oil immersion

lens allowed for more detailed investigations of the parasite and by 1890 it was widely accepted that malaria was caused by a protozoan parasite that invaded and replicated within erythrocytes (Cox, 2010). In addition, after much debate, three species which cause human malaria were identified, *Laverania malariae*, *Haemamoeba vivax* and *Haemamoeba malariae*, currently now known as *Plasmodium falciparum, P. vivax* and *P. malariae*, respectively (Cox, 2010). A fourth species, *P. ovale*, was discovered later by John Stephens while working in Africa (Cox, 2010). In 1897, mosquitoes were identified as vectors of avian malaria by Dr. Ronald Ross, a British medical officer stationed in India (Cox, 2010; Tuteja, 2007). Italian scientists, including Prof. Giovanni Battista Grassi, later showed that human malaria was transmitted solely by *Anopheles* mosquitoes (Cox, 2010; Tuteja, 2007). It was initially believed that only the four abovementioned *Plasmodium* species could cause human malaria. However, Singh *et al*. (2004) recently showed that almost a third of severe human malaria infections in the Malaysian Borneo region, were misdiagnosed as *P. malariae,* but were actually caused by *P. knowlesi*. Thus, *P. knowlesi*, which usually causes malaria in long-tailed macaque monkeys, has been identified as the fifth human malarial parasite.

1.2. THE LIFE CYCLE OF *P. FALCIPARUM*

Of the five species of *Plasmodium* that cause malaria in humans, *Plasmodium falciparum* is the most prevalent and virulent, accounting for the large majority of deaths (Francis *et al*., 1997). The life cycle of *P. falciparum* is quite complex (Figure 1.1) with the asexual stage of development occurring within a vertebrate host and the sexual stage occurring in a female *Anopheles* mosquito (Francis *et al*., 1997).

The asexual developmental phase (reviewed by (Chiang *et al*., 2006; Cowman & Crabb, 2006; Francis *et al*., 1997; Tuteja, 2007; van Agtmael *et al*., 1999)) begins when a malaria-infected anopheline mosquito feeds on a human, thereby transferring infectious sporozoites in its saliva to the human bloodstream. The sporozoites travel through the blood ultimately reaching the liver and infecting hepatocytes. This liver stage of parasite development lasts 9-16 days and is asymptomatic as the sporozoites mature into schizonts within the hepatocytes. The schizonts eventually burst, releasing thousands of liver merozoites into the blood where they proceed to infect erythrocytes. This intra-erythrocytic developmental stage is an asexual, 48-hour cycle and is the cause of the clinical manifestations of malaria. Following invasion of the erythrocyte, the merozoites progress to the ring stage of parasite growth which lasts approximately 24 hours and is characterised by limited metabolic activity. The rings develop

into trophozoites which display marked metabolic activity that includes import and glycolysis of vast amounts of glucose, endocytosis of host cytoplasm and subsequent digestion of haemoglobin, as well as the synthesis of DNA, RNA and proteins. The trophozoites mature into schizonts which, in turn, go through four to five rounds of binary divisions to produce 16-32 daughter merozoites. The pathophysiology of *falciparum* malaria is believed to be due to the adherence of trophozoite- and schizont-infected erythrocytes to the endothelium of small blood vessels, a process known as sequestration. The schizonts rupture, releasing the merozoites back into the bloodstream to infect other erythrocytes and begin a new cycle of asexual development. This intra-erythrocytic developmental phase is highly synchronised with the majority of the parasite population in the bloodstream being in the same developmental stage at any one time (Tuteja, 2007; van Agtmael *et al*., 1999). Thus, rupture of schizont-infected erythrocytes occurs at roughly the same time and is believed to release malaria toxins which are proposed to initiate a cytokine response by the host, resulting in the classic malaria symptoms of fever, malaise and chills (Tuteja, 2007; van Agtmael *et al*., 1999).

Apart from asexual development, some intra-erythrocytic stages of the parasite may differentiate into male and female sexual forms known as gametocytes, thereby initiating the sexual stage of development (reviewed by (Chiang *et al*., 2006; Cowman & Crabb, 2006; Cox, 2010; Ghosh *et al*., 2000; Tuteja, 2007)). When a female *Anopheles* mosquito feeds on a malaria-infected individual, the gametocytes enter the insect gut where they develop into gametes and eventually fuse to produce zygotes. The zygote transforms into a motile ookinete which forces its way into the epithelial lining of the midgut where it differentiates into an oocyst. After a complex developmental process which lasts several days, the oocyst releases thousands of sporozoites into the mosquito‘s body cavity. The sporozoites migrate to the salivary glands and, during the mosquito‘s next blood meal, can be transmitted via the saliva to another human host where the cycle begins again.

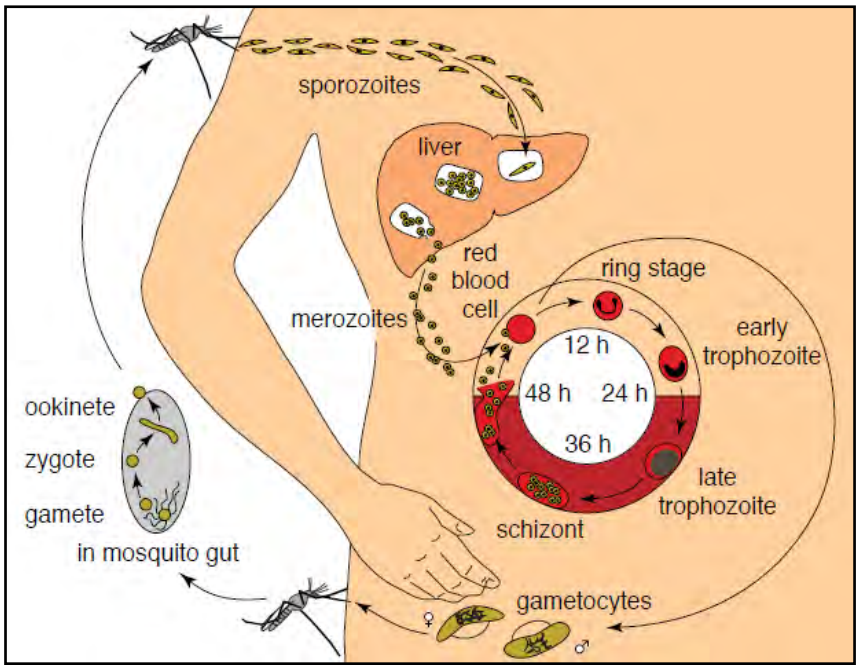

Figure 1.1: Life cycle of the *Plasmodium falciparum* malaria parasite (van Agtmael *et al*., 1999).

1.3. PATHOGENESIS AND CLINICAL SYMPTOMS OF *P. FALCIPARUM* MALARIA

Most malaria-infected individuals present with uncomplicated malaria which is characterised by non-specific, flu-like symptoms such as fever, headache, vomiting and diarrhoea and is often easily resolved either via host immune responses or antimalarials (Beeson & Brown, 2002; Mackintosh *et al*., 2004). About 1% of malaria-infected individuals exhibit severe malaria (Mackintosh *et al*., 2004), a life-threatening form of the disease which was previously thought to manifest as either cerebral malaria or severe anaemia but is now recognised as a complex disorder that affects multiple organs, similar to sepsis (Mackintosh *et al*., 2004; Miller *et al*., 2002). Severe malaria arises when the parasite progresses to the intra-erythrocytic stage of development (Rasti *et al*., 2004) and a unique feature of this developmental phase of *P. falciparum* malaria, relative to other human malarias, is sequestration (Beeson & Brown, 2002; Mackintosh *et al*., 2004; Miller *et al*., 2002; Rasti *et al*., 2004).

Sequestration is the ability of mature parasitised red blood cells (RBCs) to adhere to uninfected RBCs, endothelial cells and the placental cells of pregnant women (Beeson & Brown, 2002; Mackintosh *et al*., 2004; Miller *et al*., 2002; Rasti *et al*., 2004; Tuteja, 2007).

The parasite achieves sequestration by trafficking parasite proteins, such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) to the surface of the host RBC, and these proteins mediate adherence by interacting with receptors on the surface of various host cells (Beeson & Brown, 2002; Mackintosh *et al*., 2004; Miller *et al*., 2002; Rasti *et al*., 2004; Tuteja, 2007). Sequestration prevents parasite-infected RBCs from being destroyed in the spleen of the host, however, it also threatens the life of the host by blocking the small blood vessels of vital organs and tissues, thereby directly contributing to the pathogenesis of severe malaria (Beeson & Brown, 2002; Mackintosh *et al*., 2004; Miller *et al*., 2002; Rasti *et al*., 2004). For example, sequestration of the parasite-infected RBCs in the small vessels of the brain may result in cerebral malaria which causes unrousable coma and convulsions in the patient and is believed to be the cause of the majority of malaria deaths (Beeson & Brown, 2002; Tuteja, 2007).

Severe anaemia in malaria-infected individuals is also a key feature of severe malaria and is primarily due to parasite-mediated rupture of infected RBCs and increased destruction of infected and non-infected RBCs by the spleen and macrophages of the host (Beeson & Brown, 2002; Mackintosh *et al*., 2004; Miller *et al*., 2002; Rasti *et al*., 2004). Furthermore, free radicals produced by host macrophages in response to pro-inflammatory cytokines, and the release of malaria pigment following rupture of infected RBCs, may impair RBC production in the bone marrow thereby contributing to anaemia during malaria infection (Rasti *et al*., 2004). Interestingly, severe anaemia may have a beneficial effect on the host during malaria infection by reducing blood viscosity and thus improving circulation in small vessels, hence allowing for increased oxygen and nutrient supply to tissues (Rasti *et al*., 2004).

Metabolic acidosis, which leads to respiratory distress, is the strongest indicator of imminent death in severe malaria, and even though the pathogenesis of this acidosis is still unclear, lactic acidosis is proposed to play a central role (Mackintosh *et al*., 2004; Miller *et al*., 2002). Hypovolemia and dehydration are observed in patients with severe malaria and together with microvascular blockage and severe anaemia may reduce tissue perfusion and thus give rise to anaerobic metabolism and hence lactic acidosis (Mackintosh *et al*., 2004; Miller *et al*., 2002). Metabolic acidosis has also been implicated in a reduction of the deformability of uninfected RBCs, and though the pathogenesis of this effect remains unclear, it may further compromise tissue perfusion in individuals with severe malaria (Miller *et al*., 2002; Rasti *et al*., 2004).

Other contributors to metabolic acidosis in severe malaria are the cytokine-mediated failure of oxygen utilisation and parasite-mediated production of lactic acid (Mackintosh *et al*., 2004; Miller *et al*., 2002). Renal dysfunction and failure, spontaneous bleeding and hypoglycaemia have also been observed in cases of severe malaria (Beeson & Brown, 2002). Thus, current knowledge suggests that severe malaria results from a combination of key pathogenic processes, namely microvascular obstruction, the destruction of infected and uninfected RBCs and host inflammatory responses which together decrease tissue perfusion (Miller *et al*., 2002). This may subsequently cause downstream cellular events which aggravate the situation further.

Multiple infections with malaria lead to the development of partial immunity which protects against severe disease and only mild infections occur with little or no clinical symptoms (Beeson & Brown, 2002). Hence, due to a lack of acquired immunity, children are particularly susceptible to the disease and the prognosis is often very poor (Rasti *et al*., 2004). Pregnant women are at a greater risk of contracting the disease than non-pregnant women, and are also exceptionally vulnerable to the disease (Beeson & Brown, 2002; Tuteja, 2007). Lindsay *et al.* (2000) suggested that the increased risk of infection in pregnant women, relative to non-pregnant women, is due to an increased attractiveness to the vectors of the disease (i.e. mosquitoes) as a result of physiological and behavioural changes that occur during pregnancy. Pregnant women were found to have heightened exhalation and body temperature relative to non-pregnant women, and this was believed to result in a greater release of chemicals that likely attract mosquitoes. The pregnant women were also found to be at a greater risk of exposure to the mosquitoes since they left the safety of their bednet, in order to urinate, twice as often as non-pregnant women. Key characteristics of *P. falciparum* infection in pregnant women are sequestration and accumulation of parasites in the placental blood cavities, and maternal anaemia (Beeson & Brown, 2002; Tuteja, 2007). These pathogenic events are believed to be the cause of maternal deaths, low birthweight, premature delivery, miscarriages and stillbirths in malaria-infected pregnant women (Beeson & Brown, 2002; Tuteja, 2007).

1.4. IMPACT OF MALARIA

Malaria remains one of the world‘s most serious parasitic infections with approximately half the world‘s population in 109 countries at risk of contracting the disease (WHO, 2010a). Approximately 216 million malaria cases and 655000 deaths were reported in 2010 alone and

an estimated 81% and 91% of these cases and deaths, respectively, occurred in Africa (WHO, 2011) which carries the majority of the world malaria burden (Figure 1.2). These estimates make malaria the second leading cause of death from infectious diseases on the African continent, after HIV/AIDS (Sachs & Malaney, 2002). The primary victims are pregnant women and children younger than five, with 86% of global malaria deaths in 2010 affecting the latter group (WHO, 2011). The disease can also affect the immune system and alter the body‘s response to vaccines, thereby increasing susceptibility to other infections (Sachs & Malaney, 2002).

Figure 1.2: World malaria risk areas, 2010. ■, countries or areas experiencing malaria transmission; ■, countries or areas where risk of malaria transmission is limited (WHO, 2010b). Note: The map is intended as a visual aid only and not as a definitive source of information for malaria endemicity.

Over and above its effects on global health, malaria exacerbates poverty in many areas and is severely detrimental to the rate of economic growth in countries where it is endemic (Sachs & Malaney, 2002; Vitoria *et al*., 2009; WHO, 2010c). In general, in areas where malaria thrives, human societies have had the least success (Sachs & Malaney, 2002). For instance, in Africa alone, malaria-related illness, treatment and premature death incur costs of no less than 12 billion US dollars every year, with even greater losses to economic growth (Tuteja, 2007; WHO, 2010c). In addition, with almost 25% of household incomes spent on malaria treatment in the majority of African endemic countries, access to preventative measures and lifesaving services is limited (Vitoria *et al*., 2009). Thus, while malaria aggravates poverty,

the relationship is also reversible in that poverty promotes malaria transmission in cases where preventative measures, such as bednets or insecticides, are too costly. The scourge of malaria also discourages tourism, trade and foreign investment in countries in which it is endemic and this is severely detrimental to economic growth (Sachs & Malaney, 2002). Children are particularly susceptible to malaria morbidity and mortality since, unlike adults, they lack acquired immunity (Rasti *et al*., 2004). As a result, school absenteeism is increased and this leads to higher failure rates, drop-out rates and repetition of school years (Sachs & Malaney, 2002). In addition, malaria-infected children suffer from poor nutritional status which can be detrimental to cognitive development (Sachs & Malaney, 2002).

1.5. EFFORTS AT MALARIA ERADICATION

Until the 1850s, malaria was prevalent in most countries reaching as far north as the Arctic Circle with approximately 90% of the world‘s population at risk of contracting the disease (Mendis *et al*., 2009; WHO, 2010c). During the latter half of the 19th century, much of North America and Europe became malaria-free, likely due to changes in the agricultural use of land and better housing (Mendis *et al*., 2009). Discovery of the malaria parasite and its mosquito vector led to further suppression of the disease through the implementation of focal mosquito control and widespread availability of malaria diagnosis and treatment (Mendis *et al*., 2009). In 1955, the availability of DDT (dichlorodiphenyltrichloroethane) and chloroquine, a potent insecticide and antimalarial, respectively, led the World Health Organisation (WHO) to initiate the Global Malaria Eradication Programme in endemic areas outside of sub-Saharan Africa (Mendis *et al*., 2009; WHO, 2010c). This African region was excluded since its substantial burden of malaria and limited infrastructure made the programme unfeasible (Tanner & de Savigny, 2008). By 1978, the programme had successfully eradicated malaria in North America, the Caribbean, Europe and parts of south-central America and Asia (Tanner & de Savigny, 2008). The campaign also significantly reduced malaria cases and deaths in many other countries, including India and Sri Lanka (Mendis *et al*., 2009). However, poor maintenance of the programme and the appearance of chloroquine-resistant parasites and DDT-resistant vectors led to resurgence of the disease and by 1969, efforts to eradicate malaria were replaced by attempts to control the disease (Mendis *et al*., 2009). Furthermore, the eradication of malaria in developed countries led to a loss of interest in the disease and developing African countries, which were struggling to set up primary healthcare and broad-based health systems, received little global support for malaria control and continued to carry a heavy malaria burden (Tanner & de Savigny, 2008). By

1992, the deteriorating malaria situation and promising technical innovations sparked renewed global interest in malaria control (Tanner & de Savigny, 2008).

The Roll Back Malaria (RBM) initiative was launched by the WHO in 1998 with the goal of halving malaria deaths by 2010 and again by 2015 by improving the availability of effective treatment and prevention measures (Nabarro & Tayler, 1998). The World Malaria Report 2011 (WHO, 2011) indicated that in 46% of the countries experiencing malaria transmission, reports of malaria cases had decreased by over 50% between 2000 and 2010. In addition, the number of global cases and deaths had decreased by 17% and 26%, respectively, between 2000 and 2010. While these rates of decline fail to meet the goals set by the RBM initiative, they still reflect a major accomplishment in the fight against malaria. Taking into account the progress made by 2010, the goals of the RBM initiative were revised and the possibility of malaria eradication is back on the agenda (WHO, 2010c). Current goals of the RBM initiative are to achieve the following by 2015: (i) reduce global malaria cases from 2000 levels by 75% (ii) decrease global malaria deaths to close to zero, (iii) eliminate malaria in 10 new countries. The measures currently being employed to meet these goals include vector control, vaccine development, and therapeutic and prophylactic drugs (WHO, 2010c).

1.6. CURRENT COUNTERMEASURES

Malaria is a completely preventable and treatable disease, as long as the currently recommended interventions are correctly employed (WHO, 2011). These include vector control, chemoprophylaxis and timely diagnosis and treatment.

1.6.1. Vector control

The severity and pattern of transmission of malaria parasites, and hence the epidemiology of infection and disease, are heavily dependent on the abundance, seasonality and feeding habits of the anopheline mosquito vector (Greenwood *et al*., 2008). Malaria vector control has been a key element of successful malaria elimination strategies, such as those employed in the United States and Europe, and continues to play a leading role in current strategies (Greenwood *et al*., 2008). Malaria vector control is aimed at protecting the individual from malaria infection and decreasing the severity of local malaria transmission (WHO, 2011). The most effective and widely used forms of vector control are indoor residual spraying (IRS) of insecticides and insecticide-treated bednets (ITNs) (Greenwood *et al*., 2008; WHO, 2011).

Currently, four chemical classes of insecticides are used for vector control, i.e. pyrethroids, organophosphates, carbamates and organochlorines (WHO, 2011). The pyrethroids are the most commonly used due to their affordability, low toxicity and rapid, long-lasting effects (WHO, 2011). Any of the abovementioned insecticide classes can be used for IRS, however, only the pyrethroids are approved for use in ITNs (Greenwood *et al*., 2008; WHO, 2011). In addition, in cases where mosquito breeding sites are minimal and easily identifiable, IRS and ITNs may be supplemented with larval control (WHO, 2011). In 2010, 82 countries, including 38 African countries, provided ITNs either free or at a subsidised price, and a total of 185 million individuals were protected from malaria vectors by IRS (WHO, 2011). However, the widespread use of a single class of insecticide (i.e. the pyrethroids) has enhanced the risk of mosquitoes acquiring resistance and, to date, 27 African countries have reported cases of resistance (WHO, 2011). Given the significant role of vector control in the fight against malaria, maintaining the susceptibility of malaria vectors to currently available insecticides through controlled use, and the development of novel vector control measures, is crucial (Greenwood *et al*., 2008; WHO, 2011). Novel vector control measures under investigation are sterile insect technique and transgenic techniques. The former technique involves the sterilisation of male mosquitoes via gamma radiation or chemical sterilants (Damodaran *et al*., 2011). The rationale being that since female anopheline mosquitoes mate only once in their lifetime, the release of vast numbers of sterile male mosquitoes would significantly decrease reproduction rates of the mosquito population (Damodaran *et al*., 2011). Transgenic vector control involves the expression of a dominant lethal gene in male mosquitoes which can be passed on to the offspring and cause embryonic death (Damodaran *et al*., 2011).

1.6.2. Vaccine development

Vaccines have the potential to generate long term immunity and thus only one or two doses can impart life-long protection against a particular infection (Chiang *et al*., 2006). Vaccines are also the most affordable health intervention for a variety of infectious diseases (Todryk & Hill, 2007) and the development of a successful malaria vaccine would be a major step towards eradication of the disease. The idea that it may be possible to produce a malaria vaccine is supported by numerous observations such as the fact that immunity to the disease can develop naturally after repeated exposures or through immunisation with irradiated sporozoites (Todryk & Hill, 2007). However, the exact mechanisms by which these immune responses decrease or prevent malaria are still a mystery and humans display remarkable

heterogeneity in their immune responses to the disease (Richie & Saul, 2002). In addition, the various parasite stages express different antigens and thus a vaccine directed against liver-stage parasites will likely have little effect on blood-stage parasites (Todryk & Hill, 2007). These factors make the development of a malaria vaccine quite challenging, but not impossible.

Malaria vaccines currently under development fall into three categories (Richie & Saul, 2002; Todryk & Hill, 2007): (i) pre-erythrocytic vaccines which target the sporozoite and/or liver stages of the parasite thereby preventing progression to blood-stage infection and circumventing all manifestations of the disease, (ii) erythrocytic vaccines that reduce the clinical severity of malaria since they are directed against the asexual blood-stage parasites which cause the clinical manifestations of the disease, and (iii) transmission-blocking vaccines which block development of the sexual stages of the parasite in the mosquito. SPf-66 was the first malaria vaccine developed against *P. falciparum* to be assessed in clinical trials in the 1990s and preliminary results of this pre-erythrocytic vaccine in South America were promising, however, later studies in Africa indicated decreased efficacy (Damodaran *et al*., 2011). To date, there are still no licensed malaria vaccines, however, several are under development, with the RTS,S/AS01 vaccine already undergoing phase III clinical trials and an estimated 20 other projects in phase I or phase II trials (WHO, 2011).

The RTS,S/AS01 vaccine was developed by GlaxoSmithKline and the Program for Appropriate Technology in Health Malaria Vaccine Initiative mainly for use in infants and young children in sub-Saharan Africa (White, 2010; WHO, 2011). It is a pre-erythrocytic vaccine which blocks parasite invasion of liver hepatocytes (Todryk & Hill, 2007). It consists of a hepatitis B surface antigen fused to a recombinant antigen derived from a portion of the malaria parasite‘s circumsporozoite protein (Todryk & Hill, 2007; White, 2010; WHO, 2011). This protein coats the sporozoite stage of the parasite which is injected into the human host by the anopheline mosquito before proceeding to infect the liver hepatocytes (Todryk & Hill, 2007). The adjuvant AS01, which consists of the immunostimulants monophosphoryl lipid A and QS-21, is also included in the vaccine (Richie & Saul, 2002). Preliminary results of phase III clinical trials with RTS,S/AS01 have indicated that during the 12 months post-vaccination, incidences of clinical malaria were reduced by approximately 50% in children aged between 5 and 17 months (WHO, 2011). If the vaccine continues to perform well it may

soon be included as one of the WHO-recommended measures for malaria prevention (WHO, 2011).

1.6.3. Therapeutic and prophylactic drugs

With mosquito vectors developing resistance to insecticides and malaria vaccines still under development, therapeutic and prophylactic drugs are currently the primary means of combating malaria (Dubey *et al*., 2009; Grimberg & Mehlotra, 2011). During the last six to seven decades, since the development of synthetic antimalarials, only a few compounds have been identified as suitable for clinical use and fall into three broad categories known as the quinolines, antifolates and artemisinins (Dubey *et al*., 2009; Grimberg & Mehlotra, 2011).

1.6.3.1. Quinolines

In the early 17th century, Jesuit priests introduced the Peruvian Cinchona bark into Europe as a treatment for periodic malaria-like fevers known as the ague (Meshnick & Dobson, 2001). Thus, quinine and its quinoline derivatives, which occur naturally in the bark of Cinchona trees, were recommended by physicians long before malaria was recognised as a distinct disease (Meshnick & Dobson, 2001). These antimalarials are believed to exert their antimalarial action by targeting the food vacuoles of blood-stage parasites (Dubey *et al*., 2009; Grimberg & Mehlotra, 2011). The quinine derivative, chloroquine, is the most successful, affordable and thus the most extensively used antimalarial since the 1940s, effective for both therapy and prophylaxis (Dubey *et al*., 2009; Grimberg & Mehlotra, 2011; Meshnick & Dobson, 2001). However, the emergence of chloroquine-resistant strains of the malaria parasite has significantly reduced the usefulness of this drug (Dubey *et al*., 2009; Grimberg & Mehlotra, 2011; Meshnick & Dobson, 2001). Mefloquine is a quinoline methanol derivative used as both a therapeutic and prophylactic antimalarial, while primaquine is a therapeutic quinoline antimalarial active against the liver stages of the parasite (Dubey *et al*., 2009). Halofantrine and lumefantrine are structural analogs of quinine used to treat multidrug-resistant *P. falciparum* malaria (Grimberg & Mehlotra, 2011).

1.6.3.2. Antifolates

Unlike the quinolines, the antifolate class of antimalarials is not plant-derived and originated from synthetic compounds generated via knowledge of synthetic medicinal chemistry and cell biology (Ridley, 2002). These antimalarials target nucleic acid synthesis in the parasite and consist of various combinations of dihydropteroate synthase inhibitors (e.g. dapsone and

sulphadoxine) and dihydrofolate reductase enzyme inhibitors (e.g. proguanil and pyrimethamine) (Dubey *et al*., 2009; Grimberg & Mehlotra, 2011). Atovaquone, an inhibitor of the parasite's electron transport chain, is used in combination with proguanil for malaria prophylaxis. As with chloroquine, parasite resistance to the antifolates, particularly the sulphadoxine-pyrimethamine combination, has limited the usefulness of these antimalarials in many malaria endemic regions.

1.6.3.3. Artemisinins

Artemisinins are sesquiterpene lactone endoperoxides derived from the Chinese herb *Artemisia annua* and are the latest and most effective addition to the antimalarial drug arsenal (Klayman, 1985; Liao, 2009). In the 1980s, worldwide attention was drawn to China where artemisinin and its derivatives were being used for the successful treatment of thousands of malaria patients (Liao, 2009). The discovery of artemisinin has since been lauded as a major breakthrough in the fight against malaria (Liao, 2009). This extraordinary class of antimalarials has several advantages over other established antimalarials. The broad range of activity of artemisinins encompasses both sexual and asexual stages of *P. falciparum*, as well as strains which are no longer sensitive to conventional drugs such as chloroquine (White, 2008; Woodrow *et al*., 2005). Efficacy of artemisinins against the sexual stages of the parasite (i.e. gametocytes) reduces the transmission potential of infected individuals (Grimberg & Mehlotra, 2011; WHO, 2010a). A key feature of artemisinin treatment is its prompt onset of action, with parasite numbers in the blood being reduced by a factor of 10000 in each 48-hour cycle of asexual development of the parasite, which is far more efficient than other established antimalarials (van Agtmael *et al*., 1999; WHO, 2010a). Artemisinins are the only established antimalarials to which the parasite has not yet developed widespread resistance and they form the cornerstone of the current antimalarial treatment regimens known as artemisinin-based combination therapies (ACTs) (Grimberg & Mehlotra, 2011).

1.6.3.4. Antibiotics

In 1997, a plastid-like organelle, now known as an apicoplast, was identified in apicomplexan parasites including *P. falciparum* (Kohler *et al*., 1997). The apicoplast is believed to have originated via secondary endosymbiosis of an alga and subsequent preservation of the algal plastid (Kohler *et al*., 1997). It carries out housekeeping processes such as DNA replication, transcription and translation, as well as anabolic processes such as the synthesis of fatty acids,

haem and isoprenoid precursors (Ralph *et al*., 2004). Due to its endosymbiotic origin, the metabolic processes of the apicoplast are prokaryotic in nature and are therefore distinct from those of the eukaryotic host thereby providing ideal drug targets for malaria drug discovery (Ralph *et al*., 2004). Antibiotics which target protein synthesis in the apicoplast, such as clindamycin, tetracycline and doxycycline, have been shown to display slow-acting but effective antimalarial activity (Dahl *et al*., 2006; Goodman *et al*., 2007). These antibiotics are recommended by the WHO as second-line antimalarial treatments to be used in combination with fast-acting counterparts such as quinine and artesunate (WHO, 2010a). Thus far, there have been no reported cases of resistance to this form of antimalarial therapy in malaria-endemic areas (Ekland *et al*., 2011).

1.6.4. The current treatment strategy: combination therapies

Resistance of *P. falciparum* to all conventional antimalarials, particularly chloroquine and sulphadoxine-pyrimethamine, has been a major contributor to the current malaria crisis (WHO, 2010a). Extensive and indiscriminate usage of antimalarials as monotherapies has caused the parasites to develop resistance in response to strong selective pressure (WHO, 2010a). Thus, to circumvent resistance of *P. falciparum* to monotherapies and enhance treatment outcomes, antimalarial combination therapies are currently the preferred form of treatment (WHO, 2010a). Antimalarial combination therapy is the concurrent use of two or more blood schizonticides with different mechanisms of action and thus independent biochemical targets (WHO, 2010a). The rationale being that a drug combination is usually more effective than monotherapies and, in the rare event that the parasite does develop resistance to one of the drugs, it can still be killed by the partner drug (WHO, 2010a).

Artemisinin-based combination therapies are currently recommended by the WHO as the first-line treatment for uncomplicated *P. falciparum* malaria (WHO, 2010a). This treatment strategy uses artemisinin or one of its derivatives (e.g. artesunate, artemether, dihydroartemisinin) in combination with partner drugs such as mefloquine, amodiaquine or lumefantrine (WHO, 2010a). The choice of ACTs used in a particular region depends on the existing level of parasite resistance to the partner drug in that area. The ACTs have been, and continue to be, highly successful against malaria with cure rates comparable to those of chloroquine three decades ago (Malenga *et al*., 2005). An added advantage is that ACTs can diminish gametocyte transmissibility, thereby reducing the risk of infectiousness in individuals taking ACT (Grimberg & Mehlotra, 2011; Malenga *et al*., 2005; WHO, 2010a).

All African countries with extensive *P. falciparum* malaria have replaced older, less effective antimalarials with ACTs as a frontline treatment (WHO, 2009). Over the last decade, ACTs together with other malaria control measures, have played a major role in reducing the number of malaria cases and deaths throughout the world (Dondorp *et al*., 2011).

1.7. MALARIA DRUG DISCOVERY

Successful malaria elimination programmes of the past have all relied on the concurrent use of effective therapeutic drugs and vector control measures, and the development of resistance to one or both of these control measures makes the goal of malaria elimination unachievable (Dondorp *et al*., 2011). A prime example is the Global Malaria Eradication Programme of the 1960s which failed partly as a result of malaria parasites and vectors developing resistance to the respective antimalarial and insecticide used at the time (i.e. chloroquine and DDT) (Mendis *et al*., 2009; Tanner & de Savigny, 2008). Thus, in order for current plans to eliminate malaria to be successful, the long-term availability of effective antimalarials is crucial. Consequently, the ongoing development of novel antimalarials is vital in order to circumvent resistance and ensure a continued supply of reliable malaria therapies.

1.7.1. Emerging resistance to ACTs and the need for novel antimalarials

The ACTs, together with increased mosquito control measures, have made a significant contribution to decreasing the world malaria burden since their introduction in 2001 (WHO, 2011). There is no evidence, thus far, of artemisinin-resistant strains of *P. falciparum* parasites in Africa (Grimberg & Mehlotra, 2011; Kachur *et al*., 2010). However, there are fears that reduced susceptibility to artemisinins in Southeast Asia may lead to resistant strains which would spread to Africa and hamper the success of ACTs (Kachur *et al*., 2010). The south-eastern border of Thailand with Cambodia has long been recognised as the epicentre of drug-resistant malaria (Dondorp *et al*., 2009; Kachur *et al*., 2010; WHO, 2007). It began with chloroquine-resistant malaria several years ago, followed by resistance to sulphadoxine-pyrimethamine and recently, resistance to mefloquine (WHO, 2007). Thai and Cambodian malaria control programmes which routinely monitor these areas and assess therapeutic efficacy have led to the discovery that ACTs are failing on both sides of the border (WHO, 2007). Delayed parasite clearance and abnormally high failure rates have been observed with both artemether-lumefantrine and artesunate-mefloquine combinations (WHO, 2007). In addition, *in vitro* tests have shown that susceptibility to artemisinin derivatives and their partner drugs have diminished in this area (WHO, 2007). The alarmingly high failure rate of

artemisinins in these regions amounts to a local and global emergency due to the fact that these drugs are currently the only established group of antimalarials with significant efficacy against malaria (WHO, 2007). Even though parasite sensitivity to artemisinins appears to be reduced, the clinical and parasitological efficacy of ACTs has not yet been affected (WHO, 2011). Nevertheless, the ever-evolving malaria parasite may develop resistance to either component of the drug combination at any time.

The risk of artemisinin resistance developing in Southeast Asia and spreading to other countries is disturbingly high and Africa, which carries the majority of the world malaria burden and already experiences resistance to other antimalarials, would be particularly susceptible (WHO, 2007). A high prevalence of artemisinin-resistant malaria parasites in Africa would not only halt malaria eradication efforts, but would also cause a large number of childhood deaths, as was the case in the previous century when chloroquine-resistant parasites emerged (Dondorp *et al*., 2011). Hence, while ACTs are making a major contribution to alleviating the malaria burden, there are constant fears that resistance to ACTs may develop in the foreseeable future as well as concerns regarding side-effects during prolonged use (Jambou *et al*., 2005). Malaria research worldwide is therefore constantly focussed on the development of new antimalarials to supplement ACTs, in order to improve efficacy and pharmacokinetic properties and circumvent resistance (Greenwood *et al*., 2008).

1.7.2. Current approaches to malaria drug discovery

There is an urgent need for new antimalarials to supplement the ACTs in order to circumvent resistance and ensure their continued success in the fight against malaria. Ideally, novel antimalarials should be safe, exhibit efficacy that extends to drug-resistant strains, allow for oral administration as well as for parenteral use during coma, cure malaria within 3 days, be suitable for use in young children and pregnant women and, of particular importance, be inexpensive (Na-Bangchang & Karbwang, 2009; Nwaka & Ridley, 2003). It may take more than 5 years to discover a truly new antimalarial drug, however, approaches using existing antimalarials may produce a new drug within 3-5 years (Dubey *et al*., 2009). Six major drug discovery approaches are currently employed in the search for novel, effective malaria treatments.

(i) Re-optimising the use of existing antimalarials

The first means of re-optimising the use of existing antimalarials is drug replacement or rotation. This involves the withdrawal of a drug to which parasites have developed resistance in the hopes that drug-sensitive strains will replace the drug-resistant strains in the majority of the parasite population, thereby restoring the efficacy of the drug in question (Grimberg & Mehlotra, 2011). The rationale behind this approach is that drug-resistant parasite strains will probably be less fit than their wildtype counterparts in the absence of the drug selection (Grimberg & Mehlotra, 2011). This approach was successful with sulphadoxine-pyrimethamine in Peru and with chloroquine in Malawi, however, sensitivity to the respective drugs took 5 and 10 years to return (Grimberg & Mehlotra, 2011; Hobbs & Duffy, 2011). In contrast, the withdrawal of sulphadoxine-pyrimethamine from Venezuela and Cambodia, for 8 and 20 years, respectively, was unsuccessful in restoring sensitivity to this drug (Grimberg & Mehlotra, 2011). An alternative strategy for re-optimising existing antimalarials involves combining drugs. The rationale is that since the activity of antimalarials is usually pleiotropic, drug resistance may be a result of various mechanisms, each open to reversal by drug combination (Grimberg & Mehlotra, 2011). Thus, resistance to a particular drug may be reversed by co-administration with a second drug which chemosensitises the parasite to the first drug (Grimberg & Mehlotra, 2011). For example, chloroquine-resistance in patients was found to be reversed in the presence of chlorpheniramine (Hobbs & Duffy, 2011; Na-Bangchang & Karbwang, 2009; Rosenthal, 2003).

(ii) Repurposing drugs that are presently used to treat other diseases

The rationale behind this approach is that a compound which displays activity against a particular organism may be active against orthologs of its target in another organism, such as the malaria parasite (Rosenthal, 2003). In most developing countries malaria is co-endemic with human immunodeficiency virus (HIV), hence many malaria-infected individuals are exposed to antiretrovirals (Grimberg & Mehlotra, 2011; Skinner-Adams *et al*., 2004). This prompted Skinner-Adams *et al*. (2004) to investigate the *in vitro* effects of antiretroviral protease inhibitors on *P. falciparum*, and ritonavir, saquinavir and indinavir were found to be highly effective antimalarials. However, the high cost, potential toxicity and limited knowledge with regard to interactions with established antimalarial drugs means that these antiretrovirals are unlikely to become first-line antimalarials in the near future (Skinner-Adams *et al*., 2004). Anticancer drugs are also under investigation as potential antimalarials. For example, the anticancer agents, SU-11274 and Bay 43-9006, have shown potent activity

against *P. falciparum* (Grimberg & Mehlotra, 2011). As with the antiretrovirals, the major limitation in using anticancer drugs as antimalarials is their high level of toxicity (Grimberg & Mehlotra, 2011).

(iii) Chemical modification of existing antimalarial compounds

Derivatisation is a widely used strategy in drug discovery and involves chemical alteration of an existing drug in the hope of preserving or increasing potency whilst simultaneously ameliorating adverse traits of the parent compound (Na-Bangchang & Karbwang, 2009; Rosenthal, 2003). This strategy has produced several of the currently available antimalarials, such as mefloquine and chloroquine which are derivatives of quinine (Na-Bangchang & Karbwang, 2009; Rosenthal, 2003). Another route for producing new antimalarials from existing compounds is through hybridisation which involves the covalent binding of two compounds (Rosenthal, 2003). These hybrid molecules have the potential to postpone or circumvent the development of resistance and lower the risk of drug-drug interaction, the latter of which is often seen with combination therapies (Rosenthal, 2003). An example of a potent hybrid antimalarial is the artemisinin-quinine hybrid which showed potent *in vitro* activity against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* (Rosenthal, 2003). The activity was greater than that of the individual drugs alone or a 1:1 combination of the drugs.

(iv) Exploring natural products

Historically, antimalarial drug discovery efforts have greatly benefited from investigating natural compounds and the most successful antimalarials to date, chloroquine and artemisinin, are plant-derived (Grimberg & Mehlotra, 2011; Rosenthal, 2003). This approach uses indigenous knowledge of medicinal plants to identify a plant product with clinical activity against malaria which can then be used as a starting point for a medicinal chemistry effort (Rosenthal, 2003). However, a disadvantage of this approach is that the mechanism of action of plant-derived antimalarials is usually unknown (Grimberg & Mehlotra, 2011). Additional challenges include screening procedures, species selection criteria and fractionation processes (Grimberg & Mehlotra, 2011). Nonetheless, it is hoped that once the activity of a natural compound in humans is characterised, it can be used to identify novel molecular scaffolds upon which the next generation of antimalarials can be based (Grimberg & Mehlotra, 2011).

(v) High-throughput screening of diverse chemical libraries

Recent years have seen numerous drug-screening groups producing a plethora of compounds which may provide novel starting points for potential antimalarials with new modes of action (Grimberg & Mehlotra, 2011). Consequently, a powerful approach used currently in malaria drug discovery is the large-scale screening of these diverse chemical libraries using high-throughput assays (Grimberg & Mehlotra, 2011; Hobbs & Duffy, 2011). For example, Plouffe *et al*. (2008) used a high-throughput cell-based method to screen ~1.7 million compounds and identified ~6000 small molecules with strong antimalarial activity. Gamo *et al*. (2010) used a similar approach to screen ~2 million compounds from GlaxoSmithKline's chemical library and found that ~13500 of these displayed antimalarial activity. Virtual screening is an *in silico*, speedier approach for screening these libraries (Grimberg & Mehlotra, 2011). It involves the rapid evaluation of large libraries of chemical structures in order to identify those which are most likely to bind a pre-selected drug target, typically an enzyme or protein receptor (Grimberg & Mehlotra, 2011). This approach, either on its own or together with high-throughput screening, is becoming more popular in malaria drug discovery (Grimberg & Mehlotra, 2011).

(vi) Parasite genome-based discoveries

The entire genomes of the prevalent malaria parasites *P. vivax* and *P. falciparum*, and the human host are now known (Grimberg & Mehlotra, 2011). This has allowed for the comparison of the *Plasmodium* and human genomes, in an effort to identify novel, parasite-specific molecular targets for malaria therapy and prevention (Grimberg & Mehlotra, 2011; Na-Bangchang & Karbwang, 2009). Ongoing drug discovery projects at the Medicines for Malaria Venture, using this genome-based approach, have identified several molecular targets in *P. falciparum* including falcipains, kinases and dihydrofolate reductase (Grimberg & Mehlotra, 2011).

1.7.3. Current methods used to screen candidate antimalarials

The efficacy of candidate antimalarial compounds relies heavily on their capacity to kill malaria parasites, thereby preventing their proliferation and allowing the human immune system to completely clear dead and damaged parasites from the bloodstream (Noedl *et al*., 2003). Conventional antimalarial susceptibility assays measure the effect of candidate compounds on the growth and development of the malaria parasite in *in vitro* cultures (Noedl *et al*., 2003). In most assays the principle is the same – the measurement of parasite growth in

drug-exposed cultures relative to a drug-free control (Noedl *et al*., 2003). However, the assays differ in the procedure used to quantify parasite growth and this is achieved by various approaches including microscopy-, isotopic-, enzymatic-, ELISA- and fluorescence-based methods (Co *et al*., 2010; Kalra *et al*., 2006; Noedl *et al*., 2003).

The Giemsa-stained slide technique is an affordable means of assessing the antimalarial activity of a modest number of candidate drugs (Kalra *et al*., 2006). It involves the measurement of parasitaemia (i.e. percentage of infected RBCs) by light microscopy in Giemsa-stained thin-blood smears prepared from control and drug-treated *in vitro* cultures (Kalra *et al*., 2006). While the method is affordable and simple to perform, it is also time-consuming and highly subjective since one has to distinguish between viable and non-viable parasites and the parasite undergoes several morphological changes during its intra-erythrocytic development (Noedl *et al*., 2003). The isotopic ^{3}H-hypoxanthine assay indirectly measures parasite growth by measuring the uptake of tritium-labelled hypoxanthine which is used by the parasite for purine salvage and DNA synthesis (Kalra *et al*., 2006). This is one of the most commonly used assays for antimalarial susceptibility testing, however, it is expensive, complex and relies on the use of radioactive material (Kalra *et al*., 2006; Noedl *et al*., 2003).

Another popular assay is the colorimetric, enzymatic plasmodial lactate dehydrogenase (pLDH) assay which is based on the LDH-catalysed conversion of lactate to pyruvate (Kalra *et al*., 2006; Noedl *et al*., 2003). The assay relies on the knowledge that pLDH activity is distinguishable from LDH activity of the host RBC because the parasite enzyme is capable of utilising the cofactor APAD (3-acetylpyridine adenine nucleotide), while the human enzyme lacks this capability (Kalra *et al*., 2006; Noedl *et al*., 2003). The reaction produces reduced APAD which converts nitro blue tetrazolium to a purple formazan product which is spectrophotometrically measured (Noedl *et al*., 2003). The pLDH assay has several advantages over the ^{3}H-hypoxanthine assay in that it is non-radioactive, simpler to perform and relies on equipment and reagents that are relatively inexpensive (Co *et al*., 2010).

Rapid techniques using antibody-dependent ELISAs (enzyme-linked immunosorbent assays) for the quantification of malaria parasites in blood are also available and have been adapted for use in antimalarial drug susceptibility evaluations (Co *et al*., 2010). One such assay assesses parasite growth by measuring the levels of plasmodial histidine-rich protein 2 (Co *et*

al., 2010; Noedl *et al*., 2003). These assays are straightforward, highly sensitive and relatively inexpensive, however, they require long incubation times and multiple washing steps (Co *et al*., 2010; Noedl *et al*., 2003). Fluorescence-based assays exploit the fact that mature host RBCs, which are infected by the malaria parasite, lack DNA and RNA (Co *et al*., 2010). Thus, parasite growth can be measured by using fluorescent nucleic acid intercalating stains (e.g. PicoGreen, SYBR green I, YOYO-1) together with flow cytometry to identify parasite-infected RBCs (Co *et al*., 2010). Fluorescence-based assays are fairly straightforward, highly sensitive and reliable, and provide a precise measurement of parasitaemia based on each cell rather than the population measurement which is provided by isotopic assays (Co *et al*., 2010).

1.7.4. A novel assay for malaria drug discovery

Apart from safety, and efficacy against drug-resistant *P. falciparum* malaria, another key characteristic recommended for novel antimalarials is that they be rapid-acting (Na-Bangchang & Karbwang, 2009; Nwaka & Ridley, 2003). According to the Medicines for Malaria Venture, the gold standard for the treatment of acute uncomplicated malaria should be a radical cure which can be administered as a single dose or, at most, a three-day regimen (MMV, 2010). The rationale behind this recommendation is that minimum-dose regimens which rapidly clear parasites from the bloodstream and relieve symptoms will improve patient compliance and reduce costs (MMV, 2010). This is especially important in impoverished areas that carry the brunt of the malaria burden and have limited access to clinical treatment and supervision. Furthermore, rapid cure rates reduce the risk of drug resistance emerging and are also essential in cases of severe malaria, particularly cerebral malaria, where prompt treatment is critical to avoid death (WHO, 2010a). The importance of rapid-acting antimalarials is further illustrated by the major contribution of ACTs to reducing the world malaria burden which has been largely attributed to the rapid-acting nature of the artemisinin component (Balint, 2001; van Agtmael *et al*., 1999). Hence, it is highly preferable that novel antimalarials be rapid-acting and thus assessing the rate of activity of candidate compounds is crucial to current drug discovery efforts. Although current assays used to screen candidate antimalarials (as discussed in section 1.7.3) give a good approximation of antimalarial activity of test compounds, they do not reveal an important additional property of candidate antimalarials – the rate at which the compounds compromise parasite viability.

