

UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA VEGETAL



NOVEL APPLICATIONS OF A FLOW CYTOMETRIC
SENSITIVITY ASSAY FOR *PLASMODIUM* SPP.:
COMPOUND SCREENING AND GAMETOCYTE
DETECTION

Carolina Isabel Glória Tempera

DISSERTAÇÃO

MESTRADO EM MICROBIOLOGIA APLICADA

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Abstract

Malaria is caused by a parasite of the genus *Plasmodium* and remains the most important parasitic disease. The emergence of *Plasmodium falciparum* parasites resistant to all known antimalarial drugs is of major concern. New antimalarial drugs are needed, not only a drug that overcome the undesirable side effects of current antimalarial drugs but new highly active ones against the asexual stages, as well as, drugs that could also eliminate the transmissible sexual form of the parasite to the mosquito.

To test the susceptibility of the parasite to drugs a variety of sensitivity assays can be used to screen new compounds, such as: hypoxanthine incorporation, ELISA based assays like pLDH and HRP2, fluorometric and flow cytometric assays. Recently, a novel flow cytometric sensitivity assay based on hemozoin detection was described. Using this novel sensitivity assay new antimalarial compounds were screened at 1 and 3 μM . The SYBR green DNA staining assay and the HRP2 were also performed as mean of comparison. Results showed that none of the tested compounds presented inhibitory activity against *P. falciparum* strains 3D7 and Dd2 at 1 μM , independently of the method used. Only one of the compounds showed more than 50% inhibition at 3 μM .

The flow cytometric hemozoin detection method was also assessed for its potential to detect gametocytes. Gametocyte may have a different depolarizing profile, based on the underlying hemozoin distribution. Thus, we further investigated if they could be distinguished from other parasitic forms based on their higher degree of depolarization. A culture enriched in gametocytes was FACS sorted by selecting the higher depolarizing population. Results showed that gametocytes were selectively present only in the high depolarizing population and not in the middle and non-depolarizing events.

Therefore, this recently described sensitivity assay based on hemozoin detection can be used as a novel approach to screen for new antimalarial drugs. This approach has as major advantages the fact that results can be obtained in only 24 h of incubation and no additional reagents or additional incubation times are required. Another important characteristic of this method is that it might be able to detect gametocytes based on the particular hemozoin distribution in these forms, which can lead to the use of this method to test antimalarial transmission blocking drugs.

Key-words: Malaria, antimalarial drugs, antimalarial drug resistance, *in vitro* sensitivity assays, hemozoina, flow cytometry.

Resumo

A malária é uma doença que segundo os mais recentes dados da Organização Mundial de Saúde (OMS) foi responsável por cerca de 660 000 mortes e 219 milhões de casos em 2010. É a doença parasitária que mais mortes causa sendo a região mais afetada por esta doença a África subsariana, e com uma maior incidência em crianças até aos 5 anos de idade.

A malária é causada pelo parasita do género *Plasmodium*. As cinco espécies que podem causar doença no humano são: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* e *P. knowlesi*. No entanto *P. falciparum* é a espécie responsável pelo maior número de casos e mortes por malária sendo também o que pode levar a uma maior severidade da doença. A malária manifesta-se por períodos de febres altas e calafrios, mal-estar generalizado, torpor e dor de cabeça, náusea e dor abdominal, por vezes até vómitos e diarreia. No caso de doença severa esta implica anemia severa, malária cerebral, síndrome de dificuldade respiratória aguda, insuficiência renal e nos casos mais graves a morte.

Segundo estes factos, a malária é uma doença que levanta preocupação e tem impacto a nível mundial. Isto porque, por várias circunstâncias se pensou que estaríamos no caminho da erradicação da doença e no entanto, apesar de o número de casos e mortes ter diminuído na última década, esta continua a afetar milhões de indivíduos. Hoje em dia, um dos maiores problemas face a esta doença prende-se com o facto de *P. falciparum* já ter apresentado resistência a todos os fármacos utilizados no tratamento da doença. Entre os antimaláricos já usados encontram-se a quinina, a cloroquina, a primaquina, a pirimetamina conjugada com a sulfadoxina, a mefloquina entre outros. No entanto, as atuais directrizes para o tratamento da malária correspondem a terapias de combinação com recurso a derivados da artemisinina (“Artemisinin-based combination therapies” – ACTs). Alguns exemplos destas combinações são: artesunato combinado com amodiaquina, artesunato com mefloquina, arteméter e lumefantrina, entre outros.

