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MALARIAL PIGMENT (HEMOZOIN): FLOW CYTOMETRIC DETECTION OF HEMOZOIN IN INFECTED ERYTHROCYTES TO MONITOR DRUG-EFFECTS

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Dissertação orientada pelo Professor Doutor Thomas Hanscheid e pela Professora Doutora Ana Maria Reis

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ABSTRACT

Malaria remains the most important parasitic disease. The emergence and spread of drug resistant *Plasmodium falciparum* is a subject of great concern and requires monitoring of the parasite susceptibility. Currently there are several sensitivity assays for *Plasmodium falciparum* but they are associated with important limitations, such as: long turn-around times (30 h - 96 h), use of expensive equipment and sophisticated or labour-intensive methodologies.

Malaria parasites produce hemozoin (Hz), in order to detoxify heme after hemoglobin degradation. Hz content increases during parasite maturation, thus constituting an optimal growth indicator. Due to its physical property (birefringence) it can be detected by optical methods, like flow cytometry. Using a purpose built flow cytometer, it was possible to detect infected red blood cells (iRBCs) containing different amounts of Hz.

The principal goal of this project is to develop a rapid and reliable sensitivity assay. This kind of assay will surely be a great tool for the assessment of parasite susceptibility and for the high throughput screening of new compounds.

Blood from *Plasmodium berghei* infected mice was incubated (*in vitro*) with several antimalarial drugs (chloroquine, quinine, mefloquine, artemisinin and pyrimethamine), over 24 hours. Analysing the percentage of iRBCs containing high amounts of Hz, denominated as highly depolarizing RBCs (hdRBCs), it was possible to detect the inhibitory effect of several drugs. In the untreated samples the amount of Hz within iRBCs increased, as assessed by the percentage of hdRBCs, due to parasite maturation. Contrary to this, no increase was detected in samples treated with chloroquine, quinine, mefloquine and artemisinin. Pyrimethamine effect could not be detected.

The inhibitory effect of all antimalarial drugs tested, excluding pyrimethamine, could be detected already after 4 hours of incubation.

These preliminary results suggest that this method could be developed into a rapid, objective and automatable sensitivity test, without the need for reagents.

Keywords: hemozoin; antimalarial drug resistance; *in vitro* sensitivity assay; antimalarial drugs; flow cytometry.

RESUMO

A malária é uma doença que tem sido uma preocupação constante para a humanidade ao longo de séculos, continuando a ser responsável por cerca de 1 milhão de mortes por ano.[84] O impacto da malária é elevado estende-se muito além das taxas de mortalidade e morbilidade associadas.[20]

A malária em humanos é causada por cinco espécies de parasitas: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* e *Plasmodium knowlesi.* O parasita *Plasmodium falciparum* é responsável pela maioria dos casos em África e dos casos de malária severa. Estas cinco espécies diferem na distribuição geográfica, características microscópicas, características clínicas e no potencial para desenvolver resistência aos anti-maláricos.

O ciclo de vida do parasita é complexo e envolve um vector e um hospedeiro vertebrado. Durante o ciclo de vida, no hospedeiro, existem duas fases principais: fase hepática e a fase sanguínea. Durante a fase sanguínea o parasita invade eritrócitos, desenvolve-se e multiplica-se no seu interior. Esta fase é a responsável pela sintomatologia associada à infecção por malária.

Durante o desenvolvimento do no interior dos eritrócitos o parasita degrada a hemoglobina para obter aminoácidos e para regular a pressão ósmotica.[28] Como consequência desta degradação é produzido heme livre. Este, tal como acontece em organismos superiores, é tóxico para o parasita.[78] Para ultrapassar este obstáculo o parasita desintoxica o heme livre transformando-o em hemozoína (Hz), também denominado por pigmento malárico.[39] O conteúdo de Hz aumenta à medida que o parasita matura constituindo, assim, um óptimo indicador de crescimento.[7]

Vários anti-maláricos interferem com a produção de Hz. Fármacos que contêm quinolina, como a cloroquina, quinino, mefloquina, amodiaquina e halonfantrina têm como principal mecanismo de acção a inibição da produção de Hz, levando à morte do parasita, como uma consequência da acumulação de heme livre tóxico.[28,69,78] Outro grupo de fármacos que também pode estar envolvido na inibição da produção de Hz é eventualmente a artemisina e os seus derivados.[55] Apesar de existirem algumas provas que apontam para o envolvimento da Hz, o mecanismo de acção deste fármacos é um tema controverso, e ainda não está completamente esclarecido.

Nas últimas décadas tem-se vindo a observar um aumento da resistência a determinados anti-maláricos. A resistência aos anti-maláricos é um grave problema de saúde publica, uma vez que está associado à propagação da malária a novas áreas e ao reaparecimento em regiões onde fora erradicada.[6]

Perante este cenário torna-se imprescindível ter conhecimento das susceptibilidades do parasita aos diferentes anti-maláricos.

Actualmente existem diversos testes de sensibilidade. Basicamente, podemos agrupá-los em: ensaios *in vivo* e ensaios *in vitro*. Estes testes são úteis para definir *guidelines* para politicas de saúde pública, são ferramentas essenciais para o desenvolvimento de novos fármacos e para a epidemiologia da resistência a anti-maláricos. Por outro lado possuem diversas limitações, como o longo *turn-around time* (30 h - 96 h), o uso de equipamento sofisticado e de reagentes dispendiosos e difíceis de adquirir. Estas limitações contribuem para o desenvolvimento de novos métodos capazes de as ultrapassar.

Este projecto tem como principal objectivo o desenvolvimento de um teste de sensibilidade com base na detecção de Hz por citometria de fluxo. Devido às propriedades físicas da Hz (birrefringente), esta pode ser detectada por métodos ópticos, como por citometria de fluxo. Recorrendo a um citómetro modificado foi possível detectar eritrócitos infectados (Ei) contendo diferentes quantidades de hemozoína.

Amostras de sangue, colhidas de ratinhos infectados com *Plasmodium berghei,* foram incubadas *(in vitro)* com vários anti-maláricos (cloroquina, quinino, mefloquina, artemisinina e pirimetamina), durante 24 horas de incubação. Analisando a percentagem de Ei contendo uma elevada quantidade de Hz, denominadas "eritrócitos infectados que depolarizam muito" (Ei-dm), foi possível detectar o efeito inibitório dos fármacos.

A avaliação da percentagem de Ei-dm, possibilitou a detecção de um aumento da quantidade de Hz no interior dos Ei, consequência da maturação do parasita. Contrariamente, nas amostras tratadas com cloroquina, quinino, mefloquina e artemisinina não foi detectado nenhum aumento, indicando que os fármacos inibiram o crescimento do parasita e, consequentemente, não foi produzida mais Hz.

Curiosamente nas amostras tratadas com cloroquina detectou-se uma diminuição acentuada na percentagem de Ei-dm nas primeiras horas de incubação. Este resultado inesperado revelou-se ser consequência da agregação dos cristais de Hz.

Este fenómeno de agregação, denominado "*clumping*", foi descrito anteriormente, onde na presença de cloroquina os cristais de Hz formam um agregado.[76]

A detecção de Ei-dm resulta da avaliação da depolarização da luz que é dispersada lateralmente (depolarized *side scattered light: depolarized*-SSC). Este parâmetro, SSC, é um indicador da granulosidade celular.[64] Deste modo, é muito provável que o *depolarized*-SSC possa também ser influenciado pela granulosidade celular. Neste caso, se um Ei possuir cristais de Hz dispersos e outro tiver um agregado de Hz, o sinal de *depolarized*-SSC será menor no Ei com um agregado de Hz no seu interior, fazendo com que estes Ei deixem de pertencer à população de Ei-dm. Justificando-se, assim, a diminuição inicial na percentagem de Ei-dm nas amostras tratadas com cloroquina.

Relativamente à pirimetamina nenhum efeito foi detectado. Este resultado não foi de todo inesperado, uma vez que a pirimetamina actua numa fase tardia da maturação do parasita (no esquizonte).[50] Deste modo, o efeito da pirimetamina é apenas detectado após um ciclo de replicação, ou seja, nas gerações seguintes de parasitas. Devido ao modelo experimental usado (murganhos infectados com *Plasmodium berghei*), a cultura de Ei testada apresenta apenas uma geração de parasitas. Isto porque *P. berghei in vitro* não é capaz de romper os eritrócitos e, consequentemente, não re infecta novos eritrócitos.[32]

A artemisina é um fármaco cujo mecanismo de acção permanece desconhecido. Possíveis mecanismos como o envolvimento na produção de hemozoína[55] e a alteração das membranas do parasita[26], têm vindo a ser sugeridos nos últimos anos. Os resultados obtidos demonstram que na presença de concentrações mais elevadas de artemisina (129 nM e 259 nM) há um decréscimo gradual da percentagem de Ei-dm, a partir das 2 horas de incubação. Este resultado pode sugerir

que tal como acontece com a cloroquina, mas não tão rapidamente, o pigmento pode começar a agregar-se, ou então, que o fármaco pode provocar a lise dos Ei. Nenhuma destas hipóteses pôde ser confirmada porque durante esta experiência não foram adquiridas contagens absolutas da concentração de eritrócitos presentes nas amostras.

A utilização de estirpes de parasitas que expressam GFP (*green fluorescent protein*) para o *screening* de anti-maláricos e determinação da do parasita a diferentes compostos sensibilidade foi descrita tanto para *P. falciparum* como para *P. berghei.*[60,61] Na maioria das experiências realizadas ao longo deste projecto foram usadas estirpes de parasita que expressam GFP para que fosse possível comparar os resultados obtidos através destas duas abordagens (detecção de parasitas que expressam GFP ou detecção de Ei-dm). O efeito do fármaco foi detectado com uma maior antecedência e evidência através da análise da percentagem de Ei-dm.

Concluindo, a detecção por citometria de fluxo de Ei contendo diferentes quantidades de Hz permitiu a avaliação do efeito inibitório de todos os anti-maláricos testados, à excepção da pirimetamina, em apenas 4 horas de incubação.

Apesar dos resultados do projecto demonstrarem o potencial deste ensaio é preciso ter em conta que o objectivo principal é desenvolver um teste de sensibilidade para *Plasmodium* spp. responsáveis pela malária em humanos e, para isso, uma cultura contínua de *P. falciparum* está a ser estabelecida. No futuro será também importante testar estirpes de *P. falciparum* resistentes de modo a optimizar o ensaio. Posteriormente, será também de interesse a recolha e análise de amostras de sangue de doentes com malária num país onde esta doença é endémica.

Palavras-chave: hemozoína; resistência a anti-maláricos; teste de sensibilidade *in vitro*; fármacos anti-maláricos; citometria de fluxo.

I - INTRODUCTION

1. MALARIA

1.1 MALARIA – THE DISEASE AND ITS CONSEQUENCES

Malaria is a parasitic disease that has been a primary concern to humanity for centuries. This disease is responsible for 300 million cases resulting in approximately 1 million deaths per year.[84] Results of effective intervention studies suggest that the true number could be even higher because of indirect effects of the disease on nutrition and other infections.[17]

Impact of malaria is immense and extends far beyond measurement of mortality and morbidity. Despite global economic development, more people die from malaria nowadays than 40 years ago. More than a third of the world's population (about 2 billion people) live in malaria-endemic areas. Only in Africa there are an estimated 200–450 million cases of fever in children infected with malaria parasites each year[20], resulting in a child death in every 40 seconds. Besides its social impact malaria has health costs and economic consequences.[58]

In humans, malaria infection is caused by one or more of five species of intracellular protozoan parasite: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae and Plasmodium knowlesi. P. falciparum* causes the majority of infections in Africa and is responsible for severe disease and high mortality rates.[20] These five species differ in geographical distribution, microscopic appearance, clinical features, and potential for development of resistance to antimalarial drugs. However they exhibit a similar life cycle with only minor variations.

The malaria parasite life cycle is complex involving an insect vector and a vertebrate host (Figure 1).

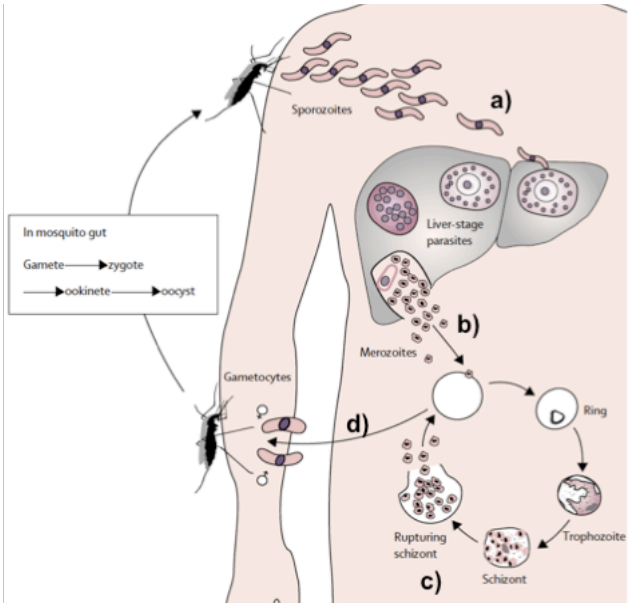

FIGURE 1: *PLASMODIUM* SPP. LIFE CYCLE. A feeding mosquito injects sporozoites into the bloodstream. The sporozoites migrate to the liver (a) and infect hepatocytes where they replicate producing merozoites, which will be released into the bloodstream (b). Merozoites invade red blood cells (RBCs) and mature into schizonts (c). Eventually, the schizont ruptures and merozoites will be released into the bloodstream, where some will infect RBCs and other will differentiate into gametocytes (d) that will be ingested by another feeding mosquito.
[adapted from Greenwood *et al.* 2005]

Of note, *P. vivax* and *P. ovale* have dormant stage (hypnozoites) which can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.

The asexual blood stage is responsible for all of the symptoms and pathologies associated with malaria. Uncomplicated malaria is linked to cyclical fevers and chills.

On the other hand, severe malaria includes multiple additional pathologies such as cerebral malaria, severe anemia and lactic acidosis.[21]

1.2. THE MALARIAL PIGMENT

During the blood stage, the malaria parasite digests hemoglobin present in red blood cells (RBCs) for its source of amino acids and iron. Interestingly, it has also been suggested that hemoglobin degradation is important for the maintenance of osmotic balance as well as to make space available for the parasite to grow and therefore, preventing premature RBC lysis.[16] As a result of hemoglobin degradation free heme is produced and, as in higher organisms, free heme is highly toxic to the parasite.[78] In order to solve this problem the parasite detoxifies the heme turning it into Hemozoin (Hz), also know as malarial pigment.[39] Its content increases as the parasite matures (Figure 2). Thus, constituting an optimal maturation indicator.[57]

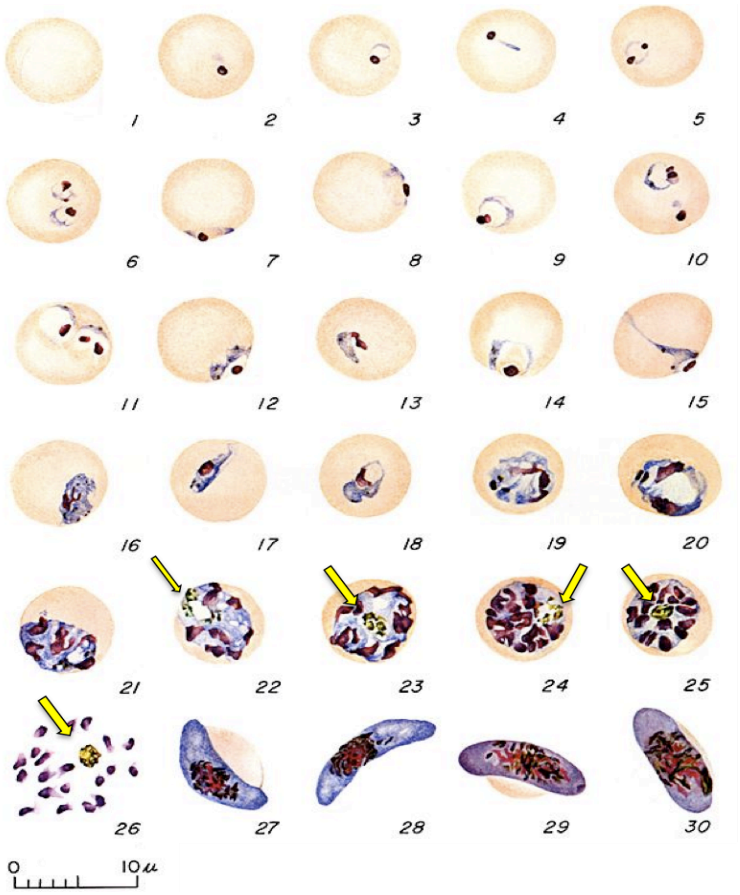

FIGURE 2: PARASITE *(Plasmodium falciparum)* MATURATION DURING THE BLOOD STAGE OF MALARIA INFECTION. 1: Normal red cell; 2-18: Trophozoites; 19-25: Schizonts; 26: ruptured schizont; 27, 28: Mature macrogametocytes (female); 29, 30: Mature microgametocytes (male). Hemozoin, brown-yellowish granules (yellow arrows) can be detected in mature trophozoites and, more easily, in schizonts. As the parasite matures the content of hemozoin increases and an aggregated of Hz crystals is formed [adapted from CDC: Diagnostic findings - Historic Images].

Several authors have reviewed the history of hemozoin.[22,28] It goes back to 1847 when Meckel, a psychiatrist, recorded innumerable black-brown pigment granules in the blood and spleen of a patient who had died in the hospital for the insane. At the time, he thought that pigment was melanin. Two years later Rudolf Virchow established the connection between the dark pigment and malaria. However, Alphonse Laveran, in 1880, was the one who established the relation between the parasite and the pigment. But, only half a century later in 1911, Brown proved that the malarial pigment was equivalent to hematin and not melanin.

The mechanism of Hz formation *in vivo* has been subject of much debate. Two decades ago it was widely assumed that Hz was merely degradation product. Lipids and proteins have been implicated in Hz formation.[51] In 1996 it was showed that histidine rich protein II (HRPII) supports Hz formation and it was also suggested that this protein might be responsible for either catalysing or initiation Hz formation *in vivo.*[69] The involvement of lipids was followed by Fitch *et al.* who observed that non-lipid components of red blood cells membranes were incapable of promoting Hz formation.[15]

In 2002 Egan described Hz formation as a process of biocrystallization.[13] Biocrystallization refers to the process by which living organisms form crystals from high molecular weight, organic materials. Both proteins and lipid membranes are typically involved in biocrystallization.[27] Recently it was found that Hz formation occurs inside lipid bodies within the parasite digestive vacuole revolutionising the idea about the process occurring in aqueous to a non-aqueous environment.[53] Nonetheless, key questions about Hz formation are still unanswered.