Traditionally, this rate has been assessed by using light microscopy to observe parasite morphological changes in Giemsa-stained thin-blood smears prepared from *in vitro* cultures at various time-points following drug treatment (Noedl *et al*., 2003). At each time-point, the fraction of parasitised red blood cells is quantified and the parasites are examined for signs of aberrant morphology. While this technique is relatively simple to perform, it is time-consuming and prone to subjective inter-operator interpretation since one has to distinguish between viable and non-viable parasites (Otten-Kuipers *et al*., 1995; Sanz *et al*., 2012). Making this distinction is particularly challenging due to the heterogeneous morphology of individual parasites under routine culture conditions, as well as the numerous morphological changes which the blood-stage parasite undergoes during its complex life cycle, as illustrated in Figure 1.3.

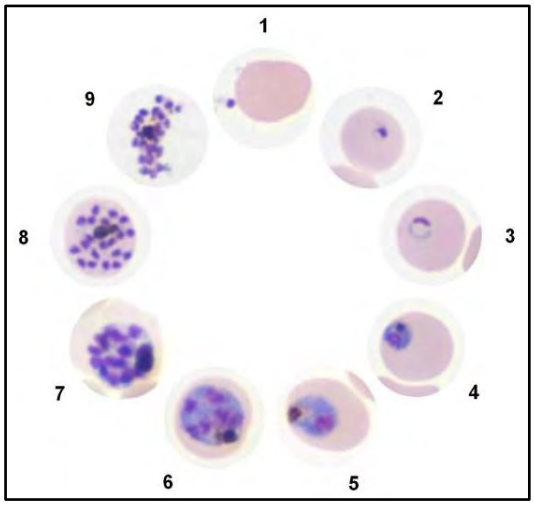

Figure 1.3: Micrographs of the various developmental stages of blood-stage *P. falciparum* parasites. Giemsa-stained thin-blood smears were prepared from an *in vitro* culture over 48 hours and photographed at 1000x magnification. Sequential parasite developmental stages are presented as numbered from 1-8: 1 – merozoite invasion of a RBC, 2 – newly invaded RBC, 3 – ring, 4 – early trophozoite, 5 – late trophozoite, 6 – early schizont, 7 – schizont, 8 – mature schizont, 9 – ruptured schizont. Note: The figure is based on an image by Tan (2008), but is compiled from own micrographs.

Another challenge is the practical difficulty of preparing uniform microscopy preparations on separate occasions for morphologic examination. As a result, assessing the rate of antimalarial action by this method is highly subjective and virtually impossible to quantitate with certainty, unless the drug-induced morphological anomalies are drastic and consistent. Quantitative procedures for assessing the rate of activity of antimalarials in *in vitro* cultures, besides subjective interpretation of morphology by light microscopy, are limited to a recently

reported viability-based assay (Sanz *et al*., 2012). This assay determines the rate of antimalarial drug action in *in vitro* cultures by directly quantifying the number of parasites capable of recovering from distinct periods of drug exposure. *In vivo* killing rates are subsequently estimated by calculating the parasite reduction ratio which is the ratio between parasitaemia at the onset of drug exposure and after one complete asexual parasite life cycle. Unfortunately, this method requires 3-4 weeks of monitoring re-growth in culture after drug exposure. Hence, there is a need for a novel, quantitative assay capable of rapidly evaluating the kinetics of drug action in culture, in order to more accurately compare the efficacy of novel antimalarials with those of existing drugs. The goal of this study was to develop such an assay.

Energy is essential for the growth, maintenance and reproduction of all living organisms and the energy currency of cells is adenosine triphosphate (ATP) (Ginsburg, 2010). Chemical reactions convert energy from one form to another at the cellular level and the energy stored in chemical bonds is released as ATP when complex molecules, such as glucose, are degraded (Ginsburg, 2010). Hence, it is conceivable that elevated ATP levels in the malaria parasite may reflect increased metabolic activity to circumvent harmful drug effects, or an inhibition of ATP-dependent anabolic pathways. Alternatively, reduced ATP may indicate a significant decline in parasite energy metabolism and viability. There are two major methods for measuring ATP in biological samples, HPLC and the bioluminescent luciferin-luciferase system, with the latter being more popular (Abraham *et al*., 2003; Bruce, 2010). Previous studies have used commercially available kits based on the luciferin-luciferase assay system for ATP measurement in cultured *P. falciparum* malaria parasites (Kanaani & Ginsburg, 1989; Peatey *et al*., 2012). Accordingly, it is plausible that the rate of antimalarial drug action may be evaluated by measuring parasite ATP levels in drug-treated cultures relative to control cultures using this bioluminescent system. Hence, this study has explored the use of the CellTiter-Glo luciferin-luciferase system (Promega) for ATP detection in cultured *P. falciparum* parasites and subsequent application of this method as a means of assessing the rate of antimalarial drug action.

Serendipitously, a potential additional methodology for determining the rate at which compounds affect parasites was discovered during research conducted by the Systems Biology Group at CSIR Biosciences. Dr. A. C. van Brummelen successfully established methods for transfecting *P. falciparum* with expression plasmids and used these to generate

transgenic parasites that express luciferase. Luciferase activity in the parasites is readily detected using a luminescence-based kit (Bright-Glo Luciferase Assay System, Promega), and for the purposes of this study this procedure has been termed the Luciferase assay. Unexpectedly, preliminary experiments indicated that luciferase activity decreased rapidly and markedly in transgenic parasites exposed to the standard antimalarials artemisinin and mefloquine. Conceivably, measurement of luciferase activity in these transgenic parasites may thus be used to determine the rate of action of antimalarials.

Thus, the aims of this study were as follows:

a) develop and optimise an *in vitro P. falciparum* ATP assay
b) gauge the utility of a *P. falciparum* ATP assay, and a Luciferase assay using transgenic parasites, as means of assessing the rate of antimalarial drug action

CHAPTER 2: DEVELOPMENT AND OPTIMISATION OF A *PLASMODIUM FALCIPARUM* ATP ASSAY

2.1. INTRODUCTION

Apart from safety and efficacy, another essential characteristic of novel antimalarials is that they be rapid-acting in order to improve patient compliance, reduce costs and lower the risk of drug resistance emerging (MMV, 2010; Na-Bangchang & Karbwang, 2009; Nwaka & Ridley, 2003). Consequently, assessing the rate of activity of candidate compounds is crucial to current drug discovery efforts. Traditional determination of this rate by morphological assessments is flawed, principally due to heterogeneous morphology of individual parasites under routine culture conditions which results in highly subjective, operator-specific interpretations (Otten-Kuipers *et al*., 1995; Sanz *et al*., 2012). Hence, there is a need for a novel, quantitative assay for determining the kinetics of drug action in culture and the goal of this study was to develop such an assay.

Adenosine triphosphate (ATP) serves as a primary source of energy for the growth, maintenance and reproduction of all living organisms (Ginsburg, 2010). Thus, it is plausible that elevated ATP levels in the malaria parasite may reflect increased metabolic activity to circumvent detrimental drug effects, while decreased ATP may indicate a significant decline in parasite energy metabolism and viability. The luciferin-luciferase system is a rapid and reliable method commonly used for measuring ATP in biological samples (Abraham *et al*., 2003; Bruce, 2010). The system is based on ATP-dependent luciferin luminescence catalysed by firefly luciferase as outlined below (Manfredi *et al*., 2002).

$$Luciferase + luciferin + ATP \rightarrow luciferase\text{-}luciferyl\text{-}AMP + PPi$$
$$Luciferase\text{-}luciferyl\text{-}AMP + O_2 \rightarrow luciferase + oxyluciferin + AMP + CO_2 + LIGHT$$

Commercially available kits based on the luciferin-luciferase system are widely used for ATP quantification in cultured mammalian and bacterial cells (e.g. CellTiter-Glo Luminescent Cell Viability Assay, Promega; BacTiter-Glo Microbial Cell Viability Assay, Promega). Previous studies have also applied this ATP-based bioluminescent assay system to measure ATP levels in asexual (Kanaani & Ginsburg, 1989) and sexual stages (Peatey *et al*., 2012) of cultured *P. falciparum* parasites. Accordingly, it is conceivable that the rate of activity of antimalarial

drugs may be assessed by quantifying parasite ATP in drug-treated cultures relative to control cultures.

Hence, this study has explored the use of the bioluminescent CellTiter-Glo kit (Promega) for ATP quantification in cultured *P. falciparum* parasites and subsequent application of this method as a means of assessing the rate of antimalarial drug action. The kit is highly sensitive, detecting as few as 4-15 cells which would be advantageous for measuring parasite ATP in samples of malaria cultures which contain a fairly minimal parasite biomass. In addition, the homogeneous "add-mix-measure" format of the kit allows for rapid generation of a luminescent signal which is proportional to the amount of ATP present and has a half-life of more than five hours. With this in mind, a preliminary method was developed which allows the measurement of parasite ATP levels in control and drug-treated *P. falciparum* cultures using the CellTiter-Glo kit (Prof. H.C. Hoppe, CSIR Biosciences). The method requires isolation of intact parasites from host RBCs in order to remove of all traces of RBC ATP which would otherwise obscure the measurement of parasite-associated ATP. This chapter describes the optimisation of this isolation step and various other aspects of the experimental protocol. Additionally, application of the optimised assay for determining the extent to which parasite ATP levels vary as a function of parasite developmental stages is described.

2.2. MATERIALS AND METHODS

2.2.1. Cultivation of *P. falciparum* parasites

2.2.1.1. Cell culture

Plasmodium falciparum 3D7 parasites were cultured in human RBCs using a method adapted from Trager and Jensen (1976). Cultures were maintained in complete malaria culture medium (CMCM; Appendix 1.1) at a haematocrit (percentage of RBCs in the culture medium) of approximately 3%. Culture flasks were suffused with a special gas mixture (5% CO_2, 5% O_2, balance N_2; Air Products, RSA), sealed and incubated at 37°C. Medium changes were carried out daily under aseptic conditions. The culture was transferred to a sterile 50 mL conical tube and centrifuged at 2000 rpm for 5 minutes before aspirating off the spent medium. Before adding fresh CMCM, a thin-blood smear was prepared from the infected RBC pellet. The smear was fixed with methanol and stained with freshly prepared Giemsa stain (Appendix 1.2). Parasite morphology was assessed by viewing the smear by light microscopy using a 100x oil-immersion objective. During its 48-hour intra-erythrocytic life-cycle, ring-stage parasites are present for the first 20-24 hours and develop into trophozoite- and schizont-stage parasites during the remaining 24 hours. Thus, a typical culture alternates between ring- and trophozoite/schizont-stage parasites every 24 hours. Aliquots of ring-stage cultures were cryopreserved every 2-3 months (see section 2.2.1.3 below). The percentage of parasitised RBCs (parasitaemia) in the culture was maintained at approximately 5% by replacing 60-80% of the infected RBCs with fresh, uninfected RBCs during the trophozoite/schizont-stage. The parasitaemia was determined by counting infected and uninfected RBCs in 10-15 fields of vision. Fresh blood was obtained from healthy volunteer donors in EDTA vacutainer tubes every 3-4 weeks. The RBC pellet was washed twice in CMCM before storing at a 50% haematocrit at 4°C. Synchrony of *P. falciparum* cultures was maintained by sorbitol treatment and/or *P. falciparum* enrichment (see section 2.2.1.2 below).

2.2.1.2. Synchronisation of cultures

2.2.1.2.1. Sorbitol treatment

The sorbitol method of synchronisation (Lambros & Vanderberg, 1979) lyses late trophozoite- and schizont-stage parasites. The method was applied to mixed-stage cultures of primarily ring-stage parasites. Firstly, the infected RBCs were pelleted from the culture by centrifugation at 2000 rpm for 5 minutes. The pellet was then resuspended in a 5% sorbitol solution (Appendix 1.3) pre-warmed at 37°C. The volume of sorbitol solution used was 10-20

times that of the RBC pellet. The suspension was incubated at 37°C for 10-15 minutes and then centrifuged at 2000 rpm for 5 minutes. The supernatant was aspirated off and the pellet was resuspended in fresh CMCM (Appendix 1.1) and returned to the culture flask. The flask was gassed (5% CO_2, 5% O_2, balance N_2; Air Products, RSA), sealed and incubated at 37°C.

2.2.1.2.2. *P. falciparum* enrichment

Synchronisation by *P. falciparum* enrichment was performed according to a method adapted from Glushakova *et al*. (2007). Briefly, infected RBCs from a primarily schizont-stage culture were pelleted by centrifugation at 2000 rpm for 5 minutes under sterile conditions. The packed RBCs (300 µL) were then pipetted over 1 mL of freshly prepared 60% Percoll solution (Appendix 1.4) in sterile microfuge tubes. Each tube was centrifuged at 10000 rpm for 20 minutes with slow deceleration. This resulted in the mixture separating into two layers: uninfected and ring-infected RBCs were pelleted at the bottom of the tube and enriched trophozoite/schizont-infected RBCs were located as an upper brown layer on top of the Percoll. The upper layer was transferred to a sterile 50 mL conical tube under aseptic conditions. A slide with coverslip was prepared from the suspension and viewed by light microscope to determine if the enrichment was successful. The unstained trophozoite/schizont-infected RBCs were easily identified by the haemozoin crystals of the parasite food vacuoles. Complete malaria culture medium (10 mL; Appendix 1.1) pre-warmed at 37°C was added dropwise to the enriched RBCs while constantly swirling. The suspension was centrifuged at 2000 rpm for 5 minutes and the supernatant was aspirated off. The enriched RBCs were washed once more in 10 mL of CMCM before adding fresh RBCs in a ~1:2 ratio and resuspending the cells in CMCM at a 1% haematocrit. The suspension was transferred to a new culture flask, gassed (5% CO_2, 5% O_2, balance N_2; Air Products, RSA), sealed and incubated at 37°C for ~4 hours. Transmission of infection was then ceased by destruction of the residual trophozoite and schizont stages by sorbitol treatment (detailed in section 2.2.1.2.1).

2.2.1.3. Cryopreservation of cultures

Cultures were cryopreserved at the ring-stage of parasite development at a parasitaemia of at least 5%, using a method adapted from de Ridder (2003). Briefly, the infected RBCs were pelleted from the culture by centrifugation at 2000 rpm for 5 minutes and the supernatant was aspirated off. Ice-cold freezing solution (Appendix 1.5) was added to the infected RBCs in a 1:1 ratio by adding one drop every 10 seconds while constantly swirling. Thorough mixing

was obtained by gently pipetting up-and-down followed by transfer to a cryotube and storage at -80°C.

2.2.1.4. Thawing of cultures

Cultures were thawed according to the method described by de Ridder (2003). Thawing was achieved as quickly as possible by placing the cryotube**,** containing the frozen parasitised RBC suspension, in a water bath at 37˚C for approximately 2 minutes. The infected RBC suspension was transferred, under aseptic conditions, to a sterile 50 mL conical tube before adding the thawing solutions which were pre-warmed at 37°C. Thawing solution A (Appendix 1.6), at 0.2x the volume of the infected RBC suspension, was added dropwise while constantly swirling. Thawing solution B (Appendix 1.7), at 1.8x the volume of the infected RBC suspension, was added in the same manner. The solution was centrifuged at 2500 rpm for 5 minutes and the supernatant was aspirated off. Fresh, uninfected RBCs were added to the infected RBC pellet in a 1:1 ratio. The pellet was resuspended in 10 mL of CMCM (Appendix 1.1) pre-warmed at 37°C and transferred to a T25 culture flask. The flask was gassed (5% CO_2, 5% O_2, balance N_2; Air Products, RSA), sealed and incubated at 37°C.

2.2.2. ATP assay: the pilot assay

Phosphate-buffered saline (PBS), artemisinin and chloroquine were purchased from Sigma-Aldrich (Steinheim, Germany). The experimental pyrimidine endoperoxide, compound 4070, was kindly donated by Dr. C. J. Parkinson (CSIR, Biosciences). The CellTiter-Glo Luminescent Cell Viability Assay was purchased from Promega (Madison, WI).

Plasmodium falciparum 3D7 parasites were cultured, as described in section 2.2.1 above, to a parasitaemia of at least 15%. An early trophozoite-stage culture was used to prepare parasite suspensions, adjusted to 5% haematocrit and 10% parasitaemia. The suspensions were prepared in the following solutions: (i) CMCM (Appendix 1.1) – untreated control; (ii) PBS – positive killing control; (iii) 100 nM artemisinin in CMCM; (iv) 100 nM chloroquine in CMCM; (v) 100 nM compound 4070 in CMCM. Each suspension was transferred to a T25 flask, gassed and incubated for 8 and 12 hours. At each time-point, 3 x 1 mL samples were removed from each flask, transferred to a microfuge tube and put on ice. Each sample was centrifuged at 8000 rpm for 30 seconds and the supernatant was aspirated off. The RBCs were lysed by resuspending the pellet in 500 µL of ice-cold 0.1% saponin lysis solution (Appendix 2.3) and vortexing the mixture until translucent. The lysate was centrifuged at

8000 rpm for 30 seconds and the supernatant was aspirated off. The isolated parasites were washed twice in 500 µL of ice-cold 0.1% BSA in PBS (Appendix 2.1). Ice-cold PBS (100 µL) was added and the pellet was resuspended by pipetting up-and-down. The parasite sample was snap-frozen in liquid nitrogen and stored frozen. The processed samples were thawed at room temperature and 50 µL of each sample was transferred to a white-walled, clear-bottomed 96-well plate (Nunc, Germany). For determination of parasite-associated ATP, an equal volume (50 µL) of pre-prepared CellTiter-Glo reagent (Appendix 2.4) was added to each well. The plate was incubated in the dark for 30 minutes at room temperature and luminescence measured in a multimode plate reader (Infinite F500, Tecan, Austria).

2.2.3. ATP assay: the optimised assay

Plasmodium falciparum 3D7 parasites were cultivated as described in section 2.2.1 above. Parasite suspensions were prepared in CMCM (Appendix 1.1) at a 5% haematocrit and parasite concentration $\leq 2x10^6$ per sample. Suspensions of uninfected RBCs were prepared at a 5% haematocrit to serve as background controls. Samples (500 µL) of the suspensions were transferred to microfuge tubes and maintained on ice. The RBCs were pelleted by centrifuging at 8000 rpm for 30 seconds before adding 500 µL of 0.24% saponin lysis solution (Appendix 2.5). Complete RBC lysis was achieved by vortexing the solution for approximately 45 seconds until translucent. The lysate was pipetted over 300 µL of phthalate oil-mix (Quashie *et al*., 2010; Appendix 2.6) in a microfuge tube and centrifuged at 14000 rpm for 40 seconds. This resulted in the isolated parasites pelleting below the oil and the remaining aqueous lysate settling above the oil. The aqueous layer was aspirated off and the inner surface of the tube above the oil layer was washed with 500 µL of 0.1% BSA in PBS (Appendix 2.1). The oil layer was aspirated off and 150 µL of ice-cold PBS was pipetted over the parasite pellet without resuspending the pellet. The sample was snap-frozen in liquid nitrogen and stored at -20°C. Processed samples were thawed at room temperature and the parasite pellet was resuspended by pipetting up-and-down before transferring 50 µL to duplicate wells of a white Lumitrac 96-well plate (Greiner, Germany). Samples of ice-cold PBS (50 µL) were included as negative controls. For detection of ATP in the samples, 50 µL of pre-prepared CellTiter-Glo reagent (Appendix 2.4) was added to each well. The plate was briefly agitated and then incubated in the dark for 10 minutes at room temperature before measuring luminescence in a multimode plate reader (Infinite F500, Tecan, Austria).

2.2.4. Data analysis

Results were expressed as means ± SD from triplicate samples, unless otherwise stated. Assay precision was determined by calculating coefficients of variation (CVs) and Z‘ factors using the following equations: (i) $Z' = 1 - 3*((SD_{sample} + SD_{negative/background\ control})/(Mean_{sample} - Mean_{negative/background\ control}))$; (ii) $CV = (SD/Mean)*100$.

2.3. RESULTS

2.3.1. The pilot ATP assay

In order to explore parasite ATP levels as a means of assessing drug-induced parasite stress, a pilot assay was developed (Prof. H.C. Hoppe, CSIR Pharmacology) to measure parasite-associated ATP in *P. falciparum* cultures using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). Trophozoite-stage parasite cultures were prepared at a haematocrit (percentage of RBCs in the culture medium) and parasitaemia (percentage of parasitised RBCs) of 5% and 10%, respectively. The cultures were incubated for 8 and 12 hours in PBS, culture medium or in culture medium containing 100 nM of artemisinin, chloroquine or the experimental pyrimidine endoperoxide, compound 4070. (Note: Effective treatment concentrations were based on median inhibition concentrations **(**IC50s) and/or test concentrations both from the current study and previous reports (detailed in Table 5.1, Appendix 5). Samples (1 mL) of the cultures were taken at each time-point and the infected RBCs were pelleted by centrifugation. The parasites were released from RBCs by saponin treatment and RBC cytoplasm and debris were removed by five microfuge washing steps using 0.1% BSA in PBS. The intact parasites were resuspended in PBS and transferred to a development plate before ATP was measured using the CellTiter-Glo reagent (Figure 2.1; details in section 2.2.2).

In control parasites, ATP levels increased by 231% between the 8 and 12 hour time-points (Figure 2.1b). In contrast, parasites exposed to PBS showed an 88% and 98% decline in ATP levels relative to controls after 8 and 12 hours, respectively (Figure 2.1a). PBS lacks glucose normally found in culture medium and thus presumably blocks ATP synthesis by glycolysis. The chloroquine treatment caused a 139% and 113% increase in ATP levels relative to controls after 8 and 12 hours of incubation, respectively. In comparison to controls, ATP levels of artemisinin-treated parasites increased by 114% and 40% after 8 and 12 hours of incubation, respectively. Conversely, compound 4070 caused a 22% and 33% decrease in ATP levels relative to controls after 8 and 12 hours, respectively.

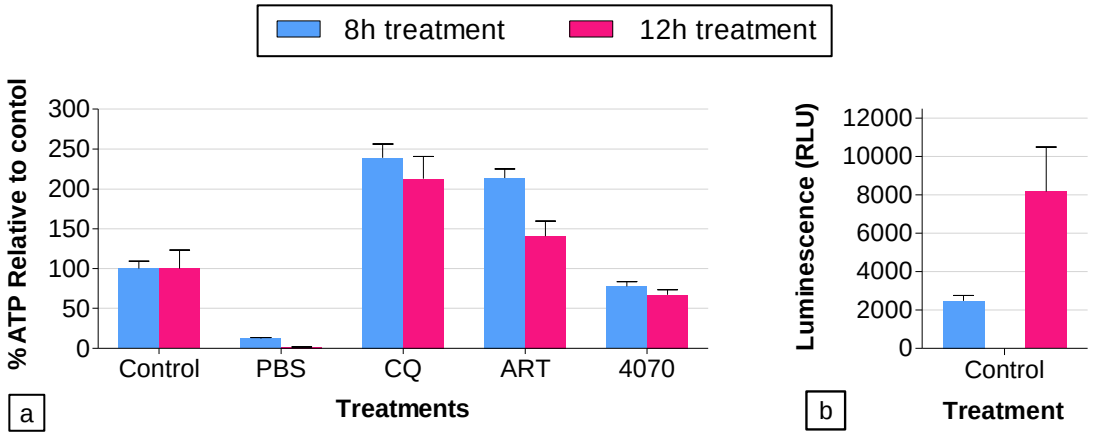

Figure 2.1: The pilot ATP assay. Parasite-associated ATP in *P. falciparum* cultures following an 8 and 12 hour incubation in PBS, culture medium (Control) or in culture medium containing 100 nM of chloroquine (CQ), artemisinin (ART) or compound 4070. Results are presented as a percentage relative to the control (Fig. 2.1a) or as absolute values measured as relative light units (RLU) (Fig. 2.1b). Each data point represents mean ± SD, n = 3.

Thus, the pilot assay showed that parasite ATP levels in *P. falciparum* cultures can be readily detected using a commercial luminescence assay, as demonstrated by the large difference in signal between control parasites and those incubated in PBS. In addition, the very significant alteration in signal during drug treatment suggests that ATP levels may represent a potential diagnostic measure of drug-induced parasite stress. This motivated the pursuit of the primary aim of this thesis, i.e. to develop a *P. falciparum* ATP assay and to explore its utility for assessing the kinetics of drug action in culture. The first step to achieving this goal was to produce an optimal protocol for measuring malaria parasite ATP by refining various aspects of the pilot assay, as described in the following sections.

2.3.2. Using a phthalate oil-mix to speed up washing and improve yields of isolated parasites

In the pilot experiment, saponin lysis of RBCs was followed by five microfuge washing steps to separate intact parasites from RBC cytoplasm (detailed in section 2.2.2). This is necessitated by the presence of ATP in normal and infected RBCs which would otherwise obscure the quantification of parasite-associated ATP. This washing process was found to be both time-consuming and impractical due to the numerous precise liquid handling and centrifugation steps. When preparing the parasite pellets, it is likely important to work quickly to prevent changes in parasite ATP levels during the pellet preparation. An additional concern was the potential for parasite lysis during the multiple centrifugation steps which, in

retrospect, may have been assessed by microscopic examination of the supernatant for signs of parasite rupture (e.g. free haemozoin crystals). Therefore, with the intention of speeding up isolation of intact parasites from RBC lysates and improving yield of intact parasites, the use of a phthalate oil-mix, as described by Quashie *et al*. (2010), was explored.

Briefly, four samples of parasite suspension were prepared at a 5% haematocrit from a trophozoite-stage *P. falciparum* culture. The volume and parasitaemia of each sample was 500 µL and 2% respectively, as opposed to the 1 mL, 10% parasitaemia samples used in the pilot assay. This amendment to the procedure allowed for more experiments to be done from a single parent culture. Two samples were processed as described in the pilot assay by removing RBC lysate through centrifugation washing steps. The remaining two samples were processed in the same manner with the exception that intact parasites were isolated from the RBC debris by centrifuging the RBC lysate through a phthalate oil-mix (dibutyl phthalate: dioctyl phthalate; 5:4) at 14000 rpm for 40 seconds. This resulted in the intact parasites pelleting below the oil and the remaining aqueous lysate, which included RBC cytoplasm and debris, settling above the oil – as illustrated in Figure 2.2 below.

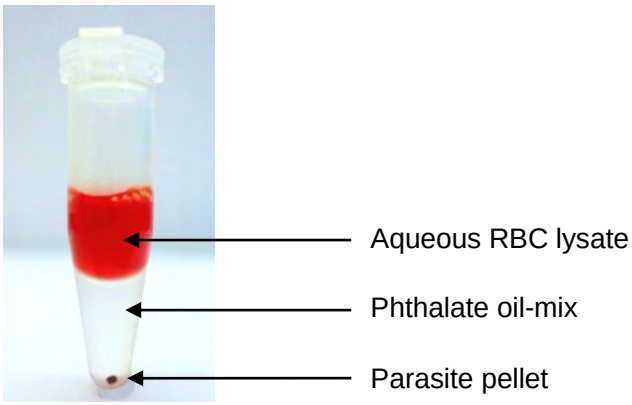

Figure 2.2: Photograph of a sample after centrifugation of the RBC lysate through the phthalate oil-mix. In order to measure parasite-associated ATP in *P. falciparum* culture samples, parasites were released from RBCs by saponin treatment. The intact parasites were isolated from RBC debris by centrifugation of the lysate through a phthalate oil-mix, as described by Quashie *et al*. (2010). This resulted in the intact parasites pelleting below the oil and the remaining aqueous RBC lysate settling above the oil.

The pellet was resuspended in PBS and parasite-associated ATP was measured using the CellTiter-Glo reagent, with culture medium as a negative control (detailed in section 2.2.2). Results are shown in Figure 2.3. Parasites isolated from the RBC lysate via the phthalate oil-

mix produced an average luminescence of 18878 RLU, while parasites isolated by microfuge washing in 0.1% BSA in PBS generated an average luminescence of 3897 RLU. When samples were compared to negative controls (culture medium), average Z‘ factors (indication of dynamic range and signal robustness – detailed in section 2.3.5) of 0.88 and 0.95 were obtained for the oil-mix and PBS wash samples, respectively. This suggests that 500 µL samples of malaria cultures at a haematocrit and parasitaemia of 5% and 2%, respectively, comfortably contain sufficient parasites to produce a quantifiable ATP signal which can be confidently distinguished from negative controls. In addition, ATP levels of parasites isolated via the phthalate oil-mix were approximately 5-fold greater than those of parasites isolated by microfuge washing, possibly due to excessive parasite rupture and non-specific adhesion of parasites to microfuge tube walls during repeated pelleting. Furthermore, use of the phthalate oil-mix was found to be far less time-consuming than repeated washing of the parasite pellet in 0.1% BSA in PBS. Therefore, in all future experiments parasites were isolated from RBC debris using the phthalate oil-mix rather than microfuge washing of the parasite pellet in 0.1% BSA in PBS.

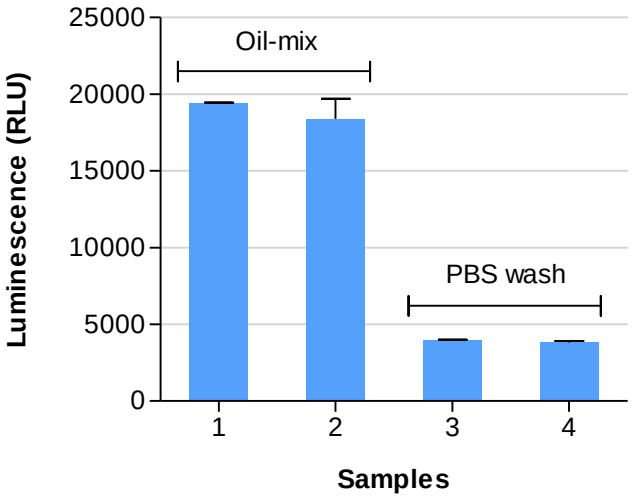

Figure 2.3: Comparison of ATP levels in parasite samples isolated from RBC lysates via a phthalate oil-mix and PBS wash. Trophozoite-stage *P. falciparum* parasites, released from RBCs by saponin treatment, were isolated from the RBC lysate by: (i) Oil-mix – centrifugation of the lysate through an oil-mix (dibutyl phthalate: dioctyl phthalate; 5:4) (samples 1-2), or (ii) PBS wash – five microfuge washing steps in 0.1% BSA in PBS (samples 3-4). Parasite ATP levels, measured as relative light units (RLU), are presented as means ± SD, n = 2.

2.3.3. Minimising contamination of the parasite pellet by RBC cytoplasm

While exploring the phthalate oil method, it was observed that samples were occasionally slightly pink in colour after resuspension of the parasite pellet in PBS. This was attributed to possible contamination of the parasite pellet by RBC cytoplasm and/or intact RBCs that escaped lysis during saponin treatment. The ATP assay procedure should, ideally, remove all traces of RBC cytoplasm, and hence RBC ATP, during isolation of intact parasites from culture samples. In order to determine whether residual RBC cytoplasm may be present in pellets after sample processing, suspensions of uninfected RBCs were prepared at a 5% haematocrit and subjected to the ATP assay procedure, using the phthalate oil-method as described in section 2.3.2.

Various adjustments were made to the experimental protocol to ensure that the final sample was free of RBC cytoplasm. The RBC pellet was lysed using a lysis solution with saponin concentration of 0.24%, as opposed to 0.1% used in previous experiments, in order to ensure complete RBC lysis. Subsequent centrifugation of the lysate through the phthalate oil-mix produced an infinitesimal, transparent ―pellet". The aqueous and oil layers were removed and 150 µL of PBS was pipetted over the ―pellet" before the sample was snap-frozen in liquid nitrogen and stored frozen. Processed samples were thawed and the ―pellet" was resuspended immediately before transfer to the development plate. Resuspension was achieved either by vortexing the mixture or pipetting up-and-down. The ―vortexed" samples appeared slightly pink in colour while ―pipetted" samples were colourless. The CellTiter-Glo reagent was used to measure ATP levels in the samples and results are shown in Figure 2.4. The residual RBC ATP present in ―vortexed" (pink) samples (mean RLU = 152472) was 76x higher than that of ―pipetted" (colourless) samples (mean RLU = 2015).

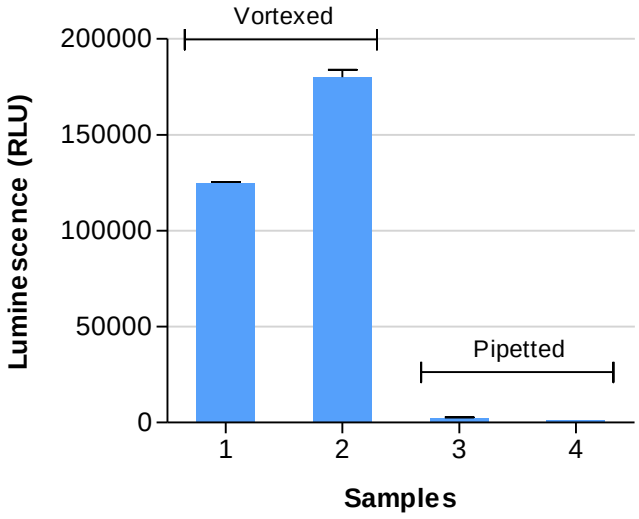

Figure 2.4: Comparison of residual RBC ATP levels in samples resuspended by vortexing and pipetting. To determine whether residual RBC cytoplasm may be present after sample processing, suspensions of uninfected RBCs (samples 1-4) were subjected to the ATP assay procedure. Resuspension of the sample "pellet" in PBS was achieved either by vortexing (1 and 2) or pipetting up-and-down (3 and 4). ATP levels, measured as relative light units (RLU), are presented as means ± SD, n = 2.

The elevated ATP levels and pink colour of the vortexed samples suggests that the suspension may have mixed with haemoglobin-containing RBC cytoplasm, and hence RBC ATP, as it travelled up the sides of the tube during vortexing. This suggests that residual RBC cytoplasm may be present on the walls of the sample tube after removal of the aqueous and oil layers and may contaminate the parasite pellet during processing of *P. falciparum* culture samples. Therefore, an additional washing step was considered to remove all traces of RBC ATP from the walls of the sample tube. To determine the effectiveness of this washing step in removing residual RBC ATP, ATP levels were measured in parallel suspensions of uninfected and trophozoite-infected RBCs. The samples were prepared at a haematocrit and parasitaemia of 5%. Samples were processed in the same manner as the uninfected RBC samples in the previous experiment, with the following modifications: (i) after centrifugation of the RBC lysate through the phthalate oil-mix and removal of the aqueous layer, the inner surface of the sample tube and the surface of the oil layer were washed with 500 µL of 0.1% BSA in PBS before removing the oil layer; (ii) resuspension of all sample pellets was achieved by pipetting the mixture up-and-down, as opposed to vortexing. Results are shown in Figure 2.5.

Residual RBC ATP (mean RLU = 1499) was present in the uninfected RBC samples, however, this accounted for less than 0.5% of the ATP measured in the trophozoite parasite samples (mean RLU = 332876). However, inter-sample variation of uninfected RBC samples was substantial (CV = 57.6%), therefore uninfected RBC suspensions were included as background controls in all subsequent experiments.

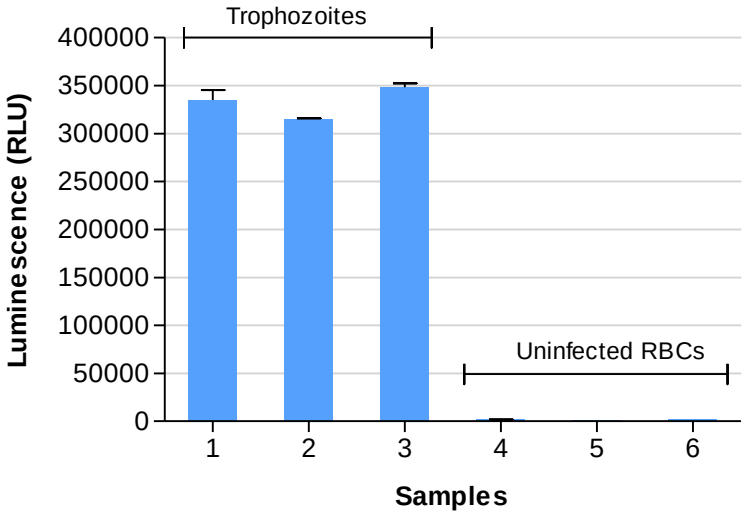

Figure 2.5: Comparison of ATP levels in samples of uninfected and trophozoite-infected RBCs subjected to the optimised ATP assay procedure. In order to minimise residual RBC ATP in the parasite sample, the ATP assay was optimised by an additional washing step and resuspension of samples by pipetting as opposed to vortexing. The efficacy of these modifications was assessed by comparing ATP levels in samples of trophozoite-infected RBCs (samples 1-3) and uninfected RBCs (samples 4-6) subjected to the modified procedure. ATP levels, measured as relative light units (RLU), are presented as means ± SD, n = 2.

Therefore, based on the results in this section, the following adjustments were made to the ATP assay procedure to minimise contamination of the parasite pellet by RBC cytoplasm, and hence RBC ATP:

i. The concentration of saponin in the lysis solution was increased from 0.1% to 0.24% to ensure complete RBC lysis;

ii. After centrifugation of the RBC lysate through the phthalate oil-mix and removal of the aqueous layer, the inner surface of the tube was washed with 500 µL of 0.1% BSA. The purpose of this additional washing step was to remove any residual RBC cytoplasm adhering to the tube walls and surface of the oil layer, before removing the layer.

iii. To the pellet, 150 µL of PBS was added before snap-freezing the sample. The pellet was resuspended immediately before transfer to the development plate by pipetting the mixture up-and-down, as opposed to vortexing the sample.

Thus, after extensive experimental refinement, an optimised protocol for measuring parasite ATP levels in *P. falciparum* culture samples was achieved (detailed in section 2.2.3). Thereafter, the linearity, sensitivity and precision of the assay were assessed to gauge the suitability of the method.

2.3.4. Linearity and sensitivity of the ATP assay

In order to determine the strength of the correlation between measured luminescence signals and parasite ATP levels, parasite suspensions were prepared from culture samples and serially diluted before measuring ATP levels. Briefly, 500 µL samples of parasite suspensions at a haematocrit and parasitaemia of 5% were prepared from trophozoite-stage *P. falciparum* cultures. The parasite pellet was isolated and resuspended in 200 µL of PBS before performing 5 x 2-fold serial dilutions to a final volume of 200 µL in PBS and measuring ATP levels (see section 2.2.3 for details). Parasite numbers in each sample were calculated from parasitaemia, and RBC concentration which was determined using a haemacytometer. The experiment was repeated on a separate occasion using higher parasite concentrations. Results are shown in Figure 2.6.

Luminescence signals showed excellent linear correlation with parasite numbers ranging from 1.23×10^5 to 1.97×10^6, evident by the corresponding linear regression correlation coefficients (r^2 values) which ranged from 0.95 to 0.99 (Figure 2.6a). When higher parasite concentrations were used in the assay, luminescence signals became saturated and deviated from linearity at parasite numbers greater than 2.19×10^6 (Figure 2.6b). Exclusion of the deviating data points produced excellent linear correlation between luminescence signals and parasite numbers ranging from 5.47×10^5 to 2.19×10^6 with r^2 values of 0.98-1.00 (Figure 2.6c), although care should be taken with this interpretation since only 3 data points were included in the linear regression. Thus, for parasite concentrations over the range 1×10^5–2×10^6 per sample, the luminescence signals produced by the *P. falciparum* ATP assay strongly correlate with parasite-associated ATP levels.

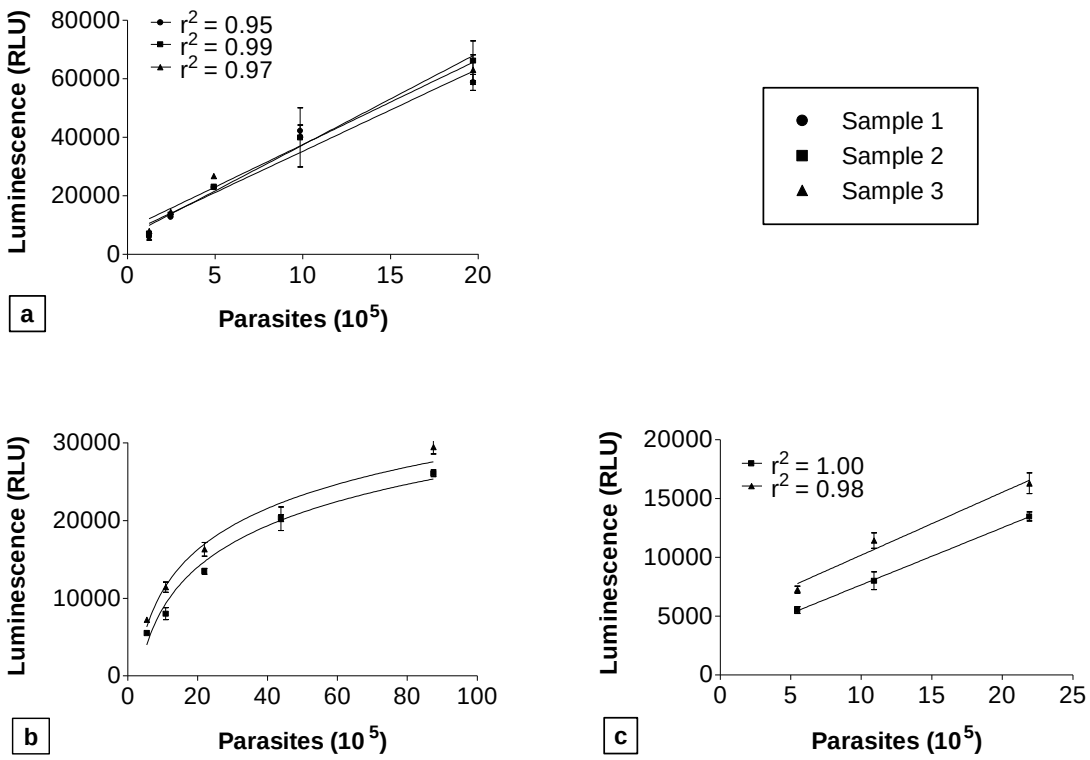

Figure 2.6: Relationship between parasite ATP levels and parasite concentrations for the *P. falciparum* ATP assay. The strength of the correlation between measured luminescence signals and parasite ATP levels was assessed by measuring ATP levels in samples with different parasite concentrations. Two parasite concentration ranges were employed (i.e. $1.23x10^5$–$1.97x10^6$ (Fig. 2.6a) and $5.47x10^5$–$8.75x10^6$ (Fig. 2.6b) and three (Fig. 2.6a) or two (Fig. 2.6b) samples of each parasite concentration were processed in parallel on each occasion. Note: Fig. 2.6c is plotted from the same data as 2.6b, with higher parasite numbers excluded. Each data point represents the mean ± SD, n = 2.

2.3.5. Precision and reproducibility of the ATP assay

The ATP assay procedure involves several centrifugation and pipetting steps which increase the likelihood of variation among ATP levels in parallel samples. Thus, in order to determine the precision of results produced by the *P. falciparum* ATP assay, coefficients of variation (CVs) and Z‘ factors were calculated (detailed in section 2.2.4) for experiments performed during optimisation of the assay, as described in this chapter. The Z‘ factor is a unitless statistical parameter that indicates the useful width of the assay window (Iversen *et al*., 2006; Zhang *et al*., 1999a). The broader this window, the more confidently a positive signal can be distinguished from a background/negative signal. A Z‘ value: (i) < 0.5 = marginal assay; (ii) ≥ 0.5 = excellent assay; (iii) = 1 = ideal assay. Results are summarised in Table 2.1.

Table 2.1: Summary of coefficients of variation (CVs) and Z' factors obtained using the *P. falciparum* ATP assay.

Figure	Parasites	Number of samples	Average % CV		Average Z'
			Intra-sample	Inter-sample	
2.3	Trophozoites	4	2.8	3.2	0.91*
2.5	Trophozoites	3	1.5	5.0	0.95#
2.6a	Trophozoites	15	6.0	7.6	0.81*
2.6b	Trophozoites	10	4.7	13.3	0.86*
2.7	Rings	3	0.7	11.5	0.98*
2.8a	Rings	3	1.3	28.7	0.95#
2.8a	Trophozoites	3	0.8	18.9	0.97#
		Total: 41	Average: 2.5	Average: 12.6	Average: 0.92

Note: (i) The "Figure" column contains the number of the figure in this chapter which corresponds to the experiment from which the data was extracted to do the calculations; (ii) The "Number of samples" refers to the number of parasite pellets that were subjected to the assay in parallel and does not take into account control samples presented in the figures. ATP luminescence in each pellet was read in duplicate wells (n=2); (iii) Intra-sample CVs were calculated from results obtained from duplicate wells containing the same test sample while inter-sample CVs were calculated from results obtained from replicate test samples (i.e. parasite pellets prepared in parallel); (iv) Z' factors were calculated by comparing the parasite samples to negative controls (medium only)* or background controls (uninfected RBCs) #.

The results indicated that for a total of 41 samples processed on different occasions in the ATP assay, the average intra-sample and inter-sample coefficients of variation were 2.5% and 12.6%, respectively, and were deemed acceptable for a cell-based assay of this nature. In addition, an average Z' factor of 0.92 was obtained, signifying an excellent assay window. Hence, the *P. falciparum* ATP assay has a broad dynamic range and is an excellent tool for measuring parasite-associated ATP in culture samples with a reasonable degree of precision.