O principal objectivo destas terapias é eliminar as formas do parasita que circulam no sangue, pois é este estadio que conduz à manifestação da doença. *Plasmodium* é um parasita que apresenta um ciclo de vida complexo. É transmitido ao humano através da picada de um mosquito infectado fêmea do género *Anopheles*. Durante a picada, o parasita que existe nas glândulas salivares do mosquito é injetado na corrente sanguínea do humano, e dirige-se até ao fígado. No fígado vários parasitas vão invadir os hepatócitos, dentro dos quais maturam e replicam antes de serem libertados novamente na corrente sanguínea. Esta fase hepática é assintomática. Uma segunda vez na corrente sanguínea, os parasitas vão invadir eritrócitos dentro dos quais vão maturar e replicar antes de lisarem o eritrócito, libertando novos parasitas para invadir novos eritrócitos. Uma vez dentro de um eritrócito, um parasita segue um ciclo de maturação que compreende as seguintes formas: forma de anel, trofozoíto, e esquizonte. No final, irá libertar vários merozoítos. No entanto, ocasionalmente alguns destes merozoítos, quando invadem um eritrócito vão originar formas sexuadas do parasita, os gametócitos. Os gametócitos apresentam-se sob a forma de percursores masculinos e femininos, e são estas formas que quando em

circulação podem ser ingeridas por um mosquito durante uma nova picada. Após ingeridas pelo mosquito, estas formas vão recombinar geneticamente, dando origem a uma nova descendência que será novamente transmitida ao humano, recomeçando este ciclo.

Colocam-se assim dois grandes objectivos à erradicação da malária: (i) um passa pelo desenvolvimento de novos agentes antimaláricos, devido à evidência de resistência de *P. falciparum* a todos os actuais antimaláricos, incluindo os derivados da artemisinina que compreendem as atuais diretrizes de tratamento da doença; (ii) o outro prende-se com a eliminação das formas sexuadas do parasita em circulação no hospedeiro, pois eliminando estas formas, quebra-se o ciclo de transmissão hospedeiro-vector.

Para a contínua pesquisa de novos compostos antimaláricos é necessário ensaios que detectem a sensibilidade dos parasitas aos diferentes fármacos. Os ensaios de sensibilidade actualmente existentes são: (i) o teste de microscopia da OMS; (ii) o teste por incorporação de hipoxantina; os testes baseados na detecção de anticorpos (Enzyme-Linked Immunosorbent Assay – ELISA) para quantificação de proteínas do parasita como a (iii) lactase desidrogenase (Parasite lactate dehydrogenase – pLDH e a (iv) proteína rica em histidina 2 (Histidine Rich Protein - HRP2); testes baseados na detecção do DNA do parasita por (v) ensaios fluorométricos e por (vi) citometria de fluxo.

No entanto, como até agora não existe o teste de sensibilidade ideal, e devido a várias limitações de cada ensaio, novos ensaios são desenvolvidos. Um dos mais recentes baseia-se na detecção de hemozoína por citometria de fluxo. A hemozoína, também denominada pigmento malárico, é um bioproducto que resulta da destoxificação de heme livre produzido após a metabolização da hemoglobolina pelo parasita, e acumula-se no interior do eritrócito durante a maturação do mesmo. A hemozoína é um cristal com propriedades birefringentes que levam à despolarização da luz. Devido a esta propriedade, foi desenvolvido um ensaio de citometria de fluxo que detecta a hemozoína presente no interior de eritrócitos infectados. Assim, com o acumular de hemozoína ao longo do tempo, a detecção dos parasitas vai aumentando, havendo um maior número de eventos a despolarizar entre as 24h-30h de um primeiro ciclo de maturação, altura em que a maioria dos parasitas se apresenta como esquizonte, o estadio que também tem maior quantidade de hemozoína. Este ensaio permitiu detectar a sensibilidade de *P. falciparum* a vários antimaláricos e foi agora utilizado para testar a sensibilidade do parasita a novos antimaláricos cedidos pelo grupo do Professor Doutor Rui Moreira da Faculdade de Farmácia.

A pesquisa da actividade dos novos compostos (“screening”) a 1 μ M e a 3 μ M foi realizada em duas estirpes de laboratório de *P. falciparum*, a 3D7 (sensível à cloroquina) e a Dd2 (resistente à cloroquina). Após incubar a estirpe Dd2 em presença dos vários compostos, a sua inibição foi avaliada por citometria de fluxo, utilizando o ensaio da detecção de hemozoína e pelo ensaio da detecção de parasitas cujo DNA foi corado com SYBR green. Os resultados de ambos os ensaios demonstraram que nenhum dos 18 compostos testados a uma concentração de 1 μ M levou à inibição do parasita. Com a estirpe 3D7 foram testados 24 novos compostos, não só utilizando os dois métodos de citometria de fluxo já referidos como também através de HRP2-ELISA. Os resultados demonstraram que independentemente do ensaio utilizado nenhum dos novos

compostos apresentou actividade inibitória a 1 μM . Porém os compostos foram testados a uma concentração mais elevada, e a 3 μM um dos compostos demonstrou actividade inibitória (o composto 321). Determinou-se a concentração à qual este composto inibe 50 % do crescimento do parasita (CI_{50}) de acordo com os três ensaios para detectar a sensibilidade do parasita obtendo-se um valor à cerca de 2 μM . No entanto, esta concentração é demasiado alta para o composto poder ser posteriormente testado como agente antimalárico na fase sanguínea do parasita, pois os actuais antimaláricos actuam na ordem dos nanomolar (nM).