2. ANTIMALARIAL DRUGS

2.1. HISTORY OF ANTIMALARIAL DRUGS

Quinine, extracted form the bark of cinchona tree, was used as an antimalarial agent as early as 1632 and by the 19th century it was still the only known antimalarial agent. Along with its dextroisomer quinidine it has been the last resource for the treatment of malaria, in particular severe disease.[70]

Chloroquine was first synthesised in 1934 but it was only recognised as a potent antimalarial in the 1940s. It became the most widely used antimalarial drug. It has been the drug of choice to treat non-severe or uncomplicated malaria and for chemoprophylaxis.[43]

Artemisinin is extracted from the plant *Artemisia annua*. It has been used in China for the treatment of fever for over a thousand years. Several other compounds used as antimalarial drug derivate from artemisinin, such as artesunate, artemether and artemotil. Combination therapy with these drugs has shown to contribute to the inhibition of drugs resistance's intensification and to the decreased malaria transmission levels observed in South-East Asia.[81]

There are some drugs that resulted from combinations of dihydrofolate-reductase inhibitors (pyrimethamine, proguanil, and others) and sulfa drugs (dapsone, sulfadoxine, and others). These drugs were developed during the Second World War. Even though all these drugs have antimalarial activity when used alone, when used in combination the synergistic effect on the parasite is more effective.[6]

2.2 CURRENTLY USED ANTIMALARIALS – MECHANISM OF ACTION

The number of drugs that can be used to treat or prevent malaria is becoming scarcer, due to the advent of resistance. Table 1 summarizes the available drugs to treat and/or prevent malaria.

The latest guideline for the treatment of uncomplicated malaria is the artemisinin-based combination therapy (ACT). On the other hand, to treat severe malaria it is recommended to use several antimalarial drugs, such as artesunate, quinine, artemether, as a single-therapy during 24 hours and, thereafter, complete the treatment with ACTs or with combination therapy of antimalarial drugs (artesunate, quinine) with antibiotics (doxycycline, clindamycin).[85]

TABLE 1: ANTIMALARIAL DRUGS USED TO TREAT AND/OR PREVENT MALARIA (adapted from Bloland 2001).

	CHEMICAL CLASS	DRUG NAME	USE
Single-agent therapy	Aminoalcohols	Quinine	▪ Treatment of: (i) severe malaria (ii) multidrug resistant *P. falciparum* (iii) malaria during the 1st trimester of pregnancy
		Mefloquine	▪ Treatment of non-severe *P. falciparum* infection thought to be CQ and SP resistant ▪ Chemoprophylaxis in areas with CQ resistant
		Halofantrine	▪ Treatment of suspected multidrug-resistant *P. falciparum*
	Amino-quinolones	Chloroquine	▪ Treatment of: (i) non *P. falciparum* infections (ii) *P. falciparum* infections where CQ remains effective (iii) Chemoprophylaxis in areas where CQ remains effective
		Primaquine	▪ Treatment of *P. vivax* infections ▪ Gametocytocidal agent
		Amodiaquine	▪ Treatment of non-severe *P. falciparum* infection thought to be CQ resistant
	Folate Antagonists	Sulfadoxine/ Pyrimethamine	▪ Treatment of non-severe *P. falciparum* infection thought to be CQ resistant
	Artemisinin compounds	Artesunate	▪ Treatment of multidrug resistant *P. falciparum* infections ▪ Treatment ofsevere and moderately severe malaria (administrated intravenously and rectal, respectively)
		Artemisinin	
		Artemether	
	Antibiotics	Tetracycline/ Doxycycline	▪ In combination with QN can increase efficacy of treatment in QN resistance ▪ Prophylaxis
Combination therapy		Atovaquone/ Proguanil	▪ Treatment of multidrug resistant *P. falciparum* infections
		Mefloquine + Artusunate	▪ Treatment of non-severe *P. falciparum* infection thought to be CQ and SP resistant
		SP + Artusunate	▪ Treatment of non-severe *P. falciparum* infection thought to be CQ resistant
		Lumefantrine + Artemether	

LEGEND: CQ = chloroquine; QN = quinine; SP = sulfadoxine/pyrimethamine.

Commonly used antimalarial drugs target different stages of the malaria parasite and exhibit different modes of action.[50] Traditionally, antimalarial agents are classified by the stages of the malaria life cycle that are targeted by the drug:

- Blood schizonticides (act on the asexual intraerythrocytic stages)
- Tissue schizonticides (kill hepatic schizonts)
- Hypnozoiticides (kill hypnozoites of *P. vivax* and *P. ovale*)
- Gametocytocides (destroy intraerythrocytic sexual forms).

When it comes down to the mechanism of action: some inhibit nuclei acid synthesis, others the hemozoin formation and some whose mechanism of action remains unknown.

Folate antagonists class includes sulfadoxine and pyrimethamine. These drugs act by inhibiting enzymes of the folate pathway, indirectly blocking the synthesis of nucleic acids in the malaria parasite.[50]

One the other hand, quinoline-containing drug class includes the most common antimalarial drugs like chloroquine, amodiaquine, quinine, quinidine, mefloquine and halofantrine. The mechanism of action of these drugs is far from being resolved and several mechanisms have been proposed for the action of these compounds.[48] However, it has been reported that interaction with heme is central to the activity of this class of compounds.[44] Thus, it was suggested that the interference with parasite heme detoxification and, consequently, the inhibition of hemozoin formation is a possible mechanism. In this case the death of the parasite results from the accumulation of cytotoxic free heme.

The mechanism of action of artemisinin compounds is incompletely understood. Several studies suggest that these compounds also interfere with hemozoin formation.[55] Recently it has also been shown that artemisinin accumulates within neutral lipids causing parasite membrane damage.[26]

To come to the point, consensus about the mechanism of action of some antimalarials has not yet been achieved. Still, many of the currently used antimalarial such as chloroquine, amodiaquine, mefloquine, quinine, halofantrine and probably others, like artemisinin compounds, interfere somehow with hemozoin formation.

3. Resistance to antimalarial drugs

3.1. Importance of resistance

Antimalarial drug resistance is defined as the “ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal or higher than those usually recommended but within tolerance of the subject”. Later this definition was modified to specify that the drug in question “must gain access to the parasite or the infected erythrocyte for the duration necessary for its normal action”.[6] Antimalarial drug resistance is a major public health problem, which hampers the efforts made to control malaria. Several factors such as drug-use patterns, characteristics of the drug itself, human host factors, parasite and vector biology, within others contribute to the development and spread of drug resistance.[82]

Antimalarial drug resistance has been described for *P. falciparum* and *P.vivax*. *P. falciparum* has developed resistance to nearly all antimalarials currently used, while *P.vivax* has only been shown to be resistant to chloroquine and primaquine (Table 2*)*.[6]

Table 2: Dates of introduction and first reports of antimalarial drug resistance (adapted from Wongsrichanalai C. *et al* 2002)

Antimalarial drug	Introduced	First reported resistance	Difference (years)	Location
Quinine	1632	1910	278	▪ Thai-Cambodian border
Chloroquine	1945	1957	12	▪ Thai-Cambodian border ▪ Columbia
Sulfadoxine-pyrimethamine	1967	1967	0	▪ Thai-Cambodian border
Mefloquine	1977	1982	5	▪ Thai-Cambodian border

P. falciparum clinical resistance to quinine (QN) monotherapy is detected sporadically in South-East Asia and Western Oceania. Data from *in vitro* assays suggested that it is less common in South America and Africa.

Chloroquine (CQ)-resistant *P. falciparum* has been described everywhere that *P. falciparum* malaria is transmitted except for malarious areas of Central America and Caribbean.[82]

Nowadays, high-level resistance to sulfadoxine-pyrimethamine (SP) frequently occurs in South-East Asia and South America. It is also becoming more prevalent in Africa.[6]

Mefloquine (MQ) resistance is frequent in some areas of South-East Asia and has been reported in the Amazon region of South America but the degree of resistance in South America is still far below those of South-East Asia. Even though an *in vitro* study, have suggested the presence of *P. falciparum* with low sensitivity to MQ[33], clinical resistance is rare[82]. Multi-drug resistance of *P. falciparum*, normally referring to resistance to both CQ and SP, occurs in Amazonia and South-East Asia (Figure 3).[82]

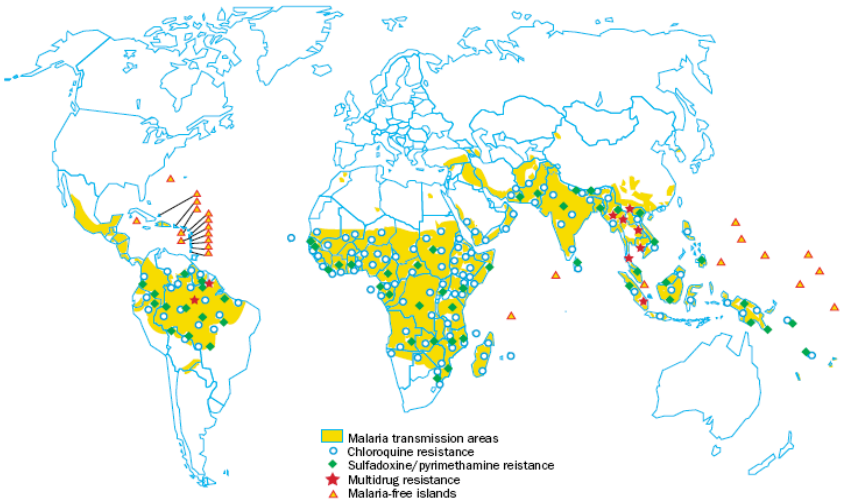

FIGURE 3: WORLD DISTRIBUTION OF *P. falciparum* RESISTANCE. Areas with reduced susceptibility of *P. falciparum* to chloroquine and sulfadoxine-pyrimethamine and areas designated as multidrug resistant according to World Health Organization (Wongsrichanalai C. *et al* 2002).

Drug resistance has become an issue of great concern since it has been implicated in the spread of malaria to new areas and re-emergence of malaria in areas where the disease had been eradicated.[6] In general, the effects of resistance to antimalarial drugs on malaria morbidity and mortality are underestimated.[68] The single well-documented study to date on the effect on mortality of resistance to chloroquine concluded that the development of resistance had resulted in a four to eight fold increase in mortality.[71]

3.2. DETECTION OF RESISTANCE

In this scenario, knowledge of the parasite's susceptibility to antimalarials is essential for treatment decisions and for establishing adequate therapeutic guidelines. The currently available assays can be divided as: *in vivo* or *in vitro* assays (Figure 4).

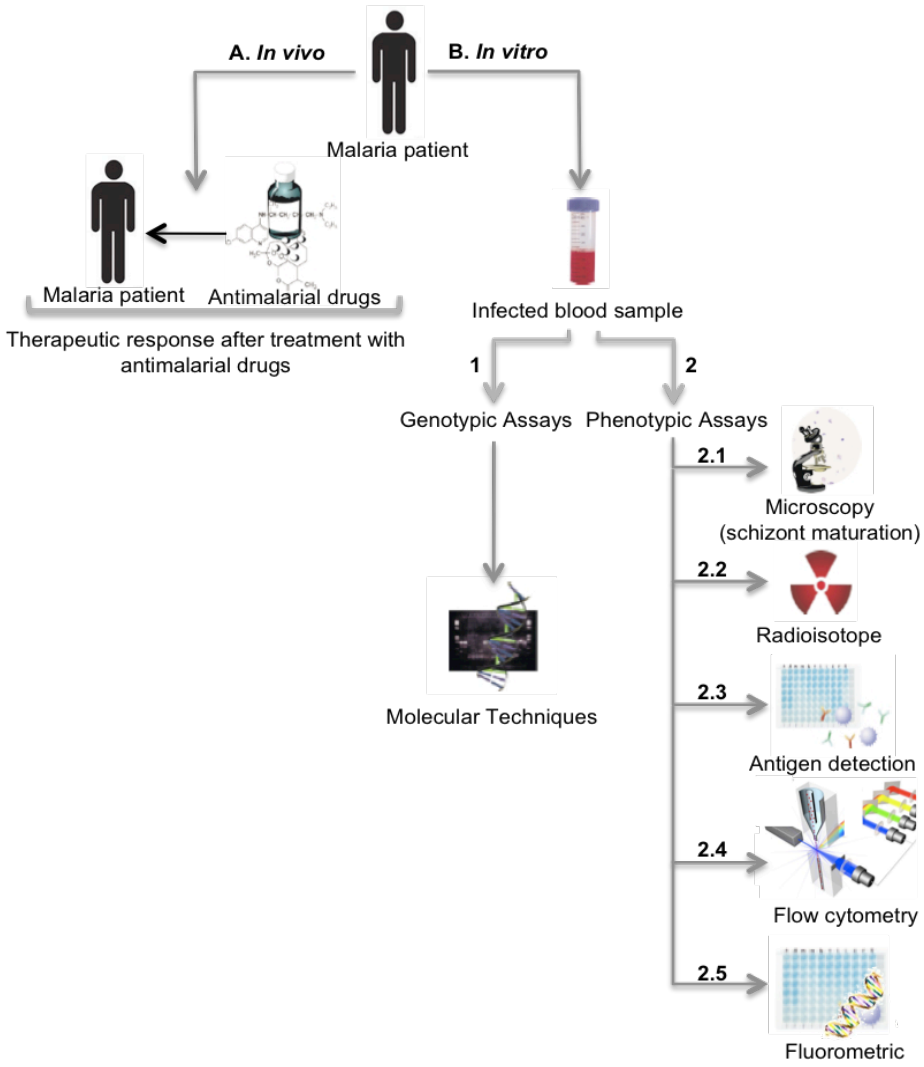

FIGURE 4: AVAILABLE SENSITIVITY ASSAYS FOR ANTIMALARIAL DRUGS (adapted from Noedl *et al* 2003).

Assay A (Figure 4). The *in vivo* assay: was originally defined by the World Health Organization, in 1973, in terms of parasite clearance as being sensitive or resistant (within three degrees of resistance: RI, RII and RIII). Although it is a valid approach for areas with low or no malaria transmission, it is difficult to apply to areas with intensive transmission. In order to overcome this issue, some modifications to the original method have been done (Table 3).[82] An *in vivo* test consists of the treatment of a group of symptomatic and parasitemic individuals with known doses of drugs and subsequent monitoring of the parasitological and/or clinical response over time. This approach reflects the true biological nature of treatment response but do not necessarily reflect the true level of pure antimalarial resistance, once the therapeutic efficacy can be diminished by several factors such as host immunity, variation of drug absorption and other.[70] A quantitative assessment of the drug sensitivity is not possible and, obviously, there are significant organizational challenges and evident ethical problems associated with this practice.

TABLE 3: CLASSIFICATIONS OF *IN VIVO* ANTIMALARIAL SENSITIVITY TEST OUTCOMES ACCORDING TO THE ORIGINAL AND MODIFIED PROTOCOL (adapted from Wongsrichanalai *et al* 2002)

CLASSIFICATION	DEFINITION
Original Classification (1973)	
Sensitive (S)	Reduction to <25% of initial parasitemia on day 2 with smears negative for malaria from day 7 to the end of follow-up (28 days or longer for drugs with a long half-life, such as mefloquine).
Resistance response I (RI)	Initial clearance of parasitemia, a negative smear on day 7, followed by recrudescence at day 8 or more days after treatment.
Resistance response II (RII)	Initial clearance or substantial reduction of parasitemia (<25% of the initial count on day 2) but with persistence or recrudescence of parasitemia during days 4–7.
Resistance response III (RIII)	No significant reduction of parasitemia.
Modified Classification (1996)	
Early treatment failure (ETF)	Aggravation or persistence of clinical symptoms in the presence of parasitemia during the first 3 days of follow-up.
Late treatment failure (LTF)	Reappearance of symptoms in the presence of parasitemia during days 4–14 of follow-up.
Adequate clinical response (ACR)	Absence of parasitemia on day 14 irrespective of fever, or absence of clinical symptoms irrespective of parasitemia, in patients not meeting ETF or LTF criteria.

Assays B (Figure 4). *In vitro* drug sensitivity assays: are all based on the inhibition of the growth and development of malaria parasites by different concentrations of a given drug relative to drug-free controls. Unlike the *in vivo* assay, *in vitro* tests are designed to assess parasites response to drugs without the confounding effects of host factors, such as acquired immunity and pharmacokinetic profiles (poor absorption, biotransformation, rapid clearance). Therefore providing a direct and quantitative assessment of drug resistance.[37]

In vitro tests can be grouped as (Figure 4, assays B):

- Genotypic Assays – Molecular techniques
- Phenotypic Assays
  - Microscopic examination
  - Radioisotope methods
  - Antigen detection
  - Flow cytometry
  - Fluorometric methods

Genotypic Assays (Figure 4, assay 1)

Molecular techniques. Due to the availability of molecular techniques, such as polymerase chain reaction (PCR), several markers associated with antimalarial drug resistance have been investigated. The introduction of real-time PCR into malaria research show interesting perspectives for the future, suggesting that it could be possible to monitor the effectiveness of antimalarial drugs and, also to determine objectively the parasitemia in different samples.[38]

Molecular techniques may as well indicate the presence of mutations encoding biological resistance to antimalarial drugs. Currently the association between pyrimethamine resistance and point mutations on the dihydrofolate reductase (*dhfr*) gene, and sulfadoxine resistance and point mutations on the dihydropteroate synthetase (*dhps*) gene, are the best documented.[82] Multiple mutations in *pfcrt* gene were identified as the central determinant of chloroquine resistant *P. falciparum*.[79] These markers can be used for surveillance of resistance. However the number of validated resistance markers is limited.

Phenotypic Assays (Figure 4, assays 2)

Microscopic examination (Figure 4, assay 2.1)**.** In 1971, Rieckmann *et al*. described an *in vitro* drug-sensitivity test for *P. falciparum* based on the measurement of schizont maturation (see figure 1).[56] In other words, it measured the ability of the parasite to develop from early ring stage to mature schizonts when challenged by increasing concentrations of chloroquine. A decade later the assay was further developed to a microculture procedure, known as the "microtest". This microtechnique was later designed into a field-applicable microtest, under World Health Organization (WHO) sponsorship, being known as the WHO test. This assay remains one of the most common techniques to assess drug sensitivity. However this is a labour-intensive procedure, which requires highly trained personnel to limit individual variability in assessing the developmental stages of the parasite.[4, 46]

Radioisotope methods (Figure 4, assay 2.2)**.** Several radiolabelled precursors have been used to assess parasite maturation and [^{3}H]hypoxanthine, a DNA precursor, is the preferred purine base for radiolabelling parasite DNA and for isotope microtests. This test is currently considered as the "gold standard" for *in vitro* sensitivity tests.[1] Desjardins *et al.* developed the first isotopic microtest, based on the measurement of the quantity of tritium-labelled hypoxanthine incorporated into the parasites nucleic acids.[11] The use of [^{3}H]hypoxanthine in drug assays allows an accurate, sensitive determination of the antimalarial drug inhibitory effect on parasite maturation.[67] It is an automated method and test results are automatically read. Thus, diminishing the influence of the variability caused by human factors. Though, a major limitation of this assay is the use of isotopic material and the high purchase cost of the equipment.