Inter-assay reproducibility was assessed by determining the extent to which RLU values differ when the *P. falciparum* ATP assay was carried out on separate occasions. This was achieved by calculating the CV for mean RLU values of corresponding time 0 control samples obtained on different occasions during experiments to assess the kinetics of drug action, as described in Chapter 3. Results are shown in Table 2.2.

Table 2.2: Inter-occasional reproducibility of the *P. falciparum* ATP assay.

Figure	Mean RLU
3.1a	12101
3.2a	158882
3.3a	221520
3.4a	338279
3.5a	315409
Inter-assay	
Average	209238.2
SD	131789.1
CV	63.0

Note: (i) The "Figure" column contains the number of the figure in Chapter 3 (see section 3.3.1) which corresponds to the experiment from which the data was extracted to do the calculations, however, the Mean RLU values used in the table are not shown on the figures; (ii) The "Mean RLU" column contains RLU values measured in corresponding time 0 control samples using the *P. falciparum* ATP assay.

The results indicated that inter-assay RLU values of time 0 control samples varied considerably (CV = 63%) for reasons that have not been explored further. Therefore, absolute values obtained on separate occasions from analogous experiments cannot be directly compared except relative to internal controls, unless the purpose of the comparison is to assess stage variability.

2.3.6. Assessing the capacity of the assay to measure ATP levels in ring-stage parasites

The pilot ATP assay was performed using only trophozoite-stage *P. falciparum* parasites which are characterised by rapid growth and marked metabolic activity (Lazarus *et al*., 2008; Zolg *et al*., 1984) and therefore, as expected, contained ATP levels that were readily detectable by the assay. In contrast, ring-stage parasites display low metabolic activity and are far less voluminous than trophozoites. Nevertheless, the measurement of ATP levels in ring-stage parasites was attempted (experimental details in section 2.2.2). Briefly, 500 µL samples of parasite suspensions at a haematocrit and parasitaemia of 5% were prepared from ring-stage *P. falciparum* cultures. The infected RBCs were lysed using a 0.1% saponin lysis solution and intact parasites were isolated from RBC debris using the phthalate oil-mix, as described before. Unlike the clearly visible dark brown pellet produced by trophozoite-stage parasites (see Figure 2.2), the ring-stage parasite pellet was minimal and transparent. Therefore, sample tubes containing ring-stage parasites were always positioned in the microfuge with caps facing out to ensure that the orientation of the pellet was known. The

pellet was resuspended in 150 µL of PBS and parasite-associated ATP was measured using the CellTiter-Glo reagent, with culture medium as a negative control. The results (Figure 2.7) indicated that luminescence signals obtained with ring-stage parasites (mean RLU = 37459) were considerably higher than negative controls (mean RLU = 106) by a factor of ± 355, with an average Z‘ factor of 0.98.

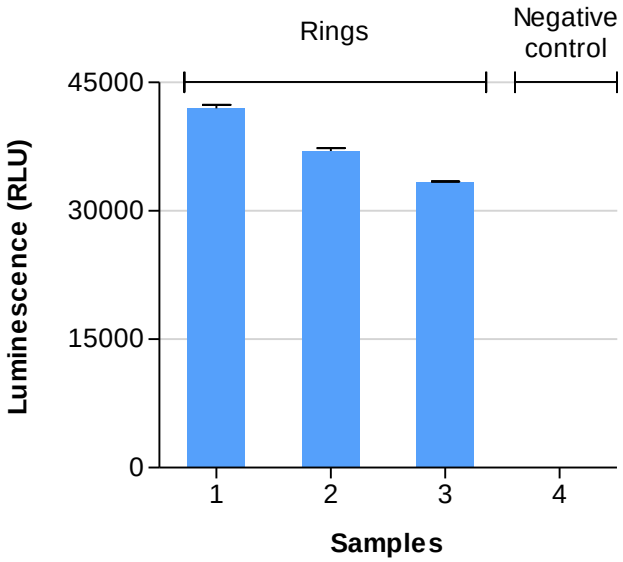

Figure 2.7: ATP levels in ring-stage *P. falciparum* parasites. Three samples (samples 1-3) of a ring-stage culture were subjected to the ATP assay procedure. Parasite ATP levels, measured as relative light units (RLU), were measured using the CellTiter-Glo reagent, with culture medium as a negative control (sample 4). Results are presented as means ± SD, n = 2.

Thus, the ATP assay appears capable of measuring ATP levels in ring-stage parasites. However, the above experiment was performed before the introduction of the RBC removal modifications, as described in section 2.3.3. To confirm that the ATP signals attributed to the rings were not due to RBC contamination, the experiment was repeated using the optimised ATP assay procedure with uninfected RBCs as controls. Trophozoite samples were processed in parallel to compare the intensity of ATP signals in the two stages. Results are shown in Figure 2.8.

As expected, ATP levels of trophozoite-stage parasites (mean RLU = 352947; Figure 2.8a) were more than 25x higher than the ATP levels of a corresponding number of ring-stage parasites (mean RLU = 13122). Luminescence signals of the ring-stage parasites were nonetheless considerably higher (13-fold) than those of background controls (uninfected

RBCs; mean RLU = 993; Figure 2.8b) with an average Z‘ factor of 0.95. This represents a very comfortable assay window for assessing changes in ATP levels in ring-stage parasites.

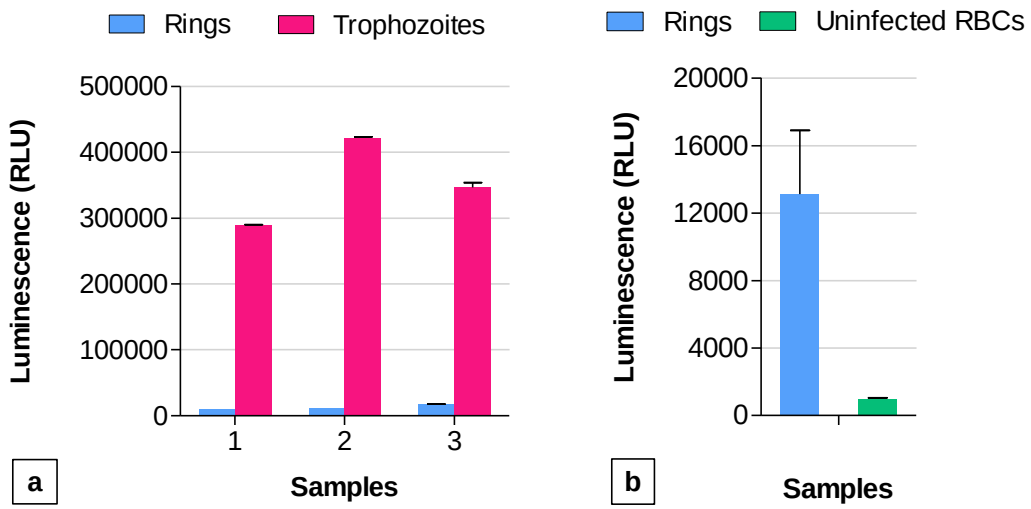

Figure 2.8: Comparison of ATP levels in ring- and trophozoite-stage *P. falciparum* parasites. Triplicate aliquots (samples 1-3) of parallel ring- and trophozoite-stage cultures were subjected to the ATP assay procedure (Fig. 2.8a). Parasite ATP levels, measured as relative light units (RLU), are presented as means ± SD, n = 2. The ATP levels of the ring-stage parasites (n = 3) are compared to the residual RBC ATP of uninfected RBC controls (n = 2) in Fig. 2.8b.

2.3.7. Determining the extent to which parasite ATP levels vary as a function of trophozoite development

In order to determine the extent to which the ATP levels of trophozoite-stage parasites vary over time, samples of culture were taken at regular intervals and parasite-associated ATP was measured. Briefly, an early-mid trophozoite-stage culture synchronised by sorbitol treatment (detailed in section 2.2.1.2.1) was prepared at a haematocrit and parasitaemia of 5%. Triplicate samples (500 µL) were taken every 2 hours over a 10-hour period and subjected to the optimised ATP assay procedure (detailed in section 2.2.3). In addition to measuring parasite ATP levels, Giemsa-stained thin-blood smears were prepared at each time-point and assessed for morphological changes.

The ATP results (Figure 2.9) indicated a substantial (62%) decrease in parasite ATP levels within the first 2 hours. This large decrease in ATP levels between the 0- and 2-hour time-points was consistently observed in all time-course experiments (e.g. Figure 2.12). The source is not clear but likely artifactual and due to exposure of parasites to non-culture

conditions during preparation for the experiment which may have caused elevated ATP levels at time 0. This was followed by a moderate (25%) decline between the 2- and 4-hour time-points. Parasite ATP levels remained unchanged between the 4- and 6-hour time-points and decreased by 39% during the last 4-hours of incubation.

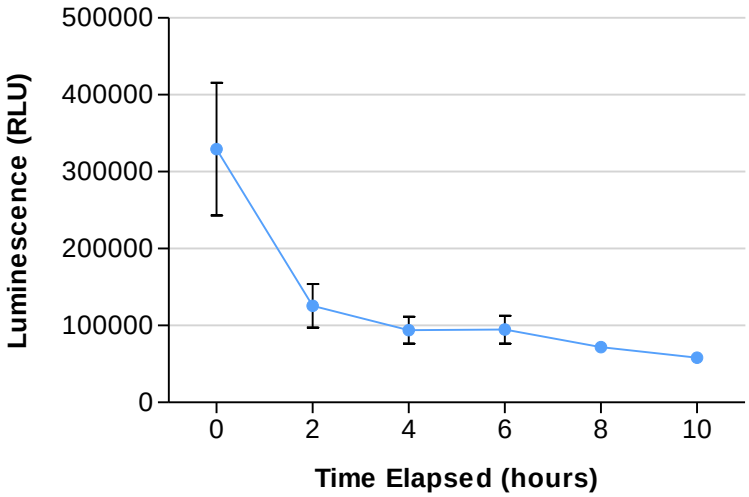

Figure 2.9: Changes in ATP levels of trophozoite-stage parasites over time in a sorbitol-synchronised culture. Samples of a sorbitol-synchronised trophozoite-stage *P. falciparum* culture were taken every 2 hours over a 10-hour period and subjected to the ATP assay procedure. ATP levels, measured as relative light units (RLU), are presented as means ± SD, n = 3.

Rapid growth and marked metabolic activity are characteristic of trophozoite-stage parasites (Lazarus *et al*., 2008; Zolg *et al*., 1984), therefore the decrease in parasite ATP levels over the remaining 8 hours was unexpected because the culture contained early-mid trophozoite-stage parasites at time 0. Hence, parasite ATP levels were expected to increase as this developmental stage matured. Examination of the thin-blood smears prepared at each time-point (Figure 2.10) provided some insight into these unexpected results. It was found that at the 6- and 8-hour time points the majority of the parasite population were schizonts, while mostly ring-stage parasites were observed at the 10-hour time-point.

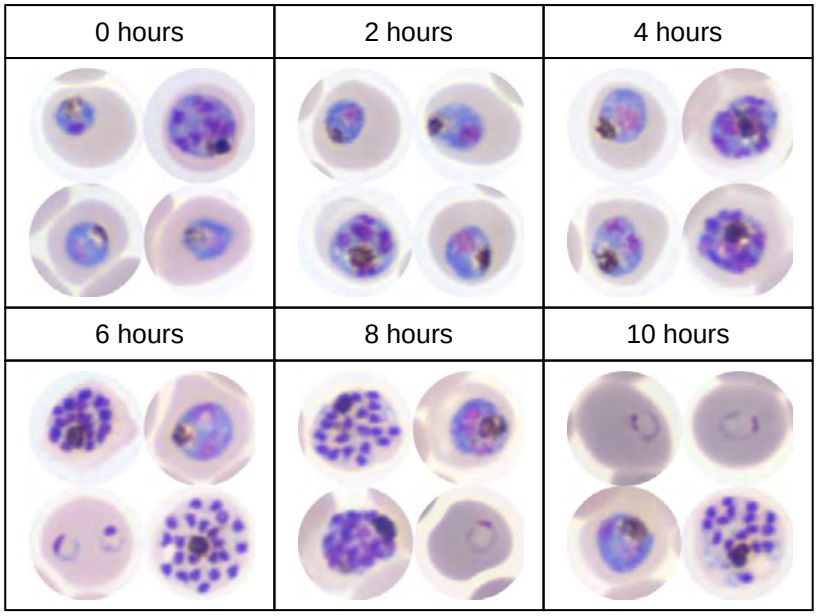

Figure 2.10: Morphological changes in *P. falciparum* parasites over time in a sorbitol-synchronised culture. Giemsa-stained thin-blood smears were prepared from a sorbitol-synchronised trophozoite-stage *P. falciparum* culture every 2 hours over a 10-hour period. Photomicrographs of the thin-blood smears were taken by light microscopy with an Olympus BX41 upright microscope equipped with a digital camera CC-12 soft imaging system (U-CMAD3, Olympus, Tokyo, Japan) using a 100x oil-immersion objective and analysis LS Report software.

In order to obtain a more accurate account of the different developmental stages present at each time-point, a total of 100 parasites were counted in each smear and the percentage of each parasite stage determined (Figure 2.11). The results indicated that the majority of parasites in the initial culture were early-mid trophozoites (74%), while the remaining parasite population consisted of early schizonts (26%). By the 4-hour time-point, the majority of parasites (66%) had reached the schizont stage and remained in this stage for the next 4 hours. As parasites transition from the trophozoite to the schizont stage, a moderate decrease in metabolic activity is expected as growth ceases and the parasite undergoes four to five rounds of binary fission to produce daughter merozoites which proceed to infect fresh RBCs. Thus, the modest decrease in ATP levels between the 2- and 4-hour time-points is likely a result of the parasites transitioning from the trophozoite to the schizont stage. Ring-stage parasites are characterised by low metabolic activity (Lazarus *et al*., 2008; Zolg *et al*., 1984) and were observed from the 6-hour time-point onward. This developmental stage accounted for 21% and 76% of the parasite population at the 8- and 10-hour time-points, respectively. Thus, the decline in ATP levels during the last 4 hours of incubation may be attributed to the

rupture of schizont-stage parasites and subsequent invasion of fresh RBCs to form rings. The percentage of schizonts in the parasite population declined markedly (66% to 20%) between the 8- and 10-hour time-points to produce the less metabolically active ring-stage parasites, however, overall ATP levels only decreased by about 20%. Each schizont ultimately produces 16-32 rings (van Agtmael *et al*., 1999), thus even though a large number of schizonts ruptured to produce less metabolically active parasites (i.e. rings), the overall decrease in ATP was not considerable since each schizont was replaced by multiple rings.

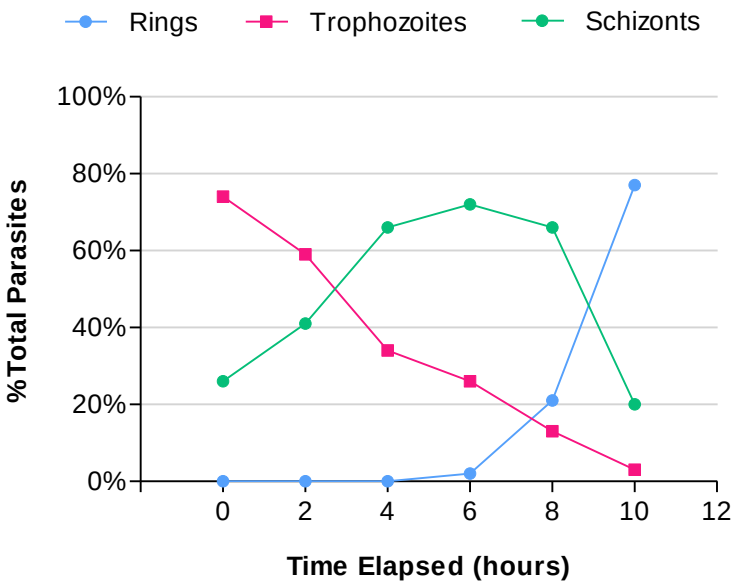

Figure 2.11: The percentage of each developmental stage present in a sorbitol-synchronised *P. falciparum* culture over a 10-hour incubation period. Giemsa-stained thin-blood smears were prepared from a sorbitol-synchronised trophozoite-stage parasite culture every 2 hours over a 10-hour period and the percentage of each developmental stage (i.e. rings, trophozoites, schizonts) was approximated by counting a total of 100 parasites.

Thus, the results suggest that a sorbitol-synchronised early-mid trophozoite-stage culture with seemingly low levels (~26%) of schizont-stage parasites at time 0 will inevitably contain multiple developmental stages over a 10-hour period. Hence, a sorbitol-synchronised culture is not suitable for assessing ATP changes in trophozoite-stage parasites during a 10-hour incubation. In order to obtain a truer reflection of the extent to which ATP levels of trophozoite-stage parasites vary over time, a more tightly synchronised culture of early-trophozoite stage parasites is required. Therefore, a Percoll-based *P. falciparum* enrichment procedure (detailed in section 2.2.1.2.2) was used to produce such a culture before repeating the experiment. As in the previous experiment, ATP results (Figure 2.12) indicated a major

(79%) decline in parasite ATP levels within the first 2 hours. This was followed by an even greater (108%) increase in parasite-associated ATP levels during the next 4 hours with a moderate (34%) decrease occurring in the remaining 4 hours.

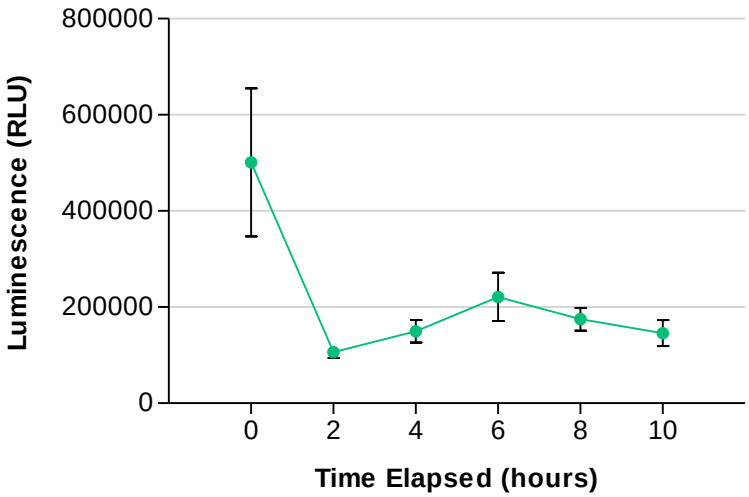

Figure 2.12: Changes in the ATP levels of trophozoite-stage parasites over time in a Percoll-synchronised culture. Samples of a Percoll-synchronised trophozoite-stage *P. falciparum* culture were taken every 2 hours over a 10-hour period and subjected to the ATP assay procedure. ATP levels, measured as relative light units (RLU), are presented as means ± SD, n = 3.

Examination of the thin-blood smears (Figure 2.13) indicated that parasites were at the trophozoite stage of development throughout the experiment. Rapid growth of parasites was observed between the 2- and 6-hour time-points as the parasites progressed from the early- to mid-trophozoite stage of development, which likely explains the increase in ATP levels between these time-points. Late trophozoite-stage parasites were present in the last 2 hours of incubation, thus the decrease in ATP levels during this time may be a result of a slowing down in parasite growth as the parasites prepared to enter the schizont stage of development.

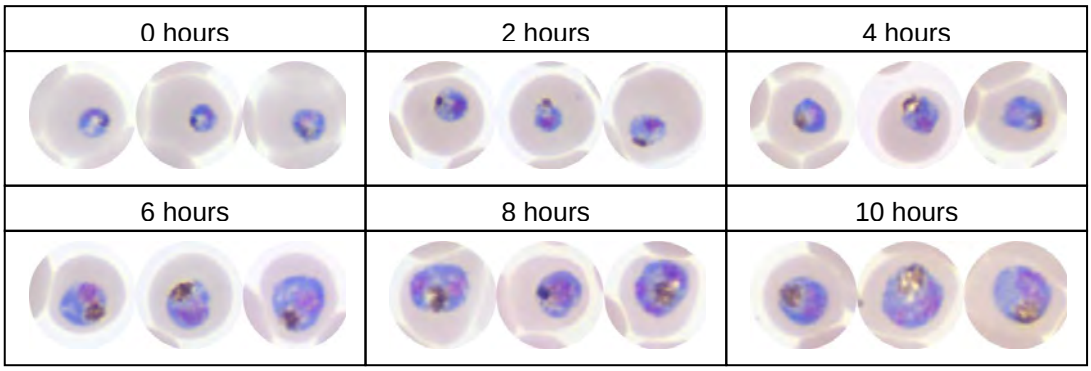

Figure 2.13: Morphological changes in trophozoite-stage parasites over time in a Percoll-synchronised culture. Giemsa-stained thin-blood smears were prepared from a Percoll-synchronised trophozoite-stage *P. falciparum* culture every 2 hours over a 10-hour period. Photomicrographs of the thin-blood smears were taken by light microscopy with an Olympus BX41 upright microscope equipped with a digital camera CC-12 soft imaging system (U-CMAD3, Olympus, Tokyo, Japan) using a 100x oil-immersion objective and analysis LS Report software.

The results indicated that the ATP levels of trophozoite-stage parasites fluctuate considerably (~2-3 fold) during a 10-hour incubation, even in the absence of drug treatment. In addition, inter-experimental absolute RLU values obtained also display considerable differences (e.g. compare the luminescence values in Figures 2.9 and 2.12). Therefore, if drug-induced parasite stress were to be assessed by measuring parasite ATP levels in drug-treated cultures over a 10-hour period, an untreated control culture should be included and all results should be expressed relative to the control.

2.4. DISCUSSION

In an effort to develop a novel, quantitative assay for assessing drug-induced parasite stress, the measurement of parasite-associated ATP using the luminescence-based CellTiter-Glo reagent (Promega, Madison, WI) was explored as a means of diagnosing abnormal parasite metabolism. The pilot assay involved the measurement of parasite ATP in samples of trophozoite-stage *P. falciparum* cultures incubated for 8 and 12 hours in PBS or drug-treated culture medium. The ATP levels of control parasites increased more than 3-fold between the 8 and 12 hour time-points as shown in Figure 2.1b. This was likely due to rapid growth and marked metabolic activity which are characteristic of trophozoite-stage parasites (Lazarus *et al*., 2008; Zolg *et al*., 1984). The metabolic activities of trophozoite-stage parasites include haemoglobin digestion and the synthesis of DNA, RNA and proteins (Lazarus *et al*., 2008; Zolg *et al*., 1984). There was a massive decline in the ATP levels of parasites incubated in PBS at both time-points, likely a result of the lack of glucose to fuel glycolysis which is the metabolic pathway by which the parasite anaerobically produces ATP (Olzewski & Llinás, 2010). At both time-points, the ATP levels of chloroquine- and artemisinin-treated parasites increased considerably, while those of parasites exposed to compound 4070 decreased moderately, relative to controls. The elevated ATP levels may be a result of increased metabolic activity by the parasite in an effort to counteract harmful drug effects, while the reduced ATP levels may be indicative of compromised parasite energy metabolism and/or viability. These are general interpretations of the above results in light of the fact that the results were obtained using the pilot assay. Detailed interpretations of how parasite ATP levels respond to drug-induced stress are discussed in Chapter 3 with results obtained using a fully optimised *P. falciparum* ATP assay protocol.

Nevertheless, results of the pilot assay have indicated that parasite ATP levels in *P. falciparum* cultures can be readily detected using a commercial luminescence assay and may represent a potential diagnostic measure of drug-induced parasite stress. Therefore, the primary aim of this study was to develop an optimised and fully functional *P. falciparum* ATP assay and to explore the utility of the assay for assessing the kinetics of drug action in culture. The first step to achieving this goal was to produce an optimal protocol for detecting malaria parasite ATP by refining various aspects of the pilot assay.

In the pilot assay, five microfuge washing steps were used to isolate intact parasites from the RBC lysate, and hence RBC ATP, following saponin treatment. This washing process was

both time-consuming and impractical due to the numerous precise liquid handling and centrifugation steps involved. Additional concerns were the possibility of parasite lysis due to the multiple centrifugation steps, as well as the potential variation in parasite ATP levels during the lengthy exposure of the parasites to non-culture conditions. Therefore, the use of a phthalate oil-mix, as described by Quashie *et al*. (2010), was explored as an alternative method for parasite isolation from RBC lysates. The ATP levels of parasites isolated via the phthalate oil-mix were approximately 5x higher than those of parasites isolated by microfuge washing in 0.1% BSA in PBS. This was possibly a result of the latter method causing parasite loss due to excessive parasite rupture and non-specific adhesion of parasites to microfuge tube walls during repeated centrifugation. Furthermore, use of the phthalate oil-mix was found to be far less time-consuming than microfuge washing of the parasite pellet. Thus, the improved ATP levels may be due to reduced variation in parasite ATP levels as a result of shorter exposure to non-culture conditions, and/or improved yield of intact parasites as a result of fewer centrifugation and liquid handling steps.

It was noted that samples were slightly pink in colour after resuspension of the parasite pellet in PBS. This was ascribed to contamination of the parasite pellet by haemoglobin-containing RBC cytoplasm, and/or intact RBCs which may have escaped lysis during saponin treatment. The ATP assay procedure should, ideally, remove all traces of RBC cytoplasm during isolation of intact parasites from culture samples. Therefore, several adjustments to the ATP assay procedure were explored in order to minimise the aforementioned contamination before repeating the experiment. The first amendment to the procedure was the increased saponin concentration of the lysis solution from 0.1% to 0.24% in order to ensure complete RBC lysis. The effectiveness of this modification was evaluated by subjecting uninfected RBCs to the ATP assay using the modified lysis solution. This resulted in a minute, transparent pellet which may represent RBC ―ghosts" i.e. post-haemolytic remains of RBCs consisting primarily of membrane and cytoskeletal elements and devoid of cytoplasmic components (Schwoch & Passow, 1973). The transparent colour of the pellet suggested that the 0.24% saponin lysis solution achieved complete haemolysis. Resuspension of the pellet in PBS was achieved by either vortexing or pipetting the sample up-and-down. ―Vortexed" samples were slightly pink in colour while ―pipetted" samples were colourless. The pink colour of vortexed samples was assumed to be a result of the suspension travelling up the sides of the tube and mixing with residual haemoglobin-containing RBC cytoplasm during vortexing. This assumption was supported by the ATP results which showed that vortexed samples (of RBC

material alone) contained 76x more residual RBC ATP than "pipetted" samples. Therefore, all future experiments were carried out using the improved lysis solution (i.e. 0.24% rather than 0.1% saponin) and resuspension of the parasite pellet was achieved by pipetting, rather than vortexing, the sample immediately before transfer to the development plate. Furthermore, an additional washing step was included in the procedure to minimise contamination of the parasite pellet by RBC cytoplasm, and hence RBC ATP, during processing of culture samples.

This additional step, performed after centrifugation of the RBC lysate through the phthalate oil-mix, involved washing the inner surface of the sample tube with 0.1% BSA in PBS to remove any residual RBC cytoplasm before removing the oil layer. The ATP levels in parallel suspensions of uninfected and trophozoite-infected RBCs were measured using the ATP assay with this additional washing step. Results indicated that residual RBC ATP was still present in uninfected RBC samples, even after inclusion of the washing step. Even though the residual RBC ATP accounted for less than 0.5% of the ATP measured in parasite samples, inter-sample variation of uninfected RBC samples was considerable (CV = 57.6%). Therefore, uninfected RBC suspensions were included as background controls in all subsequent experiments using the ATP assay. Thus, after extensive experimental refinement, an optimised protocol for measuring parasite ATP in levels in *P. falciparum* culture samples was produced. A detailed description of the protocol is given in section 2.2.3 and is summarised below.

Infected RBCs were pelleted from *in vitro* cultures by centrifugation and resuspended in culture medium at a volume, haematocrit and parasite concentration of 500 µL, 5% and $\leq 2x10^6$ per sample. Uninfected RBCs were prepared in the same manner to serve as background controls. Triplicate 500 µL aliquots of each suspension were transferred to microfuge tubes and maintained on ice as samples were individually processed. Each tube was centrifuged to pellet the RBCs which were lysed by a 0.24% saponin solution. The RBC lysate was centrifuged through a phthalate oil-mix, resulting in the intact parasites pelleting below the oil and the remaining aqueous lysate settling above the oil. The aqueous layer was removed and the inner surface of the tube was washed before removing the oil layer. Ice-cold PBS was added to the parasite pellet before snap-freezing the sample. Processed samples were thawed and the parasite pellet was resuspended by pipetting up-and-down before transferring the suspension to a white 96-well plate. Parasite-associated ATP was detected by

adding CellTiter-Glo reagent and measuring the resulting luminescence in a multimode plate reader.

Once the experimental protocol of the *P. falciparum* ATP assay was optimised and characterised, an analysis of the linearity, sensitivity and precision of the assay was performed. The strength of the correlation between measured luminescence signals and parasite ATP levels, i.e. assay linearity, was determined by measuring ATP levels in parasite suspensions with different parasite concentrations. Luminescence signals showed excellent linear correlation with parasite numbers over the range $1.23x10^5$–$2.19x10^6$ parasites per sample. When higher parasite concentrations were used in the assay, luminescence signals became saturated and deviated from linearity. This implies that, for parasite concentrations over the range $1x10^5$–$2x10^6$ per sample, the luminescence signals produced by the *P. falciparum* ATP assay strongly correlate with parasite-associated ATP levels.

Assay precision represents the degree of scatter among a series of measurements obtained from multiple samples under identical conditions (Chesher, 2008) and is usually expressed as the coefficient of variation (CV). An additional means of evaluating the quality of an assay is to calculate the Z‘ factor which is a unitless statistical parameter that reveals the useful width of the assay window, i.e. the dynamic range between background/negative and positive control signals (Iversen *et al*., 2006; Zhang *et al*., 1999a). The wider this window, the more confidently a positive signal can be distinguished from a background/negative signal. A Z‘ value: (i) less than 0.5 indicates a marginal assay; (ii) greater than 0.5 signifies an excellent assay; (iii) equal to 1 is the maximum possible Z‘ value and represents an ideal assay. For a total of 41 samples processed on separate occasions in the ATP assay, the average intra-sample and inter-sample CVs were 2.5% and 12.6%, respectively. These were considered to be acceptable given the cell-based nature of the assay. An average Z‘ factor of 0.92 was achieved, suggesting that the positive signals (parasite-associated ATP) can be differentiated from background (RBC-associated ATP) or negative controls (culture medium) with confidence. Hence, the *P. falciparum* ATP assay has a high dynamic range and is an excellent tool for detecting parasite ATP in culture samples with an acceptable degree of precision.

During optimisation of the assay, trophozoite-stage parasites were easily tracked during sample processing due to haemozoin crystals in the food vacuoles which rendered the parasite pellet a clearly visible dark brown colour. An added advantage of working with

trophozoites was that these parasites produced ATP levels which were readily detectable by the ATP assay, likely due to marked metabolic activity which is characteristic of this developmental stage (Lazarus *et al*., 2008; Zolg *et al*., 1984). On the other hand, ring-stage parasites lack food vacuoles, are far less voluminous than trophozoite-stage parasites and display low metabolic activity. Nonetheless, samples of ring-stage parasite cultures were subjected to the ATP assay in order to ascertain whether measurement of parasite-associated ATP was possible in this developmental stage. The resulting parasite pellet was minimal and transparent, and therefore extremely difficult to track during processing of samples. Even so, the ring-stage parasite samples produced signals that were readily detectable.

Comparison of ATP levels in parallel samples of ring- and trophozoite-stage parasites indicated that the ATP levels of trophozoite-stage parasites were appreciably higher than those of a corresponding number of ring-stage parasites. Luminescence signals of the ring-stage parasites were nonetheless considerably higher than those of uninfected RBC background controls. This suggests that the *P. falciparum* ATP assay is capable of detecting, and distinguishing, ATP levels in both ring- and trophozoite-stage parasites. Thus, the ATP assay could potentially be used to assess drug-induced changes in the ATP levels of ring-stage parasites. This is highly advantageous given that morphological changes in rings are extremely difficult to judge unambiguously by light microscopy. Even though trophozoite-stage parasites were used for all subsequent experiments in this study, the results suggest that it may be possible to compare drug effects in parallel samples of ring- and trophozoite-stage cultures by measuring parasite ATP levels.

The *P. falciparum* parasite undergoes rapid morphological changes and varying levels of metabolic activity as it proceeds through the 48-hour intra-erythrocytic phase of its life cycle. Ring-stage parasites are present for the first 20-24 hours and develop into trophozoite- and schizont-stage parasites during the remaining 24 hours. In order to determine the extent to which the ATP levels of trophozoite-stage parasites vary over time, parasite ATP was measured in samples of a sorbitol-synchronised early-mid trophozoite-stage culture taken every 2 hours over a 10-hour period. In addition, Giemsa-stained thin-blood smears were prepared at each time-point. A substantial decline in parasite ATP levels was observed within the first 2 hours. Exposure of parasites to non-culture conditions (i.e. removal from culture, centrifugation, etc.) during preparation for the experiment may have initiated a stress response which could explain the elevated ATP levels at time 0. Hence, the decline in ATP

levels may represent a return to ~~"n~~ormal" levels as culture conditions were restored. Parasite ATP levels showed a gradual decline over the remaining 8-hour incubation. Marked metabolic activity and rapid growth are characteristic of trophozoite-stage parasites (Lazarus *et al*., 2008; Zolg *et al*., 1984). Therefore, the decline in parasite ATP levels over the remaining 8 hours was unexpected, as initial cultures were at the early-mid trophozoite stage and parasite ATP levels were expected to increase as the parasites matured. The thin-blood smears prepared at each time-point were examined and the percentage of each developmental stage in the parasite population was approximated. The results provided an explanation for the unexpected ATP results.

The majority of the parasite population in the initial culture consisted of early-mid trophozoites with the remaining parasites made up of early schizonts. Over the 10-hour incubation period, the mid-trophozoites present at time 0 developed into schizonts. These newly-developed schizonts, together with the schizonts already present at time 0, ultimately produced merozoites which invaded fresh RBCs and developed into rings. Unlike trophozoites which are characterised by marked metabolic activity, schizonts and rings display moderate and low metabolic activity, respectively. Accordingly, the decline in ATP levels over the remaining 8-hour incubation was attributed to the development of schizont and ring-stage parasites. Thus, the results showed that over a 10-hour period a sorbitol-synchronised trophozoite culture inevitably contains multiple developmental stages at varying concentrations which significantly affect results when attempting to evaluate ATP changes in trophozoite-stage parasites. Therefore, to obtain a truer reflection of the extent to which the ATP levels of trophozoites fluctuate over time, synchronisation was achieved by *P. falciparum* enrichment. This involved the incubation of Percoll-enriched schizonts with uninfected RBCs, and once newly-invaded RBCs were observed residual schizonts were destroyed by sorbitol treatment. This produced a highly synchronised culture of early trophozoite-stage parasites before repeating the experiment.

Examination of the thin-blood smears indicated that the parasite population was in the trophozoite stage throughout the experiment. As in the previous experiment, a considerable decline in parasite ATP levels was observed within the first 2 hours. This supports the notion that exposure of parasites to non-culture conditions at time 0 may trigger a stress response which results in elevated ATP levels. This suggests that an increase in parasite-associated ATP represents a rapid and sensitive indicator for parasites under stress. Parasite ATP levels

increased during the next 4 hours, likely due to rapid growth of the parasite as observed in the Giemsa-stained thin-blood smears. A moderate decrease in parasite-associated ATP levels occurred in the remaining 4 hours which may be attributed to a decrease in parasite growth as the parasites entered the late trophozoite stage of development. The results have shown that there is considerable variation (up to 2-3 fold) in the ATP levels of trophozoite-stage parasites over a 10-hour period, even in the absence of drug treatment. Thus, if the *P. falciparum* ATP assay were to be used to assess the kinetics of drug action by measuring parasite ATP levels in drug-treated cultures over a 10-hour period, an untreated control culture should be included at all time-points and all results expressed relative to the control.

2.5. CONCLUSION

After extensive experimental refinement and characterisation, a rapid and fully functional *P. falciparum* ATP assay was developed. The assay has a broad dynamic range and is capable of measuring parasite-associated ATP in culture samples with a high degree of precision and sensitivity. Furthermore, results obtained in a pilot study and during time-course experiments suggest that the assay may be useful in assessing the rate of activity of antimalarial drugs by measuring ATP levels in drug-treated cultures over time.

CHAPTER 3: ASSESSING THE UTILITY OF THE *P. FALCIPARUM* ATP ASSAY FOR EVALUATING THE RATE OF DRUG-INDUCED PARASITE STRESS

3.1. INTRODUCTION

Assessing the rate of activity of candidate antimalarials is crucial to current drug discovery efforts due to the need for novel, rapid-acting drugs, as discussed in Chapter 2 (see section 2.1). Quantitative procedures for assessing the rate of activity of antimalarials in *in vitro* cultures, besides the time-consuming and highly subjective interpretation of morphology by light microscopy, are limited to a recently reported viability-based assay (Sanz *et al*., 2012). This assay directly quantifies the number of parasites capable of recovering from distinct periods of drug exposure *in vitro*, unfortunately, this involves 3-4 weeks of monitoring re-growth. Hence, there is a need for a novel, quantitative assay which is capable of rapidly determining the kinetics of drug action in culture. Adenosine triphosphate (ATP) serves as a primary source of energy for the growth, maintenance and reproduction of all living organisms (Ginsburg, 2010). Our hypothesis was that parasite ATP levels may represent a sensitive and readily detectable means of diagnosing abnormal parasite metabolism induced by drug treatment. Thus, we have developed and optimised a sensitive assay for measuring parasite ATP levels in *P. falciparum* cultures using a luminescence-based kit (see Chapter 2).

The utility of the assay as a means of determining the rate of antimalarial drug action was then explored by characterising the response of parasite ATP levels to a panel of antimalarials, namely chloroquine, DFMO (DL-α-difluoromethylornithine), mefloquine, artemisinin and ritonavir. These antimalarials were selected for their varying modes of action (as discussed in section 3.4. below) which, presumably, afford them different rates of activity that are suitable for gauging the utility of the ATP assay as a means of assessing the kinetics of drug action. Chloroquine, mefloquine and artemisinin are antimalarials currently in clinical use while DFMO and ritonavir are experimental compounds with known antimalarial activity. In patients receiving therapeutic doses of the clinical antimalarials, peak plasma concentrations achieved *in vivo* tend to vary from person to person (chloroquine: 39-582 nM (Telgt *et al*., 2005); mefloquine: 1.3-3 μM (Mimica *et al*., 1983); artemisinin: 1.1-2.7 μM (Räth *et al*., 2004)). Thus, for this study, effective treatment concentrations for each drug

were based on *in vitro* median inhibition concentrations **(**IC50s) and/or test concentrations both from the current study and previous reports (detailed in Table 5.1, Appendix 5). The goal was to select a concentration which causes sufficient antiplasmodial activity via direct drug action rather than secondary downstream effects and for this reason approximately 5 x IC50 was chosen as a working concentration for each drug.

3.2. MATERIALS AND METHODS

3.2.1. Drugs/compounds used in this study

Chloroquine diphosphate, mefloquine hydrochloride, artemisinin and ritonavir were purchased from Sigma-Aldrich (Steinheim, Germany). DL-α-difluoromethylornithine (DFMO) was kindly provided by P. Woster (Wayne State University, MI). Stock solutions were prepared as follows: chloroquine – 10 mM in water, mefloquine – 10 mM in methanol, artemisinin – 10 mM in DMSO, ritonavir – 100 mM in DMSO. The proteasome inhibitors lactacystin and MG-132 were obtained from Merck (Germany) and prepared as 10 mM stocks in water and DMSO, respectively. For experiments, stocks were diluted to the desired concentration in CMCM (Appendix 1.1).

3.2.2. Measurement of ATP levels in drug-treated parasites

Plasmodium falciparum 3D7 parasites were cultivated as described in Chapter 2 (section 2.2.1) and synchronised using Percoll-based *P. falciparum* enrichment (detailed in section 2.2.1.2.2). An early trophozoite-stage culture was used to prepare a 32 mL, 5% haematocrit, 2% parasitaemia suspension in CMCM (Appendix 1.1). The suspension was split into 2 x 15 mL cultures and treated with drug and solvent control solutions, respectively. Immediately after treatment, 3 x 500 µL samples of the suspensions were transferred to microfuge tubes and maintained on ice (time 0 samples). The suspensions were then transferred to T25 culture flasks, gassed (5% CO_2, 5% O_2, balance N_2; Air Products, RSA), sealed and incubated at 37°C. Thereafter, 100 µL and 3 x 500 µL samples were taken every 2 hours over a 10-hour period. At each time-point, ATP levels were measured in the 500 µL samples as described in Chapter 2 (section 2.2.3 – optimised ATP assay). Giemsa-stained thin-blood smears were prepared from the 100 µL samples, as described in Chapter 2 (section 2.2.1.1), and assessed for morphological changes. Significant differences between the parasite-associated ATP levels of control and treatment samples were determined using a two-tailed, paired Student's T-test (Microsoft Excel 2007). A probability level of $P \leq 0.05$ was considered statistically significant. Photomicrographs of the thin-blood smears were taken by light microscopy with an Olympus BX41 upright microscope equipped with a digital camera CC-12 soft imaging system (U-CMAD3, Olympus, Tokyo, Japan) using a 100x oil-immersion objective and analysis LS Report software.

3.2.3. Plasmodial lactate dehydrogenase (pLDH) assay

Plasmodial lactate dehydrogenase activity was measured using a method adapted from Makler *et al.* (1993). *Plasmodium falciparum* 3D7 parasites were cultured as described in Chapter 2 (section 2.2.1). A trophozoite-stage culture was used to prepare a 2% haematocrit, 2% parasitaemia suspension in CMCM (Appendix 1.1). Drug and control solutions at twice the final test concentrations were added to a 96-well tissue culture plate at 100 µL per well. An equal volume of the parasite suspension (100 µL) was then added to each well. The positive and background controls comprised of untreated and chloroquine-treated (1 µg/mL) parasite suspensions, respectively. The plate was transferred to an airtight chamber suffused with a special gas mixture (5% CO_2, 5% O_2, balance N_2; Air Products, RSA) and incubated at 37°C for 48 hours. Following incubation, the plates were frozen before further processing. The plates were then thawed at room temperature and the culture wells were resuspended before transferring 20 µL to a clear, flat-bottomed 96-well plate pre-loaded with 100 µL of Malstat solution (Appendix 3.1). To each well, 25 µL of NBT/PES solution (Appendix 3.2) was added and air bubbles were removed with a hairdryer. The plate was left to develop in the dark before measuring absorbance at 620 nm in a multimode plate reader (Infinite F500, Tecan, Austria). Percentage parasite viability (%PV) was determined using the following equation: $\%PV = [(Absorbance_{sample} - Mean\ Absorbance_{background})/(Mean\ Absorbance_{positive} - Mean\ Absorbance_{background})] \times 100]$. For dose-response assays, median inhibition concentrations (IC50s) were determined by non-linear regression analysis of log dose-response curves using GraphPad Prism software (v.5.02 for Windows, San Diego California, USA). Significant differences between IC50 values were determined using a two-tailed, homoscedastic Student's T-test (Microsoft Excel 2007). A probability level of $P \leq 0.05$ was considered statistically significant.

3.2.4. The Recovery assay

Plasmodium falciparum 3D7 parasites were cultured *in vitro*, as described in Chapter 2 (section 2.2.1). An early trophozoite-stage culture was used to prepare a 72 mL 2% haematocrit, 2% parasitaemia suspension in CMCM (Appendix 1.1) which was distributed into 6-well plates at 2.5 mL per well. Each plate was treated with drug and solvent control solutions, each in triplicate. An additional plate was prepared with untreated parasite suspension and uninfected RBCs in triplicate wells to serve as positive and backgrounds controls, respectively. The plates were transferred to an airtight chamber suffused with a special gas mixture (5% CO_2, 5% O_2, balance N_2; Air Products, RSA) and incubated at 37°C

for 6 hours. Following incubation, the contents of each well was mixed well and duplicate 800 µL samples were transferred to sterile microfuge tubes. The first set of samples were transferred to a 96-well tissue culture plate at 200 µL per well (plate A) and stored frozen. For the second set of samples, the tubes were centrifuged at 2500 rpm for 3 minutes and the supernatant was aspirated off. The pellet was washed thrice in 1 mL of CMCM (Appendix 1.1) pre-warmed at 37°C and then resuspended in fresh CMCM at a haematocrit of 1%. The suspensions were then transferred to a 96-well tissue culture plate at 200 µL per well (plate B). Plate B was placed in an airtight chamber, gassed and incubated at 37°C for 48 hours and thereafter stored frozen. Plasmodial lactate dehydrogenase (pLDH) activity was measured in plates A and B, as described in section 3.2.3 above. Significant differences between the parasite viability of control and treatment samples were determined using a two-tailed, paired Student‘s T-test (Microsoft Excel 2007). A probability level of $P \leq 0.05$ was considered statistically significant.

3.3. RESULTS

3.3.1. Assessing the utility of the ATP assay for evaluating the rate of drug-induced parasite stress

A sensitive assay for measuring parasite ATP levels in *P. falciparum* cultures using a luminescence-based kit was developed and optimised (see Chapter 2). The utility of the assay as a means of determining the rate of antimalarial drug action was explored by characterising the response of parasite ATP levels to a panel of antimalarials known to have varying rates of activity. This was achieved by preparing parallel control and drug-treated cultures and extracting samples from each culture every 2 hours over a 10-hour period. At each time-point the samples were used to measure parasite-associated ATP and prepare Giemsa-stained thin-blood smears to assess parasite morphology. Note: Effective treatment concentrations for each drug were based on median inhibition concentrations **(**IC50s) and/or test concentrations both from the current study and previous reports (detailed in Table 5.1, Appendix 5).