Para erradicar a malária, a eliminação de gametócitos é um dos principais objectivos, de modo a quebrar o ciclo de transmissão da doença. Porém, existem muito poucos tratamentos que inibam estas formas sexuadas, e por isso é necessário continuar a investigar novos fármacos que actuem com mais impacto neste estadio do parasita. Para tal, uma vez mais são necessários ensaios que possam averiguar a sensibilidade dos gametócitos a diversos fármacos. No entanto, não existem tantos ensaios para tal, como os que existem para testar a sensibilidade dos estadios assexuados. Uma vez que os gametócitos também apresentam hemozoína no seu interior, pressupõe-se que o ensaio de sensibilidade baseado na detecção de hemozoína permitisse também detectar gametócitos, retirando partido do facto de a hemozoína acumulada nos gametócitos apresentar-se de forma diferente nos esquizontes, pois ocorre sob a forma de vários e pequenos fragmentos ao contrário de um único e grande aglomerado. Assim, colocou-se a hipótese de se detectar os gametócitos por citometria de fluxo, e que estes corresponderiam a uma diferente população de acordo com a despolarização da sua hemozoína.

Para corroborar esta ideia, estabeleceu-se a diferenciação de gametócitos a partir de culturas contínuas e posteriormente, através de análise por citometria de fluxo, os eventos da cultura de gametócitos foram separados ("sorting") com base na sua despolarização. Partindo do princípio que os gametócitos têm mais cristais, estes podem levar a uma maior quantidade de luz a depolarizar, logo iriam localizar-se num nível de despolarização mais elevado do que os esquizontes. Assim, três populações foram separadas do seguinte modo: (i) população que não despolariza; (ii) população que despolariza a um nível médio (semelhante ao nível de despolarização de esquizontes) e (iii) população a um nível mais elevado de despolarização. Após observação de cada uma das populações por microscopia, constatou-se que dos eventos adquiridos, os gametócitos só se encontravam na população cuja despolarização era a mais elevada.

Com este recente ensaio baseado na detecção de hemozoína foi então possível testar a actividade inibitória de novos compostos antimaláricos, obtendo as mesmas conclusões que outros dois métodos utilizados em paralelo. Este ensaio poderá assim ser utilizado no rastreio da actividade de novos compostos, como alternativa aos existentes actualmente, aos quais, há resistências desenvolvidas pelo parasita assim como o facto de conduzirem a efeitos secundários indesejáveis. Em relação aos outros ensaios de sensibilidade, este método apresenta-se como um método rápido de obter resultados (24h) e em tempo real, sem a necessidade de adição de reagentes ou tempos adicionais de incubação com reagentes. Este método permitiu ainda detetar uma população específica do parasita, os gametócitos, os quais representam um dos principais

alvos para a eliminação da malária. Assim, este método poderá também ser investigado como ensaio de sensibilidade dirigido a gametócitos.

Palavras-chave: malária, *Plasmodium* spp, resistência a antimaláricos, testes de sensibilidade *in vitro*, hemozoína, citometria de fluxo, fármacos anti-maláricos.

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1. Introduction

1.1 Introduction to Malaria

Malaria is a parasitic blood disease that according to World Health Organization latest report caused an estimated 219 million cases of malaria and 660 000 deaths in 2010 ^[1]. It affects mostly the sub-Saharan Africa territory, and has a higher incidence in children under 5 years old ^[1].

Malaria is caused by one of the five known *Plasmodium* spp in humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* ^[2]. In general, symptoms include periodic chills and fevers, malaise, lethargy, headache, nausea, abdominal pain and sometimes vomiting and diarrhea. *P. falciparum* is the major strain that can cause severe disease such as severe anemia, cerebral malaria, pulmonary edema, acute respiratory distress syndrome and renal failure, and thus is the strain causing most deaths ^[3].

Plasmodium has a complex life cycle ^[4] which comprises an asexual reproduction cycle in the human host and a sexual reproduction cycle in the mosquito vector (Figure 1). The cycle starts when an infected female *Anopheles* mosquito bites an individual, and injects sporozoites, present in mosquito's salivary glands, into the host blood stream during its blood meal. These sporozoites migrate to the liver where they mature and multiply within hepatocytes. These forms are known as schizonts (Figure 1, point 2). This extra-erythrocytic stage is asymptomatic and usually lasts 6 days to 14 days, although sometimes it can last up to several months or even years in the case of *P. vivax* or *P. ovale*. These two human *Plasmodium* species can produce hypnozoites in the liver ^[5] (Figure 1, point 2). Hypnozoites are a dormant form of the parasite, also called cryptic form, which can stay in the liver for long periods of time and are the cause of the disease relapse.

After the liver stage, tens of thousands of merozoites will be released into the blood, where they will invade and develop within erythrocytes. The blood stage of infection includes asexual forms of the parasite that undergo repeated cycles of multiplication in erythrocytes (Figure 1 point 3), causing parasite numbers to rise rapidly. This stage is responsible for the symptoms of malaria.