Antigen detection (Figure 4, assay 2.3)**.** The histidine-rich protein II (HRPII) is an ELISA-based method that has been widely used in the last years due to its ease of use. HRPII appears to be produced exclusively by *P. falciparum* in the course of its growth and multiplication. Its levels are closely related with parasite density and development[10] but they remain relatively low during the first 48 hours, reaching high levels only at 60-72h[4]. Parasite growth is assessed by measuring the production of HRP2 in a commercial available ELISA kit.[12] It is an easy test to perform, and highly specialized equipment or personnel training is not needed but, as mentioned above, it requires a 72-hour incubation time.

Parasite lactate dehydrogenase (pLDH) is a terminal enzyme in the Embden–Meyerhof pathway (glycolysis) of the malaria parasite. In 1993, Makler *et al.* developed an assay that by measuring enzymatic activity of pLDH it was possible to determine drug activity, once pLDH levels correspond to the parasite density upon initial diagnosis[41] and show a rapid decrease with the initiation of

treatment.[49] Initial parasite densities of 1-2% are required and subsequent tests with fresh isolates showed that this assay was not sensitive for field application.[3] These limitations led to the development of a new pLDH-based assay. This new approach measures pLDH levels in a double-site enzyme-linked LDH immunodetection (DELI) assay, which was made possible by the development of monoclonal antibodies. This new assay is considerably more sensitive and is also field applicable.[46] Nevertheless, limited supplies of monoclonal antibodies have constrained for further validation and application of this assay.

Flow cytometry (Figure 4, assay 2.4)**.** Several flow cytometric methods have been applied for the detection of malaria parasites.[35, 60, 61, 73] The possibility of performing drug resistance measurements employing the flow cytometric technique was first demonstrated in 1990.[73] Flow cytometric analysis can be used to detect and measure DNA content of intact parasite infected RBCs with DNA-specific fluorochromes. The principle behind the assay is the contrast between host erythrocytes, which lack DNA, and the malaria parasites, which have DNA and are thus, readily stained with dyes that show enhanced fluorescence in the presence of nucleic acids. Fluorescent intensity increases in direct proportion as the parasite matures, thus allowing the differentiation of the asexual development stage based on the amount of DNA in individual host RBCs.

Several fluorochromes have been used, including thiazole orange, acridine orange, Hoechst 33258 or 33342**,** SYBR® Green I, among others. The latter has been pointed out as the best choice because, contrary to Hoechst 33258 or 33342, it doesn't require flow cytometers equipped with an ultraviolet laser, which are quite expensive.[59] Unlike thiazole and acridine orange that bind both to DNA and RNA, SYBR® green I is a specific-double stranded DNA dye, therefore excluding the problem of the RNA present in retycolocytes (immature RBCs).[67] This technique is rapid, accurate, highly sensitive, automated and non-radioactive. On the other hand the flow cytometric analysis requires quite expensive equipment and specialized technicians for maintenance.

Fluorometric assay (Figure 4, assay 2.5)**.** Like flow cytometry, fluorometric assays are based on the principal of DNA labelling with fluorochromes such as Hoechst 33258, PicoGreen®, SYBR® Green I.[5,59,66,67] However, the fluorescence intensity, which is proportional to the amount of DNA in individual samples, is measured with a minifluorometer, fluorescence spectrophotometer or fluorescence-activated microplate reader instead of using a flow cytometer. This fact makes fluorometric assay less costly. Yet it is not as sensitive as other methods and it requires long incubation period (48-96 hours).

The fact that all these tests exist and there are still efforts to develop new ones indicates that the "ideal" sensitivity assay has not been found yet. Table 4 summarizes the advantages and limitations of the available sensitivity assays, mentioned above.

3.3. RECENT FINDINGS REGARDING THE MOST COMMONLY USED SENSITIVITY ASSAYS

In May 2010, Wein *et al.* reported that the reliability of some commonly used sensitivity assays could be influenced by the mechanism of action of the drug. Suggesting that these assays might not detect the antimalarial potential of new classes of compounds with innovative modes of action, which could become promising new antimalarial drugs.[77]

TABLE 4: ADVANTAGES AND LIMITATIONS OF THE AVAILABLE SENSITIVITY ASSAYS FOR *Plasmodium falciparum*[6,4,70]

SENSITIVITY ASSAY	ADVANTAGES	LIMITATIONS
IN VIVO	▪ reflects the true biological nature of treatment response ▪ reflects the actual clinical and epidemiological situation	▪ does not allow quantitative assessment ▪ host factors may interfere with the outcome ▪ ethical and organizational problems
IN VITRO		
Phenotypic		
DIRECT (MICROSCOPY)	▪ require little equipment	▪ labour-intensive procedure ▪ highly trained personnel ▪ 24 h – 30 h incubation
RADIOISOTOPE	▪ automatic reading of results ▪ accurate and sensitive	▪ expensive equipment ▪ radioactive ▪ 48 h (up to 72 h) of incubation
FLOW CYTOMETRY	▪ rapid, accurate, highly sensitive ▪ objective and automated ▪	▪ expensive equipment ▪ specialized technicians ▪ 48 h - 72 h of incubation
FLUOROMETRIC	▪ accurate, rapid and simple ▪ instrument less expensive than a flow cytometer	▪ not as sensitive as other methods (require higher parasitemias) ▪ 48 h - 96 h of incubation
ANTIGEN DETECTION	▪ equipment relatively cheap ▪ few personnel training	▪ 48 h – 72 h of incubation ▪ limited supplies of monoclonal antibodies
Genotypic		
MOLECULAR TECHNIQUES	▪ small amount of genetic material ▪ independence from host and environmental factors ▪ large numbers of tests can be conducted in a short period	▪ sophisticated equipment and training ▪ lack of known and validated resistance markers

4. AN ALTERNATIVE ASSAY

4.1. THE APPROACH – HEMOZOIN AS A MATURATION INDICATOR

Hz is a crystal produced in the digestive vacuole of the malaria parasites during the blood stage of malaria infection. This unique crystal constitutes an optimal growth/maturation indicator once, Hz content increases during parasite maturation (Figure 2). Moreover, as mentioned above, several antimalarial drugs interfere with the formation of Hz. Thus, the measurement of RBCs with different contents of Hz might be a useful tool to determine the inhibitory effect of antimalarial compounds. Curiously, in the 1980s a simple visual agglutination test for the detection of Hz production of malaria parasites was developed. At the end of the 48 hours of incubation, the amount of dark-pigmented precipitate (Hz) is assessed.[57] However this assay was not very accurate.[4] Still, an accurate measurement of infected red blood cells containing different amounts of Hz, by flow cytometry, might allow the assessment of the antimalarial drugs inhibitory effect.

4.2. PROPERTIES OF HEMOZOIN – HOW TO DETECT IT

Hemozoin is chemically and structurally similar to a distinctive hematin pigment, called β-hematin. Hz crystals are capable of rotating the plane of polarized light (birefringent) and generating small magnetic fields (paramagnetic). Hz crystals have a brick like morphology with 100 nm x 100 nm x 500 nm dimensions, in the case of crystals isolated from the human malaria parasite (Figure 6a). In the parasite that infects murine rodents, the Hz crystals are slightly smaller.[53]

Due to its physical property of being birefringent, Hz can be detected by optical methods, such as microscopy[31] (Figure 6) or flow cytometry[42].

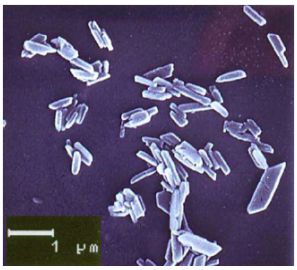

FIGURE 5: SCANNING ELECTRON MICROGRAPH OF ISOLATED *Plasmodium falciparum* HEMOZOIN (Hempelmann 2007).

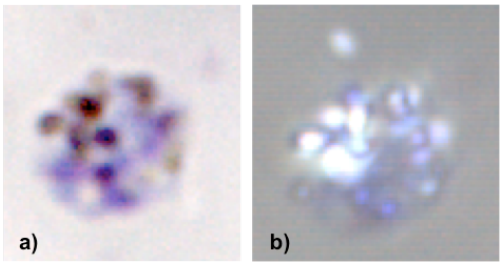

FIGURE 6: HEMOZOIN WITHIN INFECTED RED BLOOD CELLS. Giemsa-stained blood smears. a) bright-field microscopy (1000x); b) polarization microscopy (1000x). The brown pigment seen in panel a) has a bright appearance in panel b).

4.2.1. HEMOZOIN CAN BE DETECTED IN BLOOD CELLS

The malarial pigment can be easily detected inside leukocytes or parasitized RBCs in Giemsa-stained blood smears. Although, unspecific pigment like particles from the staining solution are often present, making it difficult to distinguish them from Hz crystals.

It is also possible to detect hemozoin-containing cells by flow cytometry. In fact, it has been reported that flow cytometric principles, used in automated full-blood-count analysers (Cell-Dyn 3500™, Abbott Diagnostics), can detect Hz-containing monocytes and Hz-containing neutrophils.[22,34,63] During these studies a depolarizing population of non-leucocytes events was also detected. [22,34] This distinct depolarizing population was suggested to be infected red blood cells containing Hz.

5. SOME CONSIDERATIONS

After the published work indicating that it was possible to detect pigment containing leukocytes by an haematology analyser, and that possibly infected RBCs could also be detected by flow cytometry. Probably, parasite maturation could be assed by measuring the infected RBCs containing different amounts of Hz. Once, Hz content increases during parasite maturation.

Preliminary results of flow cytometric analysis showed that it was possible to detect infected RBCs with different contents of Hz (Figure 7a), by the detection of depolarizing events. This depolarizing population is absent in the uninfected blood sample (Figure 7b).

Considering several facts such as, the increase of Hz content within infected RBCs as the parasite matures, the fact that the majority of the antimalarial drugs available act in the intraerythrocytic stage of malaria infection and, moreover, the fact that some of them act directly upon hemozoin formation, it

was thought that an accurate and reliable measurement of infected RBCs containing different amounts of Hz might allow to determine the inhibitory effect of antimalarial drugs.

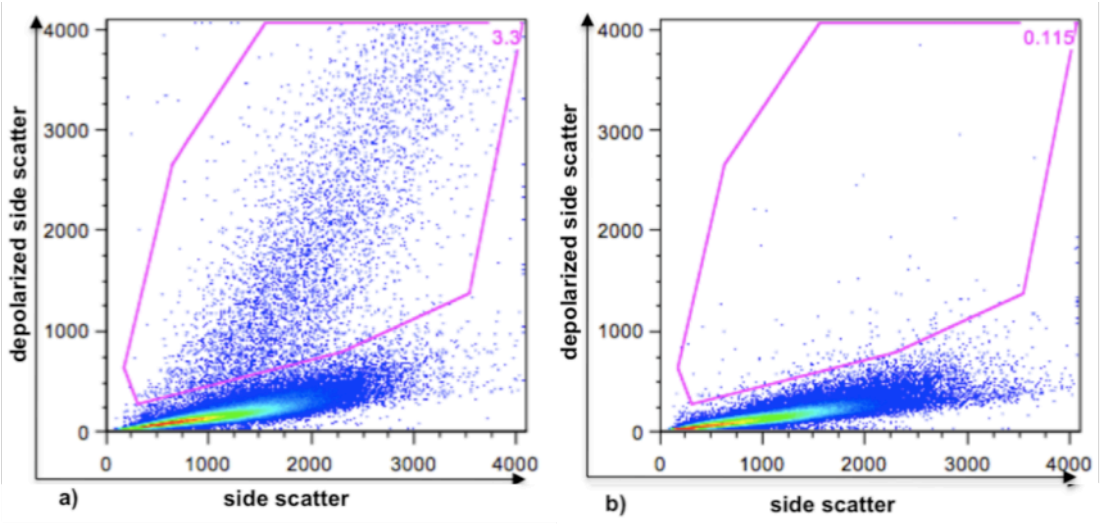

FIGURE 7: DETECTION OF DEPOLARIZING RED BLOOD CELLS IN A SAMPLE FROM AN INFECTED MOUSE. Side scatter against depolarized side scatter plots. a) blood sample from an infected mouse; b) blood sample from an uninfected mouse. Pink lines delineates the depolarizing red blood cell population.

In this study the murine strain, *Plasmodium berghei*, was the experimental model chosen due to its inability to re-infect red blood cells in *in vitro* culture. Assuring that the effect detected is based only on parasites maturation and not replication.

II - OBJECTIVES

The major goal of this project is to develop an alternative method for a novel, rapid, objective and automatable sensitivity test, without the need for reagents. This assay is based on the flow cytometric detection of infected red blood cells containing different amounts of Hz. Therefore, several experiments using a murinic model, will be conducted to assess parasite maturation by flow cytometric analysis.

III – MATERIAL AND METHODS

1. REAGENTS

1.1. RPMI COMPLETE MEDIUM. RPMI culture medium supplemented with 10% fetal calf serum, 1% non essential amino acids, 1% penicillin/streptomycin, 1% glutamine and 10 mM Hepes, pH 7.

1.2. FACS BUFFER. 2% Fetal calf serum in 1x Phosphate-Buffered Saline (PBS).

All of the reagents mentioned above were purchased from Gibco, Carlsbad, US.

1.3. GIEMSA STAIN. Giemsa's azur eosin methylene blue solution (Merck, Darsmstadt, Germany) was dilluted 1:10 in 1x PBS.

2. ANTIMALARIAL DRUGS PREPARATION

The published literature on sensitivity assays in *Plasmodium berghei* is scarce. Thus, which drug concentration should be used is not well established. According to this, the final antimalarial drugs concentrations were chosen based on the concentrations used in field applied *in vitro* sensitivity assays for *Plasmodium falciparum.*[4] The drugs tested during this project were: artemisinin, chloroquine pyrimethamine, quinine (purchased from Sigma-Aldrich, St. Louis, MO, US) and mefloquine (purchased from Novartis, Cork, Ireland).

As explained in Section 8 (Drugs Assay protocol) "red blood cell culture" was the denomination assigned to the solution obtained by mixing the drug working solution with the blood suspension. In order to have the correct final concentration in the red blood cell culture, the drug working solutionhad to be prepared twice as concentrated, because it was going to be diluted by half.

Some of the antimalarial drugs had to be prepared in methanol or ethanol. Still, the final percentage of ethanol present in the red blood cell culture never exceeded the 0.2%. This percentage was confirmed as being harmless to the red blood cell culture.

Of note, the working solutions were prepared by diluting the respective intermediate solution in RPMI complete medium.

2.1. ARTEMISININ. The stock solution of artemisinin was prepared by dissolving 18,05 mg in 50 mL pure methanol (M = 1,28 mM). Three serial dilutions of 1:10 from the stock solution were done to obtain the three intermediate solutions (Int.1, Int.2 and Int.3). The working solution at 1 nM was prepared from the Int.3. Working solution at 8 nM and 32 nM were obtained from the Int.2. From Int.1 working solutions at 64 nM, 256 nM and 518 nM were prepared.

2.2. CHLOROQUINE. A stock solution was prepared by dissolving 33 mg in 50 mL sterile distilled water (M = 659 μM). The intermediate solution was obtained by diluting the stock solution 1:100 in sterile distilled water (M = 6,59 μM). From the intermediate solution working concentrations of 10 nM, 50 nM, 100 nM and 200 nM were prepared.

2.3. MEFLOQUINE. To obtain the stock solution 13,25 mg of mefloquine hydrochloride were dissolved in 50 mL pure methanol (M = 640 μM). Two serial dilutions of 1:10 in absolute ethanol were done and intermediate solution 1 (Int.1) and 2 (Int.2) were obtained. Working solution at 10 nM was prepared from Int. 2, at 80 nM from Int.1 and at 320 nM from the stock solution.

2.4. PYRIMETHAMINE. The stock solution was prepared by dissolving 5,1 mg in 50 mL absolute ethanol (M = 410 μM). To obtain the intermediate solution the stock solution was diluted 1:100 in absolute ethanol. Working solution at 6.2 nM was prepared from the intermediate solution, while the working solution at 1600 nM was obtained directly from the stock solution.

Pyrimethamine at 51200 nM was also tested. This time it was prepared differently. Because this drug is prepared in ethanol, to obtain this final drug concentration, the percentage of ethanol present in the red blood cell culture could be harmful to the cells. So, in this case 62,4 μl of the stock solution was added (M = 410 μM) directly into a well of a 24 well plate. The plate was incubated for 24 hours until the ethanol evaporated.

2.5. QUININE. The stock solution was prepared from 46,2 mg of quinine hydrochloride dissolved in 50 mL sterile distilled water (M = 2,34 mmol). Two serial dilutions were done in order to obtain intermediate solution 1 (Int.1) and 2 (Int.2). Working solution at 800 nM was prepared from the Int.1. Working solution at 1600 nM, 3200 nM and 6400 nM were obtained from the Int.2.

3. ANIMAL MODEL

The animal model used in this project was C57BL/6 mice, which came from Charles River, Spain, and Harlan Laboratories, Spain. The animals were housed in the Instituto de Medicina Molecular (IMM) facilities and all the protocols were approved by the *Direcção Geral de Veterinária* and by the IMM Animal Care Committee.

4. GIEMSA STAINING OF BLOOD SMEARS

Air-dried blood smears were fixed in absolute methanol by dipping the slide briefly (two dips) into a 50 mL falcon with absolute methanol. Then they were air dried and stained with diluted Giemsa (1:100 in PBS 1x) for 30 minutes. After, slides were rinsed off in tap water and air-dried (according to manufacture's instructions).

The parasitemia was estimated by counting the number of total red blood cells and infected red blood cells, by bright field microscopy under oil immersion with a 100x objective.

5. *Plasmodium berghei* INFECTION

Mice were infected with two different strains: *Plasmodium berghei* ANKA and *Plasmodium berghei* ANKA expressing green fluorescent protein (GFP). Infection was done by intraperitoneal injection of a designated quantity, normally 10^6, of infected red blood cells.