The results are presented in sections 3.3.1.1–3.3.1.5 below, with parasite-associated ATP levels presented both as absolute luminescence values and relative to controls. As in previous experiments (see section 2.3.7), all time 0 samples showed elevated ATP levels (data not shown), which were attributed to exposure of parasites to non-culture conditions during preparation for the experiment, and were therefore excluded from results. Furthermore, it was found that the pattern of ATP variation of control parasites over time differed between experiments (see panel ‗a' of Figures 3.1-3.5), even though all experiments were initiated at the early trophozoite stage of development on the basis of morphological assessments. Trophozoite-stage parasites are characterised by rapid growth and marked metabolic activity (Lazarus *et al*., 2008; Zolg *et al*., 1984) and previous investigations (see Figure 2.12 of Chapter 2) showed that the ATP levels of untreated parasites fluctuated extensively during trophozoite maturation. Hence, while morphological evaluations indicated that parasites were at the same trophozoite stage at the start of each experiment, the parasites may have been at different points of maturation and thus displayed different patterns of ATP variation over time.

3.3.1.1. Chloroquine

Results indicated that parasite-associated ATP levels of chloroquine-treated parasites closely matched those of control samples ($P > 0.05$; Table 6.1, Appendix 6) over the 10-hour incubation period (Figures 3.1a and 3.1b). Morphological changes in chloroquine-treated parasites (Figure 3.1c) were gradual with overall parasite growth appearing to slow down (i.e. smaller size) relative to controls from the 6-hour time-point onward, and some abnormal and dead parasites observed at the 10-hour time-point.

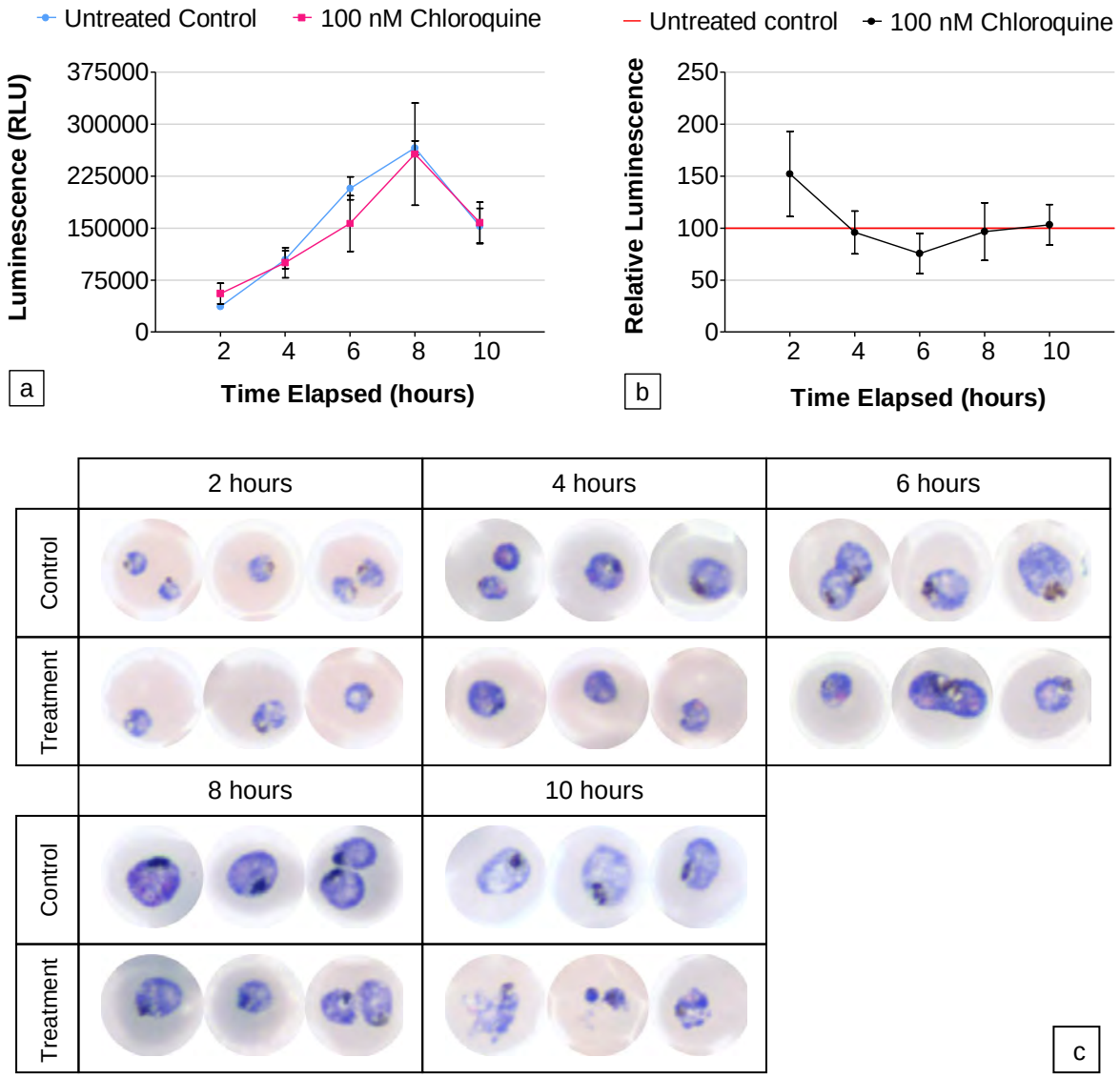

Figure 3.1: ATP levels and morphological changes in control and chloroquine-treated *P. falciparum* trophozoites over a 10-hour period. Samples of control and chloroquine (100 nM)-treated cultures were taken every 2 hours over a 10-hour period and parasite-associated ATP was measured. ATP results are presented as absolute values (Fig. 3.1a) and relative to controls (Fig. 3.1b) with each data point presented as the mean ± SD, n = 3. In addition, Giemsa-stained thin-blood smears were prepared from the cultures at each time-point. Photomicrographs of the smears (1000x magnification) are presented in Fig. 3.1c. (Note: (i) Most chloroquine-treated parasites were smaller in size relative to controls from 6 hours onward; (ii) parasites with aberrant morphology shown at the 10 hour Treatment panel represent only a small fraction of the parasite population – the remaining parasites displayed normal morphology similar to controls but were smaller in size.)

3.3.1.2. DFMO

As with chloroquine-treated samples, parasite-associated ATP levels of DFMO-treated parasites were not significantly different from those of controls ($P > 0.05$, Table 6.1, Appendix 6) over the 10-hour incubation period (Figures 3.2a and 3.2b). Examination of thin-blood smears (Figure 3.2c) indicated that morphology of DFMO-treated parasites appeared fairly normal throughout the 10-hour incubation. However, most DFMO-treated parasites were at an earlier developmental stage (judging from parasite size) than control parasites from the 8-hour time-point onward, suggesting a general slowing down of parasite growth (i.e. cell cycle delay).

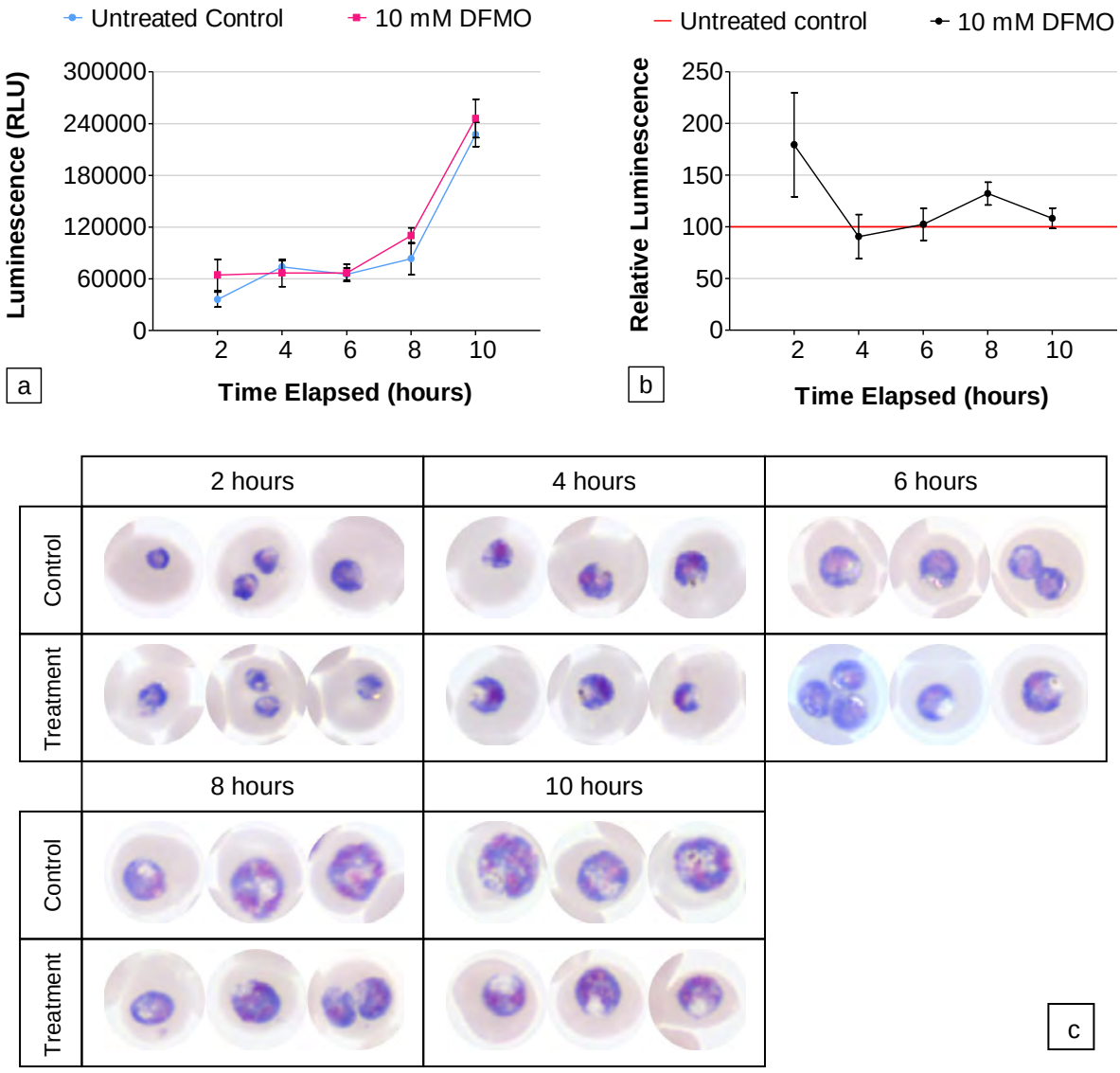

Figure 3.2: ATP levels and morphological changes in control and DFMO-treated *P. falciparum* trophozoites over a 10-hour period. Samples of control and DFMO (10 mM)-treated cultures were taken every 2 hours over a 10-hour period and parasite-associated ATP was measured. ATP results are presented as absolute values (Fig. 3.2a) and relative to controls (Fig. 3.2b) with each data point presented as the mean ± SD, n = 3. In addition, Giemsa-stained thin-blood smears were prepared from the cultures at each time-point. Photomicrographs of the smears (1000x magnification) are presented in Fig. 3.2c. (Note that the majority of DMFO-treated parasites were smaller in size than the controls from 8 hours onward.)

3.3.1.3. Mefloquine

Results showed that ATP levels of mefloquine-treated parasites were increased relative to controls from the 4-hour time-point onward (Figures 3.3a and 3.3b). The increases at the 4-hour (143%) and 6-hour (53%) time-points were statistically significant ($P < 0.05$, Table 6.1, Appendix 6) while those at the remaining time-points were not ($P > 0.05$). Even though parasite ATP levels were increased from the 4-hour time-point, aberrant parasite morphology was only observed from the 6-hour time-point. From the 6-hour time-point onward, mefloquine treatment resulted in an overall slowing down of parasite growth relative to controls (i.e. smaller size) and some abnormal parasite morphology in the form of irregularly-shaped parasites (Figure 3.3c). Evidence of parasite death was observed during the last 2 hours of incubation but only affected a small fraction of the parasite population.

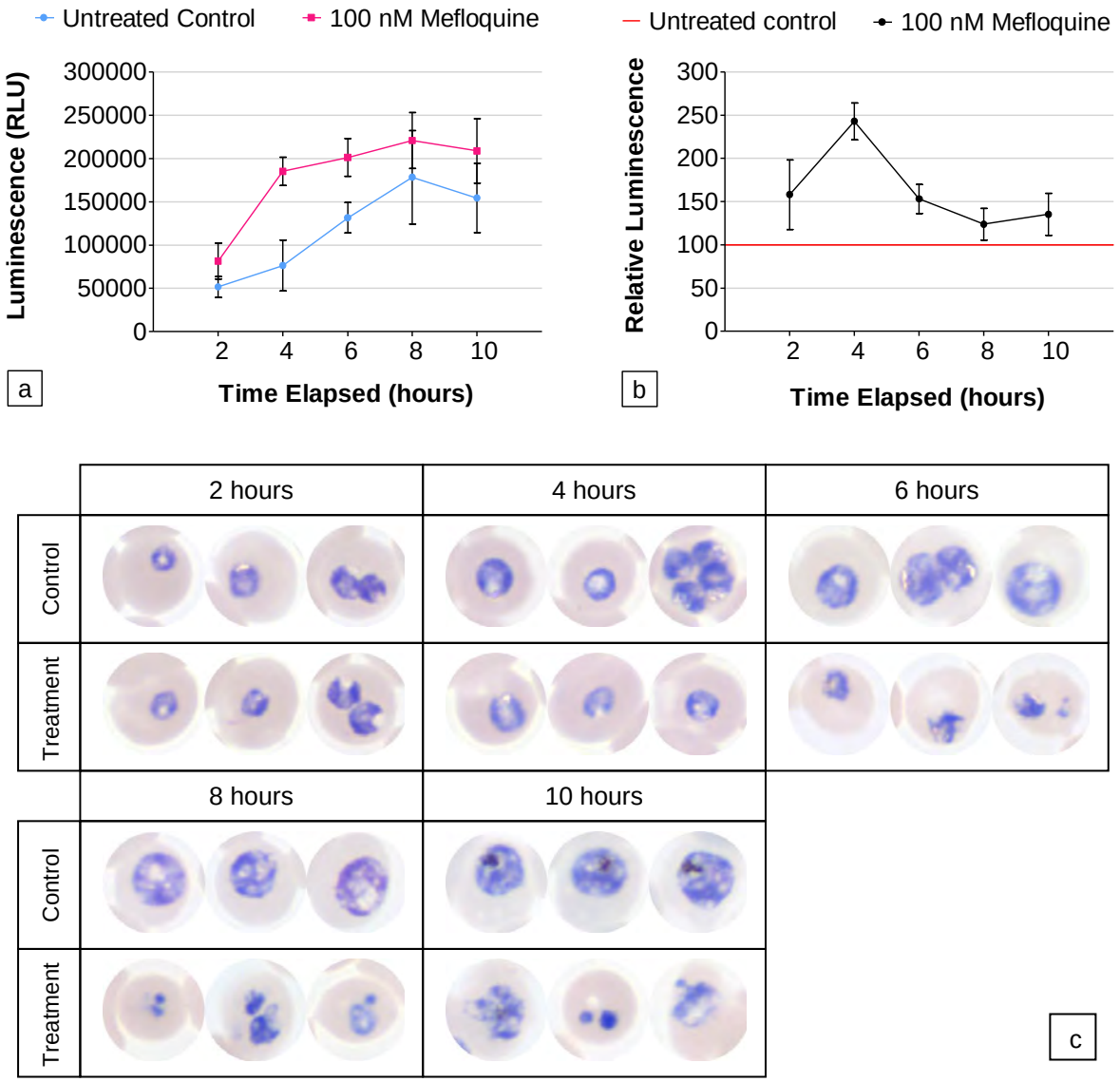

Figure 3.3: ATP levels and morphological changes in control and mefloquine-treated *P. falciparum* trophozoites over a 10-hour period. Samples of control and mefloquine (100 nM)-treated cultures were taken every 2 hours over a 10-hour period and parasite-associated ATP was measured. ATP results are presented as absolute values (Fig. 3.3a) and relative to controls (Fig. 3.3b) with each data point presented as the mean ± SD, n = 3. In addition, Giemsa-stained thin-blood smears were prepared from the cultures at each time-point. Photomicrographs of the smears (1000x magnification) are presented in Fig. 3.3c. (Note: (i) Most mefloquine-treated parasites were smaller in size relative to controls from 6 hours onward; (ii) aberrant parasite morphology and/or parasite death shown at the 6-10 hour Treatment panels represent only a small fraction of the parasite population – the remaining parasites displayed normal morphology similar to controls but were smaller in size.)

3.3.1.4. Artemisinin

Results indicated that the ATP levels of artemisinin-treated parasites were considerably (345%) higher than those of controls at the 2-hour time-point and remained elevated (80-141% higher) during the remaining 8 hours of incubation (Figures 3.4a and 3.4b). These elevations were statistically significant ($P \leq 0.05$, Table 6.1, Appendix 6) at all time-points except the 8-hour time-point ($P = 0.15$). As with the mefloquine treatment, even though elevated parasite ATP was recorded from the 2-hour time-point in artemisinin-treated parasites, abnormal parasite morphology, in the form of irregularly-shaped and dead parasites, was only observed later from the 4-hour time-point onward (Figure 3.4c). However, these morphological changes were only apparent in a small fraction of the parasite population. Furthermore, overall parasite growth of artemisinin-treated parasites was slower (i.e. smaller size) than that of control parasites from the 6-hour time-point onward.

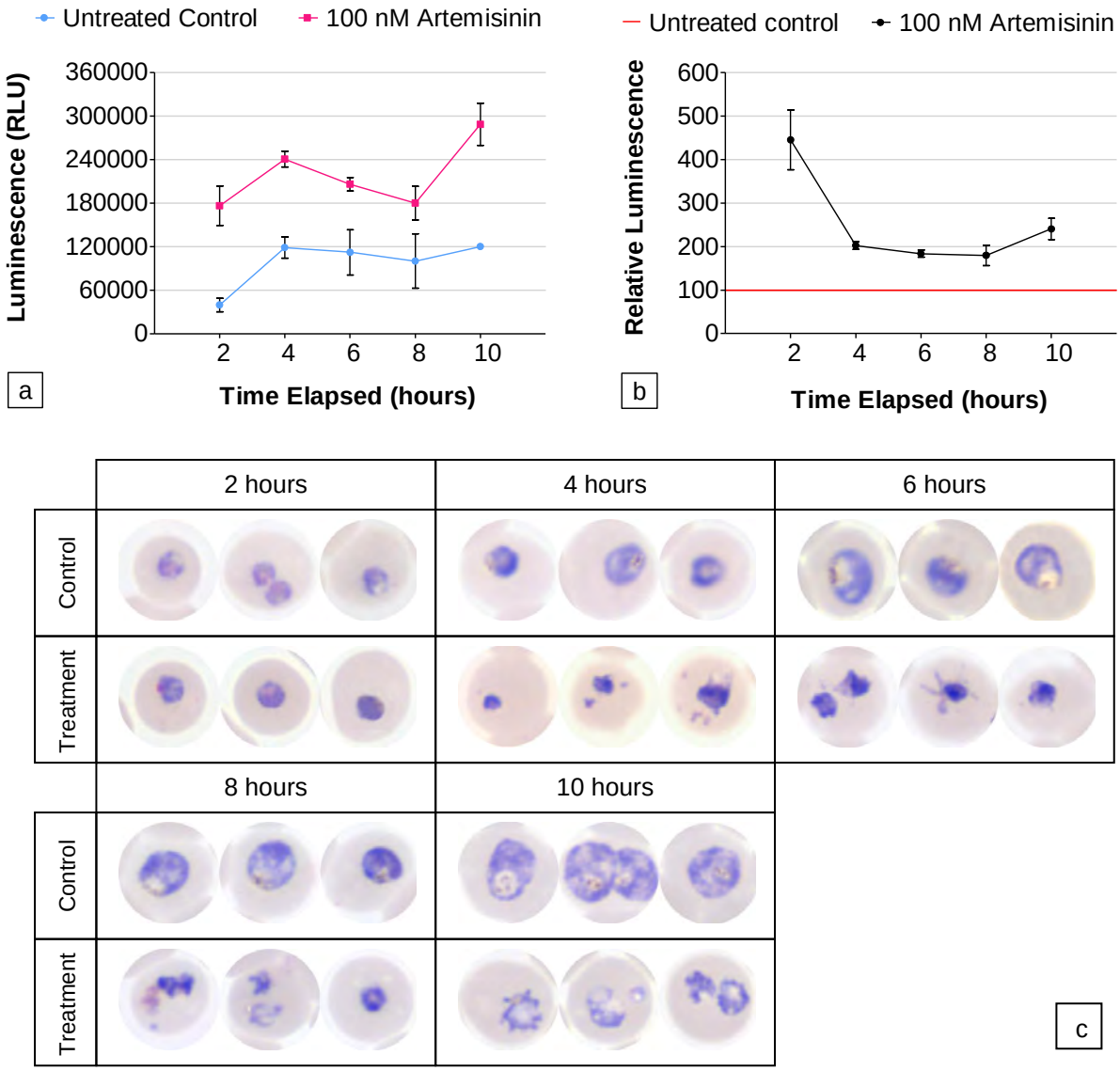

Figure 3.4: ATP levels and morphological changes in control and artemisinin-treated *P. falciparum* trophozoites over a 10-hour period. Samples of control and artemisinin (100 nM)-treated cultures were taken every 2 hours over a 10-hour period and parasite-associated ATP was measured. ATP results are presented as absolute values (Fig. 3.4a) and relative to controls (Fig. 3.4b) with each data point presented as the mean ± SD, n = 3. In addition, Giemsa-stained thin-blood smears were prepared from the cultures at each time-point. Photomicrographs of the smears (1000x magnification) are presented in Fig. 3.4c. (Note: (i) Most artemisinin-treated parasites were smaller in size relative to controls from 6 hours onward; (ii) aberrant parasite morphology and/or parasite death shown at the 4-10 hour Treatment panels represent only a small fraction of the parasite population – the remaining parasites displayed normal morphology similar to controls but were smaller in size from 6 hours onward.)

3.3.1.5. Ritonavir

Parasite-associated ATP levels of ritonavir-treated parasites were substantially decreased (≥ 96%) relative to controls from the 2-hour time-point onward (Figures 3.5a and 3.5b). These decreases were statistically significant ($P < 0.05$, Table 6.1, Appendix 6) at all time-points except the 4-hour time-point ($P = 0.09$; n = 2). Abnormal parasite morphology, delayed cell cycles and parasite death were observed from the 4-hour time point onward (Figure 3.5c) and affected most of the parasite population.

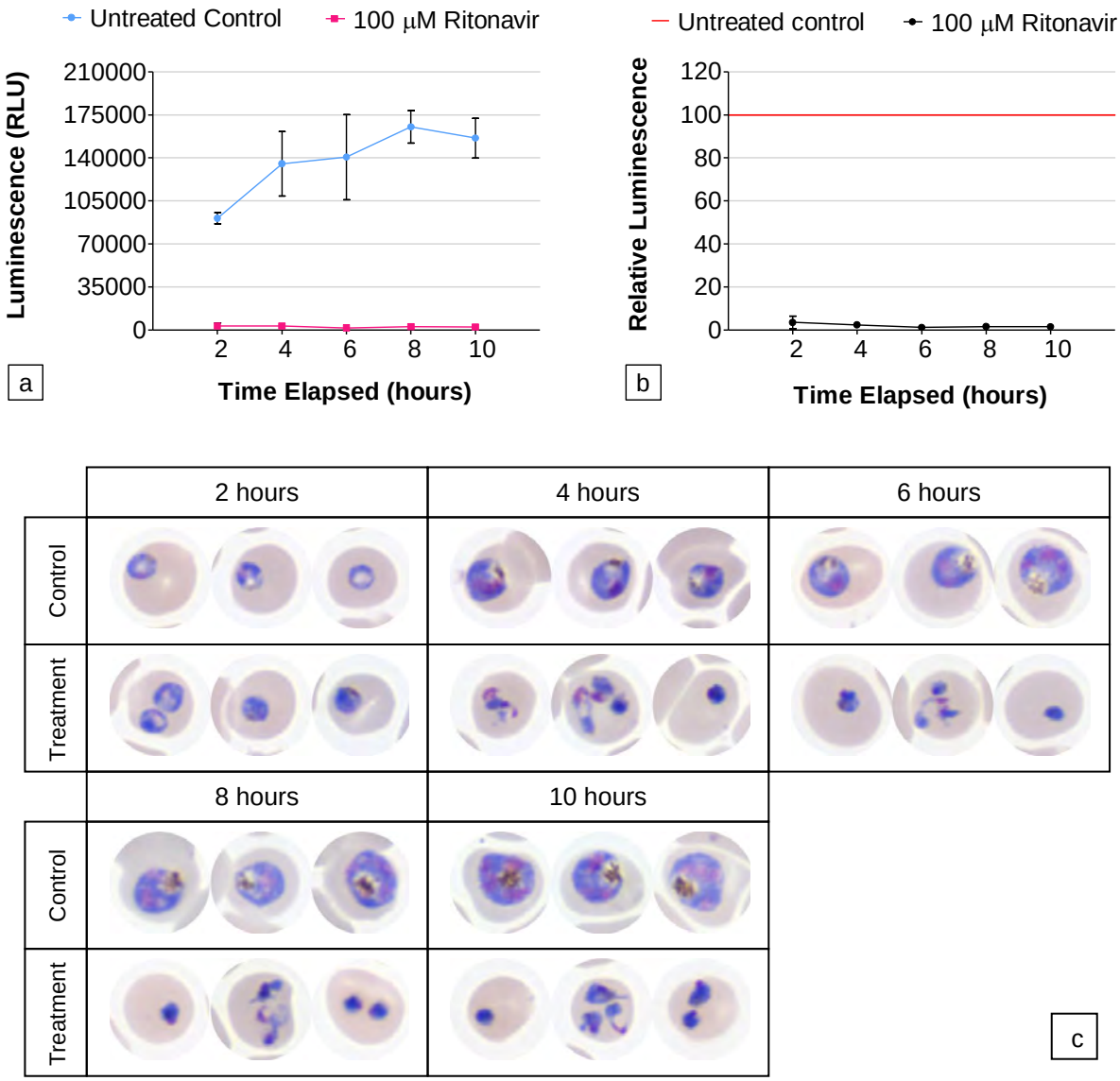

Figure 3.5: ATP levels and morphological changes in control and ritonavir-treated *P. falciparum* trophozoites over a 10-hour period. Samples of control and ritonavir (100 μM)-treated cultures were taken every 2 hours over a 10-hour period and parasite-associated ATP was measured. ATP results are presented as absolute values (Fig. 3.5a) and relative to controls (Fig. 3.5b) with each data point presented as the mean ± SD, n = 3. In addition, Giemsa-stained thin-blood smears were prepared from the cultures at each time-point. Photomicrographs of the smears (1000x magnification) are presented in Fig. 3.5c. (Note: (i) aberrant parasite morphology and parasite death shown at the 4-10 hour Treatment panels represent the majority of the parasite population – the remaining parasites displayed normal morphology similar to controls but were smaller in size from 4 hours onward.)

3.3.2. Determining whether drug-induced changes in parasite ATP levels are representative of compromised parasite viability: The Recovery assay

In order to determine whether the changes in ATP levels and parasite morphology in drug-treated cultures, described in section 3.3.1 above, were indicative of compromised parasite viability, the Recovery assay (detailed in section 3.2.4) was performed. The intra-erythrocytic phase of the parasite‘s life cycle lasts 48-hours. Therefore the purpose of the Recovery assay was to determine the percentage of cells that recover from drug treatment by completing their life cycle. This was achieved by incubating early trophozoite-stage *P. falciparum* cultures for 6 hours in control and drug-treated culture medium at concentrations equivalent to those used in the ATP assay (section 3.3.1). Following incubation, aliquots of the cultures were used to measure parasite viability by means of the pLDH assay (detailed in section 3.2.3). The medium in the remaining cultures was washed off and replaced with fresh medium and these drug-free cultures were incubated for a further 48 hours before measuring parasite viability via the pLDH assay. Results are shown in Figure 3.6.

The results indicated that following the 6-hour incubation, drug-treated cultures showed 0%-25% reduction in pLDH activity relative to controls. Ritonavir-treated parasites displayed the largest decline (25%), whilst DFMO treatment caused no decline. After the 48-hour incubation period, only 9% and 11% of chloroquine- and ritonavir-treated parasites, respectively, recovered from the 6-hour drug treatment compared to untreated controls. The DFMO-treated parasites showed the highest level of recovery with 85% of parasites still viable after the 6-hour drug treatment. In the case of mefloquine- and artemisinin-treated parasites, 56% and 46%, respectively, were still viable after the 6-hour drug treatment.

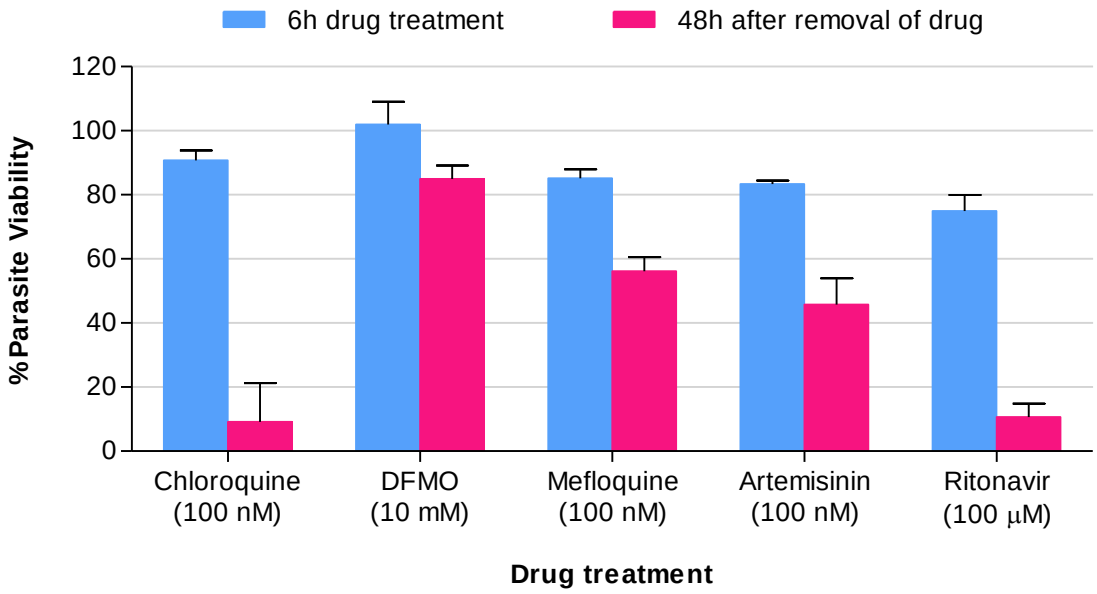

Figure 3.6: The Recovery assay: determining the extent to which a 6-hour drug treatment compromises parasite viability. Percentage parasite viability was measured using the pLDH assay in early trophozoite-stage *P. falciparum* cultures following a 6-hour drug treatment and 48 hours after washing off the drug. Viability was calculated relative to untreated control cultures. Each data point represents the mean ± SD, n = 3.

3.4. DISCUSSION

Adenosine triphosphate (ATP) serves as a primary source of energy for the growth, maintenance and reproduction of all living organisms. In most eukaryotes, ATP production involves the uptake of glucose followed by degradation to two pyruvate molecules through the process of glycolysis in the cytoplasm (Ginsburg, 2010). In the mitochondrion, pyruvate is converted to acetyl-CoA which enters the tricarboxylic acid cycle where it is fully oxidised to carbon dioxide via the process of oxidative phosphorylation (Olzewski & Llinás, 2010). This results in the production of 36 molecules of ATP, as well as biosynthetic precursors and reducing equivalents. Even though the intra-erythrocytic stages of *P. falciparum* possess mitochondria, they lack crucial complexes in the mitochondrial respiratory chain which are necessary to produce ATP aerobically via oxidative phosphorylation (Olzewski & Llinás, 2010). Consequently, similar to its host RBC, the parasite metabolises glucose anaerobically via the glycolytic pathway whereby pyruvate is ultimately fermented to lactate, yielding just two ATP molecules per molecule of glucose (Olzewski & Llinás, 2010).

The traditional *in vitro* method for evaluating the rate of antimalarial drug action by morphological assessments is highly subjective and time-consuming, and while a recently reported viability-based assay (Sanz *et al*., 2012) offers a quantitative alternative, it is far more time-consuming. Hence, there is a need for a novel, quantitative assay which is capable of rapidly determining this rate in order to more accurately compare the efficacy properties of novel antimalarials with those of existing drugs. Conceivably, parasite ATP levels may represent a sensitive and readily detectable means of diagnosing abnormal parasite metabolism brought about by drug action. Thus, a *P. falciparum* ATP assay was developed and optimised (see Chapter 2) and the utility of this assay as a means of evaluating the kinetics of antimalarial drug action was assessed. This was achieved by characterising the response of parasite ATP levels to a panel of antimalarials with varying modes of action and, presumably, rates of activity. A simultaneous assessment of parasite morphology was also carried out.

3.4.1. Effect of chloroquine on parasite ATP levels and morphology

The first drug that was evaluated was chloroquine, a 4-aminoquinoline derivative of quinine, which was the most successful, cost-effective and commonly used antimalarial since the 1940s, until the development of widespread parasite resistance rendered the drug ineffective in many malaria endemic regions (Grimberg & Mehlotra, 2011). It is widely accepted, on the

basis of several supporting studies, that chloroquine exerts its antimalarial action by inhibiting the detoxification of haem which is produced during haemoglobin digestion in the parasite‘s food vacuole (Combrinck *et al*., in press; Dorn *et al*., 1998; Egan *et al*., 1994; Ginsburg *et al*., 1998; O‘ Neill *et al.*, 2006; Sullivan *et al*., 1996; Sullivan *et al*., 1998; Zhang *et al*., 1999b).

During the intra-erythrocytic phase of its life cycle, the malaria parasite endocytoses 60-80% of the host cell cytoplasm of which 95% of the cytosolic protein is haemoglobin (Francis *et al*., 1997). The parasite degrades the haemoglobin in its food vacuole to produce amino acids and haem. Free haem has been shown to cause parasite lysis (Fitch *et al*., 1982; Orjih *et al*., 1981), and must be detoxified by the parasite. The principal means of detoxification involves the polymerisation of haem to form the chemically inert malaria pigment, haemozoin, which is stored in the food vacuole (Francis *et al*., 1997). Alternative mechanisms of haem detoxification have been proposed, e.g. degradation of haem by glutathione in the cytosol (Famin *et al*., 1999; Ginsburg *et al*., 1998; Loria *et al*., 1999; Zhang *et al*., 1999b) and peroxidative degradation of haem by hydrogen peroxide in the food vacuole (Loria *et al*., 1999), but haemozoin formation likely constitutes the major detoxification pathway (Egan *et al*., 2002).

Investigations into the antimalarial mode of action of chloroquine have suggested that, due to its weak base properties, it accumulates in the parasite‘s acidic food vacuole by pH trapping (Aikawa, 1972; Yayon *et al*., 1984; Yayon *et al*., 1985), where it binds to haem (Bray *et al*., 1999; Chou *et al*., 1980; Dorn *et al*., 1998; Sullivan *et al*., 1996). The binding of chloroquine to haem inhibits haem polymerisation (Dorn *et al*., 1998; Egan *et al*., 1994; Sullivan *et al*., 1996; Sullivan *et al*., 1998). The chloroquine-haem complexes have been suggested to cause parasite lysis (Fitch *et al*., 1982; Orjih *et al*., 1981), inhibition of haemoglobin degradative proteases (Gluzman *et al*., 1994; Vander Jaqt *et al*., 1986), and lipid peroxidation (Sugioka *et al*., 1987). Free haem has been shown to cause parasite lysis (Fitch *et al*., 1982; Orjih *et al*., 1981) likely due to its ability to alter the permeabilisation properties of membranes and disturb ion homeostasis (Chou & Fitch, 1981). In addition, Loria *et al.* (1999) found that haem displayed both peroxidase- and catalase-like activities, and therefore may play a role in the degradation of reactive oxygen species (ROS) which are produced during haemoglobin digestion in the parasite‘s food vacuole. Chloroquine was found to inhibit these enzyme-like activities which led the authors to suggest that this drug may cause parasite death by initiating

a toxic accumulation of ROS which are known to cause lipid peroxidation, DNA damage and oxidation of proteins (Bandyopadhyay *et al*., 1999). Thus, it is generally accepted that the antimalarial activity of chloroquine stems primarily from its accumulation in the parasite‘s food vacuole and inhibition of haem detoxification with a resulting toxic accumulation of haem and chloroquine-haem complexes. This theory is strongly supported by the studies of Ginsburg *et al.* (1998) and Zhang *et al*. (1999b) which showed that chloroquine treatment of *P. falciparum*-infected RBCs caused a dose-dependent inhibition of haem polymerisation with a concomitant increase in membrane-associated haem which correlated with parasite death. Similar findings were recently reported by Combrinck *et al*. (in press).

In the present study it was found that, in comparison to controls, chloroquine-treated parasites showed no noticeable change in ATP levels over a 10-hour incubation period. However, an assessment of parasite morphology revealed delayed cell cycles and some evidence of parasite death at the later time-points. Thus, chloroquine appeared to exhibit a relatively slow rate of antimalarial action. Ginsburg *et al*. (1998) found that, in chloroquine-treated *P. falciparum*-infected RBCs, parasite death was only observed after membrane-associated haem reached a critical level. Accordingly, the slow rate of antimalarial activity observed in this study may be partially due to the majority of the parasite population being at the early trophozoite-stage during the first 4 hours of incubation, with small food vacuoles and cell volumes that were relatively small relative to the host RBCs (see Figure 3.1c above). This implies a limited degree of RBC consumption by the parasite and hence low levels of free haem or chloroquine-haem complexes being produced. As time passed and the parasites consumed RBC cytoplasm more rapidly and grew in size, the accelerating generation of free haem may have only reached critical levels at the later time-points. This may explain why evidence of abnormal parasite morphology and parasite death was only observed after 10 hours of incubation. These morphological changes were not accompanied by variations in ATP levels perhaps because they did not yet translate into significant stress and an alteration in ATP homeostasis. In addition, only a small portion of parasites were abnormal or dead at the final time-point, as assessed by abnormal morphology, which may explain why a decrease in overall ATP levels was not observed.

The observed slight cell cycle delay in chloroquine-treated parasites relative to controls may be a result of the accumulation of free haem and chloroquine-haem complexes which have been shown to inhibit the proteases involved in haemoglobin digestion. The parasite relies on

its digestion of host-derived haemoglobin as a source of amino acids which are essential for its growth and development (Francis *et al*., 1997). Hence, the cell cycle delay observed in chloroquine-treated parasites may be due to inhibition of this critical process.

For the Recovery assay, *P. falciparum*-infected RBCs were exposed to drug-treated medium for 6 hours before washing off the drug and incubating parasites in drug-free medium for a further 48 hours. Results indicated that even though parasite viability measured immediately after the 6-hour chloroquine treatment was greater than 90%, only 9% of cells were truly viable and capable of completing their 48-hour life cycle after washing off the drug. This suggests that chloroquine severely compromised parasite viability during the 6-hour incubation, implying that it is a rapid acting antimalarial. However, this notion disagrees with results of the abovementioned ATP assay where an equivalent chloroquine treatment showed no major effect on ATP levels or overall morphology of parasites during the 10-hour incubation period, suggesting that chloroquine is slow-acting. These contradictory findings may be explained by the results of previous studies which have suggested that, due to its weak base properties, chloroquine is taken up by the parasite and accumulates within the acidic food vacuole by pH trapping (Aikawa, 1972; Yayon *et al*., 1984; Yayon *et al*., 1985). Accordingly, the poor performance of parasites in the Recovery assay as compared to the ATP assay is likely due to the accumulation and trapping of chloroquine in the parasite‘s food vacuole during the initial 6-hour incubation. This may have allowed the drug to escape removal during the subsequent washing step allowing it to remain in the parasite and continue to exert its toxic effects during the second 48-hour incubation, making it seem as if it acts more rapidly than it truly does.

3.4.2. Effect of DFMO on parasite ATP levels and morphology

The next drug that was investigated was DFMO (DL-α-difluoromethylornithine), a derivative of the amino acid ornithine, which inhibits polyamine synthesis through its inhibition of ornithine decarboxylase, a key enzyme in the polyamine synthetic pathway (Metcalf *et al*., 1978). Polyamines are low molecular weight nitrogenous bases essential to several cellular processes including growth, proliferation and differentiation (Clark *et al*., 2010; Sherman, 2009). Polyamine metabolism has been explored as a target for antimalarial compounds for more than two decades, however none of these compounds have reached the stage of a therapeutic drug. DFMO blocks the developmental progression of *P. falciparum* from the trophozoite to the schizont stage and this effect can be reversed by exposure of parasites to

polyamines (Assaraf *et al*., 1984; Assaraf *et al*., 1987; Gupta *et al*., 2005). Consequently, DFMO appears to have a cytostatic rather than a cytocidal effect on *P. falciparum in vitro*.

In the present study it was found that the ATP levels of DFMO-treated parasites closely matched those of controls over the 10-hour incubation period, as was the case with chloroquine-treated parasites. In addition, parasite morphology appeared normal throughout the incubation period. However, a delay in parasite growth was observed from the 8-hour time-point onward. Assaraf *et al*. (1987) found no abnormal morphology in DFMO-treated parasites after 47 hours of treatment. However, parasite growth was arrested in the trophozoite stage, which is compatible with the observations of this study. Results of the Recovery assay indicated that parasite viability was not significantly affected by a 6-hour exposure to DFMO, given that 85% of parasites were able to complete their life cycles after removal of the drug. Thus, the findings of this study agree with previous studies that suggest that DFMO has a cytostatic rather than a cytocidal effect on *P. falciparum.*

3.4.3. Effect of mefloquine on parasite ATP levels and morphology

Mefloquine, a 4-quinoline methanol derivative of quinine, is an antimalarial currently used primarily for chemoprophylaxis, as well as in combination with artemisinin for artemisinin-based combination therapy (Grimberg & Mehlotra, 2011). Even though mefloquine has been used clinically since 1975 there is still no consensus regarding its mechanism of action. Protease inhibitors have been shown to antagonise the antimalarial activity of mefloquine and chloroquine *in vitro* by blocking the activity of plasmepsin I which catalyses the initial reaction in haemoglobin degradation (Mungthin *et al*., 1998; Sullivan *et al*., 1998). This suggests that mefloquine, like chloroquine, may cause parasite death by interfering with haemoglobin metabolism which is essential for parasite growth and development. Several studies have reported antimalarial properties which mefloquine has in common with chloroquine: (i) binding to haem (Chevli & Fitch, 1982; Chou *et al*., 1980; Dorn *et al*., 1998); (ii) inhibition of haem polymerisation into haemozoin (Combrinck *et al*., in press; Dorn *et al*., 1998; Sullivan *et al*., 1998; Zhang *et al*., 1999b); (iii) inhibition of glutathione-mediated (Zhang *et al*., 1999b) and peroxidative (Loria *et al*., 1999) degradation of haem; (iv) inhibition of peroxidase- and catalase-like activities of haem (Loria *et al*., 1999). Based on these findings, it appears that mefloquine may share its mode of action with chloroquine by causing a toxic accumulation of haem or ROS.

On the other hand, there is also evidence that it may possess some additional or different mode(s) of antimalarial action compared to chloroquine. Mefloquine displays superior activity against chloroquine-resistant strains of *P. falciparum* (Geary & Jensen, 1983; Mungthin *et al*., 1998; Zhang *et al*., 1999b) and Chevli & Fitch (1982) found that mefloquine bound to membrane and purified phospholipids with high affinity, while chloroquine lacked this ability. Mefloquine and chloroquine also display differential effects on the endocytosis and digestion of host cell cytoplasm. Famin and Ginsburg (2002) found a correlation between parasite death and the accumulation of haemoglobin in chloroquine-treated parasites which was not observed with mefloquine. Instead mefloquine was found to inhibit chloroquine-mediated accumulation of haemoglobin. The authors proposed that chloroquine may exert its antimalarial effects by inhibiting haemoglobin digestion, while mefloquine may act earlier in haemoglobin metabolism by hindering ingestion of host cell cytoplasm. This theory was supported by a later study which investigated the effects of mefloquine and chloroquine on endocytosis in *P. falciparum* (Hoppe *et al*., 2004). Chloroquine treatment caused an accumulation of haemoglobin which was attributed to a block in the fusion of endocytic vesicles with food vacuoles. Mefloquine caused reduced haemoglobin levels, likely due to an inhibition of haemoglobin endocytosis. Thus, mefloquine appears to block haemoglobin metabolism at an earlier stage by blocking endocytosis of host cell cytoplasm, while chloroquine may act later by inhibiting haemoglobin digestion. This may explain mefloquine‘s superior antimalarial effect on chloroquine-resistant parasites, when compared to chloroquine.

In this study, the response of parasite ATP to mefloquine treatment was assessed by exposing *P. falciparum* cultures to the drug and measuring parasite ATP levels every 2 hours over a 10-hour period. The ATP levels of mefloquine-treated parasites were more than 2-fold higher than those of controls at the 4-hour time-point, with less pronounced elevations observed at the remaining time-points. Evidence of abnormal morphology in mefloquine-treated parasites was observed from the 6-hour time-point onward with an overall delay in parasite growth relative to controls and some irregularly-shaped parasites. Occasional parasite death was observed during the last 2 hours of incubation. In contrast, chloroquine-treated parasites showed no major change in ATP levels over the 10-hour incubation period with overall cell cycle delays from the 6-hour time-point and some abnormal and dead parasites observed only at the final time-point. These differential ATP and morphological responses imply that mefloquine acts at a faster rate than chloroquine and supports the notion that these drugs act

via different modes of action. Previous reports provide evidence that mefloquine inhibits endocytosis of host cell cytoplasm while chloroquine inhibits haemoglobin digestion (Hoppe *et al*., 2004). Thus, mefloquine appears to act at an earlier stage of haemoglobin metabolism than chloroquine which may explain why it acts at a faster rate and provokes a different ATP response in parasite cultures than chloroquine.