Within the erythrocyte, the asexual forms of the parasite passes through different sequential maturation stages: ring, trophozoite and schizont forms (Figure 2). In the end, the erythrocyte ruptures and new merozoites are released and ready to infect new erythrocytes (Figure 1, point 3). Some parasites will develop into the sexual forms, responsible for transmission, known as gametocytes (Figure 1, point 4). The female and male gametocytes (macro-gametocyte and micro-gametocyte, respectively) will be ingested by the mosquito vector during its feeding, and sexual reproduction occurs inside the mosquito midgut before the parasite is transmitted to another human host and the whole cycle starts again ^[6].

1.2 Treatment of Malaria

Most available antimalarial drugs were designed to target the symptomatic blood stages and thus act only against the sexual blood forms ^[7]. Treatment of an individual diagnosed with *P. falciparum* malaria is of great concern because contrary to the other species, it can be rapidly fatal ^[3]. However, going forward with malaria eradication objective, medicines that block parasite

transmission and drugs which eliminate the asymptomatic cryptic hepatic forms of *P. vivax* and *ovale*, are also needed ^[8].

1.2.1 History and overview of antimalarial drugs

Quinine is one of the oldest known antimalarial drugs, and occurs naturally in the bark of cinchona trees in South America. It is an alkaloid and was first isolated in 1820 and used for many decades ^[9]. During the First World War, due to quinine stocks declining, the development of the first synthetic antimalarial was conducted. Work with synthetic dyes led to the development of the acridines and the 8-aminoquinolines, such as pamaquine (and subsequently primaquine) ^[10].

In the 1940s chloroquine, a 4-aminoquinoline, was introduced as an antimalarial chemotherapy after having been synthesized in Germany. Chloroquine was not only highly effective and well tolerated as treatment but was also the main drug of choice in the WHO Global Eradication Programme of the 1950s and 1960s ^[9].

During World War II, chloroquine was only one of many antimalarials that resulted from scientific advances. Others were developed, and some of them focused on derivatives of pyrimidine. Research in this direction resulted in the development of the antifolates like proguanil. Then, pyrimethamine was developed shortly afterwards. Proguanil and pyrimethamine were used as prophylactic and therapeutic agents, despite their slow schizontocidal action. The antifolates are dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) inhibitors that disrupt folate synthesis in the parasite ^[10]. Later it was discovered that in combination with pyrimethamine, sulfa drugs like sulfadoxine, were more effective against *Plasmodium* infection. However, after the introduction of sulfa drug-DHFR inhibitor combinations, the U.S. army developed further aryl-amino alcohol derivatives from quinine, such as mefloquine and halofantrine. More recently, atovaquone was introduced as an antimalarial drug, and is used also as prophylactic agent since inhibits not only the blood stages forms of the parasite but the liver stages as well ^[11]. It is administered in synergy with proguanil ^[12].

Most of antimalarial drugs therapeutics acts upon the asexual blood stages of parasite, like quinine, chloroquine, mefloquine and the antifolates (Table 1). Primaquine is the only that acts against liver stages parasites, more specifically only against hypnozoites, that occur in *P. vivax* and *P. ovale*. Tough, primaquine also inhibits gametocyte forms.

Overall, the blood stages antimalarial drugs interfere with parasite hemoglobin degradation and heme detoxification, or with parasite folate biosynthesis. Others drugs such as tetracyclines and clindamycin inhibit protein synthesis in the apicoplast ^{[13], [14]}.