Peripheral parasitemia was quantified in a daily basis. A drop of blood from the tail was collected from infected mice and blood smears were Giemsa stained as described above (Section 4). For mice infected with a GFP-expressing strain parasitemia was also determined by flow cytometry, described below (section 7).

6. COLLECTING BLOOD SAMPLES

Mice with parasitemias between 2% and 6% were sacrificed by CO_2 asphyxiation. Blood was collected by cardiac puncture into a heparinised 1,5 mL microtube (heparin was previously diluted 1:100 in PBS 1x).

7. FLUORESCENCE DETECTION

Fluorescence was measured by quantification of the percentage of FL1 (green fluorescence) or FL3 (red fluorescence) positive cells. The percentage of FL1 or FL3 positive cells was analysed according to this protocol: an uninfected (in the case of GFP) or an unstained sample (in the case of SYBR® green I and Ter119) was analysed in the flow cytometer. Settings were established and the gain was adjusted so the acquired events would be grouped in the first log decade. Then, a gate was made excluding the acquired events (Figure 8a). After this, the infected or the stained samples were analysed. Using the gate previously established the events included within this gate are considered FL1 or FL3 positive (Figure 8b).

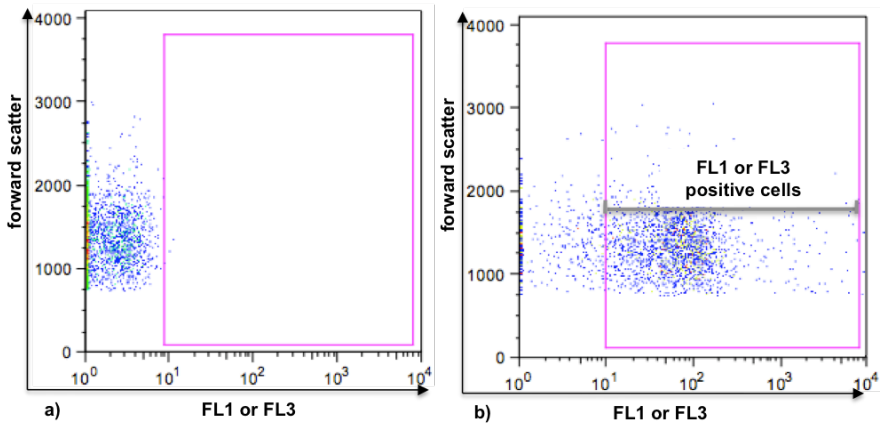

FIGURE 8: IDENTIFICATION OF FL1 OR FL3 POSITIVE CELLS. FL1 or FL3 against forward scatter plots. a) Uninfected or unstained population in the first log decade b) FL1 or FL3 positive population inside the gate previously established. Square delineated in pink represents the gate.

7.1. GREEN FLUORESCENT PROTEIN (GFP). Parasites expressing GFP were used in the majority of the experiments, in order to compare the drug effect detected by both methods: quantification of the percentage of highly depolarizing red blood cells or by analysis of the percentage of GFP positive cells (FL1 fluorescence).

7.2. SYBR® GREEN I (Invitrogen, Carlsbad, US). SYBR® Green I is a DNA stain and was only used to confirm that the events detected were in fact infected RBCs. The SYBR® Green I 10000x concentrated in DMSO was diluted in sterile PBS 1x to obtain a solution at final concentration of 10x. A volume of 2,5 μl of the SYBR Green I solution at 10x was added to the sample tube (5 μl of the red blood cell culture in 1 mL of FACS buffer) and incubated for 20 minutes, in the dark and at room temperature. The stained sample was acquired in the flow cytometer. The percentage of SYBR® green positive cells (FL1 fluorescence) was determined.

7.3. TER119 (eBioscience, San Diego, USA). Ter119-PE is an erythroid cell line marker that is detected in the red fluorescence (FL3). It was only used as confirmatory evidence that the events detected were RBCs. A volume of 2 μl of whole blood was distributed in a 96 well plate, keeping the cells on ice. Cells were washed by centrifuging them in 160 μl of FACS buffer during 3 minutes, at 1400 rpm and at 4ºC. Supernatant was discarded and cells were ressuspended in 50 μl of Fc-block (eBioscience, San Diego, USA), previously diluted 1:100 in FACS buffer, and incubated on ice for 20 minutes. Cells were washed again by adding 140 μl of FACS buffer and centrifuging them as described above (1400rpm, 3min, 4ºC). The supernatant was aspirated and the pellet was incubated

with 50 μl of a Ter119 solution previously diluted 1:500 in FACS buffer, during 20 minutes, on ice and in the dark. Another 140 μl of FACS buffer were added and cells were washed (as described above). Supernatant was discarded and cells were ressuspended in 50 μl of FACS buffer. This volume was transferred to a Cyflow tube with 950 μl of FACS buffer. The stained sample was acquired in the flow cytometer. The percentage of TER119 positive cells was determined.

8. ANTIMALARIAL DRUGS SENSITIVITY ASSAY

8.1. GENERAL PROTOCOL. Infected blood, previously collected to a heparinised 1,5 mL microtube, was diluted to 1:50 in RPMI complete medium. Then 500 μl of the prepared infected blood suspension was added to a 24 well plate. Uninfected samples were prepared the same way as the infected blood suspension. To the respective well 500 μl of each different drug was added or, in the case of the drug free control and uninfected wells another 500 μl of RPMI complete medium was added. The final suspension present in the well was defined as "red blood cell culture". The plate was incubated at 37ºC in an atmosphere of 5% CO_2. For each measurement RBCs in the wells were ressuspended and 5μl of the ressuspended solution were diluted in 1 mL of FACS buffer and immediately analyzed. The number of acquired events ranged from 50 000 to 80 000 in different experiments (Figure 9). All samples were tested in triplicate and different time points were analyzed.

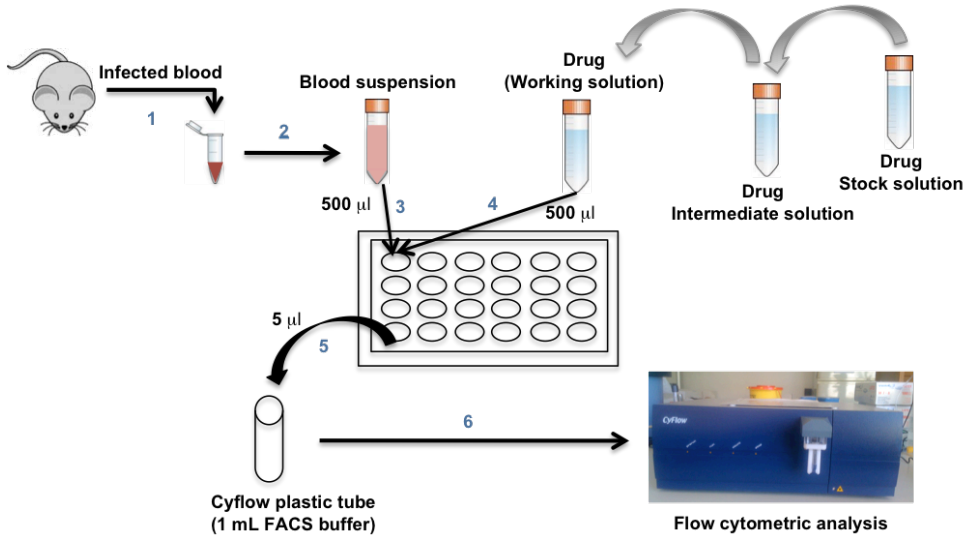

FIGURE 9: SCHEMATIC REPRESENTATION OF THE DRUGS ASSAY PROTOCOL. a) Blood from an infected mouse was collected (1). A blood suspension in RPMI complete medium was prepared (2). Several dilutions from the drug stock solution were made to obtain the intermediate solutions. The final drug working solutions were obtained by diluting the intermediate solutions in RPMI complete medium. 500 μl of blood suspension was distributed per well (3) and 500 μl of the drug working solution was added to the respective wells (4). The plate was incubated at 37ºC, 5% CO_2 atmosphere. For every measurement 5 μl of the suspension in the well was added to 1 mL of FACS buffer (5) and the sample was analysed in the flow cytometer (6).

8.2. PROTOCOL FOR INHIBITING THE EARLY DECREASE CAUSED BY CHLOROQUINE. In this case the protocol was adapted and the sample was pre-treated with quinine, before the treatment with chloroquine, as previously described by Warhurst 1972.[76] After adding 500 μl of the blood suspension to a well, from a 24 well plate, 500 μl of quinine was first added and the plate was

incubated for 15 minutes. After incubation, 7,8 µl of the intermediate solution of chloroquine (M = 6,59 µmol) was added to the same well.

8.3. DATA ANALYSIS. FlowJo (version 8.8.6) software was used to analyze the data. First, a gate was created in order to select only red blood cells (RBC). The accuracy of the gate was confirmed by staining RBCs with a marker for erythroid cells (Ter119-PE). The drug effect of the gated population was analized in a plot that shows side scatter (x-axis) against the depolarized side scatter (y-axis). Subsequently, the optimal gating strategy was established that would give the best discrimination between drug free control and drug treated samples.

9. FLOW CYTOMETRY

9.1. BASIC PRINCIPLES OF FLOW CYTOMETRY. Flow cytometry is a powerful technique for the analysis of multiple parameters of individual cells within heterogeneous populations. As a cell passes through the laser it will refract or scatter light at all angles:

1. Forward scatter (FCS): is the amount of light that is scattered in the forward direction when the laser strikes the cell. The magnitude of FCS is proportional to the size of the cell. The light scattered is quantified by a detector that converts intensity into voltage. As the cells crosses the laser, light forward scattered is collected by the detector.
2. The side scatter (SSC): is the light that is scattered to the side (at a 90º angle), reflecting the granularity and structural complexity inside the cell.
3. Depolarized side scatter: measures the polarization of light scattering (at a 90º angle). It is the same as the normal side scatter but, instead, there is a polarization filter that is perpendicularly positioned to the incident polarized laser beam (Figure 10b).

TABLE 5: FLOW CYTOMETER BASIC COMPONENTS OVERVIEW

COMPONENT	FUNCTION
Fluidic system	Presents the cells to the laser beam one at a time by injecting the sample stream into a flowing stream of sheat fluid.
Lasers	Are the light source for the scatter and fluorescence
Optics	Gathers and directs light to the detectors.
Detectors	Receive the light/detect the signals.
Electronic and peripheral computer system	Converses the signal from the detectors into digital data.

9.2. THE INSTRUMENT. A benchtop flow cytometer, CyFlow® (Partec®, Münster, Germany), with a 488 nm blue solid state laser, with detectors for forward scatter (FSC), side scatter (SSC), fluorescence FL1 (band pass 535/45 nm filter) and FL3 (long pass 620 nm filter), as well as a detector for depolarized side scatter was used to detect infected red blood cells containing hemozoin (Figure 10). The fluidic system consists of sheath fluid (Milli Q water filtered with a 0.22 µm filter) and the waste. In the latter 100 mL of bleach at 10% was added to inactivate the analysed biological material. A pair of electrodes is present in both bottles allowing the system to know when the sheath bottle is empty or when the waste bottle is full.

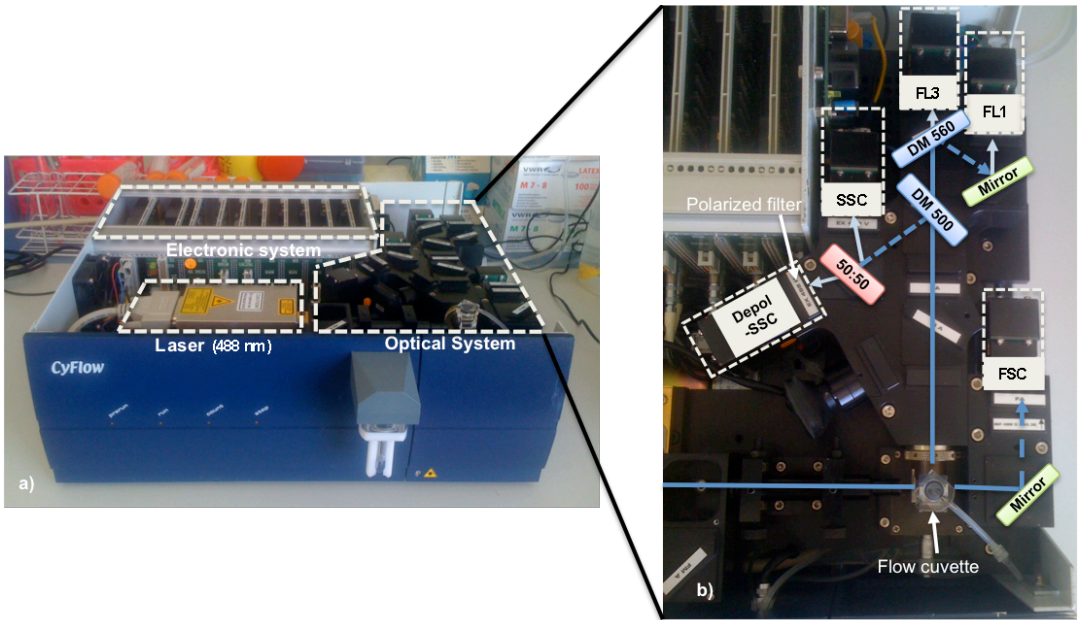

FIGURE 10: COMPONENTS OF THE INSTRUMENT - CYFLOW (PARTEC, GERMANY). a) "Inside" the instrument: the laser (source of light), the optical system and the electronic system. b) Optical bench overview: the laser beam (blue line) passes through the flow cuvette (cells from the sample are passing one at a time), and the forward scattered light is reflected to the FSC-detector. The side scattered light reaches a dichroic mirror (DM 500), light with wavelengths higher than 500 nm will pass through and light with wavelengths lower than 500 nm will be reflected. The reflected light will reach another mirror (50:50) that reflects 50% of the light to the depolarized side scatter (depol-SSC) and the other 50% to the normal side scatter (SSC). The light that passed through the DM 500 will reach another dichroic mirror (DM 560). Light with wavelengths higher than 560 nm will be detected by the red fluorescence (FL3) detector, and light with wavelengths lower than 560 nm will be reflected. The reflected light will reach a mirror and will be reflected to the green fluorescence (FL1) detector.

9.3. COUNTING ABSOLUTE EVENTS. Absolute counts were made, sporadically, as an internal control for RBCs population. A volume of 800 μl of the sample was analysed, after homogenization using a vortex. Special attention to bubbles, formed during the homogenization, is required because it might influence the absolute counts. The instrument has two electrodes, which measures a 200 μl sample volume, allowing the quantification of number of cells per volume unit (Figure 11).

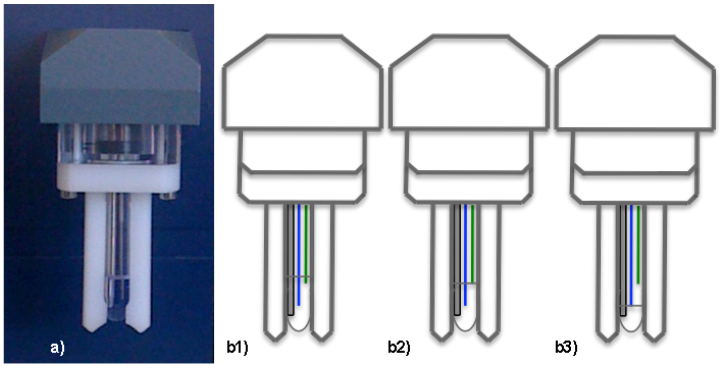

FIGURE 11: SCHEMATIC REPRESENTATION OF ABSOLUTE CELL COUNTS. a) Picture of a sample tube placed in the instrument. b) Schematic representation of absolute cell count. b1) The sample starts to be aspirated until it reaches the shorter electrode: green electrode (b2), when the instrument initiates absolute cell counting, which only ends when the sample reaches the longer electrode: blue electrode (b3). The volume between b2) and b3) is 200 μl.

IV – RESULTS

1. Detection of infected red blood cells (iRBCs)

1.1 Detection of RBCs

Red blood cells (RBCs) were easily detected by flow cytometry. Figure 14a shows the RBCs population in the forward scatter (FSC) against side scatter (SSC) plot from a blood sample of an uninfected mouse. Figure 14b shows the RBC population from a blood sample of an infected mouse. The different appearance is evident when looking at these two plots. The acquired events of the infected sample were gated, representing 98% of RBCs as confirmed by Ter119 (erythroid lineage marker) staining (Figure 12d). The two subpopulations (Figure 12c) present only in infected blood samples were also confirmed as being RBCs.

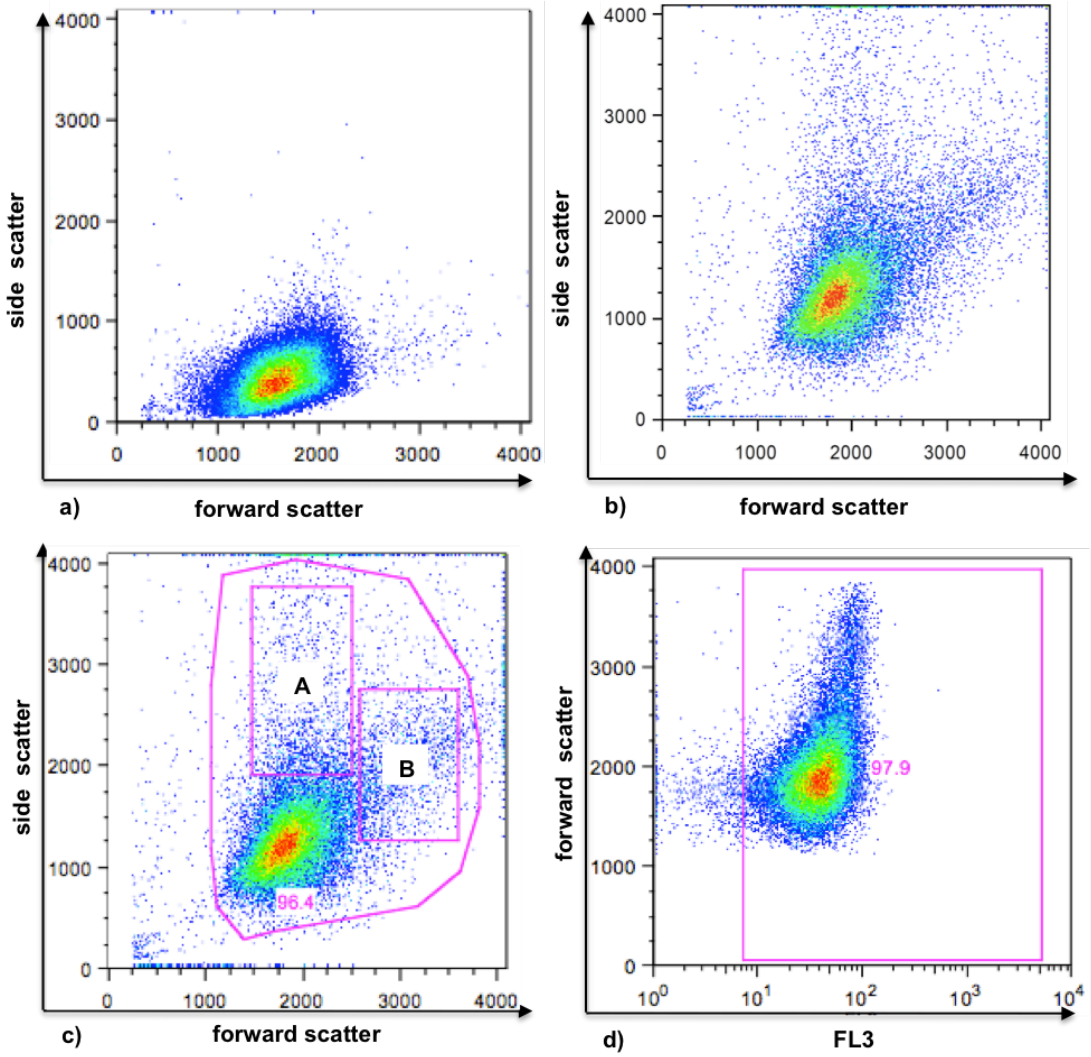

Figure 12: Flow cytometric detection of red blood cells (RBCs). a), b) and c) Forward scatter against side scatter. a) Blood sample from an uninfected mouse. b) Blood sample from an infected mouse. c) Acquired events were gated according to the RBCs specific forward (FCS) and side (SSC) light-scattering. Sub-populations A and B were included in the gate, even though they present changed features of size and granularity. Sub-populations A and B were confirmed, by staining with an erythroid marker, as being red blood cells. d) FL3 (red fluorescence) against forward scatter: the gated population in plot c) represent 97,9% of RBC (Ter119-PE positive).