Results of the Recovery assay showed that 56% of parasites recovered from a 6-hour mefloquine treatment suggesting that increased ATP levels may be indicative of compromised parasite viability. Interestingly, despite the apparent slower rate of action of chloroquine, chloroquine-treated parasites recover much less efficiently in the Recovery assay than those treated with mefloquine. As discussed previously, this may be attributable to an inability to wash out chloroquine due to the pH-trapping mechanism of food vacuole accumulation of the drug. Clearly, this does not apply equally to mefloquine, thus suggesting an additional difference between chloroquine and mefloquine action.

It is generally accepted that the antimalarial activity of chloroquine is due to an inhibition of haem detoxification. Mefloquine has been proposed to share many antimalarial properties with chloroquine and appears to interfere with haemoglobin metabolism, but its precise mode of action remains a mystery. Therefore, it is difficult to fully explain the cause for increased ATP levels in mefloquine-treated parasites, other than to make a general assumption that it reflects increased metabolic activity by the parasite as part of its stress response in order to overcome detrimental drug effects. Any form of macromolecular damage in eukaryotic cells that surpasses a set limit, regardless of the cause, initiates what is known as the cellular stress response (Kültz, 2003). This response is intended to momentarily increase tolerance limits to macromolecular damage using a phylogenetically conserved array of genes and pathways that regulate the stabilisation and repair of macromolecules in order to promote cellular integrity (Kültz, 2003). Several highly conserved stress proteins are involved in this process, including molecular chaperones, DNA repair proteins and cell cycle regulators (Kültz, 2003). Some of the most well recognised stress proteins are the molecular chaperones known as heat shock proteins (HSPs) which mediate the correct folding, processing and functioning of proteins in an ATP-dependent manner (Acharya *et al*., 2007; Pesce & Blatch, 2009; Shonhai *et al*., 2007; Vonlaufen *et al*., 2008). The major chaperone classes of HSP40, HSP60, HSP70 and HSP90 have been identified in malaria parasites and are thought to be involved in normal cell functioning, as well as stress responses (Acharya *et al*., 2007; Pesce & Blatch, 2009; Shonhai

et al., 2007; Vonlaufen *et al*., 2008). These chaperones are ATPases and hence require ATP to drive the folding of client proteins (Acharya *et al*., 2007; Pesce & Blatch, 2009; Shonhai *et al*., 2007). Hence, the increased ATP levels in mefloquine-treated parasites observed in this study may reflect an enhanced production of ATP to meet the requirements of the stress response.

3.4.4. Effect of artemisinin on parasite ATP levels and morphology

The next drug under investigation in this study was artemisinin, a sesquiterpene trioxane lactone. Artemisinin and its derivatives are currently the only class of established antimalarials to which the parasite has not yet developed widespread resistance. These drugs form the cornerstone of artemisinin-based combination therapies which have significantly reduced the number of malaria cases worldwide since their introduction as a first-line treatment for uncomplicated *P. falciparum* malaria (Greenwood *et al*., 2008). A key feature of artemisinin treatment is that it clears blood-stage parasites far more efficiently than other established antimalarials (van Agtmael *et al*., 1999; WHO, 2010a). Despite their success, the precise antimalarial mode of action of artemisinins is still under discussion and numerous theories have been put forward.

The endoperoxide bridge of artemisinin, which has the potential to produce free radicals via homolytic cleavage of the weak oxygen peroxide bond (Jung, 1997), is thought to play a key role in its antimalarial activity since artemisinin derivatives which lack this peroxide bridge display poor antimalarial activity *in vitro* (Brossi *et al*., 1988). Consequently, for several decades, oxidative stress has been thought to play a key role in the antimalarial activity of artemisinin due to its chemical ability to produce free radicals. This view is supported by findings that antioxidants antagonise the antimalarial activity of artemisinin both *in vitro* (Krungkrai & Yuthavong, 1987) and *in vivo* (Levander *et al*., 1989). Several studies have demonstrated the formation of artemisinin-haem adducts (Loup *et al*., 2007; Meshnick *et al*., 1991; Paitayatat *et al*., 1997; Pandey *et al*., 1999; Robert *et al*., 2005; Robert & Meunier, 1997) which would allow for cleavage of the drug‘s endoperoxide bridge by the iron centre of haem, thereby producing toxic free radicals. Glutathione (GSH) is an important antioxidant of the malaria parasite and it has been suggested that, in addition to producing free radicals, artemisinin may promote oxidant stress in the parasite by reducing free GSH levels due to its ability to form adducts with glutathione (Wang & Wu, 2000). In addition, dihydroartemisinin,

the active metabolite of artemisinin, has been shown to decrease the levels of GSH and other antioxidants in *P. falciparum*-infected RBCs (Ittarat *et al*., 2003).

Evidence supporting the iron-mediated production of free radicals from artemisinin was provided by Meshnick and colleagues (1993) who showed that: (i) incubation of artemisinin with iron resulted in the production of free radicals; (ii) iron chelators antagonised the antimalarial effects of artemisinin *in vivo* and of its derivative, arteether, *in vitro*; (iii) a combination of artemisinin and hemin resulted in the oxidation of RBC membrane protein thiols, an effect that was reduced by iron chelators and free radical scavengers. These results, as well as supporting evidence from other studies, led Meshnick (1994) to suggest that the antimalarial action of artemisinins relies on two sequential steps, activation and alkylation. Activation being the iron-catalysed cleavage of the peroxide bridge to produce a free radical, followed by alkylation of malaria proteins through interaction with the activated drug to form covalent adducts. Artemisinin and dihydroartemisinin, have been shown to alkylate malarial proteins in culture (Asawamahasakda *et al*., 1994) and haemoproteins in solution (Yang *et al*., 1994), respectively.

In addition to the formation of artemisinin-haem adducts resulting in a toxic accumulation of free radicals, this interaction may disrupt haem polymerisation in the parasite (Hong *et al*., 1994; Kannan *et al*., 2005; Loup *et al*., 2007; Pandey *et al*., 1999). Furthermore, free artemisinin has been shown to degrade haemozoin (Pandey *et al*., 1999). Thus, in addition to causing a toxic accumulation of free radicals, artemisinin may exert its antimalarial activity by causing a toxic accumulation of haem. An additional mode of antimalarial action proposed for artemisinin is a disruption of haemoglobin metabolism in the parasite by inhibiting its ability to degrade haemoglobin (Pandey *et al*., 1999) and endocytose host cell cytoplasm (Hoppe *et al*., 2004). However, artemisinin is active against the intra-erythrocytic ring stages of *P. falciparum* which lack haemozoin and do not degrade haemoglobin (White, 2008; Woodrow *et al*., 2005). Therefore, an inhibition of haemoglobin metabolism is clearly not the only means by which artemisinin exerts its antimalarial activity.

The parasite‘s mitochondrion, which is essential for pyrimidine biosynthesis (van Dooren *et al*., 2006), has recently been implicated as a potential target of artemisinin. Using a yeast model, Li *et al*. (2005) found that artemisinin inhibited growth and depolarised mitochondrial membrane potential. In addition, deletion and overexpression of NADH dehydrogenases,

which play a role in the mitochondrial electron transport chain, resulted in increased resistance and sensitivity to artemisinin in the respective mutant strains. The artemisinin-resistant mutant strains produced fewer ROS than their artemisinin-sensitive counterparts, implying that the mitochondrial electron transport chain is involved in activating artemisinin to produce free radicals. Based on these observations the authors suggested that, in the malaria parasite, artemisinin may be activated by the mitochondrial electron transport chain resulting in a local production of free radicals which may damage the mitochondrial membrane, causing a loss in membrane potential and ultimately parasite death. A recent ultrastructural study of *P. falciparum* by Crespo *et al*. (2008) revealed that a 4-hour artemisinin treatment had no obvious effect on the membrane potential or morphology of mitochondria in trophozoite-stage parasites. In contrast, the mitochondria of ring-stage parasites displayed partially intact membrane potential and significantly disrupted membrane structure following a 24-hour artemisinin treatment. On the basis of these findings, the authors suggested that disruption of mitochondrial membrane potential may be a late-stage effect of artemisinin and may not be its primary mode of antimalarial action.

The sarco-endoplasmic reticulum calcium ATPase (SERCA) of the malaria parasite has also been proposed as a specific protein target of artemisinin. Calcium is required by *P. falciparum* for RBC invasion, intracellular signalling and development within the host cell, and SERCAs are responsible for maintaining calcium ion concentrations by actively directing calcium ions into membrane-bound stores (Alleva & Kirk, 2001; Nagamune *et al*., 2008). Thapsigargin is a specific inhibitor of SERCA and based on its structural similarity to artemisinin, Eckstein-Ludwig *et al*. (2003) hypothesised that the antimalarial activity of activated artemisinins may involve selective and specific inhibition of the *P. falciparum* SERCA, PfATP6. Artemisinin was found to specifically eliminate the calcium-dependent ATPase activity of PfATP6 expressed in oocytes, while artemisinin competed with fluorescently tagged thapsigargin for binding to intraparasitic sites. Subsequent studies have both supported and opposed these findings. Jambou *et al*. (2005) showed that resistance to the artemisinin derivative, artemether, was associated with a polymorphism in the PfATP6 gene, thereby supporting the notion that PfATP6 may be targeted by artemisinins. A three-dimensional docking simulation study by Jung *et al*. (2005) demonstrated an interaction of artemisinin and its derivatives with a model of the thapsigargin binding site in PfATP6, supporting the notion of a shared site of action for these compounds. In contrast, a similar docking study by Garah *et al*. (2009) found no correlation between the *in silico* binding

affinity of artemisinin for PfATP6 and its *in vitro* antimalarial activity. In addition, Crespo *et al.* (2008) found that the antimalarial activity of artemisinin was not antagonised by thapsigargin, thus suggesting that these two compounds act on different sites within the parasite.

In the current study, artemisinin treatment resulted in increased parasite ATP levels throughout a 10-hour incubation period, as was the case with mefloquine-treated parasites. However, while elevated ATP levels were observed from the 4-hour time-point onward in mefloquine-treated parasites, in the case of artemisinin this increase was observed from the earlier 2-hour time-point onward. This suggests that artemisinin is a rapid-acting antimalarial which acts at a faster rate than mefloquine, which is agreement with previous *in vitro* (Gamo *et al*., 2010; Sanz *et al*., 2012) and *in vivo* (Karbwang *et al*., 1992; Myint & Shwe, 1987; White, 1994; White *et al*., 1992) studies that have shown artemisinins to be more rapid-acting than quinines. Some evidence of abnormal parasite morphology and parasite death in artemisinin-treated parasites was observed from the 4-hour time-point onward, while a delay in overall parasite growth relative to control parasites was observed from the 6-hour time-point onward. Results of the Recovery assay indicated that close to half the parasite population (46%) recovered from a 6-hour exposure to artemisinin, implying that increased ATP levels may be indicative of compromised parasite viability, as was the case with mefloquine. In addition, the ATP levels of artemisinin-treated parasites were increased to a greater degree than those of mefloquine-treated parasites. Peak ATP levels of artemisinin- and mefloquine-treated parasites were 4.5-fold and 2.4-fold higher than those of controls, respectively, implying that artemisinin may cause a greater level of parasite stress than mefloquine.

As discussed previously, a substantial body of evidence supports the notion that oxidative stress plays a key role in the antimalarial action of artemisinin, due to its capacity to produce free radicals via cleavage of its endoperoxide bridge (Jung, 1997; Krungkrai & Yuthavong, 1987; Levander *et al*., 1989; Loup *et al*., 2007; Meshnick, 1994; Meshnick *et al*., 1991; Meshnick *et al*., 1993; Paitayatat *et al*., 1997; Pandey *et al*., 1999; Robert *et al*., 2005; Robert & Meunier, 1997). Consequently, the increased ATP levels of artemisinin-treated parasites observed in this study may reflect efforts by the parasite to increase its antioxidant defences in order to overcome oxidative stress. The malaria parasite is exposed to high levels of oxidant stress during the intra-erythrocytic stage of its life cycle (Müller, 2001; Sherman,

2009). Its haemoglobin-containing host RBC is rich in oxygen and iron which play key roles in the production of ROS via the Fenton reaction (Müller, 2001; Sherman, 2009). In addition, the parasite endocytoses much of the host cell cytoplasm and free haem is released upon digestion of haemoglobin within its acidic food vacuole (Francis *et al*., 1997). This leads to spontaneous oxidation of haem iron from its ferrous form to its ferric form, during which electrons are released which react with molecular oxygen to produce superoxide anions (Francis *et al*., 1997; Müller, 2001; Sherman, 2009). Furthermore, free haem and superoxide anions are prerequisites for the generation of more ROS, such as hydroxyl radicals and hydrogen peroxide, via the Fenton reaction (Müller, 2001; Sherman, 2009).

Thus, during its intra-erythrocytic development, the malaria parasite is exposed to several ROS which are kept at low intracellular levels by a multiplex of enzymatic and non-enzymatic antioxidants (reviewed by (Müller, 2001; Nickel *et al*., 2006; Rahlfs *et al*., 2002; Sherman, 2009)), as illustrated in Figure 3.7. Superoxide dismutase, the first line of defence against ROS, converts superoxide anions to hydrogen peroxide which is further reduced by peroxiredoxins and a GSH peroxidase-like protein which obtain reducing equivalents from the redox-active thioredoxin (TRX). The NADPH-dependent TRX reductase subsequently regenerates reduced TRX which, in turn, restores oxidised glutathione disulphide (GSSG) to its reduced form, GSH, thereby serving as a link between the GSH and TRX redox systems. Glutathione, the most abundant antioxidant in malaria parasites, has been shown to reach a cytosolic concentration of ~2 mM in *P. falciparum* trophozoites via *de novo* synthesis, which involves two ATP-dependent reactions. The GSH antioxidant system detoxifies ROS via the oxidation of glutaredoxin which obtains reducing equivalents from GSH which is, in turn, replenished via the NADPH-dependent reduction of GSSG catalysed by GSH reductase. Glutathione also serves as a cofactor for enzymatic antioxidants, a reductant for antioxidant vitamins and is capable of reducing ROS non-enzymatically. The parasites two major redox systems (GSH and TRX) are NADPH-dependent and reduced NADPH is regenerated via the pentose phosphate pathway which requires glucose-6-phosphate that is produced from glucose via an ATP-dependent, hexokinase-catalysed reaction (Barrett, 1997).

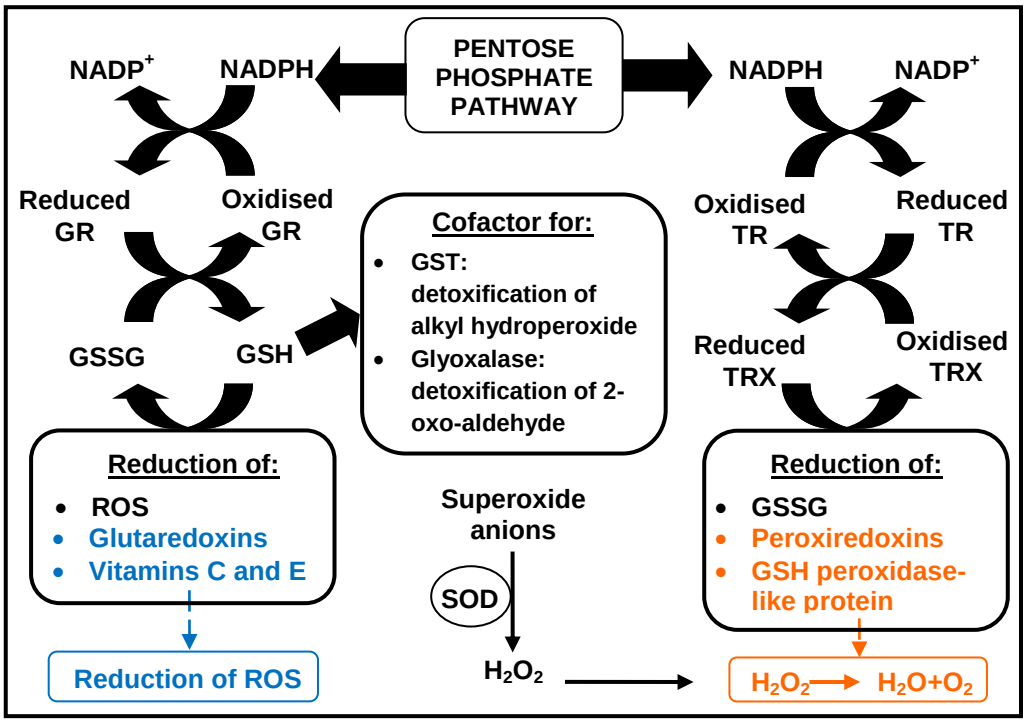

Figure 3.7: The antioxidant defence mechanisms of *P. falciparum*. NADPH, Nicotinamide adenine dinucleotide phosphate hydrogen; $NADP^+$, oxidised NADPH; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulphide; ROS, reactive oxygen species; GST, glutathione-S-transferase; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; H_2O, water; O_2, oxygen; TRX, thioredoxin; TR, thioredoxin reductase.

Hence, the elevated ATP levels of artemisinin-treated parasites observed in this study may reflect efforts by the parasite to overcome oxidative stress induced by the drug. This likely necessitates increased production of ATP to fuel synthesis of the enzymes, substrates and co-factors involved in its various antioxidant defence systems. In particular, its most abundant antioxidant GSH is synthesised *de novo* via two ATP-dependent reactions and the regeneration of NADPH relies on the ATP-dependent production of glucose-6-phosphate.

An alternative justification for the increased ATP levels in artemisinin-treated parasites may be associated with the proposed artemisinin-mediated inhibition of the *P. falciparum* SERCA, PfATP6 (Eckstein-Ludwig *et al*., 2003; Jambou *et al*., 2005; Jung *et al*., 2005). The SERCA regulates intracellular calcium stores by transporting two molecules of calcium from the parasite‘s cytoplasm to the lumen of the endoplasmic reticulum with the concomitant hydrolysis of an ATP molecule (Nagamune *et al*., 2008). Large bursts of calcium ions are involved in intracellular signalling, therefore SERCAs are essential to parasite survival

because they allow for recurring events of intracellular signalling by rapidly restoring calcium concentrations (Alleva & Kirk. 2001; Haynes & Krishna, 2004; Nagamune *et al*., 2008). During drug exposure the parasite attempts to overcome detrimental drug effects and it is likely that there is an increase in intracellular signalling and hence a concomitant increase in ATP production in order to fuel the activity of the SERCA. Thus, the increased ATP levels in artemisinin-treated parasites may be due to inhibition of the *P. falciparum* SERCA, PfATP6, which would prevent its ATP-mediated function resulting in an accumulation of ATP.

Finally, the increased ATP levels may reflect increased ATP production by the parasite in order to fuel an increased production and function of stress proteins in response to drug stress, in particular the HSPs which are ATP-dependent (as discussed previously for mefloquine).

3.4.5. Effect of ritonavir on parasite ATP levels and morphology

In addition to characterising the ATP response of *P. falciparum* to conventional antimalarials (i.e. chloroquine, mefloquine and artemisinin) and a cytostatic drug (i.e. DFMO), ritonavir, a compound which is not used clinically as an antimalarial but displays potent antimalarial activity, was also investigated. Ritonavir is a protease inhibitor developed to target the aspartyl protease of HIV type-1 and has significantly contributed to improving the outcome of HIV disease in developed countries (Chandwani & Shuter, 2008). In 2003, the WHO launched its ~~"3~~ by 5" initiative aimed at providing antiretrovirals to 3 million individuals infected with HIV-1, particularly in developing countries, by 2005. In most developing countries malaria is co-endemic with HIV, hence this initiative resulted in many malaria-infected individuals being exposed to antiretrovirals. This prompted Skinner-Adams *et al*. (2004) to investigate the *in vitro* effects of antiretroviral protease inhibitors on *P. falciparum,* and ritonavir was found to directly inhibit parasite growth at clinically relevant concentrations. Later studies confirmed the antimalarial activity of ritonavir against *P. falciparum in vitro* (Andrews *et al*., 2006; He *et al*., 2010; Parikh *et al*., 2005; Parikh *et al*., 2006) and demonstrated its *in vivo* activity against *P. chabaudi* in mice (Andrews *et al*., 2006). Despite evidence of its antimalarial activity, ritonavir is unlikely to become a first-line antimalarial due to its high cost, potential toxicity and limited knowledge with regard to interactions with established antimalarial drugs (Skinner-Adams *et al*., 2004).

In this study, it was found that ritonavir almost completely depleted the ATP levels of *P. falciparum* parasites. This effect was seen from the 2-hour time-point and persisted for the remainder of the 10-hour incubation. Abnormal parasite morphology, delayed cell cycles and parasite death were observed from the 4-hour time point onward in the majority of the parasite population. Furthermore, it was observed during sample processing that, relative to controls, the parasite pellet isolated from ritonavir-treated culture samples was minimal up to the 4-hour time-point, and barely visible at the remaining time-points. These observations suggested that the depleted ATP levels were likely due to severely compromised parasite viability, and parasite death. This was confirmed by results of the Recovery assay which showed that only 11% of parasites were truly viable and capable of completing their intra-erythrocytic life cycle after a 6-hour exposure to ritonavir.

The exact mechanism of antimalarial action of ritonavir is unclear, however, based on the fact that it was developed to target an aspartyl protease of HIV-1, Andrews *et al*. (2006) hypothesised that the drug targets the parasite‘s plasmepsins (PMs) which are also aspartyl proteases. Ten different PM isoforms have been identified in *P. falciparum* and the most well-characterised are PM-I, PM-II, PM-IV and histoaspartic protease (HAP) which are involved in haemoglobin digestion (Sherman, 2009). Andrews *et al*. (2006) used modelling and ligand docking methods to show that ritonavir has the potential to bind to the active sites of *P. falciparum* plasmepsins, PM-II and PM-IV. In addition, they found that ritonavir inhibited the activity of these enzymes *in vitro*. In contrast, Parikh *et al.* (2006) found that there was no significant difference between the antimalarial activity of ritonavir against *P. falciparum* wildtype parasites and those with PM-I, PM-II, HAP or PMI/IV knocked out. The very rapid action of ritonavir, as reported here, also makes an inhibition of haemoglobin digestion as the primary mode of action unlikely. During its intra-erythrocytic development the malaria parasite exports hundreds of proteins which alter cytoadherence, nutrient uptake and structural integrity of its host cell in order to support parasite survival (Sherman, 2009). Plasmepsin V was recently identified as an essential enzyme in this process (Boddey *et al*., 2010) and ritonavir was found to inhibit its activity. Hence it is possible that the antimalarial effects of ritonavir observed in this study may have been due to a disruption of the parasite‘s protein trafficking ability through inhibition of PM-V.

3.5. CONCLUSION

Taken together, the ATP results suggest that chloroquine and DMFO are slow-acting antimalarials, mefloquine and artemisinin have moderate rates of antimalarial action, and ritonavir is a rapid-acting antimalarial. With regard to chloroquine, mefloquine and artemisinin which are established antimalarials, these findings are in agreement with the expected rates of activities of these drugs. This supports the notion that measurement of parasite ATP levels may be a potential tool for assessing the rate of antimalarial drug action. The results of this study suggesting that ritonavir is a rapid-acting antimalarial are, to our knowledge, the first reports of the rate of activity of this antimalarial.

With chloroquine and DFMO, parasite ATP levels did not differ significantly from untreated controls over a 10-hour treatment period. This agrees with the very minor morphological changes observed and, in the case of DFMO, with the Recovery assay results. The marked activity of chloroquine in the Recovery assay is likely due to its entrapment in the parasite‘s food vacuole and prolonged exposure of the parasites to the drug beyond the 6-hour treatment window, and thus not a true reflection of its rate of action. In the case of artemisinin and mefloquine treatments, which caused rapid increases in overall parasite ATP levels, the Recovery assay showed that more or less half the parasite population was viable following a 6-hour drug exposure. This implies that elevated parasite ATP levels may be diagnostic of some degree of eventual loss of viability. Ritonavir caused a rapid and considerable decrease in overall parasite ATP accompanied by morphological evidence of parasite death in the majority the parasite population. These observations agreed with the Recovery assay results which showed that only about a tenth of ritonavir-treated parasites remained viable following a 6-hour exposure. These findings suggest that depleted ATP levels may be indicative of severely compromised parasite viability.

Based on the above discussion, the current working hypothesis would thus be that unchanged ATP levels reflect a lack of significant drug action, increased ATP levels a stress response to drug action and indicative of some loss of viability, while significantly reduced ATP is indicative of severe loss of parasite viability. Cell cycle delays observed in the drug-treated parasites relative to controls may be a survival mechanism whereby the parasite slows down its metabolism, resulting in a decrease in potential metabolic drug targets, such as haem (Veiga *et al*., 2010). This reduced metabolism may also allow for the specific induction of drug transporters to extrude the drug, thereby averting its toxic effects.

Examination of Giemsa-stained thin-blood smears is the traditional method of assessing the rate of antimalarial activity. In the case of mefloquine- and artemisinin-treated parasites, rapid increases in ATP levels were observed implying that these drugs are rapid-acting. While cell cycle delays were observed in the majority these parasites, instances of abnormal morphology and parasite death were only observed in a small fraction of the parasite population. Thus, examination of parasite morphology without concurrent measurement of parasite ATP may have led to the erroneous conclusion that these antimalarials are more slow-acting than is actually the case. This further illustrates the subjective and unreliable nature of this traditional method of assessing the kinetics of drug action. In addition, even though morphological abnormalities affected the majority of the parasite population in the ritonavir treatments, initial changes in ATP levels were not accompanied by immediate changes in parasite morphology, which were only observed at later time-points. These findings imply that measuring parasite ATP levels provides a more appropriate reflection of the earliest parasite responses to drug action than examining parasite morphology, particularly in the case of fast-acting antimalarials.

By contrast, parasite ATP levels do not appear to be as sensitive to cell cycle delays. For example, in comparison to controls, both chloroquine- and DFMO-treated parasites displayed morphological cell cycle delays with fairly unchanged ATP levels. In addition, occasional parasite death was observed in chloroquine-treated parasites after 10 hours of treatment without any change in parasite ATP in comparison to controls. Hence, in the case of a slow-acting antimalarial where parasite death may only affect a fraction of the parasite population, or where drug action results in a slow inhibition of parasite growth, measuring parasite ATP alone may not provide a complete representation of parasite response to drug action. Therefore, we conclude that the best approach for assessing the rate of antimalarial drug action would be a simultaneous evaluation of parasite ATP levels and parasite morphology to take advantage of their complimentary roles vis-à-vis rapid- and slow-acting drugs.

CHAPTER 4: EXPLORING A LUCIFERASE ASSAY USING TRANSGENIC PARASITES AS AN ALTERNATIVE METHODOLOGY FOR EVALUATING THE RATE OF DRUG-INDUCED PARASITE STRESS

4.1. INTRODUCTION

Transfection involves the transfer of exogenous nucleic acid sequences into an organism (Waterkeyn *et al*., 1999). The development of transient and stable transfection technologies for blood-stage *P. falciparum* parasites has allowed for the expression of transgenes and aided the functional study of parasite proteins via the disruption, modification or substitution of the genes that encode them (de Koning-Ward *et al*., 2000; O'Donnell *et al*., 2001). This, in turn, leads to a better understanding of parasite biology, thereby revealing novel drug targets and vaccine candidates (de Koning-Ward *et al*., 2000; O'Donnell *et al*., 2001). Luciferase is commonly used as a reporter for promoter characterization and transcriptional regulation studies in transfected mammalian cells (Kim *et al*., 2009; Yee *et al*., 2009) and *Plasmodium* parasites (Helm *et al*., 2010; Sunil *et al*., 2008). Thus, in order to investigate transcriptional regulation in *P. falciparum*, the Systems Biology Group at CSIR Biosciences successfully established methods for transfecting *P. falciparum* with expression plasmids and used these to generate luciferase-expressing transgenic parasites. Luciferase activity in these parasites is readily detected using a luminescence-based kit. Unexpectedly, preliminary experiments indicated that luciferase activity decreased rapidly and markedly in the transgenic parasites when exposed to the standard antimalarials artemisinin and mefloquine (data not shown). Conceivably, luciferase activity measurements in these transgenic parasites may thus be used to assess the rate of action of antimalarials. To investigate this further, these parasites were made available for this study and the luciferase system was explored as an alternative to the ATP assay (see Chapters 2 and 3) as a means of evaluating the rate of antimalarial drug action using a panel of antimalarials with varying mechanisms of action and, presumably, rates of activity.

4.2. MATERIALS AND METHODS

4.2.1. Transfection

Frozen stocks of transgenic *P. falciparum* parasites stably transfected with episomal luciferase expression plasmids were kindly donated by Dr. A.C. van Brummelen (CSIR Biosciences). The transfection protocol used to prepare the parasites is described in Appendix 4.1. Two luciferase-expressing parasite lines were acquired, each with a different 5‘ promoter region: (i) HSP-Luc (heat shock protein 86 promoter), (ii) LYS-Luc (lysophospholipase promoter, PlasmoDB gene ID: PF14_0017).

The plasmid construct (Figure 4.1) was comprised of *Photinus pylaris* (firefly) luciferase and human dihydrofolate reductase (hDHFR) expression cassettes in a head-to-head orientation in a pHTK (Duraisingh *et al*., 2002) backbone. The luciferase coding region was flanked by the 5‘ promoter region and the *Plasmodium berghei* 3‘ termination region. The hDHFR coding region was flanked by the 5‘ calmodulin promoter and the histidine-rich protein 2 3‘ termination region. By generating resistance to the antifolate WR99210, hDHFR acted as a selection marker that allowed transgenic parasites to be obtained from transfected parasite populations. The frozen parasite stocks were thawed and cultured, as described in Chapter 2 (section 2.2.1), using CMCM (Appendix 1.1) supplemented with 5 nM WR99210.

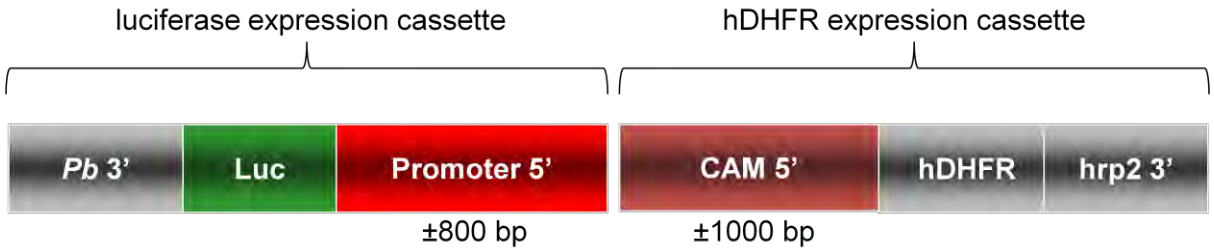

Figure 4.1: Diagram of the plasmid construct used to generate transgenic luciferase-expressing parasites. *Pb* 3‘ – *Plasmodium berghei* 3‘ termination region; Luc – *Photinus pylaris* (firefly) luciferase; CAM 5‘ – 5‘ calmodulin promoter; hDHFR – human dihydrofolate reductase; hrp2 3‘ – histidine-rich protein 2 3‘ termination region.

4.2.2. The Luciferase assay

Luciferase activity in parasites was measured using a luminescence-based kit (Bright-Glo Luciferase Assay System, Promega, Madison, WI). Briefly, parasite suspensions with 5% haematocrit, 2% parasitaemia were prepared in CMCM (Appendix 1.1) supplemented with 5 nM WR99210. A suspension of uninfected RBCs (5% haematocrit) was prepared as a background control. Samples (200 µL) of the suspensions were transferred to microfuge tubes and centrifuged at 8000 rpm for 30 seconds. The supernatant was aspirated off and the infected RBC pellet was resuspended in 100 µL of Glo lysis buffer (Promega, Madison, WI) pre-warmed at 22°C. The sample was incubated for 5 minutes at room temperature before mixing the lysate and transferring 100 µL to a white Lumitrac 96-well plate (Greiner, Germany). Pre-prepared Bright-Glo reagent (100 µL, Appendix 4.2), pre-warmed at ≤ 25°C, was added to each well and luminescence was measured in a multimode plate reader (Infinite F500, Tecan, Austria).

4.2.3. Measurement of luciferase activity in drug-treated transgenic parasites

Transgenic luciferase-expressing HSP-Luc parasites were prepared and maintained as described in section 4.2.1 above. An early trophozoite-stage culture was used to prepare a 5% haematocrit, 2% parasitaemia suspension in CMCM (Appendix 1.1) supplemented with 5 nM WR99210. Five identical test plates were prepared as follows: (i) parasite suspension (200 µL) was added to triplicate wells of each plate before adding the drug or solvent control solution; (ii) uninfected RBCs at 5% haematocrit (200 µL) were added to triplicate wells as background controls. The plates were then transferred to an airtight chamber suffused with a special gas mixture (5% CO_2, 5% O_2, balance N_2; Air Products, RSA) and incubated at 37°C. At 2-hour intervals, one plate was carefully removed from the chamber without disturbing the settled RBCs and 150 µL of supernatant was removed from all wells and discarded. Luciferase activity was then measured as described in section 4.2.2 above. Significant differences between the luciferase activity of control and treatment samples were determined using a two-tailed, paired Student‘s T-test (Microsoft Excel 2007). A probability level of $P \leq 0.05$ was considered statistically significant.

4.3. RESULTS

Apart from the ATP assay discussed in Chapter 3, the Luciferase assay was investigated as an alternative means of assessing the rate of antimalarial activity. The assay relies on transgenic luciferase-expressing *P. falciparum* parasites which were kindly prepared and donated by Dr. A. C. van Brummelen of the CSIR Systems Biology group (see Appendix 4.1 for details of the transfection protocol). The Luciferase assay involves the rapid detection of luciferase activity of the transgenic parasites using the luminescence-based Bright-Glo Luciferase Assay System (Promega, Madison, WI). Briefly, samples of trophozoite-stage transgenic cultures were prepared at a 5% haematocrit and 2% parasitaemia. Infected RBCs were lysed using the Glo lysis buffer (Promega, Madison, WI) and luciferase activity in the lysate was detected using the Bright-Glo reagent (details in section 4.2.2 above). Linearity and sensitivity of the assay were analysed before testing the suitability of the assay as a means of assessing the rate of antimalarial drug action.

4.3.1. Linearity and sensitivity of the Luciferase assay

With the aim of gauging the strength of the correlation between measured luminescence signals and parasite luciferase activity, HSP-Luc parasite suspensions were prepared from culture samples and serially diluted before measuring luciferase activity. Briefly, an 800 µL suspension of trophozoite-stage transgenic parasites was prepared at a haematocrit and parasitaemia of 5%. The haematocrit was maintained while performing 8 x 2-fold serial dilutions to a final volume of 800 µL before measuring luciferase activity (as described in section 4.2.2). Parasite numbers in each sample were calculated from the parasitaemia, and RBC concentration which was determined using a haemacytometer. Results are shown in Figure 4.2. Luminescence signals correlated excellently with parasite numbers ranging from $55x10^3$ to $70x10^5$, evident by the corresponding linear regression correlation coefficient (r^2 value) of 0.98. Thus, for parasite numbers ranging from $55x10^3$ to $70x10^5$ per sample, the luminescence signals produced by the Luciferase assay strongly correlate with parasite luciferase activity. However, the connecting line in Figure 4.2 could suggest a modest deviation from linearity at the highest parasite concentrations, which may indicate substrate limitation and/or upper limits of luciferase detection being approached at these parasite numbers. In addition to analysing the linearity and sensitivity of the Luciferase assay, the precision and reproducibility of the assay were also assessed.

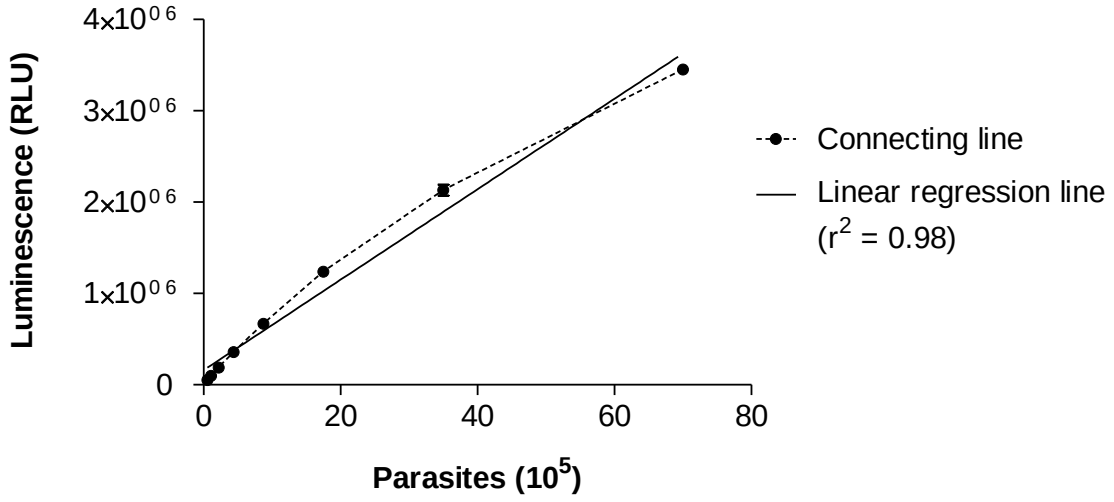

Figure 4.2: Relationship between parasite luciferase activity and parasite concentrations for the Luciferase assay. The strength of the correlation between measured luminescence signals and parasite luciferase activity was assessed by measuring luciferase activity in samples with different concentrations of HSP-Luc parasites. Each data point represents the mean ± SD, n = 2.

4.3.2. Precision and reproducibility of the Luciferase assay

As with the ATP assay, the precision of results obtained with the Luciferase assay was assessed by calculating coefficients of variation (CVs) and Z‘ factors (details in section 2.2.4 of Chapter 2) for experiments described in this chapter. Results are summarised in Table 4.1.

Table 4.1: Summary of coefficients of variation (CVs) and Z' factors obtained using the Luciferase assay.

Figure	Number of samples	Average % CV	Average Z'
4.2	8	2.3	0.93
4.3a	10	1.6	0.95
4.4a	10	1.0	0.97
4.5a	10	2.4	0.93
4.6a	10	2.6	0.92
4.6c	10	2.6	0.92
4.7a	10	4.5	0.86
4.9	2	1.9	0.94
4.12	10	2.3	0.93
	Total: 80	Average: 2.4	Average: 0.93

Note: (i) The "Figure" column contains the number of the figure in this chapter which corresponds to the experiment from which the data was extracted to do the calculations; (ii) The "Number of samples" refers to the number of parasite samples that were subjected to the assay in parallel and does not take into account control samples presented in the figures. Luciferase activity in each pellet was read in duplicate wells (n=2); (iii) CVs were calculated from results obtained from replicate test samples; (iv) Z' factors were calculated by comparing samples to background controls (uninfected RBCs).

The results showed that for a total of 80 samples processed on separate occasions in the Luciferase assay, an excellent overall CV of 2.4% was achieved. Furthermore, the average Z' factor of 0.93 indicates an excellent assay window. Therefore, the Luciferase assay has a broad dynamic range and is an appropriate means of measuring luciferase activity in culture samples with a reasonable level of precision.

Inter-occasional reproducibility of the Luciferase assay was analysed by establishing the degree to which RLU values varied when the assay was performed on separate occasions. This was accomplished by calculating the CV for mean RLU values of corresponding (i.e. 2-hour time-point) control samples obtained on different occasions during experiments to assess the kinetics of drug action, as described later in this chapter (see section 4.3.3). Results are shown in Table 4.2 below.

Table 4.2: Inter-occasional reproducibility of the Luciferase assay.

Figure	Mean RLU
4.3a	1139813
4.4a	1151288
4.5a	1099264
4.6a	1986252
4.6c	1148399
4.7a	799961
Inter-assay	
Average	1220829.5
SD	398604.0
CV	32.7

Note: (i) The "Figure" column contains the number of the figure in this chapter from which the experimental data was extracted to do the calculations; (ii) The "Mean RLU" column contains RLU values measured in corresponding (i.e. 2-hour time-point) control samples using the Luciferase assay (detailed in section 4.3.3).

The results indicated that inter-assay RLU values of corresponding control samples varied considerably (CV = 32.7%) for reasons that have not been investigated further. Therefore, absolute values recorded on different occasions from corresponding experiments cannot be directly compared except relative to internal controls, unless the purpose of the comparison is to assess stage variability.

4.3.3. Assessing the utility of the Luciferase assay for evaluating the rate of drug-induced parasite stress

As with the ATP assay (see Chapter 3), the effectiveness of the Luciferase assay as a means of assessing the kinetics of antimalarial drug action was tested by characterising the response of parasite luciferase activity to a panel of antimalarial drugs with varying rates of activity. In the case of the ATP assay, parasites were isolated from RBCs before measuring ATP in order to exclude RBC ATP. However, isolation of parasites from RBCs was unnecessary for the Luciferase assay since the RBCs lack luciferase activity. Consequently, the experimental protocol of the Luciferase assay required fewer liquid handling steps and was thus considerably more amenable to multiple sample processing than the ATP assay (see sections 4.2.3 and 3.2.2 for details of the respective protocols). This allowed for the Luciferase assay to be performed in a 96-well plate format with several drugs assayed in a single experiment. Five identical test plates containing parallel control and drug-treated HSP-Luc cultures were prepared and luciferase activity was measured every 2 hours over a 10-hour period by

subjecting one plate to the Luciferase assay, as described in section 4.2.3. Treatment concentrations for each drug were the same as those used for the ATP assay (see Chapter 3), except in the case of artemisinin to which the transgenic HSP-Luc parasites displayed resistance. Results are presented both as absolute values and relative to controls in sections 4.3.3.1–4.3.3.5 below.

4.3.3.1. Chloroquine

Results indicated that the luciferase activity of control parasites increased throughout the incubation period with an overall increase of 115% between the 2- and 10-hour time-points (Figure 4.3a). The time-dependent increase in the luciferase activity of chloroquine-treated parasites occurred at a slower rate than that of controls (Figure 4.3a). Consequently, the luciferase activity of treated parasites was progressively lower than that of controls with an average decline of 5% between time-points (Figure 4.3b). At the final time-point luciferase activity of treated parasites was 20% lower than that of control parasites ($P < 0.05$; Table 7.1, Appendix 7.1).

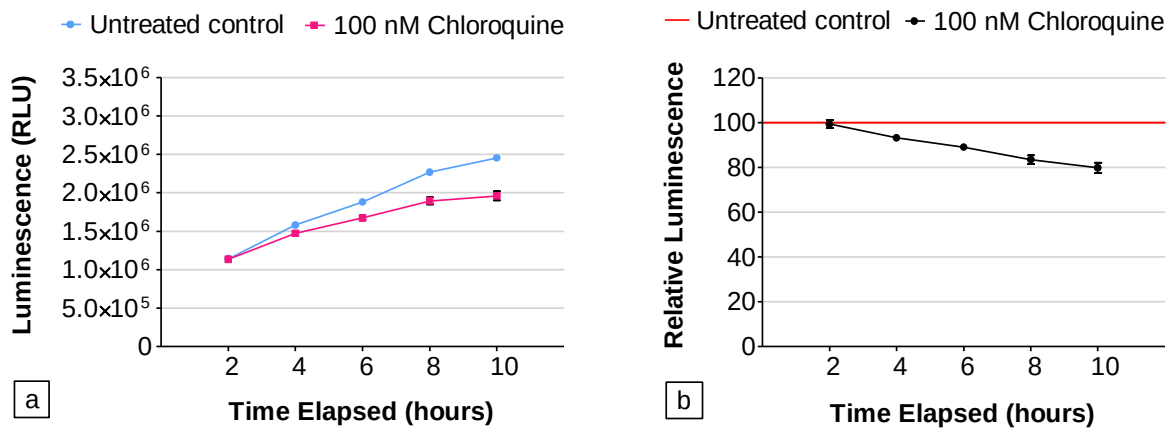

Figure 4.3: Luciferase activity in control and chloroquine-treated HSP-Luc parasites over a 10-hour period. Luciferase activity was measured in samples of control and chloroquine (100 nM)-treated cultures every 2 hours over a 10-hour period. Results are presented as absolute values (Fig. 4.3a) and relative to controls (Fig. 4.3b) with each data point presented as the mean ± SD, n = 3.

4.3.3.2. DFMO

The response of parasite luciferase activity to DFMO treatment was similar to that seen in chloroquine-treated parasites. Both control and DFMO-treated parasites showed an increase in luciferase activity with time. However, from the 6-hour time-point onward, luciferase activity of DFMO-treated parasites increased at a slower rate than that of controls (Figure 4.4a). Over the 10-hour incubation period, the overall increase in the luciferase activity of control and treated parasites was 106% and 69%, respectively. As with chloroquine, the luciferase activity of DFMO-treated parasites gradually decreased relative to controls with an average decline of 5% between time-points (Figure 4.4b). Luciferase activity of DFMO-treated parasites was 17% lower than that of controls at the final time-point ($P < 0.05$; Table 7.1, Appendix 7.1).