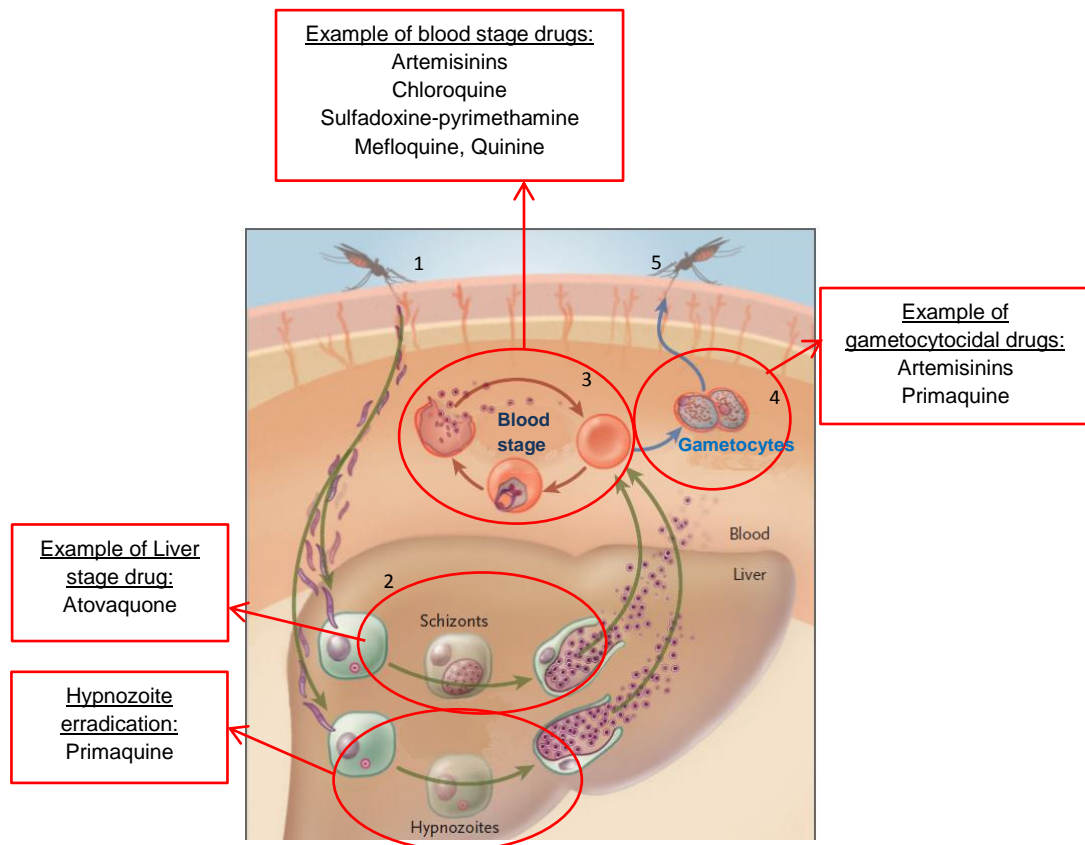


Figure 1 – *Plasmodium* life cycle and antimalarial drugs interference. *Plasmodium* life cycle from mosquito (1), through liver stage (2), blood stage (3), gametocytes (4) and these are passed again to the mosquito vector (5). Adapted from Baird, J.K. 2005 ^[7]. The antimalarial drugs can act upon liver forms, blood forms and/or gametocytes.

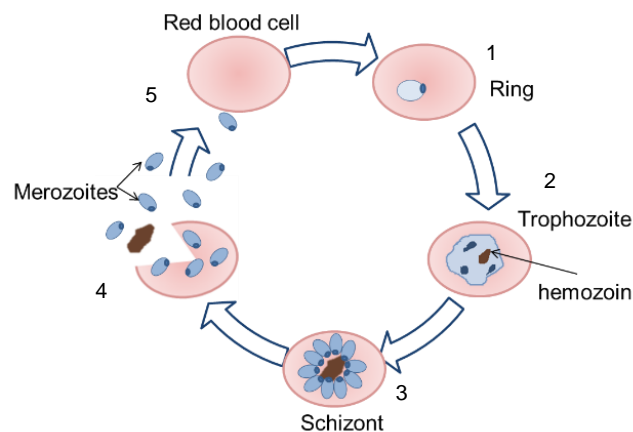


Figure 2 - *P. falciparum* intra-erythrocytic maturation cycle. Representative images of infected erythrocyte with *P. falciparum* parasite at ring stage (1), trophozoite stage (2) and schizont (3). Then the erythrocyte ruptures, freeing new merozoites as well as hemozoin (4). The new merozoites invade uninfected erythrocytes (5).

1.2.2 Current guidelines for malaria treatment

Although the guidelines may vary somewhat, especially between affluent non-endemic countries and endemic countries, the first-line treatment of uncomplicated *falciparum* malaria relies on artemisinin-based combination therapies (ACTs) ^[15]. Artemisinin comes from *Artemisia annua*

also known as qinghao, and it was long used in Chinese traditional medicine ^[16]. However, it was only in the early 1970s that its potent antimalarial activity was discovered. Artemisinin and derivatives such as artemether, artesunate and dihydroartemisinin, are associated with a high rate of recrudescence if used as monotherapy ^[17]. This is probably related with pharmacodynamic properties of these agents which include fast acting but short half-lives. Therefore, they are usually combined with longer acting antimalarials. Artemether plus lumefantrine, artesunate plus amodiaquine, artesunate plus mefloquine or/and artesunate plus sulfadoxine-pyrimethamine are examples of ACTs. Although still not clear, artemisinins seem to affect the hemoglobin catabolism during parasite maturation within the erythrocyte ^{[18], [19]}.

Artemisinin and artemisinin combination therapies also have some effectiveness at reducing gametocyte carriage ^[20], although well inferior to primaquine ^[21]. Primaquine remains the principal available drug for radical treatment to eliminate the cryptic liver stage forms of *P. vivax* or *ovale* (Figure 1, point 2). However, because of possible severe side effects like hemolytic anemia it is difficult to use in individuals with glucose-6-phosphate dehydrogenase deficiency (G6PDd) ^{[22], [23]} (Table 1).