1.2. Depolarizing cell population is present in blood samples from infected mice

Blood from uninfected mice have an insignificant percentage of depolarizing events (Figure 13a). On the other hand, in blood samples from infected mice a population of depolarizing cells is easily detected (Figure 13b).

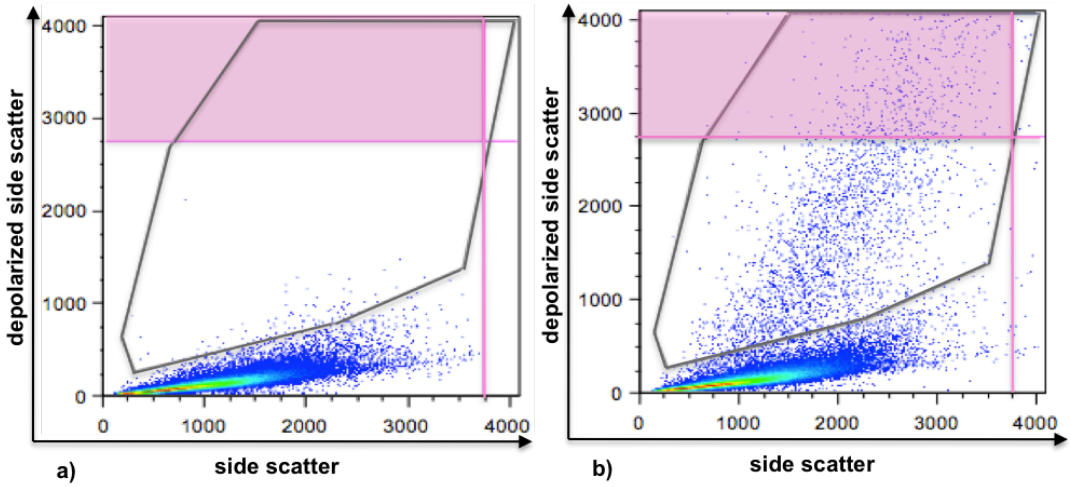

Figure 13: Depolarizing events present in samples from infected mice. Side scatter against depolarized side scatter plots. (a) Blood sample from an uninfected mouse; (b) blood sample from a *Plasmodium berghei*-ANKA infected mouse (parasitemia=5.5%). Pink rectangle highlights the highly depolarizing red blood cell population. Grey lines delineate the gate for the total depolarizing red blood cell population.

1.2.1. Depolarizing events represent infected RBCs

The depolarizing events were confirmed as being RBCs, by Ter119 (erythroid lineage marker) staining (Figure 14).

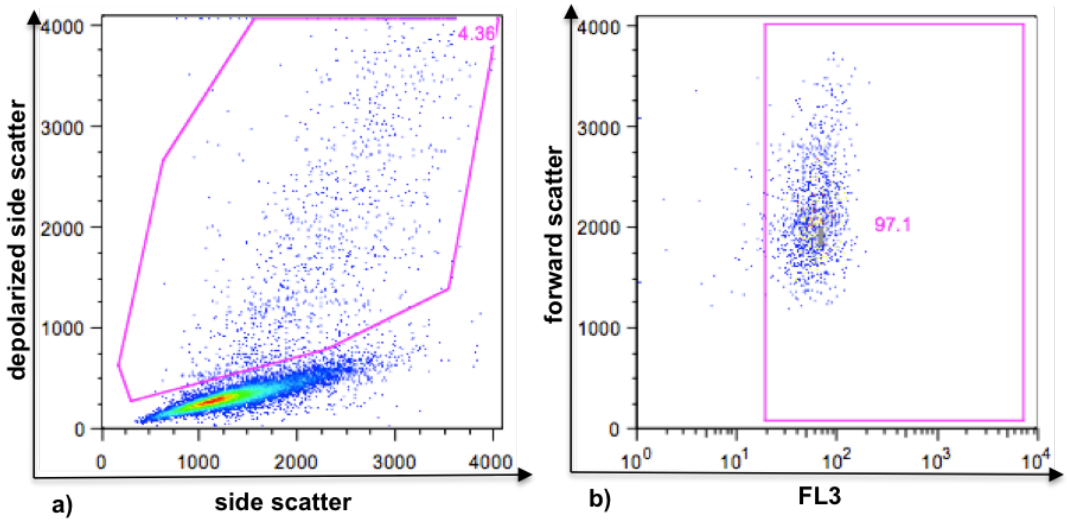

Figure 14: Detecting red blood cells. a) Side scatter against depolarized side scatter plots. b) FL3 (red fluorescence) against forward scatter. The depolarizing population gated in a) were 98.6% of red blood cells (Ter119-PE positive). Pink lines delineate the gates used to select depolarizing cells (a) and Ter119 positive cells (b).

DNA staining using SYBR® Green I, indicated that 98% of the depolarizing events were infected RBCs (iRBCs) (Figure 15).

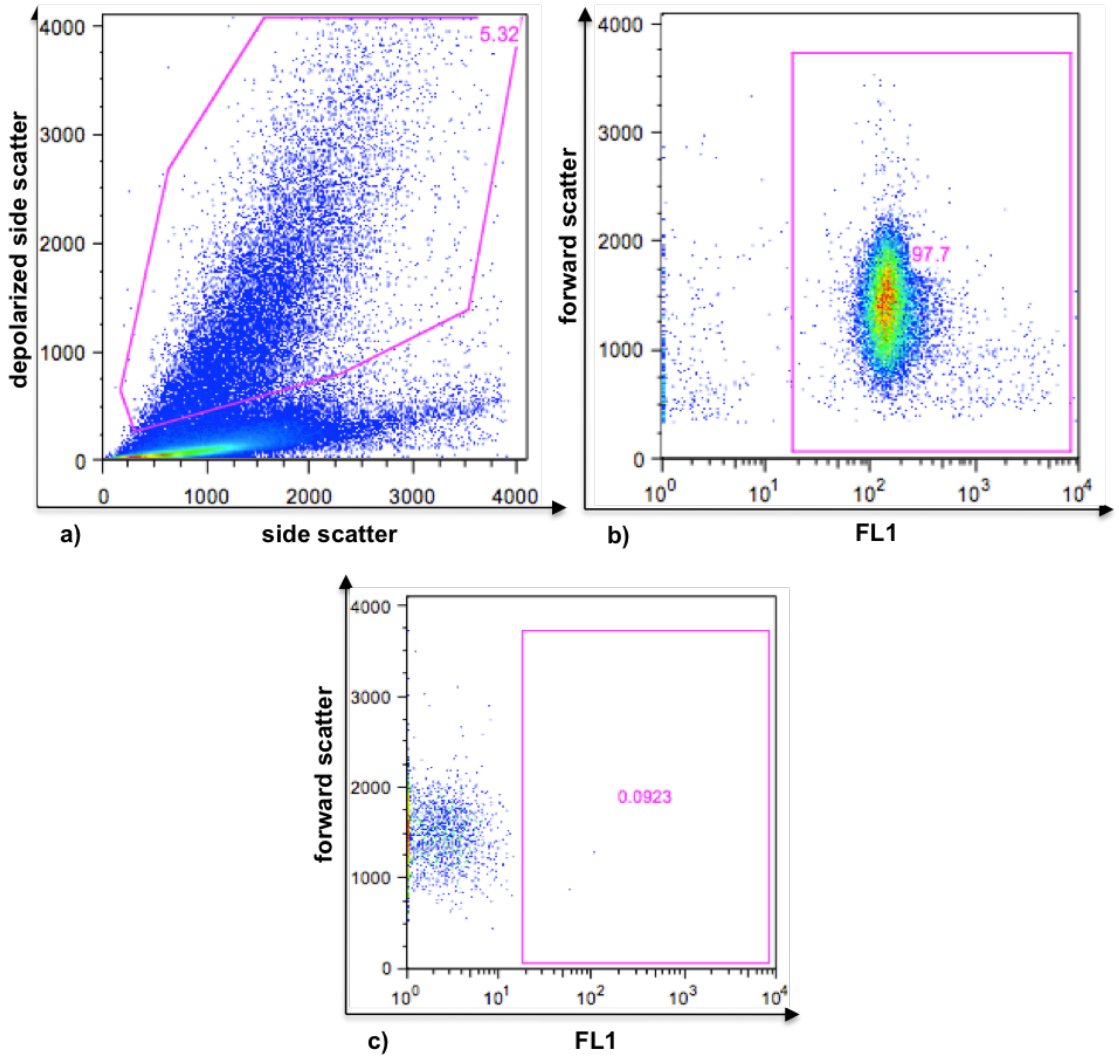

FIGURE 15: DETECTING INFECTED RED BLOOD CELLS. a) Side scatter against depolarized side scatter plot: depolarizing population. b) FL1 (green fluorescence) against forward scatter: 97,7% of SYBR® green positive cells, representing iRBCs. c) Unstained control. Pink lines delineate the several gates used.

2. GATING STRATEGY

Investigating the optimal gating strategy it was found that the best discrimination between drug free control and the untreated samples could be achieved by enumerating the percentage of highly depolarizing red blood cells (hdRBCs). Applying this strategy to the data obtained at 12 hours (Figure 16) to chloroquine treated samples, the gate represented in Figure 16f showed the biggest difference between the percentage of hdRBCs and the drug free control, represented by the higher “fold difference” (%hdRBCs drug free control / %hdRBCs drug treated samples). Thus, it was used as a basis for further analysis.

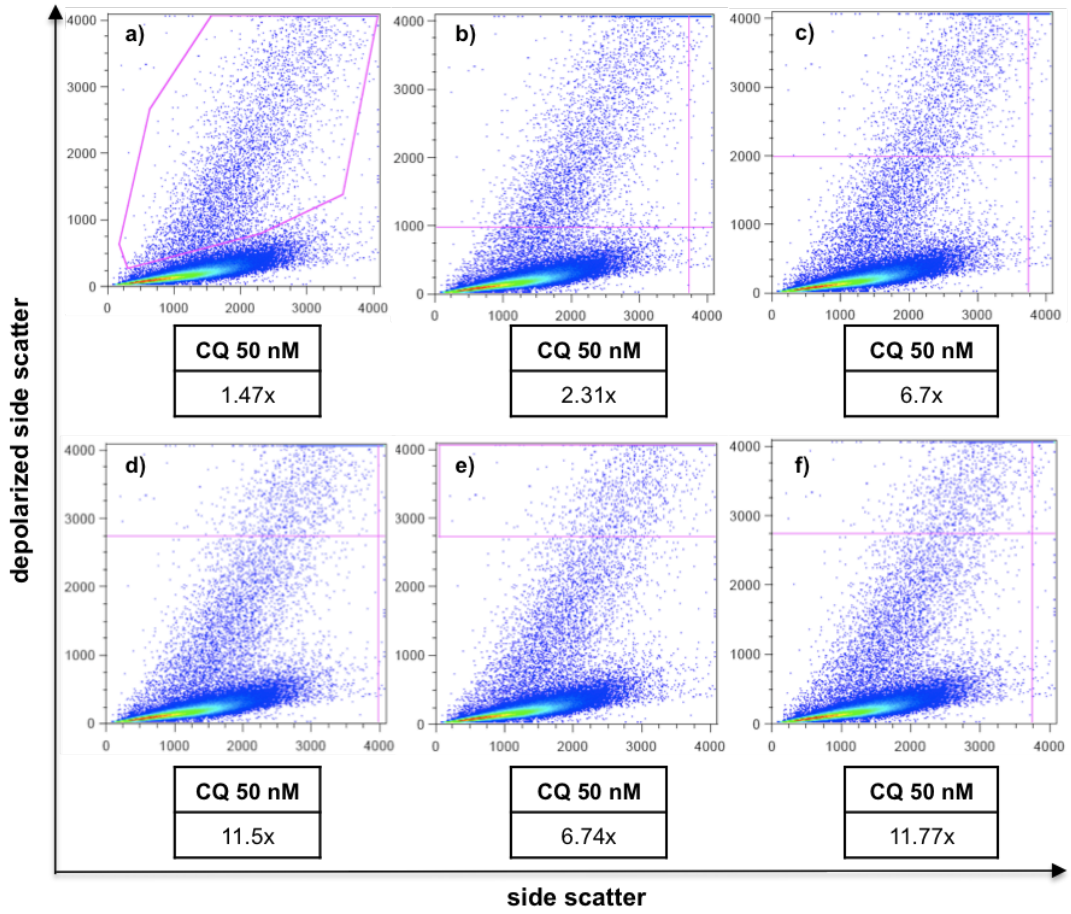

FIGURE 16: FINDING THE OPTIMAL GATING STRATEGY. The tables show the "fold decrease = %hdRBCs drug free control / %hdRBCs drug treated samples". f) the optimal gating strategy used for further analysis (the plots shown here are just an exemplification of the gates).

3. DRUGS ASSAY

3.1. ANTIMALARIAL DRUGS KNOWN TO INTERFERE WITH HEMOZOIN FORMATION

3.1.1.Chloroquine (CQ) and Quinine (QN). In the uninfected sample (negative control) highly depolarizing red blood cells (hdRBCs) were not detected (Figure 17, black line). Contrary to this, in the drug free control (infected sample - positive control) there is a steady increase of the percentage of hdRBCs reaching a peak at 12 hours of incubation. At this time the percentage of hdRBCs is 2.4 times higher than the percentage of hdRBCs present at time zero. After this a steady decrease is observed until 24 hours (Figure 17, red line).

The drug-treated samples are significantly different. In the samples treated with QN no change in the percentage of hdRBCs was observed until 12 hours of incubation after which it starts decreasing until 24 hours (Figure 17, green lines).

The CQ treated samples show an accentuated early decrease, already at 1 hour of incubation, after which it keeps slightly decreasing over the 24h period (Figure 17, blue lines).

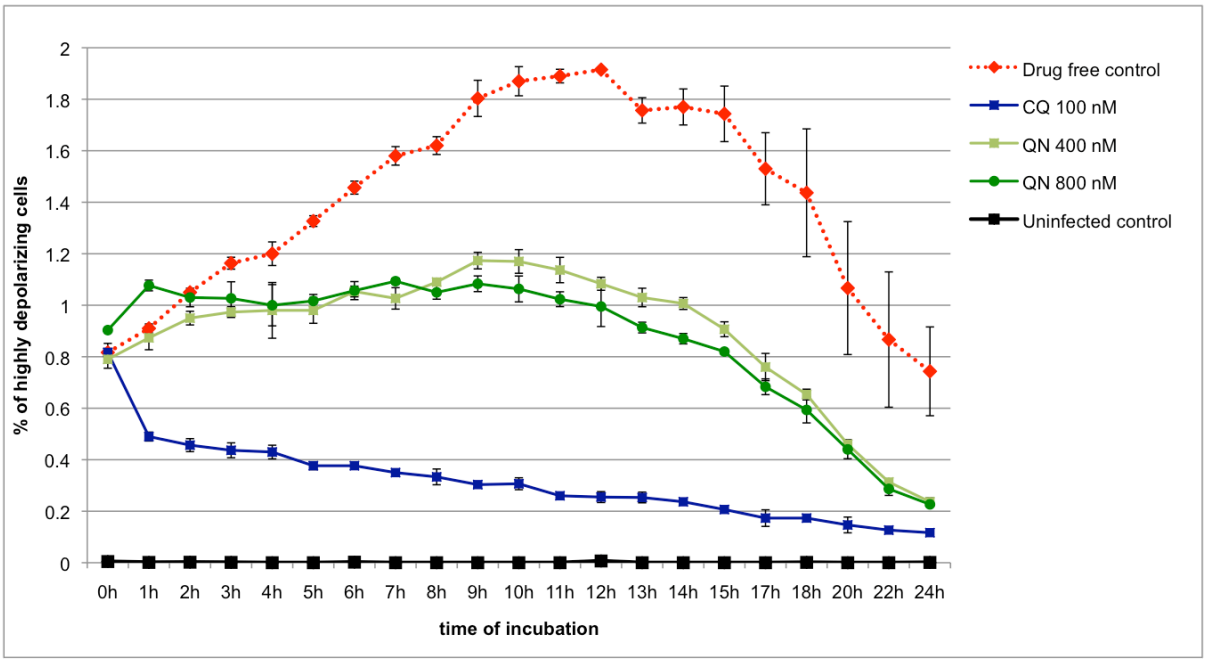

FIGURE 17: QUININE (QN) AND CHLOROQUINE (CQ) EFFECT ON THE PERCENTAGE OF HDRBCS. Drug free control (red line), treated with different concentrations of CQ (blue lines) and QN (green lines) (parasitemia = 5.7%). Measurements were done hourly, for a 24 hour period. Each time point represents the mean value of triplicate samples.