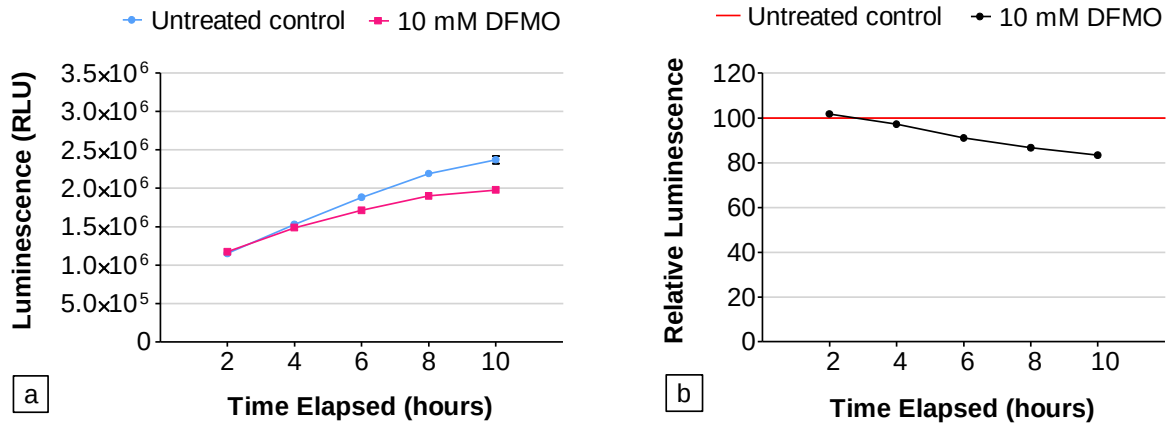

Figure 4.4: Luciferase activity in control and DFMO-treated HSP-Luc parasites over a 10-hour period. Luciferase activity was measured in samples of control and DFMO (10 mM)-treated cultures every 2 hours over a 10-hour period. Results are presented as absolute values (Fig. 4.4a) and relative to controls (Fig. 4.4b) with each data point presented as the mean ± SD, n = 3.

4.3.3.3. Mefloquine

Luciferase activity of control parasites increased throughout the 10-hour incubation period with an overall increase of 120% (Figure 4.5a). Unlike the moderate effect on parasite luciferase activity seen with chloroquine and DFMO treatments, mefloquine had a far more pronounced effect on the transgenic parasites. Luciferase activity of mefloquine-treated parasites was 44% lower than that of controls at the 2-hour time-point ($P < 0.05$; Table 7.1, Appendix 7.1) and remained relatively unchanged at the remaining time-points (Figure 4.5a). Consequently, the luciferase activity of treated parasites was, on average, 66% below that of controls during the last 6 hours of incubation (Figure 4.5b; $P < 0.05$, Table 7.1, Appendix 7.1).

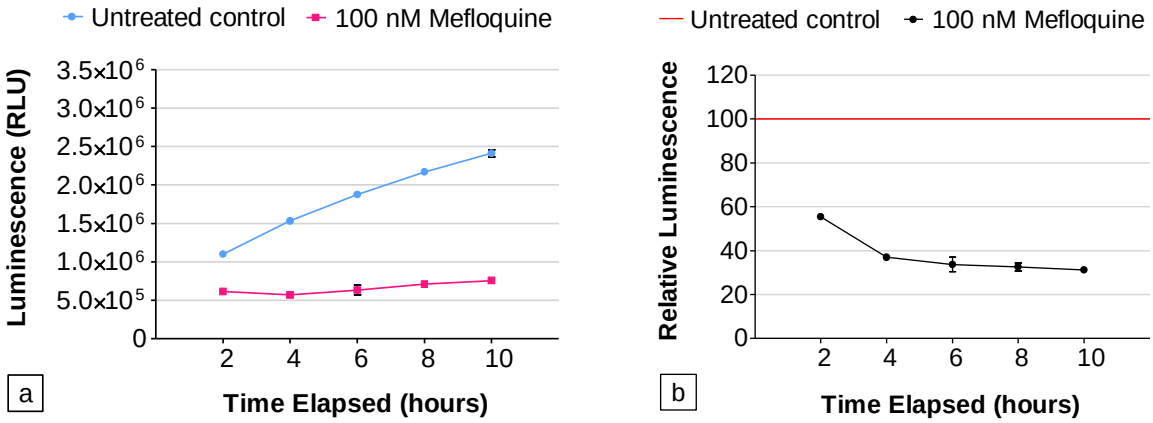

Figure 4.5: Luciferase activity in control and mefloquine-treated HSP-Luc parasites over a 10-hour period. Luciferase activity was measured in samples of control and mefloquine (100 nM)-treated cultures every 2 hours over a 10-hour period. Results are presented as absolute values (Fig. 4.5a) and relative to controls (Fig. 4.5b) with each data point presented as the mean ± SD, n = 3.

4.3.3.4. Artemisinin

The luciferase activity of control parasites did not increase throughout the 10-hour incubation period (Figure 4.6a), as was the case in previous experiments (see Figures 4.3a, 4.4a and 4.5a). This may be due to a higher degree of synchronisation of the developmental stages of the parasite population in the previous experiments. Luciferase activity of HSP-Luc parasites treated with 100 nM artemisinin closely matched that of untreated parasites throughout the 10-hour incubation period (Figures 4.6a and 4.6b). This was unexpected since the 100 nM artemisinin treatment had caused significantly elevated ATP levels in the wildtype parasites used in the ATP assay (see section 3.3.1.4 of Chapter 3). Consequently, the IC50s of artemisinin against the wildtype and HSP-Luc parasites were compared and it was found that the transgenic parasites were less sensitive to artemisinin (see section 4.3.4 below). Accordingly, the Luciferase assay was repeated using a higher concentration of artemisinin (500 nM).

The 500 nM artemisinin treatment was found to have a significant effect on parasite luciferase activity, similar to that obtained with mefloquine. The luciferase activity of control parasites increased throughout the 10-hour incubation period with an overall increase of 111% (Figure 4.6c). In contrast, the luciferase activity of artemisinin-treated parasites decreased by 71% between the 2- and 4-hour time-points and remained relatively unchanged during the remainder of the incubation (Figure 4.6c). As a result, the luciferase activity of treated parasites was 67% below that of controls at the 2-hour time-point and, on average, 96% lower than that of controls at the remaining time-points (Figure 4.6d; $P < 0.05$, Table 7.1, Appendix 7.1). This pattern of decreasing luciferase activity in artemisinin-treated parasites, relative to controls, was similar to that observed with mefloquine, however, the artemisinin treatment caused more pronounced decreases.

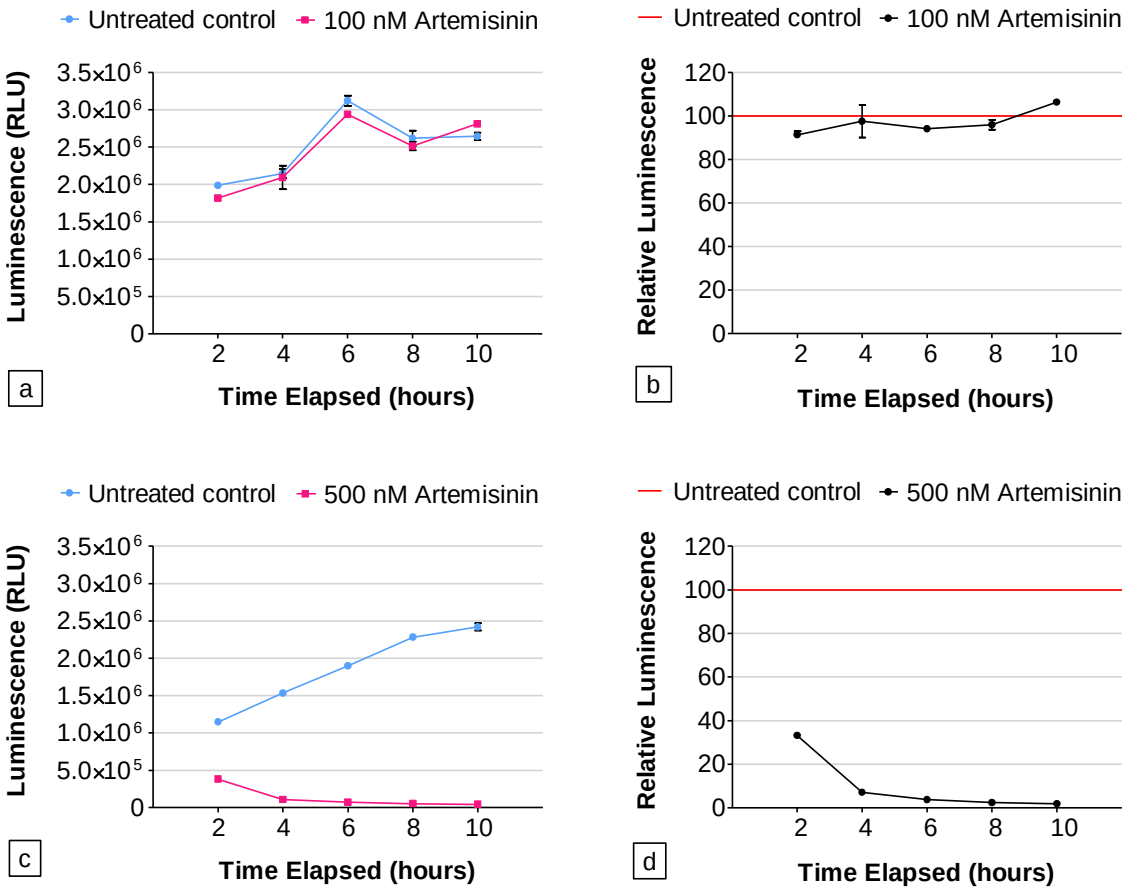

Figure 4.6: Luciferase activity in control and artemisinin-treated HSP-Luc parasites over a 10-hour period. Luciferase activity was measured in samples of control and artemisinin-treated cultures every 2 hours over a 10-hour period in two separate experiments with respective artemisinin concentrations of 100 nM and 500 nM. Results are presented as absolute values (Fig. 4.6a and 4.6c) and relative to controls (Fig. 4.6b and 4.6d) with each data point presented as the mean ± SD, n = 3.

4.3.3.5. Ritonavir

The luciferase activity of control parasites increased substantially (256%) during the first 8 hours of incubation before decreasing by 23% during the last 2 hours (Figure 4.7a). In contrast, the luciferase activity of ritonavir-treated parasites decreased by 16% between the 2- and 4-hour time-points, with a further decrease of 92% during the remaining 6 hours of incubation (Figure 4.7a). As a result, the luciferase activity of treated parasites was 34% and 64% lower than that of controls at the 2- and 4-hour time-points, respectively and, on average, 95% below that of controls at the remaining time-points (Figure 4.7b; $P < 0.05$, Table 7.1, Appendix 7.1). This pattern of luciferase activity was similar to parasites treated with 500 nM artemisinin.

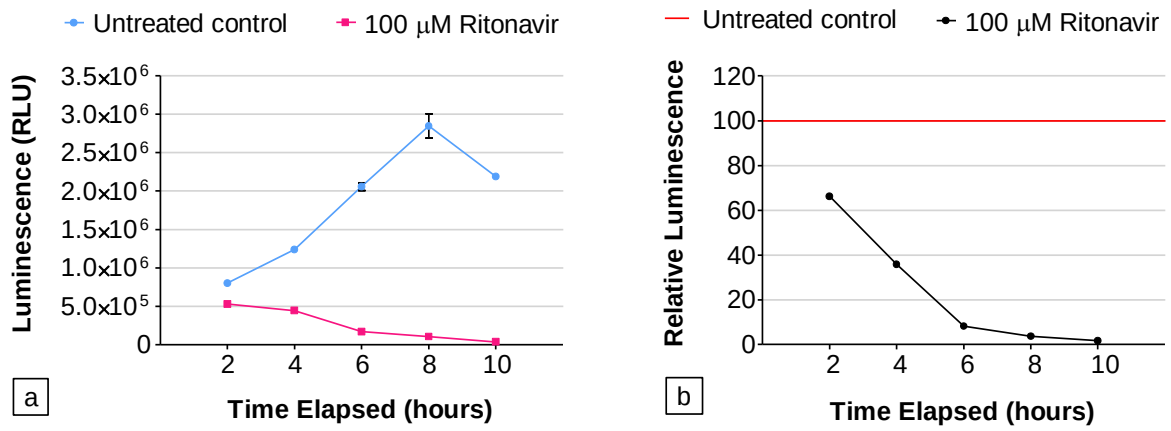

Figure 4.7: Luciferase activity in control and ritonavir-treated HSP-Luc parasites over a 10-hour period. Luciferase activity was measured in samples of control and ritonavir (100 μM)-treated HSP-Luc cultures every 2 hours over a 10-hour period. Results are presented as absolute values (Fig. 4.7a) and relative to controls (Fig. 4.7b) with each data point presented as the mean ± SD, n = 3.

4.3.4. Investigating drug resistance in luciferase transgenic parasites

Even though a 100 nM artemisinin treatment had caused significantly elevated ATP levels in wildtype parasites (see section 3.3.1.4 of Chapter 3), it had a negligible effect on the luciferase activity of luciferase transgenic HSP-Luc parasites (see section 4.3.3.4 above). Therefore, in order to ascertain whether there was a difference in the sensitivity of wildtype and HSP-Luc parasites to artemisinin, the IC50s of artemisinin against these parasite lines were determined. This was achieved using a standard pLDH assay (detailed in section 3.2.3 of Chapter 3) with a final concentration range of $1.00x10^{0}$–$1.52x10^{-4}$ µM. The results of two independent experiments (Figures 4.8a and 4.8b) showed that the IC50 of artemisinin against HSP-Luc parasites was significantly higher than that obtained against wildtype parasites ($P < 0.05$; Table 7.4, Appendix 7.2). This suggested that the HSP-Luc parasites were less sensitive to artemisinin than the wildtype parasites.

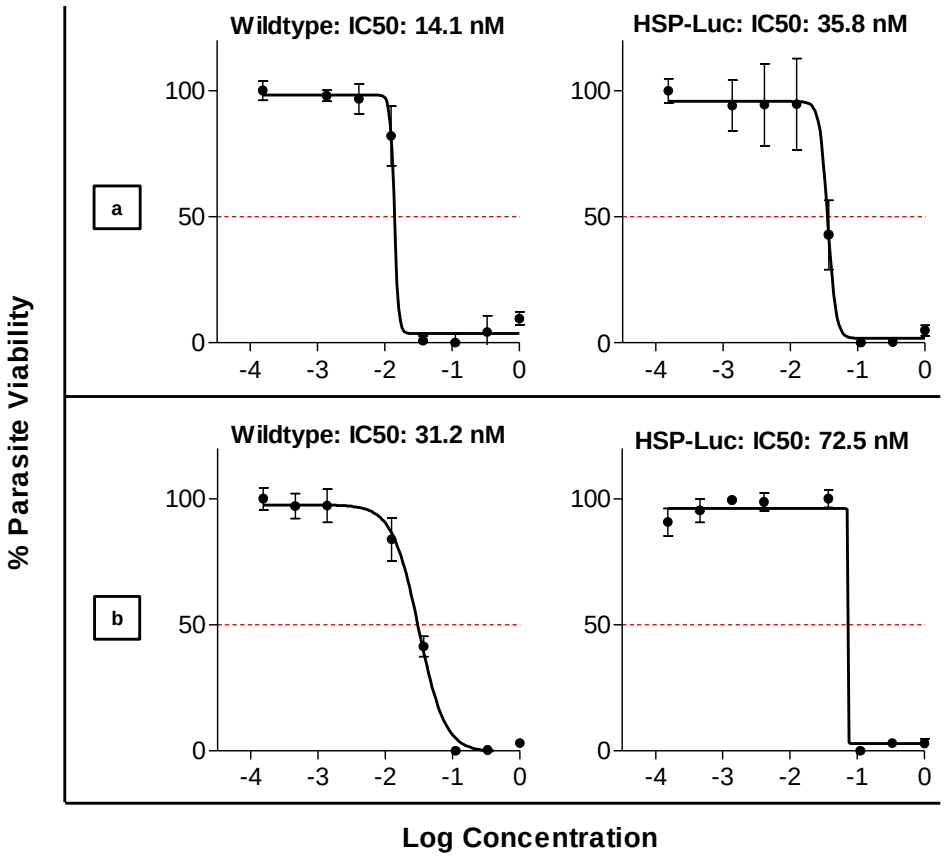

Figure 4.8: Comparing the IC50 of artemisinin against wildtype and HSP-Luc parasites.

Trophozoite-stage cultures were exposed to two-fold serial dilutions of the drug (final concentration range: $1.00x10^{0}$–$1.52x10^{-4}$ µM) for 48 hours. Percentage parasite viability was determined using the pLDH assay and the IC50 was determined from a log dose-response curve using GraphPad Prism software. The results of two independent experiments (a-b) are shown, with each data point representing the mean ± SD, n = 2.

The next step was to determine whether the apparent resistance of HSP-Luc parasites to artemisinin may be linked to their expression of luciferase. This was achieved by assessing the sensitivity of LYS-Luc parasites, which displayed luciferase activity 31x lower than that of HSP-Luc parasites (Figure 4.9), to artemisinin.

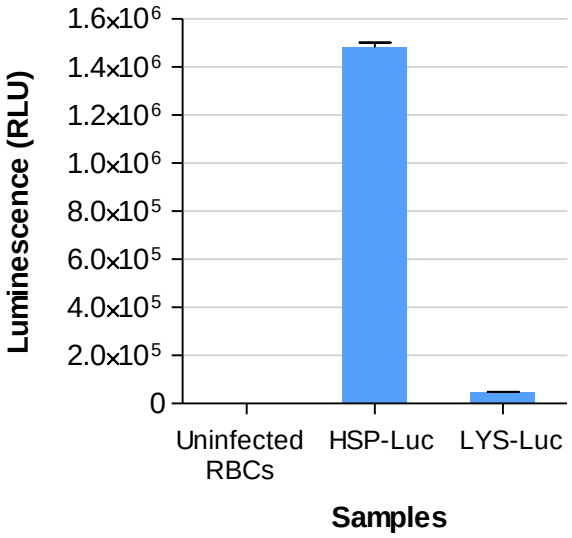

Figure 4.9: Comparing the luciferase activity of transgenic luciferase parasites with different promoters. To determine the effect of different promoters on the level of parasite luciferase activity, parallel samples of trophozoite-stage HSP-Luc and LYS-Luc cultures were subjected to the Luciferase assay. Uninfected RBCs served as negative controls. Luciferase activities, measured as relative light units (RLU), are presented as means ± SD, n = 2.

The results of IC50 determinations from two independent experiments (Figures 4.10a and 4.10b) once again showed that the IC50 of artemisinin against HSP-Luc parasites (IC50s = 64.5 nM, 50.9 nM) was substantially higher than that against wildtype parasites (IC50s = 35.8 nM, 37.5 nM). In contrast, it was found that the IC50 of artemisinin against LYS-Luc parasites (IC50s = 35.6 nM, 39.3 nM) was not significantly different ($P > 0.05$; Table 7.4, Appendix 7.2) from that of wildtype parasites. These findings suggest that, in comparison to wildtype parasites, HSP-Luc parasites and LYS-Luc parasites are less and equally sensitive to artemisinin, respectively. Since luciferase activity of LYS-Luc parasites is significantly lower ($P < 0.05$; Table 7.2, Appendix 7.1) than that of HSP-Luc parasites, and wildtype parasites lack luciferase activity, the results suggest that reduced sensitivity of HSP-Luc parasites to artemisinin may be associated with luciferase expression.

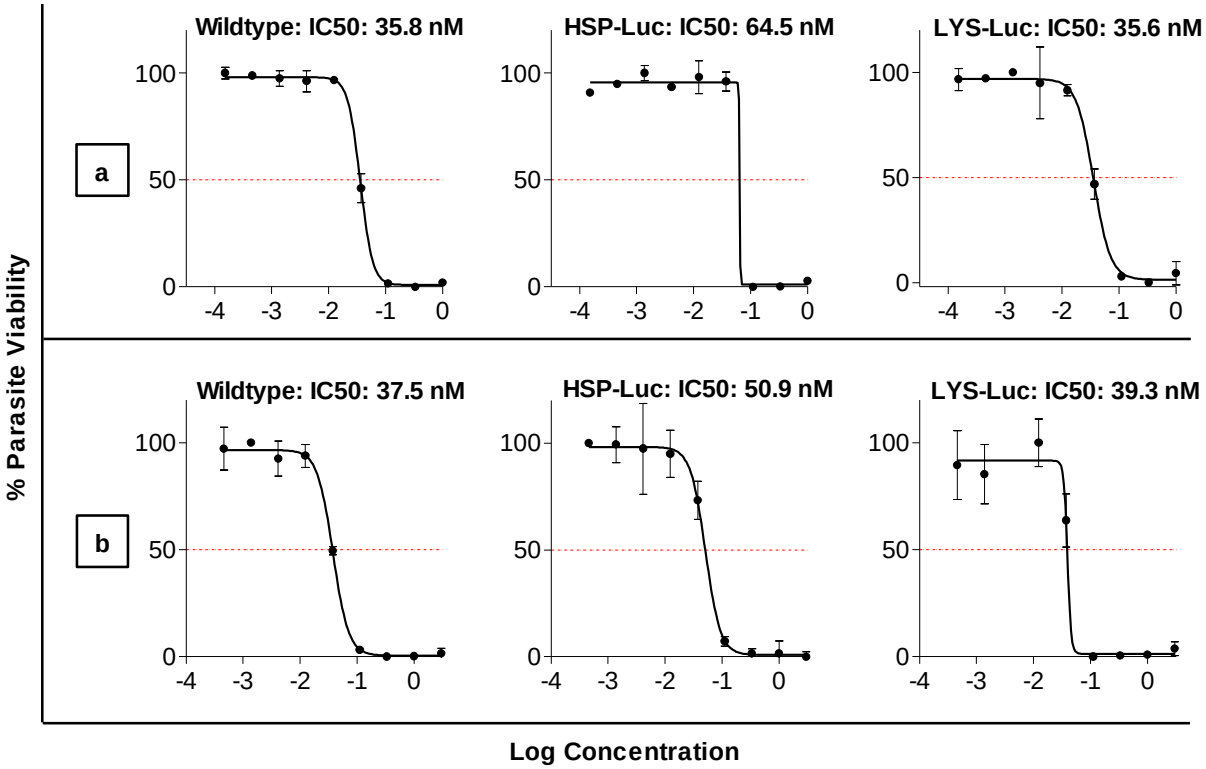

Figure 4.10: Comparing the IC50 of artemisinin against wildtype and luciferase transgenic parasites. Trophozoite-stage cultures were exposed to two-fold serial dilutions of artemisinin (final concentration ranges: 4.10a = $1.00x10^{0}$–$1.52x10^{-4}$ µM; Fig. 4.10b = $3.00x10^{0}$–$4.57x10^{-4}$ µM) for 48 hours. Parasite viability was determined using the pLDH assay and the IC50 was determined from a log dose-response curve using GraphPad Prism software. The results of two independent experiments (a-b) are shown, with each data point representing the mean ± SD, n = 2.

In order to ascertain whether the transgenic HSP-Luc parasites used in the Luciferase assay were resistant to the other established antimalarials used in this study (i.e. chloroquine and mefloquine), IC50 determinations were performed for these drugs as well. The results (Figure 4.11) showed no significant difference ($P > 0.05$; Table 7.4, Appendix 7.2) in the IC50s of chloroquine and mefloquine against wildtype and HSP-Luc parasites, suggesting that these parasite lines were equally sensitive to these respective drugs.

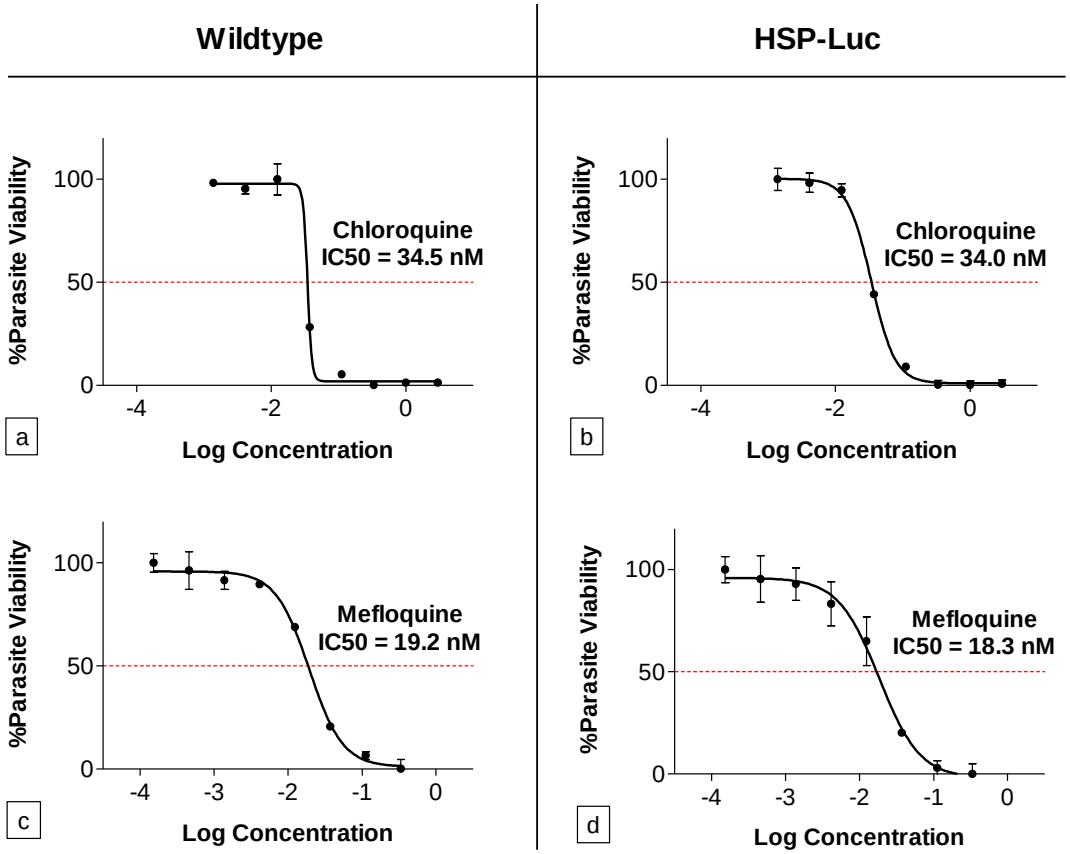

Figure 4.11: Comparing the IC50s of chloroquine and mefloquine against wildtype and HSP-Luc parasites. Trophozoite-stage cultures were exposed to two-fold serial dilutions of the drugs (final concentration ranges: Fig. 4.11a-b = $3.33x10^{-1}$–1.5210^{-4} µM; Fig. 4.11c-d = $3.00x10^{0}$–$1.37x10^{-3}$ µM) for 48 hours. Parasite viability was determined using the pLDH assay and the IC50 was determined from a log dose-response curve using GraphPad Prism software. Each data point represents the mean ± SD, n = 2.

4.3.5. Determining whether drug-induced decreases in luciferase activity are a result of proteasomal degradation

Overall results of the Luciferase assay indicated that, over a 10-hour incubation period, the luciferase activity of drug-treated parasites remained fairly unchanged and/or decreased relative to controls (see section 4.3.3 above). Several cellular processes, including the cellular stress response, rely on the correct balance of various cellular proteins, and protein degradation plays a major role in the maintenance of this balance (Myung *et al*., 2001). The eukaryotic cell possesses two proteolytic mechanisms, the non-specific lysosomal pathway and the ubiquitin-proteasome pathway (Myung *et al*., 2001). The involvement of proteasomal degradation in the antimalarial-mediated decreases of HSP-Luc luciferase activity was assessed by determining whether proteasome inhibitors could prevent these decreases. This was achieved using proteasome inhibitors Lactacystin (LC) and MG-132 (MG), and the

antimalarial mefloquine (MQ) which had decreased luciferase activity of HSP-Luc parasites by 66% after a 6-hour 100 nM treatment (see section 4.3.3.3 above). Briefly, trophozoite-stage HSP-Luc parasites were prepared at a 5% haematocrit and 2% parasitaemia. Cultures were incubated for 6 hours with MQ (100 nM), LC (5 μM) or MG (300 nM) alone, or a combination of MQ+LC or MQ+MG, before measuring luciferase activity via the Luciferase assay, as described in section 4.2.2 above. The results are presented in Figure 4.12. Note: Treatment concentrations for LC and MG were based on their IC50s against HSP-Luc parasites (Figure 8.1, Appendix 8) determined using a standard pLDH assay.

The MQ and LC treatments both caused a 58% decrease in luciferase activity while the MG treatment caused a 28% decrease, relative to controls ($P < 0.001$; Table 7.3, Appendix 7.1). Mefloquine treatment in the presence of the proteasome inhibitors LC or MG resulted in decreased luciferase activity that was 25% and 4% lower, respectively, than that observed with MQ alone. This suggests that the decreased luciferase activity of HSP-Luc parasites in the presence of antimalarials is likely not due to proteasomal degradation since this decrease persisted even in the presence of proteasome inhibitors.

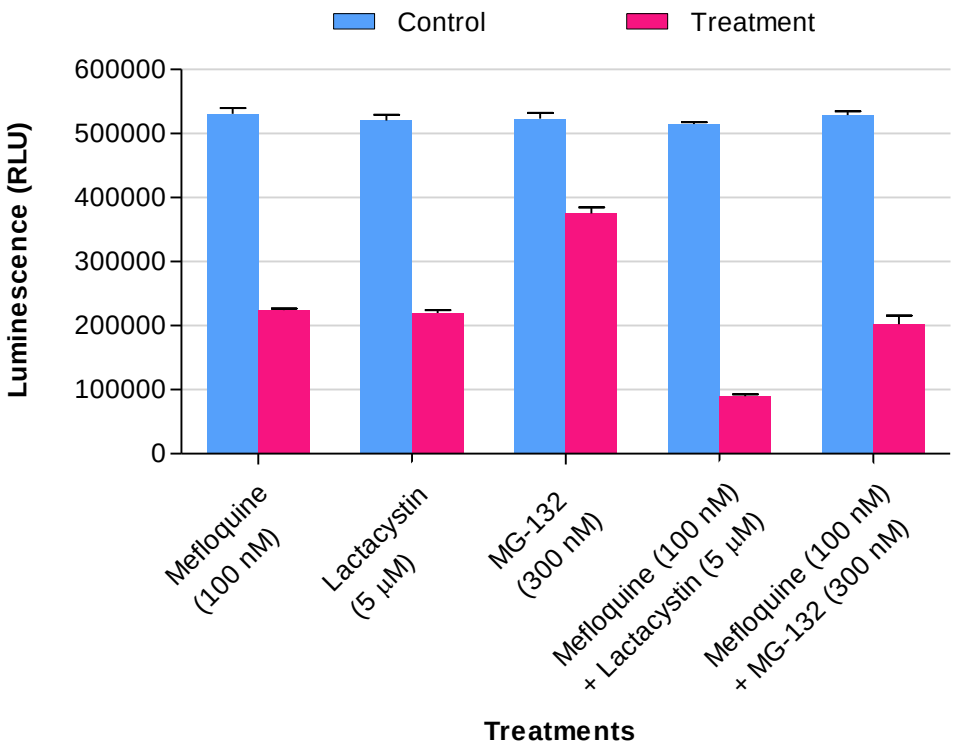

Figure 4.12: The effect of proteasome inhibitors on luciferase activity in control and drug-treated HSP-Luc parasites. To determine whether proteasomal degradation was involved in antimalarial-mediated decreases of HSP-Luc luciferase activity, samples of trophozoite-stage cultures were incubated for 6-hours in culture medium or culture medium containing mefloquine or a proteasome inhibitor, either alone or in combination. Samples were subjected to the Luciferase assay and luciferase activities, measured as relative light units (RLU), are presented as means ± SD, n = 3.

4.4. DISCUSSION

In addition to our investigations of the *P. falciparum* ATP assay (see Chapter 3) as a novel, quantitative means of assessing the rate of antimalarial drug action in *in vitro* cultures, the Luciferase assay was explored as a potential additional methodology. The assay uses a luminescence-based kit (Bright-Glo Luciferase Assay System, Promega) to detect parasite luciferase activity in luciferase-expressing *P. falciparum* transfectants. The transfectants were generated by the CSIR Systems Biology Group, and preliminary experiments by the group showed that luciferase activity in these parasites decreased rapidly and markedly during exposure to artemisinin and mefloquine (data not shown). Conceivably, measurements of luciferase activity in these transgenic parasites may thus be used to evaluate the rate of antimalarial drug action. To explore this further, the luciferase-expressing parasite line was made available for this study.

Firstly, the correlation of luciferase activity in culture samples with varying parasite concentrations was determined. The results indicated that, for $55x10^3$ to $70x10^5$ parasites per sample, the luminescence signals produced by the Luciferase assay correlated strongly with parasite numbers. This appears to be an advantage over the ATP assay (see section 2.3.4 of Chapter 2) where luminescence signals became saturated and diverged from linearity at parasite numbers greater than $22x10^5$.

The utility of the Luciferase assay as a means of assessing the rate of antimalarial drug action was evaluated in the same manner as for the ATP assay, i.e. by characterising the response of parasite luciferase activity to a panel of antimalarial drugs with varying modes of action and, presumably, rates of activity. The results are discussed below. Note: Since luciferase activity at each time-point was measured using a separate aliquot of pre-prepared Bright-Glo reagent, the discussion below focuses on the ‗relative to control' data rather than the absolute values.

4.4.1. Characterising the response of parasite luciferase activity to a panel of antimalarial drugs

The results of this study revealed that the luciferase activities of chloroquine- and DFMO-treated parasites were progressively lower than that of controls with maximum decreases of 20% and 17%, respectively, at the final time-point. This modest variation between the luciferase activities of control and treated parasites suggests that chloroquine and DFMO are slow-acting antimalarials. The luciferase activity of mefloquine-treated transgenic parasites

was found to be significantly (44%) lower than that of controls within 2 hours of treatment, with even lower (± 66%) levels observed during the remainder of the 10-hour incubation period. This is in contrast to the mild decreases caused by DFMO and chloroquine treatments, which suggests that mefloquine acts at a faster rate than these drugs.

The luciferase activity of transgenic parasites in the presence of 100 nM artemisinin closely mirrored that of the controls throughout the 10-hour incubation period. This was unexpected since the same 100 nM treatment had caused greatly elevated ATP levels in the wildtype parasites used in the ATP assay (see section 3.3.1.4 of Chapter 3). Comparison of the IC50s of artemisinin against the wildtype and transgenic HSP-Luc parasites revealed that the transgenic parasites were less sensitive to artemisinin. Consequently, the Luciferase assay was repeated using a higher concentration of artemisinin (500 nM). The results of this second experiment showed that the luciferase activity of the artemisinin-treated transgenic parasites was significantly lower (67%) than that of controls after 2 hours of incubation with even lower (± 96%) levels detected during the remaining 8 hours. This response appears to be similar to that observed with mefloquine-treated parasites, but the decreases in the luciferase activity of artemisinin-treated parasites were more pronounced. This suggests that the rate of antimalarial action of artemisinin may be faster than that of mefloquine which agrees with previous *in vitro* (Gamo *et al*., 2010; Sanz *et al*., 2012) and *in vivo* (Karbwang *et al*., 1992; Myint & Shwe, 1987; White, 1994; White *et al*., 1992) studies that have shown artemisinins to be more rapid-acting than quinines. However, care must be taken in evaluating the relative rates of action of artemisinin and the other drugs, considering the higher dose of artemisinin required due to its increased IC50 in HSP-Luc parasites, and thus the increased risk of secondary drug effects unrelated to the primary mode of action. Taken together, the Luciferase assay results suggest that chloroquine and DFMO are slow-acting antimalarials, mefloquine has a moderate rate of activity and artemisinin is rapid-acting, which is consistent with the findings of the ATP assay discussed in Chapter 3.

In addition to the traditional (i.e. chloroquine, mefloquine and artemisinin) and cytostatic (i.e. DFMO) antimalarials used in this study, a potent antimalarial compound which is not used clinically (i.e. ritonavir) was also analysed. Ritonavir-treated *P. falciparum* parasites were found to display luciferase activity that was significantly lower than that of controls within 2 (34% lower) and 4 (64% lower) hours of treatment, and ± 95% below that of controls during

the remaining 6 hours of incubation. This implies that ritonavir, like artemisinin, is a rapid-acting antimalarial which agrees with the findings of the ATP assay discussed in Chapter 3.

4.4.2. Possible explanations for the luciferase activity decreases observed in drug-treated HSP-Luc parasites

Note: Since the proposed modes of action of each drug were discussed at length in Chapter 3 (section 3.4), only information pertinent to the Luciferase assay results are mentioned here.

Due to the parasite‘s limited capacity for *de novo* amino acid synthesis, digestion of host-derived haemoglobin is critical to parasite survival as it releases amino acids which may be used for energy metabolism or incorporated into parasite proteins (Francis *et al*., 1997). A number of the drugs used in this study have been suggested to exert their antimalarial action by interfering with haemoglobin metabolism in the parasite. Chloroquine, due to its weak base properties, is believed to accumulate in the parasite‘s acidic food vacuole by pH trapping (Aikawa, 1972; Yayon *et al*., 1984; Yayon *et al*., 1985) where it binds to haem (Bray *et al*., 1999; Chou *et al*., 1980; Dorn *et al*., 1998; Sullivan *et al*., 1996) forming chloroquine-haem complexes which have been implicated in the inhibition of haemoglobin degradative proteases (Gluzman *et al*., 1994; Vander Jaqt *et al*., 1986). Artemisinin and mefloquine have been proposed to disrupt haemoglobin metabolism in the parasite by inhibiting endocytosis of the haemoglobin-rich host RBC cytoplasm (Famin & Ginsburg, 2002; Hoppe *et al*., 2004) and by blocking haemoglobin degradation (Mungthin *et al*., 1998; Pandey *et al*., 1999; Sullivan *et al*., 1998). Ritonavir has been suggested to impede haemoglobin metabolism in the parasite by binding to and inhibiting plasmepsins, the parasite‘s haemoglobin degradative proteases (Andrews *et al*., 2006). Based on this discussion, the lower levels of luciferase activity in parasites treated with chloroquine, mefloquine, artemisinin or ritonavir relative to controls observed in this study may be associated with the proposed ability of these drugs to interfere with haemoglobin metabolism. By disrupting this critical process these drugs may have limited the parasite‘s source of amino acids, thus impairing the production of parasite proteins, including luciferase which the transgenic HSP-Luc parasites used in the Luciferase assay were modified to express.

While the aforementioned plasmepsin-inhibitor theory of antimalarial action, based on its inhibition of HIV aspartic acid protease, may seem plausible for ritonavir, its very rapid action against *P. falciparum*, as reported here, makes an inhibition of haemoglobin digestion

as the principle mode of action unlikely. Furthermore, Parikh *et al.* (2006) showed that the antimalarial activity of ritonavir against wildtype and plasmepsin knockout *P. falciparum* parasites was not significantly different, which further opposes the plasmepsin-inhibitor theory of antimalarial action. Therefore, it is difficult to fully explain the decline in the luciferase activity of ritonavir-treated parasites observed in this study.

The slow rate at which the luciferase activity of chloroquine-treated parasites decreased relative to controls in this study may be partly due to the fact that most parasites were in the early trophozoite-stage of development at time 0. During this early phase of development, endocytosis and digestion of host cell cytoplasm by the parasite is limited and consequent release of haem is comparatively limited. Consequently, at the earlier time-points there may have been minimal production of chloroquine-haem complexes. As time passed, parasite consumption of host cell cytoplasm may have increased in order to create space and provide amino acids for the growing parasite. This may have led to an increased production of free haem and chloroquine-haem complexes which may explain why, in comparison to controls, the decrease in luciferase activity of chloroquine-treated parasites, though still modest, was more pronounced at the later time-points.

Studies by Famin and Ginsburg (2002) and Hoppe *et al*. (2004) suggest that mefloquine blocks parasite endocytosis of the haemoglobin-rich host cell cytoplasm, while chloroquine may act later by inhibiting haemoglobin digestion in the food vacuole. Thus, in this study, the speedier action of mefloquine relative to chloroquine on parasite luciferase activity may be because it is proposed to target an earlier stage of haemoglobin metabolism than chloroquine. Interestingly, studies have shown that mefloquine is active against chloroquine-resistant strains of *P. falciparum* (Geary & Jensen, 1983; Mungthin *et al*., 1998; Zhang *et al*., 1999b), suggesting that it may possess some additional or different mode(s) of antimalarial action compared to chloroquine. The results of this study which show a more pronounced decrease in parasite luciferase activity in mefloquine-treated parasites as compared to that seen in chloroquine-treated parasites supports the notion that these drugs may differ in their modes of action. In comparison to DFMO which has a cytostatic effect on malaria parasites (Assaraf *et al*., 1984; Assaraf *et al*., 1987; Gupta *et al*., 2005), mefloquine has a cytotoxic mode of antimalarial action which may explain why its effect on parasite luciferase activity in this study was more rapid and more potent than that of DFMO.

DFMO is proposed to inhibit the synthesis of polyamines which are essential to several cellular processes, including protein synthesis (Metcalf *et al*., 1978). Assaraf *et al*. (1984) found that DFMO treatment blocked the developmental progression of *P. falciparum* parasites from the trophozoite to the schizont stage, with a concomitant inhibition of nucleic acid and protein synthesis. Thus, the decreased luciferase activity of DFMO-treated parasites seen in this study was likely due to DFMO inhibiting polyamine synthesis, which may have led to an inhibition in the synthesis of proteins, such as luciferase which the HSP-Luc parasites were modified to express. The results also showed that the luciferase activity of DFMO-treated parasites only decreased relative to controls from the 6-hour time-point onward. This may be partially due to the majority of the parasite population being in the early trophozoite-stage of development at time 0. Assaraf *et al.* (1984) showed that in untreated *P. falciparum* parasites, the synthesis and accumulation of polyamines peaked during the early trophozoite stage of development, while protein synthesis peaked once trophozoites matured. Thus, in this study, DFMO-mediated inhibition of polyamine synthesis may have only manifested as a decrease in protein synthesis once the trophozoites had matured, i.e. from the 6-hour time-point onward.

Regarding artemisinin, the most popular notion is that its antimalarial action may be centred on oxidative stress due to its chemical ability to produce free radicals upon iron-mediated cleavage of its endoperoxide bridge (Jung, 1997). An additional means by which artemisinin may promote oxidative stress is by forming adducts with glutathione, thereby decreasing levels of this major antioxidant in the parasite (Wang & Wu, 2000). Free radicals are notorious for causing lipid peroxidation, DNA damage and oxidation of proteins (Bandyopadhyay *et al*., 1999). Artemisinin and its active metabolite, dihydroartemisinin, have been shown to alkylate malaria proteins in *P. falciparum* cultures (Asawamahasakda *et al*., 1994). This effect is believed to be due to the formation of covalent adducts upon interaction of malaria proteins with the free radicals produced from artemisinin. Conceivably, the decreased luciferase activity of artemisinin-treated transgenic parasites observed in this study may be due to artemisinin-mediated production of free radicals and alkylation of parasite proteins, such as luciferase. Alkylation of luciferase may have impaired its function as a catalyst for the luminescence-producing reaction which forms the basis of the Luciferase assay, as illustrated in Figure 4.13.

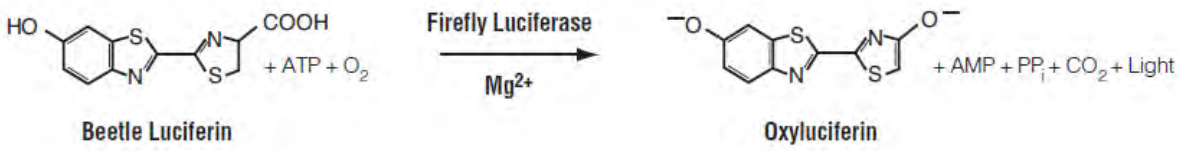

Figure 4.13: The Luciferase reaction. The Luciferase assay used in this study rapidly detects the luciferase activity of transgenic parasites using the luminescence-based Bright-Glo Luciferase Assay System which is based on the luciferase-catalysed mono-oxygenation of luciferin in the presence of magnesium, ATP and molecular oxygen (Promega, Madison, WI).

Another means by which the panel of drugs used in this study may have decreased parasite luciferase activity is by eliciting a cellular stress response, a common phenomenon in eukaryotic cells under threat of macromolecular damage (Kültz, 2003). An often typical feature of this response is a reduction in the global translation of non-essential proteins, thereby conserving cellular energy for use in the production of stress proteins, such as DNA repair and heat shock proteins, which help to restore cellular homeostasis (Holcik & Sonenberg, 2005). Furthermore, the correct balance of various cellular proteins is critical to several cellular processes, including the stress response, and protein degradation (i.e. proteolysis) may also play a role in ensuring that this balance is maintained (Myung *et al*., 2001). Amino acids released during proteolysis of non-essential proteins can be re-used in biosynthetic processes. Luciferase was not essential to the survival of the HSP-Luc parasites used in this study and its expression was under the control of an exogenous promoter in the episomal plasmid. The promoter used was a heat shock promoter (hsp86) which typically is upregulated during periods of cellular stress. Indeed, using unrelated promoters in episomal constructs, it was found that luciferase mRNA transcript levels actually increase during drug treatment of transgenic parasites, despite a rapid decline in luciferase enzyme activity (A.C. van Brummelen, unpublished results). It is therefore unlikely that the decline in luciferase activity during drug exposure is due to transcriptional suppression. More likely it reflects either inhibition of luciferase mRNA translation, or increased degradation and/or misfolding of the enzyme due to drug-induced cellular stress.

In addition to their previously described detrimental drug effects, chloroquine, mefloquine and artemisinin may have initiated a stress response in the parasite via their proposed ability to inhibit haem detoxification. Haem is the toxic byproduct of haemoglobin digestion in the parasite‘s food vacuole and is detoxified primarily via its polymerisation to inert haemozoin

(Francis *et al*., 1997). Several studies have shown that the aforementioned drugs bind haem (Bray *et al*., 1999; Chou *et al*., 1980; Dorn *et al*., 1998; Sullivan *et al*., 1996; Chevli & Fitch, 1982; Loup *et al*., 2007; Meshnick *et al*., 1991; Paitayatat *et al*., 1997; Pandey *et al*., 1999; Robert *et al*., 2005; Robert & Meunier, 1997) and inhibit haemozoin formation (Combrinck *et al*., in press; Dorn *et al*., 1998; Egan *et al*., 1994; Hong *et al*., 1994; Kannan *et al*., 2005; Loup *et al*., 2007; Pandey *et al*., 1999; Sullivan *et al*., 1996; Sullivan *et al*., 1998; Zhang *et al*., 1999b) thereby causing a toxic accumulation of haem in the parasite which may elicit a cellular stress response, resulting in increased luciferase degradation/misfolding or decreased translation.