Table 1 – Major antimalarial drugs actions

Class		Mecanisms of action	Drugs	Parasite stage
Quinolines	Arylamino-alcohols	Inhibition of haem polymerization ^[24] By π - π interactions of drug with free heme	Quinine	Blood-stage schizonticide
	Amino-Quinolones		Mefloquine	
			Chloroquine	
			Primaquine	Liver-stage schizonticide, gametocytocide
Antifolate		Dihydrofolate reductade (DHFR) and dihydropteroate synthase (DHPS) inhibitors in the folic acid synthesis pathway ^[10]	Pyrimethamine	Blood-stage
			Proguanil	
Artemisinines (sesquiterpene lactones)		Inhibition of hemozoin formation; inhibition of parasite's sarco-endoplasmic reticulum Ca2+-ATPase (SERCA); or generation of free radicals ^{[18], [25]}	Artemether	Blood-stage schizonticide, gametocytocide
			Artesunate	
			Dihydroartemisinin	
Hydroxy-napthoquine		Inhibition of electron transport ^{[11], [26]}	Atovaquone	Liver-stages Blood-stage schizonticide

Overall, there is a need for novel antimalarial drugs that overcome the limitations of the currently available antimalarials. The side effects of antimalarial drugs and their lack of activity against liver parasites and sexual stages has presented an important concern in malaria eradication.

1.2.3 Antimalarial drug resistance

Since malarial drug treatment exists, *Plasmodium* parasites have developed resistance to the most of the drugs that have been used to treat malaria ^[24]. The advent of chloroquine resistance was probably the most relevant, because chloroquine was thought to lead to malaria eradication yet, resistance appeared and spread globally ^[27]. In the late 1950s, resistance to chloroquine was noted on the Thai-Cambodian border in Colombia, than in 1980 all endemic areas in South America were affected, and by 1989 almost all Asia and Oceania. In Africa, chloroquine resistance was first documented in the east in 1978 ^[28]. Mefloquine, sulfadoxine-pyrimethamine, and atovaquone-proguanil, are antimalarial drugs to which *P. falciparum* also developed resistance shortly after their introduction (Table 2). However, the use of proguanil and pyrimethamine alone as prophylaxis had a major impact in the insurgent of parasite resistance against these drugs ^[10].

Table 2 – Dates of introduction and first reports of antimalarial drug resistance.

Antimalarials	Introduced	First reported resistance	Difference (years)
Quinine	1632	1910	278
Chloroquine	1945	1957	12
Sulfadoxine-pyrimethamine	1967	1967	0
Mefloquine	1977	1982	5
Atovaquone	1996	1996	0

(Adapted from Wongsrichanalai, C., *et al.* 2002 ^[28])

1.2.4 Artemisinin drug resistance

One of the greater concerns in malaria control is associated with the development of drug resistance, mainly because resistance of *P. falciparum* to almost all antimalarial drugs has been described, including to the first line treatment with artemisinin ^{[29], [30]}.

Resistance to artemisinin is characterized by slow parasite clearance *in vivo* ^[31] (Figure 3) with no corresponding reductions of susceptibility detected in *in vitro* ^[32].

Commonly, for other antimicrobial agents, resistance is defined by clinical failures and decreased susceptibility *in vitro* ^[32]. However, for the current definition of “artemisinin resistance”, based on delayed clearance of parasites, neither criterion is fulfilled. However, it is still an issue of big concern ^[33].

In this scenario, it is of great importance to continue the search for new antimalarial drugs, as well as the continuous surveillance of *P. falciparum* resistance, especially in endemic countries.

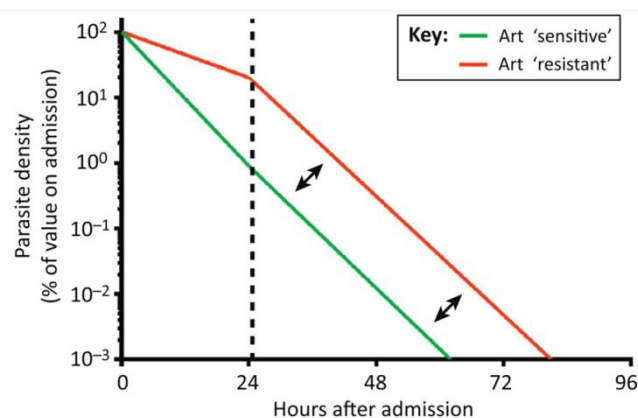


Figure 3 - Parasite clearance in artemisinin sensitive and resistant parasite populations. Parasite clearance profile of artemisinin resistant and sensitive parasite populations. A broken line at 24h shows a shift in clearance between populations, and arrows represent an identical slope thereafter. Abbreviations: Art = artemisinin (From: Ferreira, P. E., *et al.* 2013) ^[32].

1.3 Antimalarial drug research

In the antimalarial drug research area, different approaches to study *P. falciparum* drug sensitivity have been developed. There are *in vivo* tests, originally defined by the World Health Organization (WHO) in terms of parasite clearance, which comprise the follow classification: sensitive [S] or one of the three degrees of resistance [RI, RII, RIII] ^[34]. Later a modified protocol was introduced based on the clinical outcome with the following classifications: adequate clinical response, early treatment failure, and late treatment failure (Table 3). There are also phenotypic *in vitro* tests that take advantage of the possibility of continuous culture of the parasite (developed in the 1970s) to perform the assays ^[35]. Although molecular *in vitro* tests also exist, the correlation between genetic changes and resistance is not well known yet for most antimalarial drugs.