Another experiment was conducted testing higher concentrations of QN (1600 nM and 3200 nM) (Figure 18, darker green lines). No evident differences between these concentrations and the ones tested before, 400 nM and 800 nM (Figure 17, green lines) were observed.

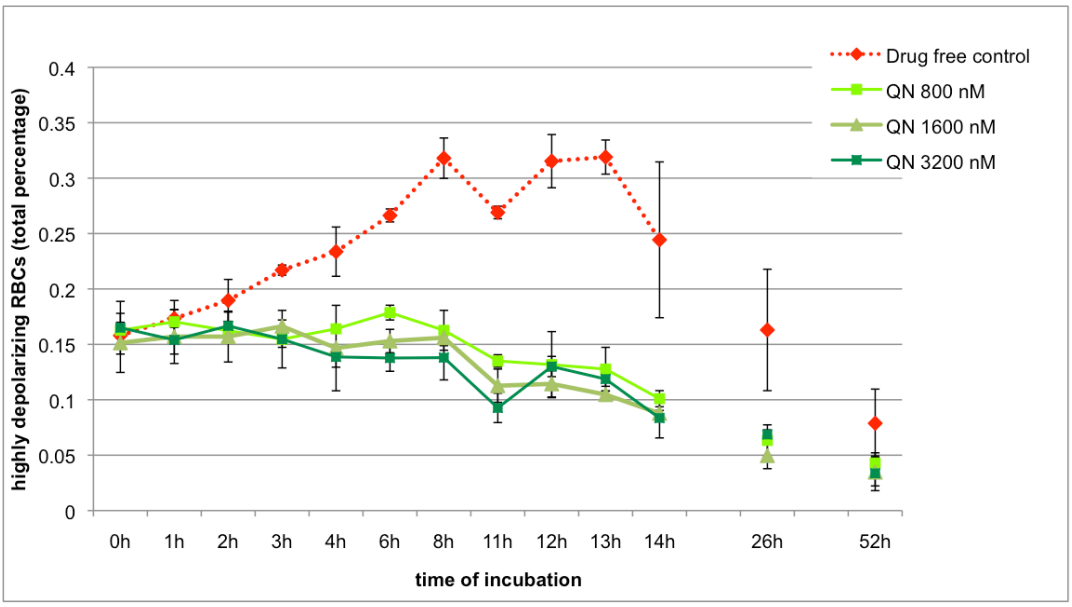

FIGURE 18: QUININE (QN) EFFECT ON THE PERCENTAGE OF HDRBCS. Drug free control (red line), treated with different concentrations of QN (green lines) (parasitemia = 5%). The first 14 hours were measured hourly, and then it was measured again at 26 hours and 52 hours after incubation. Each time point represents the mean value of triplicate samples.

3.1.2. Mefloquine (MQ). Samples treated with MQ at 5 nM could not be distinguished from the drug free control (Figure 19, pink line). On the other hand, the effect of MQ at 40 nM could be detected after 3 hours of incubation, whereas the effect of MQ at 160 nM could be observed already at 2 hours of incubation. In samples treated with MQ 160 nM no changes in the percentage of hdRBCs were observed until 8 hours of incubation.

After 8 hours of incubation samples treated with MQ at 40 nM and 160 nM showed a gradual decrease on the percentage of hdRBCs (Figure 19, purple lines).

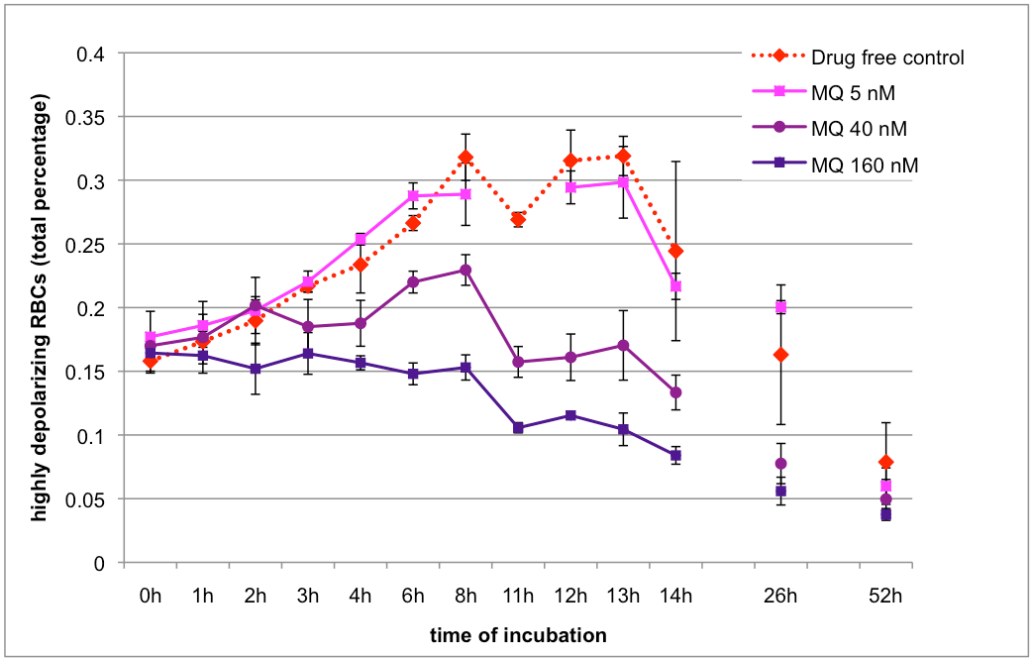

FIGURE 19: MEFLOQUINE (MQ) EFFECT ON THE PERCENTAGE OF HDRBCS. Drug free control (red line), treated with different concentrations of MQ (pink and purple lines) (parasitemia = 5%). The first 14 hours were measured hourly, then measurements were done again at 26 hours and 52 hours after incubation. Samples treated MQ 5 nM at 8 hours and 10 hours after incubation were not measured. Each time point represents the mean value of triplicate samples.

3.2. ANTIMALARIAL DRUGS WITH UNKNOWN MODES OF ACTION

3.2.1. Artemisinin (Art). The artemisinin was first tested at lower concentrations of 0.5 nM, 4 nM and 16 nM, as described in Basco 2007[4] for *Plasmodium falciparum* field sensitivity assay, but no changes were observed when compared to the drug free control (Figure 20).

Then, higher concentrations were tested. Samples treated with artemisinin at 128 nM and 259 nM showed a continuous decrease in the percentage of hdRBCs after 4 hours of incubation. After 14 hours of incubation, the percentage of hdRBCs present in the sample treated with 259 nM is ten times less than the drug free control, at the same time point. Artemisinin at 32 nM showed a slightly lower percentage of hdRBCs from 6 hours onwards, when compared with the drug free control, but after 26 hours of incubation both samples (drug free control and Art 32 nM) had a similar percentage of hdRBCs (Figure 21).

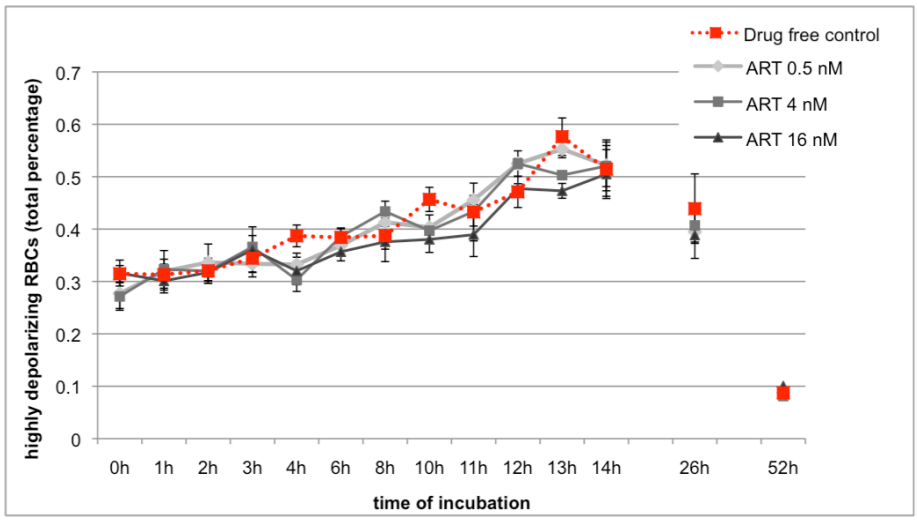

FIGURE 20: ARTEMISININ (ART) EFFECT ON THE PERCENTAGE OF HDRBCS. Drug free control (red line), treated with ART at 0.5 nM, 4 nM, 16 nM (grey lines) (parasitemia = 5%). The first 14 hours were measured hourly, then measurements were done again at 26 hours and 52 hours after incubation. Each time point represents the mean value of triplicate samples.

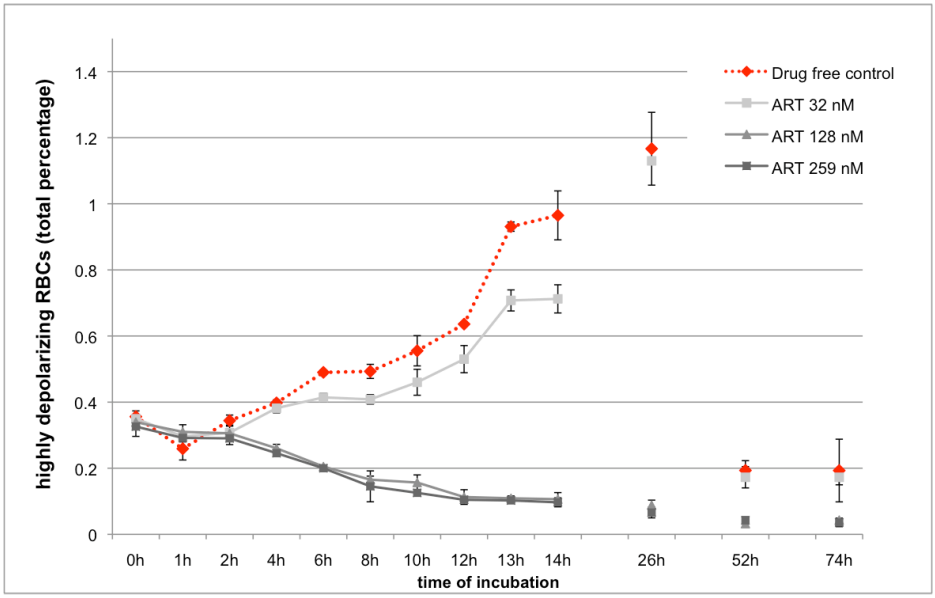

FIGURE 21: ARTEMISININ (ART) EFFECT ON THE PERCENTAGE OF HDRBCS. Drug free control (red line), treated with ART at 32 nM, 128 nM and 259 nM (grey lines) (parasitemia = 6%). The first 14 hours were measured hourly, then measurements were done again at 26 hours and 52 hours after incubation. Each time point represents the mean value of triplicate samples.

3.3 ANTIMALARIAL DRUGS THAT DO NOT INTERFERE WITH HZ FORMATION

3.3.1. PYRIMETHAMINE (PYR). For *P. falciparum* field sensitivity assay the concentrations of pyrimethamine tested are very different from each other, ranging from 0.05 nM to 51200 nM. In Figure 22, samples were treated with Pyr at 3.1 nM, 800 nM and 51200 nM. No differences in the percentage of hdRBCs compared with the drug free control were found in samples treated with Pyr at 3.1 nM and 800 nM. However, after 13 hours of incubation it was possible to detect a decrease in the percentage of hdRBCs in samples treated with Pyr at 51200 nM.

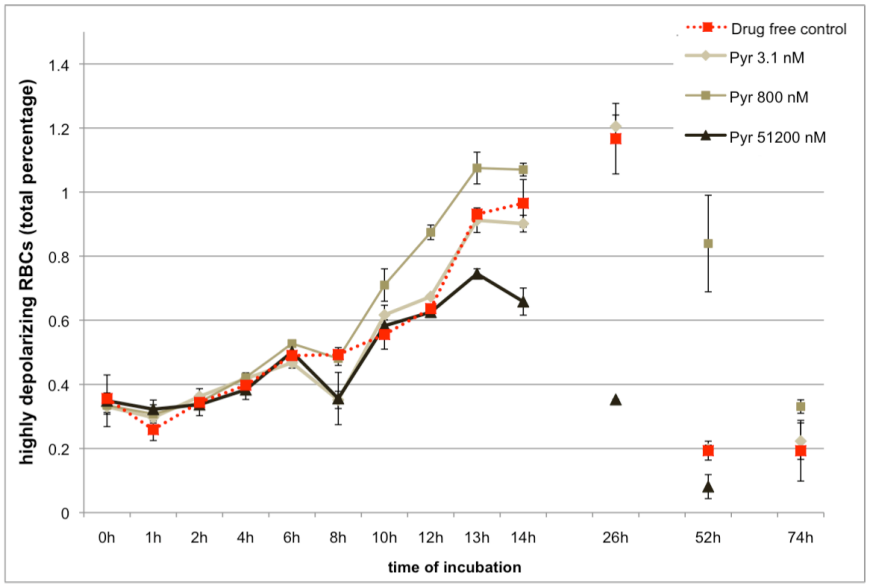

FIGURE 22: PYRIMETHAMINE (PYR) EFFECT ON THE PERCENTAGE OF HDRBCS. Drug free control (red line), treated with different concentrations of Pyr (brownish lines) (parasitemia = 6%). The first 14 hours were measured hourly, then measurements were done again at 26 hours and 52 hours after incubation. Each time point represents the mean value of triplicate samples.

4. EARLY DECREASE ON THE PERCENTAGE OF HDRBCS IN CQ-TREATED SAMPLES

In another experiment, chloroquine was tested at different concentration of: 25 nM, 50 nM and 100 nM. Once again, the early decrease could be detected after 2 hours of incubation in samples treated with CQ 50nM and CQ 100nM (Figure 23, blue lines). It was noticeable that as the concentration of CQ increases, the decrease in the first hours of incubation is more accentuated (Figure 23).

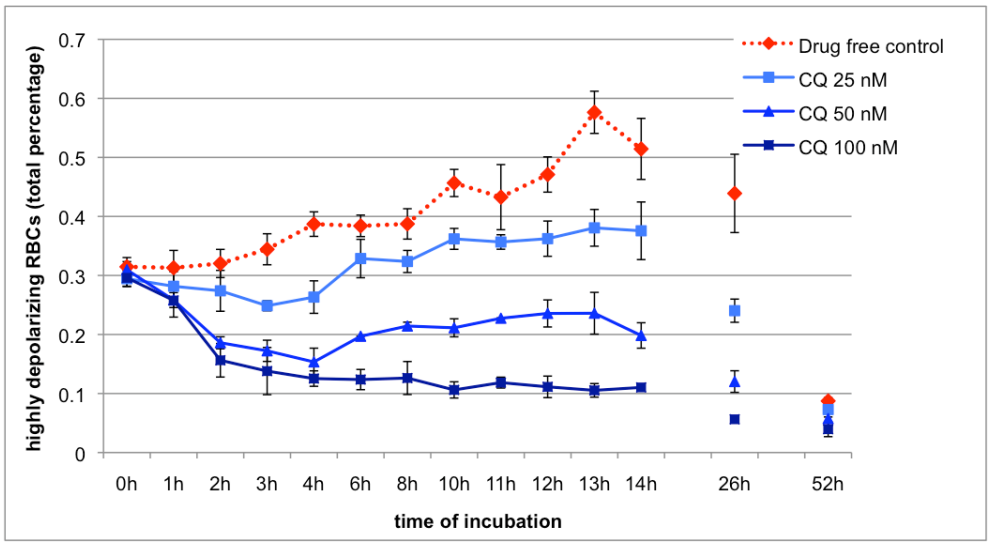

FIGURE 23: CHLOROQUINE (CQ) EFFECT ON THE PERCENTAGE OF HDRBCS. Drug free control (red line), treated with different concentrations of CQ (blue lines) (parasitemia = 5%). The first 14 hours were measured hourly, then measurements were done again at 26 hours and 52 hours after incubation. Each time point result represents the mean value of triplicate samples.

It was suggested that the early decrease observed in the samples treated with CQ (Figure 24) might be due to pigment aggregation, also described as CQ-induced pigment clumping.[75]

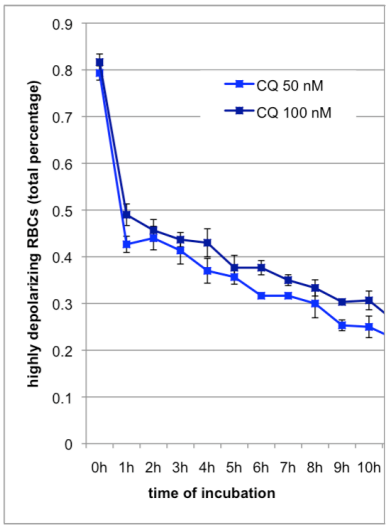

FIGURE 24: EARLY DECREASE IN BLOOD SAMPLES INFECTED WITH *Plasmodium berghei*-ANKA TREATED WITH CHLOROQUINE (CQ). Samples treated with different concentrations of CQ (blue lines) (parasitemia = 5.5%). This graph represents the first 10 hours of incubation observed on Figure 19. Note the accentuated decrease observed already after 1 hour of incubation.

Quinine (QN) was described as being an inhibitor of CQ-induced clumping.[76] Several experiments were conducted where the sample was pre-treated with quinine and, after 15 minutes of incubation, CQ was added. In figure 25 the results from one of these experiments are shown. It was possible to detect that the decrease on the percentage of hdRBCs, on the first hour of incubation, was reduced by half in samples pre-treated with QN.

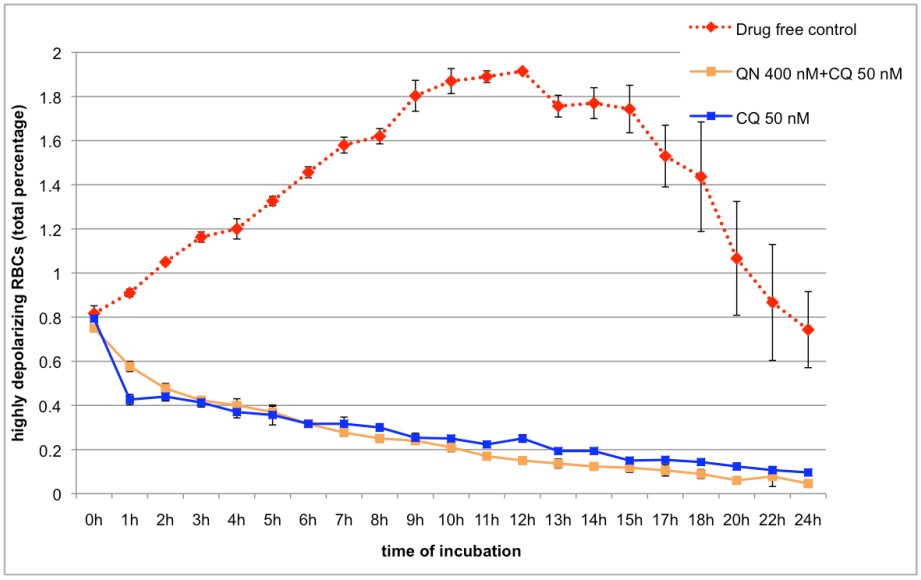

FIGURE 25: QUININE (QN) PRE-TREATMENT EFFECT ON THE PERCENTAGE OF HDRBCS. Drug free control (red line), only treated with CQ (blue line) and pre-treated with QN and 15 minutes later with CQ (orange line) (parasitemia = 5.7%). Each time point represents the mean value of triplicate samples.