4.4.3. Investigating drug resistance in the luciferase-expressing HSP-Luc parasites

While investigating the response of parasite luciferase activity to artemisinin treatment it was found that a 100 nM treatment, which had caused greatly elevated ATP levels in the wildtype parasites (see section 3.3.1.4 of Chapter 3), barely affected the luciferase activity of HSP-Luc parasites. Comparison of the IC50s of artemisinin against these parasite lines revealed that the luciferase-expressing HSP-Luc parasites were less sensitive to artemisinin than the wildtype parasites, which likely explains the contradictory results of the ATP and Luciferase assays. Additionally, IC50 determinations of chloroquine and mefloquine against these parasite lines indicated that wildtype and HSP-Luc parasites were equally sensitive to mefloquine and chloroquine, respectively.

To ascertain whether the reduced sensitivity of HSP-Luc parasites to artemisinin may be associated with luciferase expression, artemisinin sensitivity of an alternative luciferase-expressing parasite line, the LYS-Luc parasites, was investigated. The only genetic difference between the HSP-Luc and LYS-Luc parasites is their respective promoters, i.e. the heat shock protein 86 promoter and the lysophospholipase promoter. The results of IC50 determinations indicated that, relative to wildtype parasites, the HSP-Luc parasites were less sensitive to artemisinin while the LYS-Luc parasites were equally sensitive. Measurements of luciferase activity showed that the ~~–ar~~temisinin-resistant" HSP-Luc parasites displayed activity that was markedly (31x) higher than that of the ~~–ar~~temisinin-sensitive" LYS-Luc parasites and, as expected, the ~~–ar~~temisinin-sensitive" wildtype parasites lacked activity. Taken together, these results suggest that the reduced sensitivity of HSP-Luc parasites to artemisinin, relative to that of wildtype parasites, may be associated with luciferase expression, which can reach substantial levels in these transgenic parasites. The exact mechanism by which luciferase

expression may lead to artemisinin resistance in these parasites (e.g. competition for drug targets by adduct formation, altered parasite metabolism to compensate for artemisinin action, etc.) needs to be investigated further. This does raise doubts, however, that luciferase-expressing parasites can be used to perform assays with all classes of drugs.

4.4.4. Does the proteasome play a role in decreasing the luciferase activity of drug-treated HSP-Luc parasites?

Overall results of the Luciferase assay showed that the luciferase activity of drug-treated parasites remained fairly unchanged and/or decreased relative to controls (see section 4.3.3 above). The cellular stress response relies on the correct balance of various cellular proteins and proteolysis plays a major role in the maintenance of this balance (Myung *et al*., 2001). Thus, the decrease in the luciferase activity of HSP-Luc parasites following drug treatment may be due to the parasite degrading foreign and unnecessary proteins, such as luciferase, as part of its stress response. The eukaryotic cell possesses two proteolytic mechanisms: (i) the non-specific lysosomal pathway where proteins are engulfed within endocytic vesicles and transported to the lysosome where degradation is carried out by a collection of proteolytic enzymes and, (ii) the highly specific ubiquitin-proteasome pathway where the protein destined for destruction is targeted for proteasomal degradation by the attachment of multiple ubiquitin molecules (Myung *et al*., 2001). The *P. falciparum* genome has 14 genes encoding protein subunits of the proteasome, as well as a single gene encoding the ClpQ/hsIV ortholog, the prokaryotic precursor of the proteasome (Gille *et al*., 2003).

The contribution of proteasomal degradation to the antimalarial-mediated decreases of HSP-Luc luciferase activity observed in this study was assessed by determining whether proteasome inhibitors could abrogate these decreases. This was achieved using the antimalarial mefloquine (MQ), which in previous experiments had decreased parasite luciferase activity considerably, and the proteasome inhibitors Lactacystin (LC) and MG-132 (MG). The results showed that, relative to controls, the MQ and LC treatments decreased luciferase activity considerably (58%) while the MG treatments caused a less pronounced (28%) decrease. The fact that parasite luciferase activity decreased substantially when only proteasome inhibitors were present suggests that these decreases were likely not a result of proteasomal degradation of luciferase. The proteasome regulates the turnover of most eukaryotic proteins and thus plays a central role in several cellular processes, including protein quality control and signal transduction (Kreidenweiss *et al*., 2008). Therefore, it is

likely that proteasome inhibitors may threaten parasite survival and trigger a stress response, reflected by a marked reduction in luciferase activity. Furthermore, when MQ was used in combination with LC or MG, parasite luciferase activity decreased relative to controls, however, these decreases were more pronounced than those observed with MQ alone, particularly with LC. This further supports the notion that decreased luciferase activity of HSP-Luc parasites in the presence of antimalarials, is likely not due to proteasomal activity since the presence of proteasome inhibitors failed to abrogate this decrease. This makes it more likely that the activity decreases are due to either an inhibition of luciferase translation or increased misfolding/denaturation of the enzyme during cellular stress.

4.5. CONCLUSION

Overall, the Luciferase assay results imply that chloroquine and DFMO have a slow rate of antimalarial action, mefloquine has a moderate rate of activity, and artemisinin and ritonavir are rapid-acting antimalarials. For the established drugs, chloroquine, mefloquine and artemisinin, as well as the polyamine inhibitor DFMO, these observations agree with the expected rates of activity of these antimalarials, thereby supporting the use of the Luciferase assay as a potential means of evaluating the rate of antimalarial drug action. In addition, the rates of activity of the abovementioned antimalarials indicated by the Luciferase assay are in agreement with those suggested by the ATP assay discussed in Chapter 3. The findings of this study which suggest that ritonavir acts rapidly on parasite ATP and luciferase levels are, to our knowledge, the first account of this antimalarial's rate of activity.

From a technical point of view, the Luciferase assay has several advantages over the ATP assay. It is far easier to perform than the ATP assay since isolation of transgenic parasites from RBCs is unnecessary due to the lack of luciferase activity in RBCs and thus only three pipetting steps are required after drug exposure (i.e. removal of medium, addition of lysis buffer to infected RBCs and addition of luciferase reagent to the lysate). By contrast, parasite isolation is essential during the ATP assay in order to remove RBC ATP. Consequently, when 2-hour time-points are employed when assessing the rate of antimalarial activity, the Luciferase assay can be performed in a 96-well plate format and multiple drugs can be assayed in a single experiment. In contrast, due to the multiple pipetting steps involved in the ATP assay during isolation of parasites from RBCs, only a single drug can comfortably be analysed per experiment. An additional advantage of the Luciferase assay is that luciferase activity can be accurately detected in samples with parasite concentrations as high as $7x10^6$, as opposed to the ATP assay where the limit of detection is $2x10^6$ parasites per sample.

This practical advantage of the Luciferase assay should be seen in the context of some additional considerations. A major difference between the Luciferase and ATP assays is that the former assay measures the activity of a foreign protein (i.e. luciferase) which the parasites were modified to express, while the latter assay measures the natural energy source of multiple cellular processes (i.e. ATP). The results of this study showed that, relative to controls, parasite ATP levels were relatively unchanged, increased or decreased in response to drug stress. The role of ATP in metabolic processes, plus Recovery assay and morphological evaluations, led to the hypothesis that unchanged ATP levels reflect poor drug

action, increased ATP levels indicate a stress response to drug action and some loss of parasite viability, while a major decline in ATP reflects severely compromised viability. In contrast, parasite luciferase activity always decreased in response to drug stress, relative to controls, and this was broadly interpreted as a reflection of a stress response to drug action. Thus, an advantage of the ATP assay over the Luciferase assay is that more information regarding the status of parasite health may potentially be inferred from the results. Additionally, since the Luciferase assay relies on transgenic parasites, it is possible that expression of a foreign protein (i.e. luciferase) may have unanticipated effects on the parasite that affect drug sensitivity. Possible interactions of luciferase with the antimalarials used in this study are also unknown. As an example, the transgenic HSP-Luc parasites used in the Luciferase assay were less sensitive to artemisinin than the wildtype parasites. Potential resistance of these transgenic parasites to compounds under investigation would thus necessitate the performance of IC50 determinations prior to use of the Luciferase assay in order to select effective treatment concentrations. Since the ATP assay makes use of wildtype parasites, the potential shortcomings associated with the use of transgenic parasites are not a factor in this assay. Furthermore, the ATP assay may be rapidly applied to parasites isolated from the field, without the necessity of having to first generate transgenic parasites which is a time-consuming process and may alter the wild-type characteristics of the field strains.

In comparison to controls, parasite luciferase activity generally decreased in response to drug stress. Numerous cellular processes, such as the cellular stress response, rely on the correct balance of various cellular proteins and proteolysis has a leading role in the preservation of this balance (Myung *et al*., 2001). Eukaryotic cells have two proteolytic mechanisms – the non-specific lysosomal pathway and the highly specific ubiquitin-proteasome pathway (Myung *et al*., 2001). Interestingly, significant decreases in the luciferase activity of mefloquine-treated parasites persisted even in the presence of proteasome inhibitors. This suggests that proteasomal degradation of luciferase may not have contributed to the decreases in the luciferase activity of drug-treated parasites observed in this study. Instead, these decreases may be due to a decline in the translation of non-essential proteins such as luciferase, as part of a stress response initiated by drug treatment.

One aspect that was not investigated is whether altered parasite ATP levels in drug-treated parasites may influence the luciferase activity results, bearing in mind that luciferase uses ATP and luciferin as substrates. ATP is added exogenously as part of the luciferase assay

reagents. However, no attempt is made to remove/deplete parasite ATP before conducting the luciferase assay, so an effect on the read-outs is a formal possibility that may be investigated in future.

In conclusion, the results of this study suggest that the Luciferase assay may serve as a useful indicator of the rate of antimalarial action. However, possible resistance of the transgenic parasites used in the assay to the drugs being tested may be a complication which has to be taken into account.

CHAPTER 5: GENERAL CONCLUSION

The Medicines for Malaria Venture endorses a "single dose radical cure" as the ideal treatment for uncomplicated malaria since rapid clearance of blood-stage parasites and symptom relief improves patient compliance, reduces costs and limits drug resistance (WHO, 2010c). This call for rapid-acting antimalarials makes assessing the rate of activity of candidate compounds critical to malaria drug discovery. Traditional determination of this rate by morphological assessments is flawed, primarily due to heterogeneous morphology of individual parasites under routine culture conditions which result in highly subjective, operator-specific interpretations. In addition, an *in vitro* viability-based assay which determines this rate by directly quantifying the number of parasites capable of recovering from distinct periods of drug exposure was recently reported (Sanz *et al*., 2012). However, this method is very time-consuming, requiring 3-4 weeks of monitoring re-growth in culture following drug exposure. Hence, the aim of this study was to develop a novel, quantitative assay capable of rapidly determining the kinetics of drug action in culture.

A rapid protocol for detecting ATP in *P. falciparum* parasites using a luminescence-based kit was developed and optimised, and luciferase-expressing transgenic parasites, from which luciferase activity is readily detected using a similar kit, were acquired. The utility of both methods as potential means of assessing the rate of antimalarial drug action were investigated using a panel of antimalarials with varying modes of action and, presumably, rates of activity. The results indicated that parasite ATP remained unchanged, increased or decreased in response to drug stress. Assessments of parasite morphology and a recovery assay were used to aid interpretation. The results of these assessments suggested that, generally, during drug exposure unchanged parasite ATP levels reflect poor drug action, elevated ATP levels indicate a stress response and partially compromised viability, while marked decreases in ATP reflect severely compromised viability. With regard to the Luciferase assay, parasite luciferase activity decreased during drug treatment. The observed responses of parasite ATP and luciferase activity to established (i.e. chloroquine, mefloquine and artemisinin) and cytostatic (i.e. DFMO) antimalarials occurred at rates that agreed with the expected rates of activity of these compounds. Hence, measurement of parasite ATP and/or luciferase activity show potential as novel means of evaluating parasite health and the kinetics of antimalarial action. With further validation and characterisation, these assays may benefit drug discovery

as tools for comparing candidate compounds with established antimalarials in order to identify rapid-acting ―hit" compounds.

Additionally, the results suggested that the potent antimalarial, ritonavir, is rapid-acting which, to our knowledge, is the first report regarding the rate of activity of this drug. Another interesting observation was that, even though chloroquine-treated parasites showed relatively no change in ATP or luciferase activity, they failed to recover from a 6-hour drug treatment after removal of drug pressure. This supports the notion that pH entrapment in the parasite's food vacuole, which would allow for prolonged drug pressure, is involved in chloroquine's mode of action.

Decreases in the luciferase activity of the drug-treated transgenic parasites persisted in the presence of proteasome inhibitors, opposing the involvement of proteasomal degradation. The molecular basis for these decreases may be investigated further by determining the half-life of luciferase in the transgenic parasites by blocking protein translation and assessing the rate at which luciferase levels decline. A rapid decline would suggest that luciferase has a high turnover rate and that drug-induced decreases may be attributable to a global decrease in the translation of non-essential proteins, which is a characteristic feature of the eukaryotic stress response (Holcik & Sonenberg, 2005). By contrast, a gradual decline would suggest a long half-life and thus that the drug-induced decreases may be the result of selective proteasome-independent protein digestion. It should be noted, however, that changes in the levels of luciferase are being inferred from measuring its enzymatic activity. The luciferase protein used in this study (i.e. firefly luciferase) is innately unstable and thermolabile which may lead to denaturation and subsequent loss of activity under stress conditions (Tisi *et al*., 2002). Thus, western-blotting using anti-luciferase antibodies would be necessary to confirm that the observed activity decreases correspond to changes in the levels of the actual protein and not just a loss of enzymatic function due to protein denaturation or irreversible damage/inhibition during drug-induced stress. Alternatively, a more stable reporter protein may be employed such as green fluorescent protein which has a high degree of resistance to denaturation by heat and chemical denaturants, although its detection by fluorimetry is much less sensitive than luciferase (Ripp *et al*., 2011).

Regardless of the molecular basis of luciferase activity decreases, the results presented here support the use of the Luciferase assay as a justifiable means of assessing malaria parasite

stress. Further work may include evaluating the luciferase and ATP assays with an expanded set of drug or experimental compounds, to determine if there are exceptions of compound classes for which the assays do not detect rates of action. It is also important to note that the assays in their current form may be useful for ranking compounds relative to each other with regards to rate of action. But they have not been characterised sufficiently to unambiguously determine the absolute killing rates of compounds, i.e. the time-points where all parasites in the culture have been irretrievably compromised. For example, despite a near complete depletion of detectable ATP and luciferase with some of the drug treatments, some parasites were still able to recover in the recovery assay, albeit at a highly reduced rate. Further work will need to be carried out to firmly establish the correlates between total parasite inhibition and the changes in ATP or luciferase levels.

Additionally, the transgenic luciferase-expressing parasites were found to be less sensitive to artemisinin than wildtype parasites. An alternative transgenic parasite line, with lower levels of luciferase activity than that used for the Luciferase assay, did not display artemisinin resistance. This suggested that resistance may be due to an interaction between artemisinin and luciferase. Possible interactions of luciferase with the antimalarials used in this study are unknown and need to be investigated further. Another potential weakness of the Luciferase assay system is that the transgenic parasites upon which it relies carry episomal plasmids which may segregate unevenly into daughter parasites during cell division (O‘ Donnell *et al*., 2001). Consequently, these transgenic parasite populations may easily lose their plasmids and therefore lose their luciferase-expressing ability, unless selective pressure is maintained (O‘ Donnell *et al*., 2001). In order to avoid this situation, parasites carrying integrated copies of the luciferase expression cassettes may be used. These are generated by subjecting the episomally transformed parasites to a drug cycling process whereby drug pressure is removed for 2 weeks before being reinstated until only integrated forms of the transgenic parasites are present in the parasite population (O‘ Donnell *et al*., 2001).

The panel of antimalarials used in this study did not include any antibiotics which are currently used as second-line antimalarial treatments (WHO, 2010a). This class of drugs targets the apicoplast of the malaria parasite resulting in a ~~“d~~elayed-death” phenotype where the progeny of the treated parasites are killed as opposed to the treated parasites themselves (Dahl *et al*., 2006; Goodman *et al*., 2007). The asexual life cycle of the malaria parasite lasts 48 hours, thus in order to investigate the response of parasite ATP levels and luciferase

activities to antibiotics, the end-point of the ATP and Luciferase assays will need to be extended beyond the 10-hour time-point used in this study.

APPENDICES

APPENDIX 1: CULTIVATION OF *P. FALCIPARUM* PARASITES

1.1. Complete malaria culture medium (CMCM)

RPMI 1640 medium with 25 mM Hepes, 2 mM L-glutamine (500 mL; Lonza, Belgium)
Glucose (Sigma-Aldrich, Germany)
Albumax II (Invitrogen, USA)
Hypoxanthine (Sigma-Aldrich, Germany)
NaOH (1 M; Sigma-Aldrich, Germany)
Gentamicin (50 mg/mL; Lonza, Belgium)

Under aseptic conditions:
Transfer 2 g of glucose and 2.5 g of albumax to a sterile 50 mL centrifuge tube and add 20 mL of the RPMI medium. Weigh out 44 mg of hypoxanthine in a microfuge tube and dissolve in 1 mL of NaOH before adding to the mixture. Add 600 µL of gentamicin to the mixture and vortex until the glucose and albumax are completely dissolved. Filter sterilise (0.22 µm) the solution into the remaining 450 mL of RPMI medium and store at 4°C.

1.2. Giemsa stain

Giemsa solution (Sigma-Aldrich, Germany)
Phosphate buffered saline, 1X (Lonza, Belgium)

Prepare immediately before use by mixing 1 part of Giemsa solution with 10 parts of phosphate-buffered saline (v/v).

1.3. 5% Sorbitol solution

Dissolve 5 g of D-sorbitol (Sigma-Aldrich, Germany) in 100mL of autoclaved ddH_20 and filter sterilise (0.22 µm) filter. Store at 4°C.

1.4. 60% Percoll solution

Firstly, prepare 2.5X RPMI/12.5% sorbitol solution by dissolving 1.045 g of RPMI 1640 powder (Sigma-Aldrich, Germany) and 5 g of D-sorbitol (Sigma-Aldrich, Germany) in 20

mL of sterile water. Adjust the pH to 7.0 and make up volume to 40 mL. Filter sterilise (0.22 µm) and store at 4°C. Thereafter, prepare 60% Percoll solution by mixing 6 mL of Percoll (GE Healthcare, Sweden) and 4 mL of 2.5X RPMI/12.5% sorbitol solution. Prepare on the day of use and store at 4°C.

1.5. Freezing solution (28% glycerol in CMCM)

Add 280 µL of glycerol (Sigma-Aldrich, Germany) and 720 µL of CMCM (see 1.1.) to a microfuge tube and mix well. Prepare on the day of use and store at 4°C.

1.6. Thawing solution A (12% NaCl)

Dissolve 120 mg of NaCl (Sigma-Aldrich, Germany) in 1 mL of sterile water and filter sterilise (0.22 µm). Store at 4°C.

1.7. Thawing solution B (1.6% NaCl)

Dissolve 80 mg of NaCl (Sigma-Aldrich, Germany) in 5 mL of sterile water and filter sterilise (0.22 µm). Store at 4°C

APPENDIX 2: THE ATP ASSAY

2.1. 0.1% BSA in PBS

Bovine serum albumin (BSA, Albumax II, Invitrogen, USA)
Phosphate buffered saline (PBS; Sigma-Aldrich, Germany)

Dissolve 0.1 g of BSA in 100 mL of PBS and store at 4°C.

2.2. 5% Saponin solution

Dissolve 60 mg of saponin (Fluka, Germany) in 1.2 mL of 0.1% BSA in PBS (see 2.1.) and store protected from light at 4°C.

2.3. 0.1% Saponin lysis solution (0.1% saponin, 0.1% BSA in PBS)

Prepare the lysis solution on the day of use. For 10 mL of lysis solution, mix 200 µL of 5% saponin (see 2.2.) and 9800 µL of 0.1% BSA in PBS. Store protected from light at 4°C.

2.4. CellTitre-Glo reagent

CellTitre-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI) stored at -20°C.

Thaw the buffer and lyophilised substrate to room temperature, combine and aliquot. Store aliquots protected from light at -20°C for up to 21 weeks.

2.5. 0.24% Saponin lysis solution (0.24% saponin, 0.1% BSA in PBS)

Prepare the lysis solution on the day of use. For 10 mL of lysis solution, mix 476 µL of 5% saponin (see 2.2.) and 9524 µL of 0.1% BSA in PBS. Store protected from light at 4°C.

2.6. Phthalate oil-mix (Quashie *et al*., 2010)

Dibutyl phthalate (Merck, Germany)
Dioctyl phthalate (Sigma-Aldrich, Germany)

Prepare the oil-mix by combining 5 parts of dibutyl phthalate with 4 parts of dioctyl phthalate. Store protected from light at room temperature.

APPENDIX 3: THE pLDH ASSAY

3.1. Malstat solution

L-lactate (Sigma-Aldrich, Germany)
Trizma base (Sigma-Aldrich, Germany)
3-acetylpyridine nicotinamide adenine dinucleotide (APAD; Sigma-Aldrich, Germany)
Triton X-100 (Fluka, Germany)

Dissolve 4 g of L-lactate, 1.32 g of Trizma base and 22 mg of APAD in 100 mL of ddH_2O and adjust pH to 9. Add 400 µL of Triton X-100 and make up to a final volume of 200 mL and store 4°C.

3.2. NBT/PES solution

Nitro blue tetrazolium salt (NBT; Sigma-Aldrich, Germany)
Phenazine ethosulphate (PES; Sigma-Aldrich, Germany)

Dissolve 160 mg of NBT and 8 mg of PES in 100 mL of ddH_2O and store protected from light at 4°C.

APPENDIX 4: THE LUCIFERASE ASSAY

4.1. Stable transfection of *P. falciparum* parasites with luciferase expression plasmids

Transfection of parasites was performed by Dr. A.C. van Brummelen (CSIR Biosciences) using methodology adapted from the following laboratory protocols:

(i) Stable transfection of *P. falciparum* parasites, Fidock Lab, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Yeshiva University, NY, USA

(ii) Transfection of *P. falciparum*, Llinàs Lab, Department of Molecular Biology, Princeton University, NJ, USA

Note: Complete malaria culture medium (CMCM; Appendix 1.1) was used with the exception that Albumax II was replaced with 10% human serum.

4.1.1. Preparation of parasites

a. Culture *P. falciparum* parasites as described in Chapter 2 (section 2.2.1). For each transfection, 80-100 µL of healthy ring-stage parasites at 5% parasitaemia is required.
b. The day before transfecting: (i) In the morning, adjust the parasitaemia to 1.8 - 2.5% by the addition of fresh uninfected RBCs, in order to produce ~5% rings the next day; (ii) In the late afternoon, replace medium maintaining a 3% haematocrit.
c. Transfection day: (i) About 3 hours before transfection, prepare smears and replace medium; (ii) Determine whether the cells are healthy, at the correct stage (primarily large, healthy rings) and ideal parasitaemia (~5%) for transfection.

4.1.2. Preparation of the DNA:

a. Prepare plasmid constructs in NEB5α *Escherichia coli* cells (New England BioLabs, Ipswich, MA) using standard molecular biology techniques.
b. Purify plasmid DNA using the QIAGEN Plasmid Maxi Kit – 50-100 µg of DNA is required per transfection.
c. Have all plasmids in cytomix and ready for transfection stored at 4 °C, so that whenever parasites are ready, transfection can be performed. Equilibrate isolated plasmid DNA in cytomix (120 mM KCl (anhydr); 0.15 mM $CaCl_2$; 10 mM KH_2PO_4

(anhydr); 25 mM HEPES; 2 mM EGTA; 5 mM $MgCl_2$; adjust pH to 7.6 with KOH and filter sterilise (0.2µm)) for transfection using Amicon Centricon columns.

- Columns hold a maximum volume of 2.5 mL. Load up to 300 µg DNA/column and exchange buffer with cytomix for transfection.
- Parafilm the top of the column and puncture the parafilm with a sterile needle several times.
- Centrifuge the column at 3000 rpm until there is less than 300 µL of cytomix in the column. For 300 µg of DNA, start with 45 minutes, check and then add time as needed.
- Add cytomix to the column to bring up the volume to 300 uL and pipette up and down a few times without touching the filter. Thus, the final DNA concentration will be 1 µg/µL. Flip upside down into collection tube and centrifuge at 3000 rpm for 5 minutes to recover DNA from the column.
- Measure the volume of eluted DNA and determine the volume required to transfect the parasites with 100 µg of plasmid DNA (i.e. 100 µL/transfection for DNA concentration of 1 µg/µL).

4.1.3. Transfection

Note: Try and finish the entire transfection procedure in about 30 minutes from the time the parasites are harvested from the incubator to when the transformed parasites are returned to the incubator. Adhere to sterile technique.

a. Label all electroporation cuvettes, tissue culture plates and microfuge tubes and pre-set electroporator to required settings (0.31 kV, 950 µF and max capacitance) before beginning transfection.
b. Pre-prepare uninfected RBCs at 4% haematocrit in CCM and aliquot into the wells of a 6-well tissue culture plate at 3.5 mL per transfection per well.
c. Harvest 2.5 mL culture/transfection into a sterile 15 ml conical tube and centrifuge at 1300 rpm for 4 minutes at room temperature (RT).
d. Remove and discard <u>all</u> the supernatant and resuspend the cell pellet in excess cytomix.
e. Centrifuge the cells at 1300 rpm for 3 minutes at discard <u>all</u> supernatant. The cell pellet should be about 80-100 µL.

f. Add DNA and then cytomix to final volume of 400 µL. Assuming the cell pellet is 80 µL and DNA concentration is 1µg/µL, add 100 µl of DNA and 220 µL of cytomix. Make sure there are no bubbles.

g. Carefully transfer the suspension into electroporation cuvettes without creating bubbles. Insert cuvette into shocking chamber and slide into place. Press both Pulse buttons and release as soon as beep is heard. Record time constant – time constants between 7 and 11 msec are good. You will always hear a slight ~~"p~~op." If you chill the cuvettes briefly (< 30 secs) immediately before zapping, they will not pop as much into the top of the cuvette.

h. Immediately after electroporating, add 1 mL of CCM gently down the side of each cuvette, aseptically on the bench. When all electroporations are complete, transfer them back to the laminar flow hood.

i. Resuspend the cells by pipetting gently up/down in cuvette to remove all/most cells and transfer to the pre-prepared wells of the tissue culture plates, but do not take any of the lysed and dried culture around the top of cuvette. Wash cuvette with another 1 mL of CCM and add to well before transferring the plates to an airtight chamber. Suffuse the chamber with the special gas mixture (5% CO_2, 5% O_2, balance N_2, Air Products, RSA) and incubate at 37°C – this is Day 0.

j. Three to four hours post-transfection, remove cultures from wells and centrifuge at 1300 rpm for 4 minutes at RT. Discard supernatant and gently resuspend cells in 5 mL fresh CCM before transferring to a new plate and returning to incubator in gassed chamber.

4.1.4. Continuing care of transfection cultures:

a. Keep cultures stationary for the first 7 days and thereafter shake at 50 rpm until parasites come up.

b. Adding drug on Day 2: Spin down the cultures and prepare smears. The parasitaemia should be between 1-2%. If the parasitaemia is > 3%, cut the culture at 1:2 with fresh RBCs or split into 2 wells because the drug will not kill the parasites quickly enough before they crash. Resuspend in CCM supplemented with the appropriate drug – 2.5 nM WR9210 or 2.5 µg/mL blasticidin.

c. Medium changes/Smearing: Change the media every day for the first 6 days on a regular schedule. Smear on day 6 to make sure that all the parasites are dead. WR99210 kills by day 4 and blasticidin by day 6. If there are a lot of gametocytes (>

1%) in days 6-10, the culture is very stressed and it may take longer for parasites to appear. After day 6, feed every other day and smear once or twice a week to check for parasites.

d. Most important: Between days 10-14 cultures must be cut at 3:5 to 1:2 to add fresh RBCs and remove old lysing cells, and then every 7-8 days. Cultures are about to lyse if they are very dark despite having low parasitaemia, > 50% of the RBCs on the smear are blebby, or if there is a layer of reddish colour immediately over the cells (from the side view).

4.1.5. When parasites come up:

a. Smear once or twice a week, scanning the slide for evidence of life (rings!). Parasites should appear between day 20-30, but transfections may come up as early as day 10, or as late as day 45, depending on how quickly your parasite strain grows, and how well the transfection went (health of starting cultures, stress during transfection, parasitaemia on day 2, care of transfections in first 10 days, etc.).

b. As soon as parasitaemias reach ~2 % with > 50 % rings, cultures should be frozen down – two aliquots should be frozen on separate days (see Chapter 2, section 2.2.1.3 for details). Remaining culture should be expanded and harvested for genomic DNA extraction (for microsatellite analysis, plasmid rescue and PCR screening for integration).

c. At some point between days 45 - 90 integration of the plasmid into the genome may occur. These integrant parasites will grow quicker than episomally transformed parasites. When growth rates increase, genomic DNA should be extracted and PCRs performed to confirm integration of the plasmid into the genome. Genomic DNA can be extracted every 3-4 weeks to screen for integration. Integration can occur as early as day 24, and as late as day 150.

d. Once genomic DNA is extracted, the remaining culture can be down-sized to 1 or 2 ml cultures and should be kept at lower parasitaemias, such that they will only need cutting twice per week (i.e. Monday and Friday) and medium changes every other day. Note that the growth rate of episomally transformed cultures are slower than normal.

e. Stably transfected cultures are maintained in the same manner as wildtype cultures (see Chapter 2, section 2.2.1) with the exception that CCM is supplemented with

WR99210. Two aliquots of transfection cultures should be frozen down once a month (see Chapter 2; section 2.2.1.3 for details).

4.2. Bright-Glo reagent

Bright-Glo Luciferase Assay System (Promega, Madison, WI) stored at -20°C.

Thaw the buffer and lyophilised substrate to room temperature (≤ 25°C), combine and aliquot. Store aliquots protected from light at -80°C for up to 1 month.

APPENDIX 5: DETERMINING EFFECTIVE TREATMENT CONCENTRATIONS FOR THE PANEL OF ANTIMALARIAL DRUGS USED IN THIS STUDY

Based on the data in Table 5.1 below, the following treatment concentrations were selected for the ATP assay: chloroquine, artemisinin, mefloquine = 100 nM; DFMO = 10 mM; ritonavir = 100 μM.

Table 5.1: Previously reported IC50 values against *P. falciparum* for the panel of antimalarials used in this study.

Drug	*P. falciparum* strain	IC50	Test concentration	Reference
Chloroquine	3D7	8.5 nM	-	Amewu *et al*., 2006
	3D7	9.7 nM	-	Vivas *et al*., 2008
	3D7	10.6 nM	-	Biot *et al*., 2006
	3D7	11.3 nM	-	Aunpad *et al*., 2009
	3D7	14 nM	-	Penali *et al*., 2007
	3D7	22.2 nM	-	He *et al*., 2010
	3D7	31 nM	-	Jansen *et al*., 2010
	3D7	38.8 nM	-	Bero *et al*., 2009
DFMO	3D7	1 mM	5 mM	van Brummelen *et al*., 2009
	FCR-3	-	5-10 mM	Assaraf *et al*., 1984
	FCR-3	-	10 mM	Assaraf *et al*., 1987
	FCD-3	1.8 mM	-	Das *et al*., 1995
	3D7	1.25 mM	-	Gupta *et al*., 2005
Mefloquine	3D7	6.11 nM	-	Vivas *et al*., 2008
	3D7	18.2 nM	-	Aunpad *et al*., 2009
	3D7	19.3 nM	-	Wong *et al*., 2011
	3D7	26.8 nM	-	Varotti *et al*., 2008
	3D7	38.7 nM	-	Biot *et al*., 2006
	3D7	50 nM	-	Veiga *et al*., 2010
Artemisinin	3D7	3.5 nM	-	Wong *et al*., 2011
	3D7	9.5 nM	-	Amewu *et al*., 2006
	3D7	14.1 nM	-	Sanella *et al*., 2007
	3D7	15.2 nM	-	O‘ Neill *et al*., 2005
	3D7	23.7 nM	-	Jansen *et al*., 2010
	3D7	35.4 nM	-	Bero *et al*., 2009
Ritonavir	3D7	1.7 μM	-	Andrews *et al*., 2006
	3D7	5.62 μM	-	He *et al*., 2010
	3D7	10.9 μM	-	Nsanzabana & Rosenthal, 2011
	3D7	12.2 μM	-	Parikh *et al*., 2006
	3D7	15 μM	-	This study, Figure 5.1 below

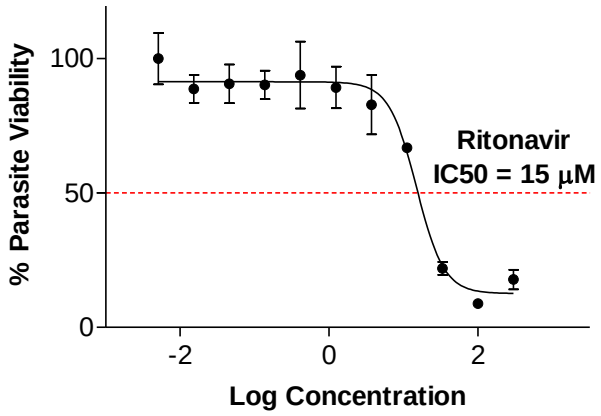

Figure 5.1: IC50 determination of ritonavir against *P. falciparum*. Trophozoite-stage cultures were exposed to two-fold serial dilutions of ritonavir (final concentration ranges: $3.00x10^{2}$–$5.08x10^{-3}$ μM) for 48 hours. Percentage parasite viability was determined using the pLDH assay (section 3.2.3) and the IC50 was determined from a log dose-response curve using GraphPad Prism software. Each data point represents the mean ± SD, n = 2.

APPENDIX 6: STATISTICAL ANALYSES (ATP ASSAY)

Table 6.1: T-test results for comparing ATP levels (measured as relative light units, RLU) of control and drug-treated parasites over a 10-hour period.

	Time (h)	Luminescence (RLU)						*P*-value
		Untreated control			Treatment			
CHLOROQUINE	0	15646	8555		7443	17381		0.9767
	2	34519	35930	39134	68756	58600	39266	0.1980
	4	103377	92133	118010	121291	100728	78276	0.8281
	6	222592	209588	189911	199055	152017	118829	0.0697
	8	262559	277111	257590	328202	261676	180959	0.8505
	10	139663	182308	137045	191697	144871	137301	0.8662
DFMO	0	180287	157005	139355	203531	202217	158971	0.0668
	2	26093	41075	40764	64920	82394	46114	0.1333
	4		75647	69944		55578	77958	0.7419
	6		63764	68167		73962	59680	0.9418
	8	82042	65484	102541	115685	99430	115298	0.0622
	10	240758	228520	212982	268358	223592	246120	0.2577
MEFLOQUINE	0	203621	257406	203532	254061	279652	213624	0.1472
	2	52559	63060	38879	101457	60111	82379	0.2117
	4	43626	100731	84531	196533	166645	192639	0.0492
	6	111475	144281	139064	178044	221681	204247	0.0031
	8	129744	236478	169154	228093	185399	249172	0.4624
	10	158935	192220	112165	177915	198484	250367	0.3242
ARTEMISININ	0	347735	300378	366726	273371	408822	316418	0.9335
	2	48136	29660	41057	152743	170442	206194	0.0161
	4	107912	135300	113482	237751	231683	253011	0.0113
	6	84468	145851	106442	216911	201695	199913	0.0512
	8	61654	101993	136782	204447	177762	158262	0.1501
	10	118667	123605	117779	321068	280007	265112	0.0101
RITONAVIR	0		313160	319906		214484	256773	0.1377
	2	95504	90450	86436	1125	6120	2310	0.0015
	4		116703	153628		3221	2916	0.0891
	6	175473	106107	140248	937	1713	2286	0.0206
	8	168103	176646	150786	2217	750	5018	0.0030
	10	157966	170946	139063	1358	2645	3211	0.0038

Table 6.2: T-test results for comparing percentage parasite viability of control and drug-treated parasites: (i) after a 6-hour treatment and (ii) 48 hours after removal of the drug.

	Drug	% Parasite Viability						*P*-value
		Control			Treatment			
Following a 6-hour drug treatment	CQ	101.98	103.64	94.38	87.24	92.44	92.69	0.1419
	DFMO	86.57	97.95	115.48	108.24	94.35	103.34	0.8636
	MEF	100.63	100.52	98.85	86.03	82.07	87.41	0.0182
	ART	90.29	107.06	102.65	82.96	82.44	84.62	0.0805
	RIT	105.24	98.39	96.37	70.54	73.90	80.33	0.0433
48 hours after removal of drug	CQ	109.37	98.41	92.22	0.40	4.07	22.99	0.0159
	DFMO	110.11	93.39	96.50	82.77	82.36	89.84	0.1398
	MEF	99.77	101.80	98.43	57.95	51.13	59.40	0.0063
	ART	98.14	96.18	105.68	47.33	53.07	36.94	0.0191
	RIT	101.59	101.84	96.56	6.75	10.11	15.07	0.0020

Note: CQ – 100 nM chloroquine; DFMO – 10 mM DL-α-difluoromethylornithine; MEF – 100 nM mefloquine; ART – 100 nM artemisinin; RIT – 100 μM ritonavir.

APPENDIX 7: STATISTICAL ANALYSES (LUCIFERASE ASSAY)

7.1. Luciferase assays

Table 7.1: T-test results for comparing luciferase activity (measured as relative light units, RLU) of control and drug-treated transgenic parasites over a 10-hour period.

	Time (h)	Luminescence (RLU)						*P*-value
		Untreated control			Treatment			
CHLOROQUINE	2	1122792	1135220	1161427	1114444	1131018	1154462	0.0334
	4	1624619	1565411	1550456	1470395	1470118	1476366	0.0460
	6	1843140	1919962	1876667	1668529	1677102	1673088	0.0090
	8	2255781	2270968	2280419	1939037	1899480	1846652	0.0081
	10	2424750	2463256	2472402	2005687	1978203	1892323	0.0088
DFMO	2	1151942	1175345	1126576	1169837	1171990	1173610	0.2951
	4	1548971	1517604	1521085	1493519	1478117	1486485	0.0206
	6	1882597	1883848	1877151	1707241	1704277	1725099	0.0026
	8	2175676	2201653	2197486	1883984	1897428	1920885	0.0008
	10	2314402	2391607	2405235	1967261	1959193	2005835	0.0040
MEFLOQUINE	2	1068543	1103156	1126093	592811	618868	619048	0.0004
	4	1525561	1536325	1534993	569568	564547	567318	0.0000
	6	1876207	1876098	1875251	560900	677688	656483	0.0008
	8	2163910	2153942	2193879	662591	731057	726984	0.0002
	10	2359820	2432798	2447317	753132	755304	755427	0.0003
100 nM RTEMISININ	2	1955654	1998676	2004426	1847839	1788982	1812592	0.0326
	4	2213200	2095381	2130932	1912448	2211172	2158983	0.7200
	6	3188196	3130931	3050952	2971665	2953970	2891520	0.0083
	8	2713456	2634770	2507764	2573214	2511762	2459613	0.0668
	10	2678600	2665420	2588218	2797483	2827012	2815603	0.0330
500 nM ARTEMISININ	2	1173540	1137779	1133876	368864	390675	383248	0.0006
	4	1539472	1530216	1532835	111012	109447	106320	0.0000
	6	1898968	1898183	1896089	73621	71271	66768	0.0000
	8	2311966	2266280	2264829	55235	53918	51062	0.0000
	10	2365821	2431478	2458806	47208	42305	42071	0.0002
RITONAVIR	2	799676	801699	798510	522527	537373	527696	0.0002
	4	1240990	1234286	1239954	447811	455675	427037	0.0002
	6	2117281	2034637	2021813	145794	170474	192287	0.0005
	8	3026346	2780699	2734315	102593	104139	109030	0.0011
	10	2192257	2206089	2169329	40117	38903	30587	0.0000

Table 7.2: T-test results for comparing luciferase activity (measured as relative light units, RLU) of HSP-Luc and LYS-Luc transgenic parasites.

Luminescence (RLU)				*P*-value
HSP-Luc		LYS-Luc		
1496187	1467729	46657	48277	0.0067

Table 7.3: T-test results for comparing the luciferase activity (measured as relative light units, RLU) of untreated transgenic HSP-Luc parasites with those of parasites treated with antimalarials and/or proteasome inhibitors.

Sample	Luminescence (RLU)						*P*-value
	Control			Treatment			
Mefloquine (MQ)	520115	535073	536490	226040	225260	220702	0.0004
Lactacystin (LC)	511996	519286	529460	511996	519286	529460	0.0001
MG-132 (MG)	514856	522357	532573	214440	220974	223318	0.0000
MQ+LC	511657	514973	517905	514856	522357	532573	0.0000
MQ+MG	524843	525538	535510	365164	375438	384686	0.0002

7.2. pLDH assays

Table 7.4: T-test results for comparing the IC50s of artemisinin, chloroquine and mefloquine against wildtype parasites with those against luciferase transgenic parasites.

Figure	Drug	IC50s						*P*-value	
		WT		HL		LL		WT vs. HL	WT vs. LL
4.8a	Artemisinin	14.16	13.42	37.08	28.56	-	-	0.0470	-
4.8b	Artemisinin	35.58	26.30	85.26	77.05	-	-	0.0149	-
4.10a	Artemisinin	34.02	37.52	60.93	47.06	37.75	33.85	0.1256	0.9919
4.10b	Artemisinin	37.04	38.03	51.70	49.85	39.51	39.23	0.0062	0.0704
4.11a,b	Chloroquine	30.09	34.48	33.50	34.48	-	-	0.5275	-
4.11c,d	Mefloquine	19.32	19.12	20.11	15.94	-	-	0.6248	-

Note: (i) The ―Figure" column contains the number of the figure in this chapter from which the experimental data was extracted to do the calculations, (ii) WT, wildtype parasites; HL, HSP-Luc parasites; LL, LYS-Luc parasites.

APPENDIX 8: DETERMINING EFFECTIVE TREATMENT CONCENTRATIONS FOR THE PROTEASOME INHIBITORS USED IN THE LUCIFERASE ASSAY

In order to determine effective treatment concentrations for the proteasome inhibitors used in the Luciferase assay (see section 4.3.5 above), median inhibition concentrations (IC50s) were determined for lactacystin and MG-132 against luciferase transgenic *P. falciparum* parasites. Based on the IC50s obtained, the following treatment concentrations were chosen for the assay: lactacystin = 5 µM; MG-132 = 300 nM.

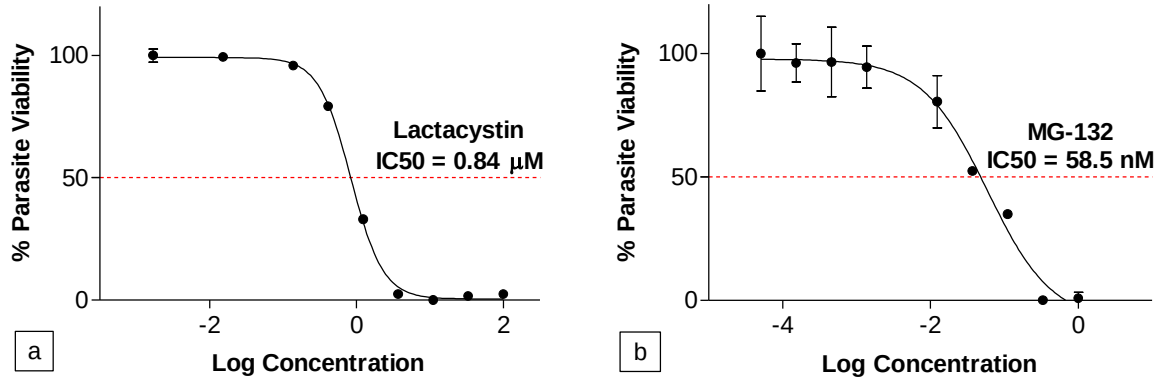

Figure 8.1: IC50 determinations of proteasome inhibitors against HSP-Luc parasites.

Trophozoite-stage cultures were exposed to two-fold serial dilutions of the drugs (final concentration ranges: (i) lactacystin = $1.00x10^{2}$–$1.69x10^{-3}$ µM; (ii) MG-132 = $1.00x10^{0}$–$5.08x10^{-5}$ µM) for 48 hours. Percentage parasite viability was determined using the pLDH assay (section 3.2.3) and the IC50s were determined from a log dose-response curve using GraphPad Prism software (Fig. 8.1a, Lactacystin IC50 = 0.84 µM; Fig. 8.1b, MG-132 IC50 = 58.5 nM). Each data point represents the mean ± SD, n = 2.

REFERENCES

Note: Articles without page numbers are from online journals.

Abraham, E. H., Salikhova, A. Y., and Hug, E. B. (2003) Critical ATP Parameters Associated With Blood and Mammalian Cells: Relevant Measurement Techniques, *Drug Development Research 59*, 152-160.

Acharya, P., Kumar, R., and Tatu, U. (2007) Chaperoning a cellular upheaval in malaria: Heat shock proteins in *Plasmodium falciparum*, *Molecular and Biochemical Parasitology 153*, 85-94.

Aikawa, M. (1972) High-Resolution Autoradiography of Malarial Parasites Treated with ^{3}H-Chloroquine, *American Journal of Parasitology 67*, 277-284.