All *in vitro* tests share the same principle: comparison of cultures incubated with different concentrations of the tested compound to a drug free control, yet, they differ considerably in the methods used to assess parasite growth or viability.

The main phenotypic *in vitro* drug sensitivity assays include ^[36] (Table 4):

- Light microscopy methods like the WHO microtest to assess parasite maturation. It requires little technical equipment, can be used for samples with low parasite densities, and usually requires only 24 hours of incubation. On the other hand, its biggest disadvantage is the fact that it is labour-intensive, subjective (inter-operator variation) and requires highly trained personal.
- Isotope incorporation assays, such as the [³H]-hypoxanthine incorporation (tritium-labeled hypoxanthine) ^[37]. It allows a high degree of automatization and therefore is faster to perform than the tests based on the morphological assessment of parasite growth. On the negative side, it only measures metabolic activity over the second half of the parasite life cycle and implies work with isotopic/radioactive material. It also requires high purchase cost for infrastructure, equipment and thus is very badly suited for field studies.

Table 3 – Classifications of *in vivo* antimalarial sensitivity test outcomes according to the original and modified protocol

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)	
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.
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.

(adapted from Wongsrichanalai *et al.* 2002 ^[28])

- Antigen detection of parasite lactate dehydrogenase (pLDH) or histidine-rich protein (HRP2), produced by malaria parasites during their growth and multiplication, using an Enzyme-Linked Immunosorbent Assay (ELISA).

➤ Parasite lactate dehydrogenase (pLDH) is a terminal enzyme in the Embden-Meyerhof pathway (glycolysis) of the malaria parasite ^[38]. Its production and accumulation can be used both for malaria diagnosis *ex vivo* and in *in vitro* *P. falciparum* cultures as indices of the presence and viability of the parasite. As a drawback, this assay requires monoclonal antibodies (mAbs) specific for pLDH, which are expensive, have limited supplies and lack an optimal specificity.



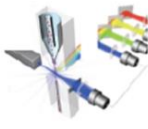
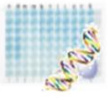
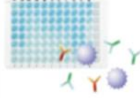
➤ Histidine- and alanine-rich protein is produced by *P. falciparum* in the course of its growth and multiplication ^[39]. Histidine-rich protein 2 (HRP2) levels are closely associated with parasite density and development, and studies have implicated HRP2 as an important factor in the detoxification of heme ^[40]. This protein stability can be considered an advantage for *in vitro* drug susceptibility assays. The assessment of this protein production can be simply measured in a commercial available, double-site sandwich, Enzyme-Linked Immunosorbent Assay (ELISA) test kit. This assay, based on the HRP2 produced by *P. falciparum* and measured using the ELISA has the advantage of requiring low parasitemia (0.05%). However is an assay that requires a time period of drug incubation of 72 h, followed by a series of steps to detect the protein through ELISA.

- Parasitemia measuring through fluorescent DNA dyes, such as SYBR green I ^[41], YOYO, PicoGreen ^[42], DAPI ^[43] and Hoechst with either spectrophotometric or cytometric readout. These assays take advantage of the lack of DNA in the mature RBCs, detecting the stained DNA from the parasite, thus assessing parasitemia in blood samples.

➤ With spectrophotometric readouts, the fluorescence intensity, which is proportional to the amount of DNA in individual samples, is measured with a mini fluorometer, fluorescence spectrophotometer or fluorescence activated microplate reader. However, this method is not as sensitive as other methods and it requires long incubation period (48 - 96 h).

➤ With the cytometric readout using a flow cytometer, which enables to assess the parasitemia percentage and perform double stain for a better evaluation of the parasite as mentioned below.

Table 4 – Advantages and limitation of antimalarial drugs sensitivity assays.

		 WHO microtest	 Isotopic assay [3H]-hypoxanthine incorporation	 Flow Cytometry	 Fluorometric assays	 Antigen detection pLDH HRP2
		Quantification of schizonts	Measurement of parasite DNA			Measurement of parasite proteins
Advantages	- Requires little equipment.	- Automatic reading of results.	- Automatic reading of results.	- Automatic reading of results.	- Simple, does not require specialized personnel.	- Simple, does not require specialized personnel.
		- Accurate and sensitive.	- Accurate and sensitive.	- Accurate and sensitive.	- Accurate.	- Low detection limit (0.05% parasitemia).
Limitations	- Subjective and labor-intensive.	- Handling of radioactive reagents.	- Additional incubation steps with reagents.	- Additional incubation steps with reagents.	- Additional incubation steps with reagents.	- Additional incubation steps with reagents.
	- Highly trained personnel.	- Additional incubation steps with reagents.				

Adapted from: Rebelo, M. *et al.* 2013 ^[49] – Supplement info

1.4 Flow Cytometry and Malaria

Flow cytometry is becoming particularly important for the study of malaria parasite growth and invasion due to the speed and amount of information it provides and also because it overcomes some of the limitation of existing non-cytometric methods. Flow cytometers have also been progressively more cost affordable and portable, conferring important features for field research.