5. ABSOLUTE COUNTS

As mention before absolute counts were done in order to control the red blood cell population. Figure 26 shows the different analysed cell populations.

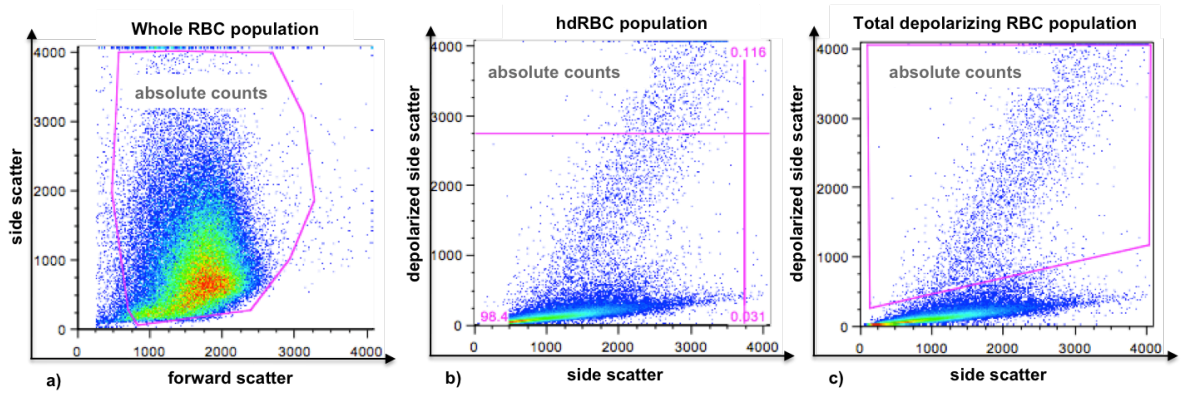

FIGURE 26: DIFFERENT APPROACHES APPLIED TO ABSOLUTE CELL COUNTS. a) Forward scatter against side scatter plot: whole red blood cell population. b) Side scatter against depolarized side scatter: highly depolarizing red blood cell population. c) Side scatter against depolarized side scatter: total depolarizing red blood cell population. Pink lines represent the gate.

The absolute counts of the whole red blood cell population did not show obvious differences over the 24 hour incubation period (Figure 27).

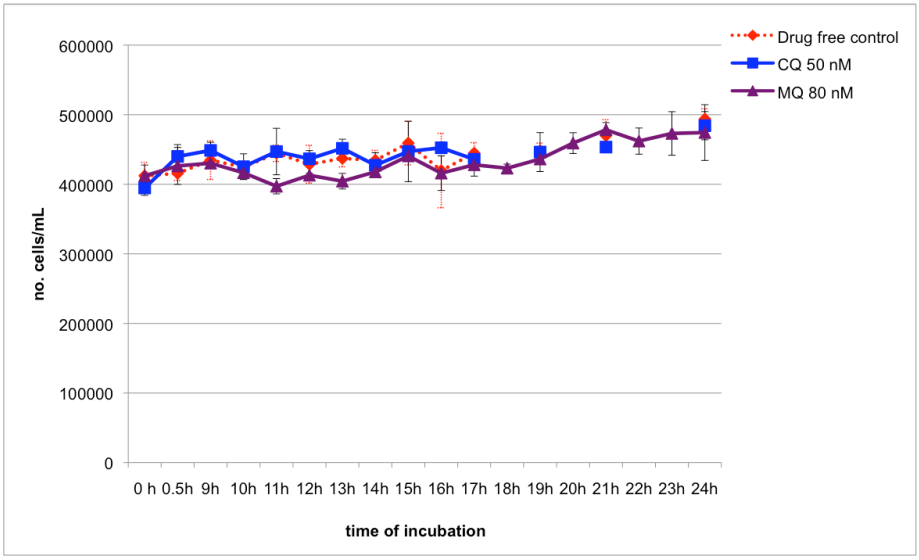

FIGURE 27: ABSOLUTE COUNTS OF RED BLOOD CELL POPULATION. Over the 24 hours of incubation the absolute counts, from the drug free control (red line) ranged from a minimum value of 412,188 to a maximum value of 493,690. Drug treated samples (blue and purple lines) showed an average change, between the maximum and the minimal value, of 85,302. [Absolute counts were obtained from the selected RBC population using the gate represented in Figure 28a)].

Analysing the absolute counts obtained from population of hdRBCs (Figure 28a) and from the total depolarizing RBC population (Figure 28b) it was possible to detect differences between the drug free control and the drug treated samples. In the drug free control the absolute counts started to decrease only after 18 hours of incubation. While, in the dug treated samples (Figure 28, blue and green lines) the absolute counts decreased already after 12 hours of incubation.

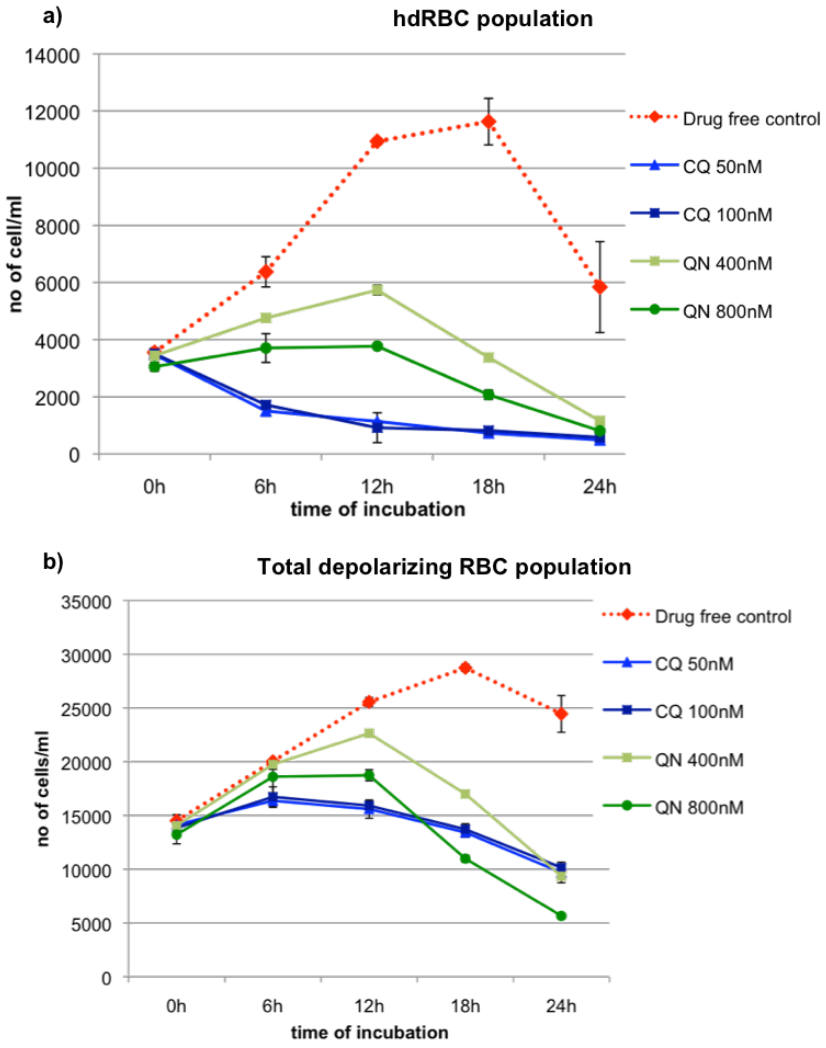

FIGURE 28: ABSOLUTE COUNTS OF DEPOLARIZING RED BLOOD CELLS. a) The absolute counts of highly depolarizing red blood cell population, which were gated as exemplified in Figure 26b); b) absolute counts of total depolarizing red blood cell population, which were gated as exemplified in Figure 26c).

6. GREEN FLUORESCENCE PROTEIN (GFP) FLUORESCENCE

The percentage of GFP positive infected RBCs was also quantified, as shown in Figure 29. The results presented are from two different experiments, one where CQ was tested, and another where QN and MQ were tested.

The time curve of the percentage of GFP positive iRBCs in the treated samples was very similar to the drug free control time curve (Figure 29).

In the CQ treated samples it was possible to detect an effect at 4 hours of incubation, comparing with the drug free control. The percentage of GFP positive iRBCs remained fairly the same over the 14-hour period. After 26 hours the percentage of GFP positive iRBCs started to decrease in both the drug free control and the CQ treated sample (Figure 29, blue and red lines).

On the other hand, differences between the drug free control and QN-treated samples were only detected after 12 hours of incubation. Samples treated with MQ showed an effect on the percentage of GFP positive iRBCs at 6 hours of incubation. Onwards it remained constant until 12 hours after incubation, when it started to decrease.

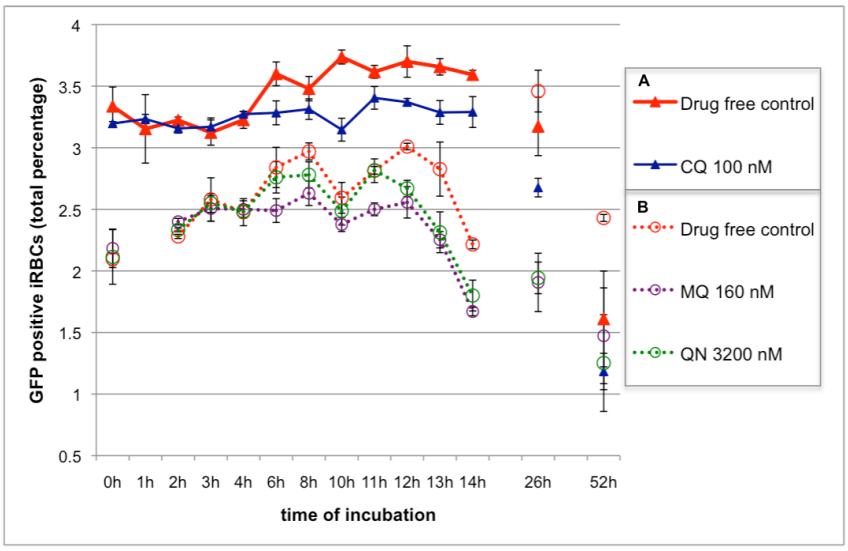

FIGURE 29: ANTIMALARIAL DRUGS EFFECT IN THE PERCENTAGE OF GFP POSITIVE IRBCS. Drug free control (red lines) samples treated with chloroquine (blue line), mefloquine (purple line) and quinine (green line). Lines represent the results from one experiment (A), and dashed lines represent results from another experiment (B).

7. COMPARING THE EFFECTS OF THE ANTIMALARIAL DRUGS TESTED

The drug effect of the several antimalarial drugs tested was assessed by comparing the difference in the percentage of hdRBCs over time with time zero ("difference over time = %hdRBCs at each time point - %of hdRBCs at 0 hours") (Figure 30).

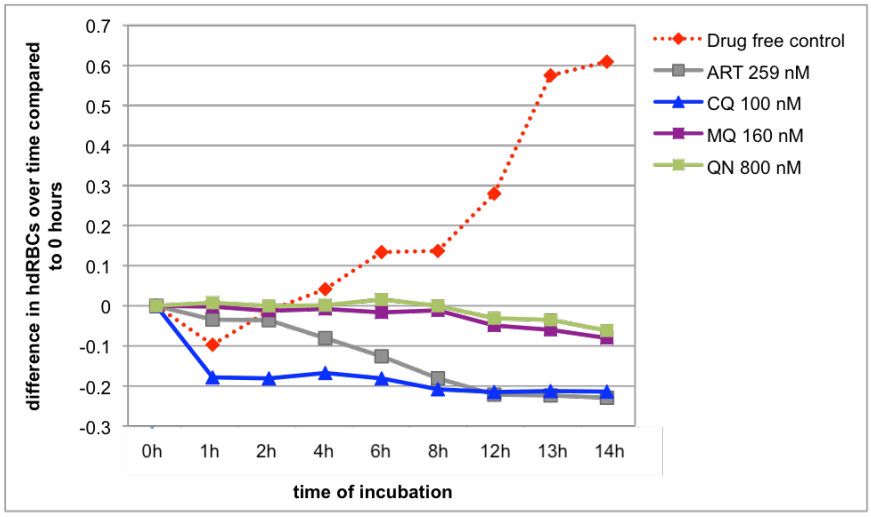

FIGURE 30: COMPARING THE EFFECT OF DIFFERENT ANTIMALARIAL DRUGS. hdRBCs = highly depolarizing red blood cells; "difference over time = %hdRBCs at each time point - %of hdRBCs at 0 hours". The difference of the percentage of hdRBCs, over time, in samples treated with artemisinin and chloroquine is higher than in the mefloquine and quinine treated samples.

The effect caused by artemisinin and chloroquine was different from the effect of quinine and mefloquine. Interestingly, in the case of artemisinin a decrease in the first 6 hours of incubation was also detected, similar to what was seen in chloroquine but less accentuated. On the other hand, mefloquine and quinine showed no significant fold differences during the first 8 hours of incubation.

8. GENERAL CONSIDERATIONS

Several experiments were conducted in order to optimize the protocol of the sensitivity assay. Results from experiments testing chloroquine, quinine, mefloquine were confirmed in several other experiments where different time points were analysed. Blood from infected mice with different parasitemias (ranging from 2% to 6%) gave similar results. In other words the time curve pattern in the several experiments was always similar. Generally, in the drug free control, the curve showed an increase in the first 12 hours, reaching a peak in between 11 and 13 hours of incubation. The peak was followed by a decrease. After 24 hours of incubation the percentage of hdRBCs was equivalent to the percentage initially observed, at time zero.

In samples treated with chloroquine the early decrease already at 1 or 2 hours of incubation was always detected.

Samples treated with quinine, as well as the ones treated with mefloquine, also showed the same pattern. A constant percentage of hdRBCs until 8 - 14 hours when it started to decrease.

Artemisinin and pyrimethamine were tested only once.

V - DISCUSSION

Currently there are different available sensitivity assays for *Plasmodium falciparum.*[11,40,45,56] These assays are well suited to provide guidelines for public health policies and indispensable tools in drug development and epidemiology of drug resistance. The WHO microtest and the isotopic assay have been used for more than two decade and have proven their reliability.[46]

However, these different assays are associated with some drawbacks: subjectivity of the results (WHO microtest), handling of radioactive material (Isotopic assay), and the majority of them are associated with long turn around-times (48 h - 96 h).[4,46,56] Recently, it has been described that the reliability of some of these assays might be linked with the mode of action of the drug.[77] These limitations contribute to the continuous effort to develop new and more rapid sensitivity assays.

An alternative sensitivity assay may be based on the detection of hemozoin (Hz). In fact, in the 80s a simple visual agglutination test was developed for the detection of Hz. At the end of the 48 hours of incubation time, the amount of dark-pigmented precipitate (Hz) was assessed.[57] However this assay did not seem to be very accurate, due to the leukocytes interference upon the precipitate formation.[4]

However, a reliable measurement of Hz might allow to assess the inhibitory effect of antimalarial drugs. Flow cytometry has proven to be able to detect and to reliably measure leukocytes containing Hz.[22,23,25,34,63] The same may apply to the detection of infected red blood cells (iRBCs) containing Hz and, thus it might become a rapid sensitivity assay, without need for reagents.

1. FLOW CYTOMETRIC DETECTION OF HEMOZOIN

In 1987, Grooth *et al.* described a new parameter in flow cytometry: polarization of light scattering.[19] Hemozoin (Hz) is a birefringent crystal and the resulting depolarization can be detected by flow cytometry.[42]

Detection of depolarizing events is based on the side scattered depolarizing light (depolarized SSC). Side scatter (SSC) detection is an indicator of cell granularity.[64] As suggested by H.M. Shapiro (personal communication), it is very likely that depolarized SSC might be also influenced by cell granularity. In other words, if two infected red blood cells (iRBCs) contain the same amount of Hz, one in form of a large aggregated of Hz crystals and, the other in form of several dispersed Hz crystals, then the one with the dispersed crystals may possibly give a stronger depolarized SSC signal.

2. DETECTION OF INFECTED RED BLOOD CELLS CONTAINING HEMOZOIN

During the 80's, due to the birefringence of malaria pigment, it was predicted that it could be used to automate the detection of malaria.[36] In 2002, Scott *et al.* showed that by full blood counts analysis in the Cell-Dyn hemoanalyser it was possible to automated detect hemozoin (Hz) within leucocytes.[63] Later on, this approach led to several publications showing its applicability as a screening test for malaria.[22,23,25,34] In some of these studies a distinct cluster population of depolarizing events with a small size signature was described, which was thought to be Hz containing-infected red blood cells (iRBCs).[22,34]

In fact, it was confirmed that iRBCs with different amounts of Hz do depolarize. By flow cytometric analysis of infected whole blood samples 98% of the depolarizing events were iRBCs, as confirmed by Ter119 and SYBR® green I staining (Figures 14 and 15).

3. DETECTION OF PARASITE MATURATION

In vivo Plasmodium berghei asexual life cycle (blood stage) lasts for 22-23 hours.[32] The parasite enters schizogony approximately after 16 hours.[9] *In vitro P. berghei* is not able to rupture iRBCs and, as a consequence, it cannot re-invade RBCs.[32] Meaning that only one generation of parasites are present in the red blood cell culture.

During maturation Hz is produced in form of dispersed crystals. When the parasites enter schizogony these dispersed crystals begin to aggregate, forming a mass, which is present in the mature schizont (Figure 2). Therefore, the mature schizont has the highest amount of hemozoin.[7] Because the depolarizing red blood cells represent iRBCs, it was likely that the schizonts would have the higher depolarized side scatter signal than the younger forms.