Alleva, L. M., and Kirk, K. (2001) Calcium regulation in the intraerythrocytic malaria parasite *Plasmodium falciparum*, *Molecular and Biochemical Parasitology 117*, 121-128.

Amewu, R., Stachulski, A. V., Ward, S. A., Berry, N. G., Bray, P. G., Davies, J., Labat, G., Vivas, L., and O' Neill, P. M. (2006) Design and synthesis of orally active dispiro 1,2,4,5-tetraoxanes; synthetic antimalarials with superior activity to artemisinin, *Organic and Biomolecular Chemistry 4*, 4431-4436.

Andrews, K. T., Fairlie, D. P., Madala, P. K., Ray, J., Wyatt, D. M., Hilton, P. M. M., P.A., Beattie, L., Gardiner, D. L., Reid, R. C., Stoermoer, M. J., Skinner-Adams, T., Berry, C., and McCarthy, J. S. (2006) Potencies of Human Immunodeficiency Virus Protease Inhibitors *In Vitro* against *Plasmodium falciparum* and *In Vivo* against Murine Malaria, *Antimicrobial Agents and Chemotherapy 50*, 639-648.

Asawamahasakda, W., Ittarat, I., Pu, Y., Ziffer, H., and Meshnick, S. R. (1994) Reaction of Antimalarial Endoperoxides with Specific Parasite Proteins, *Antimicrobial Agents and Chemotherapy 38*, 1854-1858.

Assaraf, Y. G., Golenser, J., Spira, D. T., and Bachrach, U. (1984) Polyamine levels and the activity of their biosynthetic enzymes in human eythrocytes infected with the malarial parasite, *Plasmodium falciparum*, *Biochemical Journal 222*, 815-819.

Assaraf, Y. G., Golenser, J., Spira, D. T., Messer, G., and Bachrach, U. (1987) Cytostatic effect of DL-α-difluoromethylornithine against *Plasmodium falciparum* and its reversal by diamines and spermidine, *Parasitology Research 73*, 313-318.

Aunpad, R., Somsri, S., Na-Bangchang, K., Udomsangpetch, R., Mungthin, M., Adisakwattana, P., and Chaijaroenkul, W. (2009) The effect of mimicking febrile temperature and drug stress on malarial development, *Annals of Clinical Microbiology and Antimicrobials 8*.

Balint, G. A. (2001) Artemisinin and its derivatives: An important new class of antimalarial agents, *Pharmacology and Therapeutics 90*, 261-265.

Bandyopadhyay, U., Das, D., and Banerjee, R. K. (1999) Reactive oxygen species: Oxidative damage and pathogenesis, *Current Science 77*, 658-666.

Barrett, M. P. (1997) The Pentose Phosphate Pathway and Parasitic Protozoa, *Parasitology Today 13*, 11-16.

Beeson, J. G., and Brown, G. V. (2002) Pathogenesis of *Plasmodium falciparum* malaria: the roles of parasite adhesion and antigenic variation, *Cellular and Molecular Life Sciences 59*, 258-271.

Bero, J., Ganfon, H., Jonville, M., Frédérich, M., Gbaguidi, F., De Mol, P., Moudachirou, M., and Quetin-Leclercq, J. (2009) *In vitro* antiplasmodial activity of plants used in Benin in traditional medicine to treat malaria, *Journal of Ethnopharmacology 122*, 439-444.

Biot, C., Daher, W., Chavain, N., Fandeur, T., Khalife, J., Dive, D., and De Clercq, E. (2006) Design and Synthesis of Hydroxyferroquine Derivatives with Antimalarial and Antiviral Activities, *Journal of Medicinal Chemistry 49*, 2845-2849.

Boddey, J. A., Hodder, A. N., Günther, S., Gilson, P. R., Patsiouras, H., Kapp, E. A., Pearce, J. A., de Koning-Ward, T. F., Simpson, R. J., Crabb, B. S., Cowman, A. F. (2010) An aspartyl protease directs malaria effector proteins to the host cell, *Nature 463*, 627-631.

Bray, P. G., Janneh, O., Raynes, K. J., Mungthin, M., and Ginsburg, H. (1999) Cellular Uptake of Chloroquine Is Dependent on Binding to Ferriprotoporphyrin IX and Is Independent of NHE Activity in *Plasmodium falciparum*, *The Journal of Cell Biology 145*, 363-376.

Brossi, A., Venugopalan, B., Gerpe, D. L., Yeh, H. J. C., Flippen-Anderson, J. L., Buchs, P., Luo, X. D., Milhous, W., and Peters, W. (1988) Arteether, a New Antimalarial Drug: Synthesis and Antimalarial Properties, *Journal of Medicinal Chemistry 31*, 645-650.

Bruce, J. I. E. (2010) Plasma membrane calcium pump regulation by metabolic stress, *World Journal of Biological Chemistry 1*, 221-228.

Chandwani, A., and Shuter, J. (2008) Lopinavir/ritonavir in the treatment of HIV-1 infection: a review, *Therapeutics and Clinical Risk Management 4*, 1023-1033.

Chesher, D. (2008) Evaluating Assay Precision, *Clinical Biochemistry Reviews 29*, 23-26.

Chevli, R., and Fitch, C. D. (1982) The Antimalarial Drug Mefloquine Binds to Membrane Phospholipids, *Antimicrobial Agents and Chemotherapy 21*, 581-586.

Chiang, P. K., Bujnicki, J. M., Su, X., and Lanar, D. E. (2006) Malaria: Therapy, Genes and Vaccines, *Current Molecular Medicine 6*, 309-326.

Chou, A. C., Chevli, R., and Fitch, C. D. (1980) Ferriprotoporphyrin IX Fulfills the Criteria for Identification as the Chloroquine Receptor of Malaria Parasites, *Biochemistry 19*, 1543-1549.

Chou, A. C., and Fitch, C. D. (1981) Mechanism of Hemolysis Induced by Ferriprotoporphyrin IX, *Journal of Clinical Investigation 68*, 672-677.

Clark, K., Niemand, J., Reeksting, S., Smit, S., van Brummelen, A. C., Williams, M., Louw, A. I., and Birkholtz, L. (2010) Functional consequences of perturbing polyamine metabolism in the malaria parasite, *Plasmodium falciparum*, *Amino Acids 38*, 633-644.

Co, E. M. A., Johnson, S. M., Murthy, T., Talwar, M., Hickman, M. R., and Johnson, J. D. (2010) Recent Methods in Antimalarial Susceptibility Testing, *Anti-Infective Agents in Medicinal Chemistry 9*, 148-160.

Combrinck, J. M., Mabotha, T. E., Ncokazi, K. K., Ambele, M. A., Taylor, D., Smith, P. J., Hoppe, H. C., and Egan, T. J. (in press) Insights into the role of heme in the mechanism of action of antimalarials, *ACS Chemical Biology*.

Cowman, A. F., and Crabb, B. S. (2006) Invasion of Red Blood Cells by Malaria Parasites, *Cell 124*, 755-766.

Cox, F. E. G. (2010) History of the discovery of the malaria parasites and their vectors, *Parasites and Vectors 3*.

Crespo, M. D. P., Avery, T. D., Hanssen, E., Fox, E., Robinson, T. V., Valente, P., Taylor, D. K., and Tilley, L. (2008) Artemisinin and a Series of Novel Endoperoxide Derivatives Exert Early Effects on Digestive Vacuole Morphology, *Antimicrobial Agents and Chemotherapy 52*, 98-109.

Dahl, E. L., Shock, J. L., Shenai, B. R., Gut, J., DeRisi, J. L., and Rosenthal, P. J. (2006) Tetracyclines Specifically Target the Apicoplast of the Malaria Parasite *Plasmodium falciparum*, *Antimicrobial Agents and Chemotherapy 50*, 3124-3131.

Damodaran, S. E., Pradhan, P., and Pradhan, S. C. (2011) Newer approaches to malaria control, *Tropical Parasitology 1*, 57-63.

Das, B., Gupta, R., and Madhubala, R. (1995) Combined action of inhibitors of polyamine biosynthetic pathway with a known antimalarial drug chloroquine on *Plasmodium falciparum*, *Pharmacological Research 31*, 189-193.

de Koning-Ward, T. F., Janse, C. J., and Waters, A. P. (2000) The Development of Genetic Tools for Dissecting the Biology of Malaria Parasites, *Annual Review of Microbiology 54*, 157-185.

de Ridder, J. (2003) Malaria Manual, 3rd ed., University of Pretoria, Pretoria.

Dondorp, A. M., Fairhurst, R. M., Slutsker, L., MacArthur, J. R., Breman, J. G., Guerin, P. J., Wellems, T. E., Ringwald, P., Newman, R. D., and Plowe, C. V. (2011) The Threat of Artemisinin-Resistant Malaria, *The New England Journal of Medicine 365*, 1073-1075.

Dondorp, A. M., Nosten, F., Yi, P., Das, D., Phyo, A. P., Tarning, J., Lwin, K. M., Ariey, F., Hanpithakpong, W., Lee, S. J., Ringwald, P., Silamut, K., Imwong, M., Chotivanich, K., Lim, P., Herdman, T., An, S. S., Yeung, S., Singhasivanan, P., Day, N. P. J., Lindegardh, N., Socheat, D., and White, N. J. (2009) Artemisinin Resistance in *Plasmodium falciparum* Malaria, *The New England Journal of Medicine 361*, 455-467.

Dorn, A., Vippagunta, S. R., Matile, H., Jacquet, C., Vennerstrom, J. L., and Ridley, R. G. (1998) An Assessment of Drug-Haematin Binding as a Mechanism for Inhibition of Haematin Polymerisation by Quinoline Antimalarials, *Biochemical Pharmacology 55*, 727-736.

Dubey, A. K., Kumar, N., Singh, M., Singh, A. P., and Ashok, P. K. (2009) Newer Trends In Antimalarial Chemotherapy, *The Pharma Research 1*, 143-149.

Duraisingh, M. T., Triglia, T., and Cowman, A. F. (2002) Negative selection of *Plasmodium falciparum* reveals targeted gene deletion by double crossover recombination, *International Journal for Parasitology 32*, 81-89.

Eckstein-Ludwig, U., Webb, R. J., van Goethem, I. D. A., East, J. M., Lee, A. G., Kimura, M., O' Neill, P. M., Bray, P. G., Ward, S. A., and Krishna, S. (2003) Artemisinins target the SERCA of *Plasmodium falciparum*, *Nature 424*, 957-961.

Egan, T. J., Combrinck, J. M., Egan, J., Hearne, G. R., Marques, H. M., Ntenteni, S., Sewell, B. T., Smith, P. J., Taylor, D., van Schalkwyk, D. A., and Walden, J. C. (2002) Fate of haem iron in the malaria parasite *Plasmodium falciparum*, *Biochemical Journal 365*, 343-347.

Egan, T. J., Ross, D. C., and Adams, P. A. (1994) Quinoline anti-malarial drugs inhibit spontaneous formation of *β*-haematin (malaria pigment), *FEBS Letters 352*, 54-57.

Ekland, E. H., Schneider, J., and Fidock, D. A. (2011) Identifying apicoplast-targeting antimalarials using high-throughput compatible approaches, *The FASEB Journal 25*.

Famin, O., and Ginsburg, H. (2002) Differential effects of 4-aminoquinoline-containing antimalarial drugs on hemoglobin digestion in *Plasmodium falciparum*-infected erythrocytes, *Biochemical Pharmacology 63*, 393-398.

Famin, O., Krugliak, M., and Ginsburg, H. (1999) Kinetics of Inhibition of Glutathione-Mediated Degradation of Ferriprotoporphyrin IX by Antimalarial Drugs, *Biochemical Pharmacology 58*, 59-68.

Fitch, C. D., Chevli, R., Banyal, H. S., Philips, G., Pfaller, M. A., and Krogstad, D. J. (1982) Lysis of *Plasmodium falciparum* by ferriprotoporphyrin IX and a chloroquine-ferriprotoporphyrin IX complex, *Antimicrobial Agents and Chemotherapy 21*, 819-822.

Francis, S. E., Sullivan, D. J., and Goldberg, D. E. (1997) Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*, *Annual Review of Microbiology 51*, 97-123.

Gamo, F., Sanz, L. M., Vidal, J., de Cozar, C., Alvarez, E., Lavandera, J., Vanderwall, D. E., Green, D. V. S., Kumar, V., Hasan, S., Brown, J. R., Peishoff, C. E., Cardon, L. R., and Garcia-Bustos, J. F. (2010) Thousands of chemical starting points for antimalarial lead identification, *Nature 465*, 305-310.

Garah, F. B., Stigliani, J. L., Coslèdan, F., Meunier, B., and Robert, A. (2009) Docking studies of structurally diverse antimalarial drugs targeting PfATP6: no correlation between *in silico* binding affinity and *in vitro* antimalarial activity, *ChemMedChem 4*, 1469-1479.

Geary, T. G., and Jensen, J. B. (1983) Lack of Cross-Resistance to 4-Aminoquinolines in Chloroquine-Resistant *Plasmodium falciparum In vitro*, *The Journal of Parasitology 69*, 97-105.

Ghosh, A., Edwards, M. J., and Jacobs-Lorena, M. (2000) The Journey of the Malaria Parasite in the Mosquito: Hopes for the New Century, *Parasitology Today 16*, 196-201.

Gille, C., Goede, A., Schlöetelburg, C., Preißner, R., Kloetzel, P., Göbel, U. B., and Frömmel, C. (2003) A comprehensive view on proteasomal sequences: implications for the evolution of the proteasome, *Journal of Molecular Biology 326*, 1437-1448.

Ginsburg, H. (2010) Malaria parasite stands out, *Nature 466*, 702-703.

Ginsburg, H., Famin, O., Zhang, J., and Krugliak, M. (1998) Inhibition of Glutathione-dependent Degradation of Heme By Chloroquine and Amodiaquine as a Possible Basis for Their Antimalarial Mode of Action, *Biochemical Pharmacology 56*, 1305-1313.

Glushakova, S., Yin, D., Gartner, N., and Zimmerberg, J. (2007) Quantification of malaria parasite release from infected erythrocytes: inhibition by protein-free media, *2007 6*.

Gluzman, I. Y., Francis, S. E., Oksman, A., Smith, C. E., Duffin, K. L., and Goldberg, D. E. (1994) Order and Specificity of the *Plasmodium falciparum* Hemoglobin Degradation Pathway, *Journal of Clinical Investigation 93*, 1602-1608.

Goodman, C. D., Su, V., and McFadden, G. I. (2007) The effects of anti-bacterials on the malaria parasite *Plasmodium falciparum*, *Molecular and Biochemical Parasitology 52*, 181-191.

Greenwood, B. M., Fidock, D. A., Kyle, D. E., Kappe, S. H. I., Alonso, P. L., Collins, F. H., and Duffy, P. E. (2008) Malaria: progress, perils and prospects for eradication, *The Journal of Clinical Investigation 118*, 1266-1276.

Grimberg, B. T., and Mehlotra, R. K. (2011) Expanding the Antimalarial Drug Arsenal—Now, But How?, *Pharmaceuticals 4*, 681-712.

Gupta, R. D., Krause-Ihle, T., Bergmann, B., Müller, I. B., Khomutov, A. R., Müller, S., Walter, R. D., and Luersen, K. (2005) 3-Aminooxy-1-Aminopropane and Derivatives Have an Antiproliferative Effect on Cultured *Plasmodium falciparum* by Decreasing Intracellular Polyamine Concentrations, *Antimicrobial Agents and Chemotherapy 49*, 2857-2864.

Haynes, R. K., and Krishna, S. (2004) Artemisinins: activities and actions, *Microbes and Infection 6*, 1339-1346.

He, Z., Chen, L., You, J., Qin, L., and Chen, X. (2010) *In vitro* interactions between antiretroviral protease inhibitors and artemisinin endoperoxides against *Plasmodium falciparum*, *International Journal of Antimicrobial Agents 35*, 191-193.

Helm, S., Lehmann, C., Nagel, A., Stanway, R. R., Horstmann, S., Llinás, M., and Heussler, V. T. (2010) Identification and Characterization of a Liver Stage-Specific Promoter Region of the Malaria Parasite *Plasmodium*, *PLoS ONE 5*, e13653.

Hobbs, C., and Duffy, P. (2011) Drugs for malaria: something old, something new, something borrowed, *F1000 Biology Reports 3*.

Holcik, M., and Sonenberg, N. (2005) Translational Control In Stress And Apoptosis, *Nature Reviews Molecular Cell Biology 6*, 318-327.

Hong, Y., Yang, Y., and Meshnick, S. R. (1994) The interaction of artemisinin with malarial hemozoin, *Molecular and Biochemical Parasitology 63*, 121-128.

Hoppe, H. C., van Schalkwyk, D. A., Wiehart, U. I. A., Meredith, S. A., Egan, J., and Weber, B. W. (2004) Antimalarial Quinolines and Artemisinin Inhibit Endocytosis in *Plasmodium falciparum*, *Antimicrobial Agents and Chemotherapy 48*, 2370-2378.

Ittarat, W., Sreepian, A., Srisaran, A., and Pathepchotivong, K. (2003) Effects of Dihydroartemisinin on the Antioxidant Capacity of *P. falciparum*-infected Erythrocytes, *Southeast Asian Journal of Tropical Medicine and Public Health 34*, 744-750.

Iversen, P. W., Eastwood, B. J., Sittampalam, S. G., and Cox, K. L. (2006) A Comparison of Assay Performance Measures in Screening Assays: Signal Window, Z' Factor, and Assay Variability Ratio, *Journal of Biomolecular Screening 11*, 247-252.

Jambou, R., Legrand, E., Niang, M., Khim, N., Lim, P., Volney, B., Ekala, M. T., Bouchier, C., Esterre, P., Fandeur, T., and Mercereau-Puijalon, O. (2005) Resistance to *Plasmodium falciparum* field isolates to *in-vitro* artemether and point mutations of the SERCA-type PfATPase6, *Lancet 366*, 1960-1963.

Jansen, O., Angenot, L., Tits, M., Nicolas, J. P., De Mol, P., Nikiéma, J., and Frédérich, M. (2010) Evaluation of 13 selected medicinal plants from Burkina Faso for their antiplasmodial properties, *Journal of Ethnopharmacology 130*, 143-150.

Jung, M. J. (1997) Synthesis and Cytotoxicity of Novel Artemisinin Analogs, *Bioorganic and Medicinal Chemistry Letters 7*, 1091-1094.

Jung, M. J., Kim, H., Nam, K. Y., and No, K. T. (2005) Three-dimensional structure of *Plasmodium falciparum* Ca2+-ATPase(PfATP6) and docking of artemisinin derivatives to PfATP6, *Bioorganic and Medicinal Chemistry Letters 15*, 2994-2997.

Kachur, S. P., MacArthur, J. R., and Slutsker, L. (2010) A call to action: addressing the challenge of artemisinin-resistant malaria, *Expert Review of Anti-Infective Therapy 8*, 365-366.

Kalra, B. S., Chawla, S., Gupta, P., and Valecha, N. (2006) Screening of antimalarial drugs: An overview, *Indian Journal of Pharmacology 38*, 5-12.

Kanaani, J., and Ginsburg, H. (1989) Metabolic Interconnection between the Human Malaria Parasite *Plasmodium falciparum* and Its Host Erythrocyte: Regulation of ATP levels by means of an adenylate translocator and adenylate kinase, *The Journal of Biological Chemistry 264*, 3194-3199.

Kannan, R., Kumar, K., Sahal, D., Kukreti, S., and Chauhan, V. S. (2005) Reaction of artemisinin with haemoglobin: implications for antimalarial activity, *Biochemical Journal 385*, 409-418.

Karbwang, J., Bangchang, K. N., Thanavibul, A., Bunnag, D., Chongsuphajaisiddhi, T., and Harinasuta, T. (1992) Comparison of oral artemether and mefloquine in acute uncomplicated *falciparum* malaria, *The Lancet 340*, 1245-1248.

Kim, J. K., Estève, P., Jacobsen, S. E., and Pradhan, S. (2009) UHRF1 binds G9a and participates in p21 transcriptional regulation in mammalian cells, *Nucleic Acids Research 37*, 493-505.

Klayman, D. L. (1985) Qinghaosu (Artemisinin): An Antimalarial Drug form China, *Science 228*, 1049-1055.

Kohler, S., Delwiche, C. F., Denny, P. W., Tilney, L.G., Webster, P., Wilson, R. J. M., Palmer, J. D., and Roos, D.S. (1997) A Plastid of Probable Green Algal Origin in Apicomplexan Parasites, *Science 275*, 1485-1489.

Kreidenweiss, A., Kremsner, P. G., and Mordmüller, B. (2008) Comprehensive study of proteasome inhibitors against *Plasmodium falciparum* laboratory strains and field isolates from Gabon, *Malaria Journal 7*.

Krungkrai, S. R., and Yuthavong, Y. (1987) The antimalarial action on *Plasmodium falciparum* of qinghaosu and artesunate in combination with agents which modulate oxidant stress, *Transactions of the Royal Society of Tropical Medicine and Hygiene 81*, 710-714.

Kültz, D. (2003) Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function, *The Journal of Experimental Biology 206*, 3119-3124.

Lambros, C., and Vanderberg, J. P. (1979) Synchronization of *Plasmodium falciparum* Erythrocytic Stages in Culture, *The Journal of Parasitology 65*, 418-420.

Lazarus, M. D., Schneider, T. G., and Taraschi, T. F. (2008) A new model for hemoglobin ingestion and transport by the human malaria parasite *Plasmodium falciparum*, *Journal of Cell Science 121*, 1937-1949.

Levander, O. A., Ager, A. L., Morris, V. C., and May, R. G. (1989) Qinghaosu, dietary vitamin E, selenium, and cod-liver oil: effect on the susceptibility of mice to the malarial parasite *Plasmodium yoelii*, *American Journal of Clinical Nutrition 50*, 346-352.

Li, W., Mo, W., Shen, D., Sun, L., Wang, J., Lu, S., Gitschier, J. M., and Zhou, B. (2005) Yeast Model Uncovers Dual Roles of Mitochondria in the Action of Artemisinin, *PLoS Genetics 1*, 329-334.

Liao, F. (2009) Discovery of Artemisinin (Qinghaosu), *Molecules 14*, 5362-5366.

Lindsay, S., Ansell, J., Selman, C., Cox, V., Hamilton, K., and Walraven, G. (2000) Effect of pregnancy on exposure to malaria mosquitoes, *The Lancet 355*, 1972.

Loria, P., Miller, S., Foley, M., and Tilley, L. (1999) Inhibition of the peroxidative degradation of haem as the basis of action of chloroquine and other quinoline antimalarials, *Biochemical Journal 339*, 363-370.

Loup, C., Lelièvre, J., Benoit-Vical, F., and Meunier, B. (2007) Trioxaquines and Heme-Artemisinin Adducts Inhibit the *In Vitro* Formation of Hemozoin Better than Chloroquine, *Antimicrobial Agents and Chemotherapy 51*, 3768-3770.

Mackintosh, C. L., Beeson, J. G., and Marsh, K. (2004) Clinical features and pathogenesis of severe malaria, *Trends in Parasitology 20*, 597-603.

Makler, M. T., Ries, J. M., Williams, J. A., Bancroft, J. E., Piper, R. C., Gibbins, B. L., and Hinrichs, D. J. (1993) Parasite lactate dehydrogenase activity as an assay for *Plasmodium falciparum* drug sensitivity, *The American Journal of Tropical Medicine and Hygiene 48*, 739-741.

Malenga, G., Palmer, A., Staedke, S., Kazadi, W., Mutabingwa, T., Ansah, E., Barnes, K. I., and Whitty, C. J. (2005) Antimalarial treatment with artemisinin combination therapy in Africa, *British Medical Journal 331*, 706-707.

Manfredi, G., Yang, L., Gajewski, C. D., and Mattiazzi, M. (2002) Measurements of ATP in mammalian cells, *Methods 26*, 317-326.

Medicines for Malaria Venture. (2010) New Medicines for Malaria Control and Eradication: Target Product Profiles (TPPs) for 2010. *[Online]* http://www.mmv.org/sites/default/files/uploads/docs/essential_info_for_scientists/PRODUCT_PROFILE_Sept_2010.pdf [Accessed: 26 March 2012].

Mendis, K., Rietveld, A., Warsame, M., Bosman, A., Greenwood, B., and Wernsdorfer, W. H. (2009) From malaria control to eradication: The WHO perspective, *Tropical Medicine and International Health 14*, 1-7.

Meshnick, S. R. (1994) The mode of action of antimalarial endoperoxides, *Transactions of the Royal Society of Tropical Medicine and Hygiene 88*, 31-32.

Meshnick, S. R., and Dobson, M. J. (2001) The History of Antimalarial Drugs, in *Antimalarial Chemotherapy: Mechanisms of Action, Resistance, and New Directions in Drug Discovery* (Rosenthal, P. J., Ed.), pp 15-25, Humana Press, Totowa, NJ, USA.

Meshnick, S. R., Thomas, A., Ranz, A., Xu, C. M., and Pan, H. Z. (1991) Artemisinin (qinghaosu): the role of intracellular hemin in its mechanism of antimalarial action, *Molecular and Biochemical Parasitology 49*, 181-189.

Meshnick, S. R., Yang, Y., Lima, V., Kuypers, F., Kamchonwongpaisan, S., and Yuthavong, Y. (1993) Iron-Dependent Free Radical Generation from the Antimalarial Agent Artemisinin (Qinghaosu), *Antimicrobial Agents and Chemotherapy 37*, 1108-1114.

Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P. L., and Ververt, J. P. (1978) Catalytic irreversible inhibition of mammalian ornithine decarboxylase by substrate and product analogues, *Journal of the American Chemical Society 100*, 2551-2552.

Miller, L. H., Baruch, D. I., Marsh, K., and Doumbo, O. K. (2002) The pathogenic basis of malaria, *Nature 415*, 673-679.

Mimica, I., Fry, W., Eckert, G., and Schwartz, D. E. (1983) Multiple-dose kinetic study of mefloquine in healthy male volunteers, *Chemotherapy 29*, 184-187.

Müller, S. (2001) Redox and antioxidant systems of the malaria parasite *Plasmodium falciparum*, *Molecular Microbiology 53*, 1291-1305.

Mungthin, M., Bray, P. G., Ridley, R. G., and Ward, S. A. (1998) Central Role of Hemoglobin Degradation in Mechanisms of Action of 4-Aminoquinolines, Quinoline Methanols, and Phenanthrene Methanols, *Antimicrobial Agents and Chemotherapy 42*, 2973-2977.

Myint, P. T., and Shwe, T. (1987) A controlled clinical trial of artemether (qinghaosu derivative) versus quinine in complicated and severe *falciparum* malaria, *Transactions of the Royal Society of Tropical Medicine and Hygiene 81*, 559-561.

Myung, J., Kim, K. B., and Crews, C. M. (2001) The Ubiquitin-Proteasome Pathway and Proteasome Inhibitors, *Medical Research Reviews 21*, 245-273.

Na-Bangchang, K., and Karbwang, J. (2009) Current status of malaria chemotherapy and the role of pharmacology in antimalarial drug research and development, *Fundamental & Clinical Pharmacology 23*, 387-409.

Nabarro, D. N., and Tayler, E. M. (1998) The "Roll Back Malaria" Campaign, *Science 280*, 2067-2068.

Nagamune, K., Moreno, S. N., Chini, E. N., and Sibley, L. D. (2008) Calcium Regulation and Signaling in Apicomplexan Parasites, in *Molecular Mechanisms of Parasite Invasion* (Burleigh, B. A., and Soldati-Favre, D., Eds.) 7th ed., pp 70-81, Landes Bioscience and Springer Sci, New York and Texas, USA.

Nickel, C., Rahlfs, S., Deponte, M., Koncarevic, S., and Becker, K. (2006) Thioredoxin Networks in the Malarial Parasite *Plasmodium falciparum*, *Antioxidants and Redox Signaling 8*, 1227-1239.

Noedl, H., Wongsrichanalai, C., and Wernsdorfer, W. H. (2003) Malaria drug-sensitivity testing: new assays, new perspectives, *Trends in Parasitology 19*, 175-181.

Nsanzabana, C., and Rosenthal, P. J. (2011) *In vitro* activity against *Plasmodium falciparum* of antiretroviral drugs, *Antimicrobial Agents and Chemotherapy 55*, 5073-5077.

Nwaka, S., and Ridley, R. G. (2003) Virtual drug discovery and development for neglected diseases through public–private partnerships, *Nature Reviews Drug Discovery 2*, 919-928.

O‘ Donnell, R. A., Preiser, P. R., Williamson, D. H., Moore, P. W., Cowman, A. F., and Crabb, B. S. (2001) An alteration in concatameric structure is associated with efficient segregation of plasmids in transfected *Plasmodium falciparum* parasites, *Nucleic Acids Research 29*, 716-724.

Olzewski, K. L., and Llinás, M. (2010) Central carbon metabolism of *Plasmodium* parasites, *Molecular and Biochemical Parasitology 175*, 95-103.

O'Neill, P. M., Rawe, S. L., Borstnik, K., Miller, A., Ward, S. A., Bray, P. G., Davies, J., Oh, C. H., and Posner, G. H. (2005) Enantiomeric 1,2,4-Trioxanes Display Equivalent *in vitro* Antimalarial Activity Versus *Plasmodium falciparum* Malaria Parasites: Implications for the Molecular Mechanism of Action of the Artemisinins, *ChemBioChem 6*, 2048-2054.

O'Neill, P. M., Ward, S. A., Berry, N. G., Jeyadevan, J. P., Biagini, G. A., Asadollaly, E., Park, B. K., and Bray, P. G. (2006) A medicinal chemistry perspective on 4-aminoquinoline antimalarials, *Current Topics in Medicinal Chemistry 6*, 479-507.

Orjih, A. U., Banyal, H. S., Chevli, R., and Fitch, C. D. (1981) Hemin Lyses Malaria Parasites, *Science 214*, 667-669.

Otten-Kuipers, M. A., Roelofsen, B., and Op den Kamp, J. A. (1995) Stage-dependent effects of analogs of gramicidin A on the growth of *Plasmodium falciparum in vitro*, *Parasitology Research 81*, 26-31.

Paitayatat, S., Tarnchompoo, B., Thebtaranonth, Y., and Yuthavong, Y. (1997) Correlation of Antimalarial Activity of Artemisinin Derivatives with Binding Affinity with Ferroprotoporphyrin IX, *Journal of Medicinal Chemistry 40*, 633-638.

Pandey, A. V., Tekwani, B. L., Singh, R. L., and Chauhan, V. S. (1999) Artemisinin, an Endoperoxide Antimalarial, Disrupts the Hemoglobin Catabolism and Heme Detoxification Systems in Malarial Parasite, *The Journal of Biological Chemistry 274*, 19383-19388.

Parikh, S., Gut, J., Istvan, E., Goldberg, D. E., Havlir, D. V., and Rosenthal, P. J. (2005) Antimalarial Activity of Human Immunodeficiency Virus Type 1 Protease Inhibitors, *Antimicrobial Agents and Chemotherapy 49*, 2983-2985.

Parikh, S., Liu, J., Sijwali, P., Gut, J., Goldberg, D. E., and Rosenthal, P. J. (2006) Antimalarial Effects of Human Immunodeficiency Virus Type 1 Protease Inhibitors Differ from Those of the Aspartic Protease Inhibitor Pepstatin, *Antimicrobial Agents and Chemotherapy 50*, 2207-2209.

Peatey, C. L., Leroy, D., Gardiner, D. L., and Trenholme, K. R. (2012) Anti-malarial drugs: how effective are they against *Plasmodium falciparum* gametocytes?, *Malaria Journal 11*.

Penali, L., Mulholland, D. A., Tano, K. D., Cheplogoi, P. K., and Randrianarivelojosia, M. (2007) Low antiplasmodial activity of alkaloids and amides from the stem bark of *Zanthoxylum rubescens* (Rutaceae), *Parasite 14*, 161-164.

Pesce, E., and Blatch, G. L. (2009) The Hsp40-Hsp70 chaperone machinery of *Plasmodium falciparum*, *African Journal of Biochemistry Research 3*, 154-163.

Plouffe, D., Brinker, A., McNamara, C., Henson, K., Kato, N., Kuhen, K., Nagle, A., Adrián, F., Matzen, J. T., Anderson, P., Nam, T., Gray, N. S., Chatterjee, A., Janes, J., Yan, S. F., Trager, R., Caldwell, J. S., Schultz, P. G., Zhou, Y., and Winzeler, E. A. (2008) *In silico* activity profiling reveals the mechanism of action of antimalarials discovered in a high-throughput screen, *Proceedings of the National Academy of Sciences 105*, 9059-9064.

Quashie, N. B., Ranford-Cartwright, L. C., and de Koning, H. P. (2010) Uptake of purines in *Plasmodium falciparum*-infected human erythrocytes is mostly mediated by the human Equilibrative Nucleoside Transporter and the human Facilitative Nucleobase Transporter *Malaria Journal 9*.

Rahlfs, S., Schirmer, R. H., and Becker, K. (2002) The thioredoxin system of *Plasmodium falciparum* and other parasites, *Cellular and Molecular Life Sciences 59*, 1024-1041.

Ralph, S. A., van Dooren, G. G., Waller, R. F., Crawford, M.J., Fraunholz, M. J., Foth, B. J., Tonkin, C. J., Roos, D. S., and McFadden, G. I. (2004) Tropical infectious diseases: Metabolic maps and functions of the *Plasmodium falciparum* apicoplast, *Nature Reviews Microbiology 2*, 203-216.

Rasti, N., Wahlgren, M., and Chen, Q. (2004) Molecular aspects of malaria pathogenesis, *FEMS Immunology and Medical Microbiology 41*, 9-26.

Räth, K., Taxis, K., Walz, G., Gleiter, C.H., Li, S., and Heide, L. (2004) Pharmacokinetic study of artemisinin after oral intake of a traditional preparation of *Artemisia Annua* L. (Annual Wormwood), *The American Journal of Tropical Medicine and Hygiene 70*, 128-132.

Richie, T. L., and Saul, A. (2002) Progress and challenges for malaria vaccines, *Nature 415*, 694-701.

Ridley, R. G. (2002) Medical need, scientific opportunity and the drive for antimalarial drugs, *Nature 415*, 686-693.

Ripp, S., Sayler, G., and Close, D. (2011) Mammalian-Based Bioreporter Targets: Protein Expression for Bioluminescent and Fluorescent Detection in the Mammalian Cellular Background, in *Biosensors for Health, Environment and Biosecurity* (Serra, P. A., Ed.), pp 469-498, InTech, Rijeka, Croatia.

Robert, A., Benoit-Vical, F., Claparols, C., and Meunier, B. (2005) The antimalarial drug artemisinin alkylates heme in infected mice, *Proceedings of the National Academy of Sciences 102*, 13676–13680.

Robert, A., and Meunier, B. (1997) Characterization of the First Covalent Adduct between Artemisinin and a Heme Model, *Journal of the American Chemical Society 119*, 5968-5969.

Rosenthal, P. J. (2003) Antimalarial Drug Discovery: old and new approaches, *The Journal of Experimental Biology 206*, 3735-3744.

Sachs, J., and Malaney, P. (2002) The economic and social burden of malaria, *Nature 415*, 680-685.

Sanella, A. R., Messori, L., Casini, A., Vincieri, F. F., Bilia, A. R., Majori, G., and Severini, C. (2007) Antimalarial properties of green tea, *Biochemical and Biophysical Research Communications 353*, 177-181.

Sanz, L. M., Crespo, B., De-Cozár, C., Ding, X. C., Llergo, J. L., Burrows, J. N., Garcia-Bustos, J. F., and Gamo, F. (2012) *P. falciparum In Vitro* Killing Rates Allow to Discriminate between Different Antimalarial Mode-of-Action, *PLoS ONE 7*, e39049.

Schwoch, G., and Passow, H. (1973) Preparation and Properties of Human Erythrocyte Ghosts, *Molecular and Cellular Biochemistry 2*, 197-218.

Sherman, I. W. (2009) *Advances in Parasitology: Reflections on a Century of Malaria Biochemistry*, Vol. 67, 1st ed., Academic Press, London, UK.

Shonhai, A., Boshoff, A., and Blatch, G. L. (2007) The structural and functional diversity of Hsp70 proteins from *P. falciparum*, *Protein Science 16*, 1803-1818.

Singh, B., Sung, K. L., Matusop, A., Radhakrishnan, A., Shamsul, S. S. G., Cox-Singh, J., Thomas, A., and Conway, D. J. (2004) A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings, *The Lancet 363*, 1017-1024.

Skinner-Adams, T. S., McCarthy, J. S., Gardiner, D. L., Hilton, P. M., and Andrews, K. T. (2004) Antiretrovirals as Antimalarial Agents, *The Journal of Infectious Diseases 190*, 1998-2000.

Sugioka, Y., Suzuki, M., Sugioka, K., and Nakano, M. (1987) A ferriprotoporphyrin IX-chloroquine complex promotes membrane phospholipid peroxidation, *FEBS Letters 223*, 251-254.

Sullivan, D. J., Gluzman, I. Y., Russell, D. G., and Goldberg, D. E. (1996) On the molecular mechanism of chloroquine's antimalarial action, *Proceedings of the National Academy of Sciences USA 93*, 11865-11870.

Sullivan, D. J., Matile, H., Ridley, R. G., and Goldberg, D. E. (1998) A Common Mechanism for Blockade of Heme Polymerization by Antimalarial Quinolines, *The Journal of Biological Chemistry 273*, 31103-31107.

Sunil, S., Chauhan, V. S., and Malhotra, P. (2008) Distinct and stage specific nuclear factors regulate the expression of falcipains, *Plasmodium falciparum* cysteine proteases, *BMC Molecular Biology 9*.

Tan, J. C. (2008) The dynamic structural genome of *Plasmodium falciparum*, PhD thesis, University of Notre Dame, Indiana.

Tanner, M., and de Savigny, D. (2008) Malaria eradication back on the table, *Bulletin of the World Health Organization 86*, 82-83.

Telgt, D. S., van der Ven, A. J., Schimmer, B., Droogleever-Fortuyn, H. A., and Sauerwein, R. W. (2005) Serious Psychiatric Symptoms After Chloroquine Treatment Following Experimental Malaria Infection, *Annals of Pharmacotherapy 39*, 551-554.

Tisi, L. C., White, P. J., Squirrell, D. J., Murphy, M. J., Lowe, C. R., and Murray, J. A. H. (2002) Development of a thermostable firefly luciferase, *Analytica Chimica Acta 457*, 115-123.

Todryk, S. M., and Hill, A. V. S. (2007) Malaria vaccines: the stage we are at, *Nature Reviews Microbiology 5*, 487-489.

Trager, W., and Jensen, J. B. (1976) Human Malaria Parasites in Continuous Culture, *Science 193*, 673-675.

Tuteja, R. (2007) Malaria - an overview, *FEBS Journal 274*, 4670-4679.

van Agtmael, M. A., Eggelte, T. A., and van Boxtel, C. J. (1999) Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication, *Trends in Pharmacological Sciences 20*, 199-205.

van Brummelen, A. C., Olszewski, K. L., Wilinski, D., Llinás, M., Louw, A. I., and Birkholtz, L. (2009) Co-inhibition of *Plasmodium falciparum* S-Adenosylmethionine Decarboxylase/Ornithine Decarboxylase Reveals Perturbation-specific Compensatory Mechanisms by Transcriptome, Proteome, and Metabolome Analyses, *The Journal of Biological Chemistry 284*, 4635.

van Dooren, G. G., Stimmler, L. M., and McFadden, G. I. (2006) Metabolic maps and functions of the *Plasmodium* mitochondrion, *FEMS Microbiology Reviews 30*, 596-630.

Vander Jaqt, D. L., Hunsaker, L. A., and Campos, N. M. (1986) Characterization of a Hemoglobin-degrading, Low Molecular Weight Protease from *Plasmodium falciparum*, *Molecular and Biochemical Parasitology 18*, 389-400.

Varotti, F., Botelho, A. C. C., Andrade, A. A., de Paula, R. C., Fagundes, E. M. S., Valverde, A., Mayer, L. M. V., Mendonça, J. S., de Souza, M. V. N., Boechat, N., and Krettli, A. U. (2008) Synthesis, Antimalarial Activity, and Intracellular Targets of MEFAS, a New Hybrid Compound Derived from Mefloquine and Artesunate, *Antimicrobial Agents and Chemotherapy 52*, 3868-3874.

Veiga, M. I., Ferreira, P. E., Schmidt, B. A., Ribacke, U., Björkman, A., Tichopad, A., and Gil, J. P. (2010) Antimalarial Exposure Delays *Plasmodium falciparum* Intra-Erythrocytic Cycle and Drives Drug Transporter Genes Expression, *PLoS ONE 5*.

Vitoria, M., Granich, R., Gilks, C. F., Gunneberg, C., Hosseini, M., Were, W., Raviglione, M., and De Cock, K. M. (2009) The Global Fight Against HIV/AIDS, Tuberculosis, and Malaria, *American Journal of Clinical Pathology 131*, 844-848.

Vivas, L., Rattray, L., Stewart, L., Bongard, E., Robinson, B. L., Peters, W., and Croft, S. L. (2008) Anti-malarial efficacy of pyronaridine and artesunate in combination *in vitro* and *in vivo*, *Acta Tropica 105*, 222-228.

Vonlaufen, N., Kanzok, S. M., Wek, R. C., and Sullivan, W. J. (2008) Stress response pathways in protozoan parasites, *Cellular Microbiology 10*, 2387-2399.

Wang, D., and Wu, Y. (2000) A possible antimalarial action mode of qinghaosu (artemisinin) series compounds. Alkylation of reduced glutathione by *C*-centered primary radicals produced from antimalarial compound qinghaosu and 12-(2,4-dimethoxyphenyl)-12-deoxoqinghaosu, *The Royal Society of Chemistry*, 2193-2194.

Waterkeyn, J. G., Crabb, B. S., and Cowman, A. F. (1999) Transfection of the human malaria parasite *Plasmodium falciparum*, *International Journal of Parasitology 29*, 945-955.

White, N. J. (1994) Clinical pharmacokinetics and pharmacodynamics of artemisinin and derivatives, *Transactions of the Royal Society of Tropical Medicine and Hygiene 88*, 41-43.

White, N. J. (2008) Qinghaosu (Artemisinin): The Price of Success, *Science 320*, 330-334.

White, N. J. (2010) A Vaccine for Malaria, *The New England Journal of Medicine 365*.

White, N. J., Nosten, F., Waller, D., Crawley, J., Chapman, D., Brewster, D., and Greenwood, B. M. (1992) Comparison of artemether and chloroquine for severe malaria in Gambian children, *The Lancet 339*, 317-321.

Wong, S. K., Lim, Y. Y., Abdullah, N. R., and Nordin, F. J. (2011) Assessment of antiproliferative and antiplasmodial activities of five selected *Apocynaceae* species, *BMC Complementary and Alternative Medicine 11*.

Woodrow, C. J., Haynes, R. K., and Krishna, S. (2005) Artemisinins, *Postgraduate Medical Journal 81*, 71-78.

World Health Organisation. (2007) Resistance to artemisinin derivatives along the Thai-Cambodian border, *Weekly Epidemiological Record 82*, 357-360.

World Health Organisation. (2009) World Malaria Report 2009. *[Online]* http://www.who.int/malaria/world_malaria_report_2009/en/ [Accessed: 20 May 2010].

World Health Organisation. (2010a) Guidelines for the Treatment of Malaria. *[Online]* http://www.who.int/malaria/publications/atoz/9789241547925/en/index.html [Accessed: 29 September 2011].

World Health Organisation. (2010b) World Malaria Risk Areas, 2010. *[Online]* http://gamapserver.who.int/mapLibrary/app/searchResults.aspx [Accessed: 01 March 2012].

World Health Organisation. (2010c) Roll Back Malaria Partnership: Global Malaria Action Plan. *[Online]* http://www.rbm.who.int/gmap/gmap.pdf [Accessed: 19 September 2011].

World Health Organisation. (2011) World Malaria Report 2011. *[Online]* http://www.who.int/malaria/world_malaria_report_2011/en/ [Accessed: 28 February 2012].

Yang, Y., Little, B., and Meshnick, S. R. (1994) Alkylation of Proteins by Artemisinin: Effects of Heme, pH, and Drug Structure, *Biochemical Pharmacology 48*, 569-573.

Yayon, A., Cabantchik, Z. I., and Ginsburg, H. (1984) Identification of the acidic compartment of *Plasmodium falciparum*-infected human erythrocytes as the target of the antimalarial drug chloroquine, *The EMBO Journal 3*, 2695-2700.

Yayon, A., Cabantchik, Z. I., and Ginsburg, H. (1985) Susceptibility of human malaria parasites to chloroquine is pH dependent, *Proceedings of the National Academy of Sciences 82*, 2784-2788.

Yee, S. W., Shima, J. E., Hesselson, S., Nguyen, L., De Val, S., LaFond, R. J., Kawamoto, M., Johns, S. J., Stryke, D., Kwok, P., Ferrin, T. E., Black, B. L., Gurwitz, D., Ahituv, N., and Giacomini, K. M. (2009) Identification and Characterization of Proximal Promoter Polymorphisms in the Human Concentrative Nucleoside Transporter 2 (*SLC28A2*), *The Journal of Pharmacology and Experimental Therapeutics 328*, 699-707.

Zhang, J., Chung, T. D. Y., and Oldenburg, K. R. (1999a) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays, *The Journal of Biomolecular Screening 4*, 67-73.

Zhang, J., Krugliak, M., and Ginsburg, H. (1999b) The fate of ferriprotorphyrin IX in malaria infected erythrocytes in conjunction with the mode of action of antimalarial drugs, *Molecular and Biochemical Parasitology 99*, 129-141.

Zolg, J. W., Macleod, A. J., and Scaife, J. G. (1984) The accumulation of lactic acid and its influence on the growth of *Plasmodium falciparum* in synchronised cultures, *In Vitro 20*, 205-215.