Having in mind that: (i) only one generation is being analysed and that the (ii) parasite mature form (schizont) has the highest amount of hemozoin, it was expected that in the untreated infected blood samples (drug free control) the percentage of highly depolarizing RBCs (hdRBCs) would increase over time. Actually, this increase could happen in two possible ways: the percentage of hdRBCs would increase reaching a maximum value and remaining constant after that. Meaning that all the parasites present in the red blood cell culture would reach the mature form of a schizont. Or, although less likely, the percentage of hdRBCs would increase constantly over time, meaning that parasites

would keep on maturing over 22-23 hours. In this case, parasites present in the collected blood must be very young forms, which was not the case as confirmed by microscopic examination of Giemsa stained blood smears.

As expected, in the first 12 hours of incubation the percentage of hdRBCs, in the drug free control sample, increased over time. Surprisingly, after 12-14 hours of incubation the percentage hdRBCs started to decrease (Figure 17 red line) (this decrease will be denominated further on as “late decrease”). Several possible explanations were identified and are discussed below (section 4).

4. LATE DECREASE OF HIGHLY DEPOLARIZING RBCS IN THE DRUG FREE CONTROL SAMLPES

1) The RBC population could start lysing after 12 hours of *in vitro* culture.

The absolute counts obtained from the whole RBC population did not decrease over the 24 hours (Figure 27 red line), suggesting that there was no RBC lysis.

2) Infected RBCs could start lysing.

Absolute counts obtained from the total depolarizing iRBCs showed a decrease after 18 hours of incubation (Figure 28b red line), supporting this explanation. The fact that no decrease was observed in the whole RBC population might be explained by considering that the hdRBC population only represents 1% of whole RBC population. Thus, changes detectable in the absolute counts in the hdRBCs may not be noticed in the whole RBC population.

3) Aggregation of Hz crystals.

The aggregation of fine particles of Hz into a single, dense, rounded mass occurs when the parasite enters schizogony[7], as shown in Figure 31. These larger aggregates of Hz crystals might produce a weaker depolarized side scatter signal (depolarized SSC), as explained above (section 1.) As a consequence, iRBCs with schizonts would not be present in the hdRBC population, thus the percentage of hdRBCs would decrease.

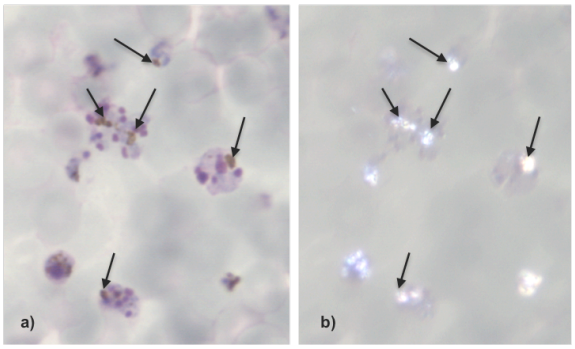

FIGURE 31: HEMOZOIN (HZ) AGGREGATES INSIDE SCHIZONTS. a) Bright-field microscopy. b) Polarization microscopy. Red blood cells infected with mature parasite (schizont). Hz crystals aggregates detected inside infected red blood cells (arrows). Giemsa stained blood smears from the drug free control after 24 hours of incubation.

Concluding, this late decrease might be explained by combining the last two explanations mentioned above. The decrease in the percentage of hdRBCs observed between 12 and 18 hours of incubation might be due to schizogony associated Hz-aggregation, because no decrease in the absolute counts from the total depolarizing RBC population was detected (Figure 28b red line). On the other hand, after 18 hours of incubation a decrease in the absolute counts of total depolarizing RBC population

was detected (Figure 28b red line). Thus, the decrease on the percentage of hdRBCs observed after 18 hours of incubation is very likely to be a consequence of iRBCs lysis.

5. LATE DECREASE OF HIGHLY DEPOLARIZING RBCS ALSO PRESENT IN DRUG TREATED SAMPLES

The late decrease of the percentage of hdRBCs, after 12-14 hour of incubation, could also be detected in samples treated with chloroquine, quinine, mefloquine, and with low concentrations of artemisinin (Figures 17, 18, 19 and 20).

Because cell maturation is inhibited by the drugs, schizogony associated Hz aggregation is an unlikely explanation.

It was thought that as a consequence of parasite death, caused by the presence of the drug, iRBCs might start lysing. Absolute counts obtained from the hdRBCs and the total depolarizing RBC populations showed a decrease already after 12 hours of incubation (Figures 28a and 28b, blue and green lines). Once again and as stated above, these changes in the absolute counts of the hdRBC population were not detected in the whole RBC population (section 4).

6. THE EFFECT OF ANTIMALARIAL DRUGS THAT INTERFERE WITH HEMOZOIN ON HDRBCS

Quinine (QN), mefloquine (MQ) and chloroquine (CQ) were described as drugs that interfere with hemozoin production.[75,76,52] In this case, one would expect that a sensitive strain in the presence of drug would stop Hz production. Consequently, the percentage of hdRBCs would not increase over time.

Samples treated with QN and MQ showed a constant percentage of hdRBCs in the first 8 hours, as expected (Figures 17, 18 and 19). Contrary to this, samples treated with CQ showed an unexpected marked decrease in the percentage of hdRBCs, already at 1-2 hours of incubation (Figures 17 and 23, blue lines).

7. CHLOROQUINE - EARLY DECREASE OF THE PERCENTAGE OF HDRBCS

Possible explanations for the early decrease of the percentage of hdRBCs observed in the CQ treated samples, include: 1) RBCs lysis; 2) dissolution of already formed Hz; and 3) aggregation of Hz.

RBCs lysis is an unlikely explanation since the absolute counts from the total depolarizing RBCs population did not decrease.

When discussing the molecular mechanism of CQ's mode of action, Sullivan mentioned that it had been noticed previously, by others, that *in vitro* CQ did not dissolve Hz.[69] If dissolution of Hz was the explanation, a decrease on the absolute counts of the total depolarizing RBC population would be expected. Still no decrease was observed during the first hours of incubation and this explanation was excluded.

Interestingly, it was found that the absolute counts of the hdRBC population decreased (Figure 28a, blue line) while the absolute counts form the total depolarizing RBC population did not (Figure 28b, blue line). Meaning that the iRBCs started to have a lower depolarized-SSC signal in the first 6 hours of incubation. These iRBCs remained in the total depolarizing RBC population but were not present in the hdRBC population anymore.

It seems that the reason for this early decrease on the percentage of hdRBCs detected in CQ-treated samples might be explained by the same principle as the late decrease observed in the drug free control, where Hz aggregation was implicated. In fact, a phenomenon denominated as CQ-induced clumping of Hz has been described. In the presence of CQ, Hz crystals start to aggregate into clumps (Figure 32). [75] Once again RBCs containing Hz clumps are likely to have lower depolarized-SSC, as described in section 1.

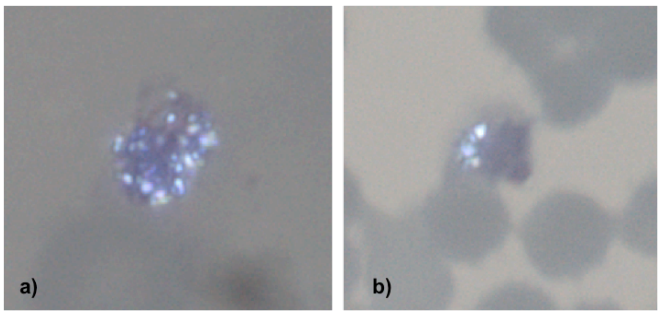

FIGURE 32: CHLOROQUINE-INDUCED HEMOZOIN CLUMPING. Giemsa stained smears from the drug free control (a), and from the chloroquine treated samples (b), after 3 hours of incubation. Note that in a) approximately 20 dispersed granules of Hz could be detected, whereas in b) only 4 bigger and less dispersed granules were observed.

It has been previously described that QN inhibits CQ-induced clumping of Hz in *Plasmodium berghei in vitro*.[76] Several experiments pre-treating the infected sample with QN were conducted. It was expected that, once inhibiting the clumping phenomenon the time curve of the sample pre-treated with QN would resemble the one from samples treated with QN or MQ, where no changes on the percentage of hdRBCs could be detected in the first hours of incubation. Instead the time curve of the sample pre-treated with QN was very similar to the one treated only with CQ (Figure 25).

CQ-induced clumping can be inhibited in two different ways by: 1) competitive inhibition or 2) non-competitive inhibition.[7]

Quinine will competitively inhibit the CQ-induced pigment clumping.[7] Meaning that these drugs (QN and CQ) interact with the same receptor site as chloroquine. In the competitive inhibition what has been described is that a certain QN concentration will inhibit 50% of CQ-induced clumping, and by increasing CQ concentration the pigment clumping can be restored.[7] The results from Figure 25 showed that samples treated with CQ at 50 nM had a 2-fold decrease between time zero and the first hour of incubation on the percentage of hdRBCs. This decrease was inhibited by half when the sample was pre-treated with QN at 400 nM. Still, from the first hour of incubation onwards it appears that the QN concentration tested was not sufficient to inhibit the pigment clumping. Because the constant decrease over the 24-hour period detected in the sample treated with CQ at 50 nM is the same as the sample pre-treated with QN at 400 nM.

Concluding, early decrease on the percentage of hdRBCs observed in samples treated with CQ seems to be a consequence of CQ-induced clumping.

8. ARTEMISININ EFFECT ON HDRBCS

Samples were treated with the same concentrations of artemisinin routinely used in *in vitro* sensitivity assays for *Plasmodium falciparum* (0.5 nM, 4 nM and 16 nM), as described in Basco 2007.[4] Samples

treated with the mentioned concentration showed no differences in the percentage of hdRBCs when compared with the drug free control (Figure 20). It was somehow intriguing that the concentrations recommended for *P. falciparum* did not seem to affect *P. berghei*. Cooper *et al.* showed that CQ-resistant *P. falciparum* lines showed a significantly greater sensitivity to artimisinin when compared with CQ-sensitive parasites.[8] In fact, it has been described that parasites resistant to CQ can display hypersensitivity to other antimalarials.[72] The same may apply to *P. berghei*, explaining the reason why no effect could be seen, when using lower artemisinin concentrations.

When higher concentrations of artemisinin were tested, already after 2 hours of incubation, an effect was detected but only in the samples treated with 128 nM and 259 nM (Figure 21). This effect was exclusively present samples treated with artemisinin.

Artemisinin mode of action is still unresolved and remains a subject of controversial debate.[48] Evaluating the artemisinin effect by analysing the percentage of hdRBCs, two hypotheses can be formulated: 1) artemisinin, like CQ, induces Hz clumping, which has never been described before; or 2) artemisinin cause iRBCs lysis. In fact, both explanations may be valid, since it has already been proposed that artemisinin might interfere with Hz production.[55] Thus it might have an effect on Hz itself that could be detected by this assay. Moreover, it has also been described that artemisinin initiates oxidation reactions that damage parasite membranes[26], which may lead to iRBCs lysis. Interestingly, the obtained results might give some clues about artemisinin and related compounds mode of action. No absolute counts were obtained during this experiment, thus no conclusions regarding this hypothesis could be drawn. As future work, besides acquiring absolute counts during the experiment, it would also be of interest to try and pre-treat the sample with a clumping inhibitor such as quinine.

9. ARTEMISININ EFFECT ON HDRBCS AND ITS THERAPEUTIC APPLICATION

The effect of the different drugs tested on the percentage of hdRBCs over time was compared (Figure 30). Interestingly, artemisinin decreased the percentage of hdRBCs at 6 hours of incubation and only in a four hour interval (between 6 and 10 hours) this percentage decreased by half.

This finding suggests that, from the different tested drugs, artemisinin seems to be the one that might have a more rapid effect on the parasite. In fact, artemisinin has been described to be the antimalarial compound that reduces parasitemia more rapidly[80], being considered a highly active antimalarial agent. This finding also correlates with artemisinin therapeutic usage. Although parasitemia might not correlate with disease severity[80], patients with hyperparasitemia (≥ 5% parasitemia) are diagnosed as a severe malaria case. [83] Meaning that the use of a rapid effective drug might be crucial for the patient outcome. This justifies why artemisinin and its relative compounds are the drugs of choice for the treatment of severe malaria.[85]

10. PYRIMETHAMINE EFFECT ON HDRBCS

Pyrimethamine is an antimalarial drug that does not directly interfere with Hz production. Its mode of action is associated with blocking DNA synthesis.[50]

The concentrations of pyrimethamine described for field *in vitro* sensitivity assays range from 0.05 nM to 51200 nM.[4] Samples treated with pyrimethamine at 3.1 nM, 800 nM showed no differences in the

percentage of hdRBCs, when compared with the drug free control (Figure 22). Contrary to this, samples treated with pyrimethamine at 51200 nM started to show an effect at 13 hours of incubation (Figure 22, black line).

Pyrimethamine main action is on the late trophozoite and schizont parasites, which are synthesizing DNA.[50] Thus, an effect over this 24-hour incubation period was not expected.

Plasmodium falciparum asexual life cycle lasts for 48 hours.[21] When testing this drug using SYBR® green I and by the pLDH (parasite lactate dehydrogenase) assays with an incubation period of 48 hours, erroneous IC50 values (concentration required to inhibit the parasite viability by 50%) were obtained.[77] On the other hand, the IC50 value was correct when the incubation period was extended for another 24 hours (72-hour incubation).[77] Meaning that the drug effect could only be detected in the second generation of the parasites.

The parasite strain used during this project, *Plasmodium berghei,* cannot re-infect RBCs. Re-infection of RBCs only occurs when schizonts rupture and release merozoites. Schizonts of *P. berghei* do not rupture spontaneously[32], therefore no re-infection will occur. Thus, the obtained results refer only to one generation of parasites. Justifying why no effect on the percentage of hdRBCs could be detected. This limits the applicability of this assay for testing drugs, which act in later stages of the parasite development, using this experimental murine model.

However, this will not be a limitation when using *Plasmodium falciparum* culture. In fact, *P. falciparum* is the only species for which all life cycle stages have been established in culture.[62]

11. ASSESSMENT OF ANTIMALARIAL DRUGS INHIBITORY EFFECT BY GFP ANALYSIS

Parasite strains expressing GFP have been tested for antimalarial drug screening. This approach was considered to be a sensitive tool.[60,61] It was successfully applied to *Plasmodium falciparum*, but a 72-hour incubation period was required.[61]

In the majority of the experiments conducted in this project, strains expressing GFP were used to compare the results obtained from both approaches (detection of hdRBCs and detection of GFP positive iRBCs). In all cases, drug effects could be detected much earlier by analysing the percentage of hdRBCs than by the analysis of GFP positive iRBCs. Moreover, fold differences between the drug treated samples and the drug free control were more evident when analyzing the percentage of hdRBCs (Table 6).

TABLE 6: COMPARISON OF THE GFP POSITIVE IRBCS ANALYSIS WITH THE ANALYSIS OF HDRBCS

Mean used to measure drug effect	Antimalarial drug tested	Earliest time when the drug effect was detected*	Highest fold difference observed during incubation**
GFP positive iRBCS	CQ 100 nM	6 hours	1.2 (at 10 hours)
	QN 3200 nM	11 hours	1.2 (at 14 hours)
	MQ 160 nM	11hours	1.2 (at 14 hours)
hdRBCs	CQ 100 nM	1-2 hours	5.5 (at 13 hours)
	QN 3200 nM	2 hours	2.9 (at 11 hours)
	MQ 160 nM	2 hours	3 (at 13 hours)

iRBCs = infected red blood cells; hdRBCs = highly depolarizing red blood cells; CQ = chloroquine; MQ = mefloquine; QN = quinine.

* Time of incubation when the drug effect could be detected for the first time.

** Fold difference between the percentage of hdRBCs or the percentage of GFP positive iRBCs compared the drug free control. The values represent the highest fold difference over the 24 hours.

12. POSSIBLE LIMITATIONS OF THE ASSAY AND GENERAL CONSIDERATIONS

There are several limitations associated with this sensitivity assay.

***Plasmodium berghei* does not re-infect RBCs**. Schizonts of *P. berghei* do not rupture spontaneously[32], therefore no re-infection occurs. Moreover this parasite strain has strong preference for immature RBCs[9], which are not abundant in the red blood cell culture. Combining this two parasite features it becomes difficult to establish a continuous culture of RBCs infected with *P. berghei*.

However, this will not be a limitation when using *Plasmodium falciparum* culture.

No reference method was used as a mean of comparison the antimalarial drug effect. The obtained results should be validated by another sensitivity assay. A major obstacle is that the different available sensitivity assays require longer incubation periods, minimum of 30 hours. Thus, the inhibitory drug effects detected at earlier time points by this alternative method, might not be detected by the other currently used methods. Still, the efficacy of each concentrations of the antimalarial drugs tested should be assessed also by another method, such as the [^{3}H]hypoxanthine incorporation which is pointed as the *gold standard* method.[1]

Parasite sensitivity to the tested antimalarial drugs was not confirmed. With the exception of chloroquine, which successfully treated mice infected with the *Plasmodium berghei* ANKA, the parasite sensitivity to the other tested drugs was not confirmed. However, the parasite strains used were described as being sensitive to chloroquine, quinine, mefloquine and artemisinin.[52,54,60] No published data regarding the *Plasmodium berghei* sensitivity to pyrimethamine was found.

Only samples from infected mice with parasitemias ranging 2% to 6% were tested. The ultimate goal of this project is to apply sensitivity assay to malaria infection in humans. A patient may have severe malaria and still the peripheral parasitemia may be as low as 0.02%.[80] This is not an uncommon scenario in malaria endemic countries. Thus, high parasitemias as the ones tested (2% - 6%) are not frequent under field-conditions. Therefore, it is important to test blood from infected mice with lower parasitemias in order to determine the limit of detection of the assay.

The minimum parasitemia required might be a limitation for some *in vitro* sensitivity assays. To obtain reliable results peripheral blood parasitemias should be higher than 0.5% (SYBR® green I and [3H] hypoxanthine[74,77]) or 1 - 2% (pLDH assay[46]).

VI – CONCLUSION

The flow cytometric detection of infected red blood cells containing hemozoin allowed to assess the inhibitory effect of several antimalarial drugs, in a short time period. Already after 4 hours it was possible to detect the inhibitory effect of the majority of the antimalarial drugs tested. Moreover the methodology is easy to establish and to perform. Certainly, the use of an expensive flow cytometer limits this method. Yet the prospect exists that cheaper instruments, like image cytometers[65] could be used instead.

Having in mind that the final goal of this project is to develop a sensitivity assay for *Plasmodium* spp. responsible for human malaria, a continuous *Plasmodium falciparum* culture has already been set up. Later on it would be of interest to set up also cultures of sensitive and resistant *Plasmodium falciparum* strains, which would be used to optimize the assay. Eventually it would also be important to test specimens collected in the field.

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