Using flow cytometry it is possible to assess blood parasitemia after specifically staining the nucleic acid (DNA) of the parasites, taking advantage of the fact that normal circulating red blood cells (RBCs) lack nucleic acids, while reticulocytes contain only RNA. Parasitemia in blood samples can therefore be determined by counting and comparing the ratio of RBCs which stain positive for DNA to the total number of RBCs analysed. As mentioned, there is an exception that can potentially confound cytometric analysis of malaria, which is the presence of reticulocytes^[44]. These are erythrocytes which have been recently released from the bone marrow and still contain small amounts of RNA and they only present at levels less than 1.5% in healthy adults. The presence of reticulocytes could be confused as a parasite if one was not using DNA specific stains. For example, acridine orange^[44] and SYTO 9^[45] are not specific, thus staining both DNA and RNA. However, the remnant RNA in the circulating erythrocytes degrades quickly and within a few days in *in vitro* cultures, leading to a small presence of this type of cell in blood samples^[46].

Flow cytometers with more than one laser also enable the use of combinations of stains^[45]^[47] to study more aspects of the malaria parasite. The variety of different nucleic acid stains as well as the membrane potential stains has been combined together with additional stains to learn more about malaria parasite biology. Shapiro^[48] described the use of a double staining to differentiate nucleic acids by combining Hoechst 33342 with a RNA selective stain and used this to increase sensitivity for cell cycle detection using flow cytometry. Pyronin Y, a homolog of acridine orange, was a better choice because it is more selective for double stranded RNA, does not fluoresce when attached to DNA, and can detect the phases of the cell growth cycle.

With flow cytometry assays it is possible to set up different approaches for the study of *Plasmodium* parasites thus obtaining information about growth and development, allowing the evaluation of current drugs, tracking levels of resistance and screening for new and effective drugs through determination of the concentration of drugs which inhibit more than 50% (IC₅₀) of the parasite growth. In these assays the goal is to determine the number of DNA positive cells in the presence of different concentrations of drugs, all of which is compared to a drug-free control.

1.5 Hemozoin detection by Flow Cytometry

To overcome some of the drawbacks of the available *in vitro* tests referred above, a novel flow cytometric sensitivity test for *P. falciparum* has been proposed recently^[49]. This test is based on the detection of hemozoin (Hz).

Hz crystals are formed during the intra-erythrocytic stage (Figure 2), when the parasite digests hemoglobin to obtain amino acids, iron and space to grow^[50]. As a result of this digestion free toxic heme is produced and detoxified by the parasite that converts it into Hz^[51].

Hz, also known as malaria pigment, is a paramagnetic, birefringent pigment that depolarizes light and therefore it can be detected using optical methods such as flow cytometry^[52].

In *P. falciparum* Hz is detectable at developmental erythrocyte stages beyond the ring form, which include trophozoites (Figure 4 - A and D), schizonts (Figure 4 - B and E), and also gametocytes (Figure 4 - C and F). However, in mature schizonts, Hz crystals aggregate as they start to appear and form a big clump. On the other hand, in the gametocytes, Hz pigment does not

form an agglomerate and instead it seems to be more disperse (Figure 4), and also present in Garnham bodies ^[53].

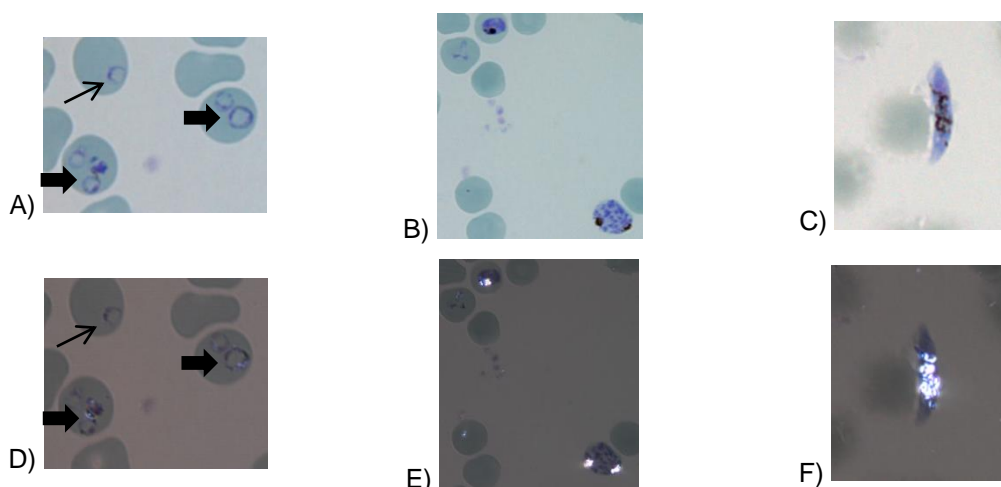


Figure 4 – Hemozoin within *P. falciparum*. In A) and D) the thin arrow point to a ring form and the thick arrows points to young trophozoites. B) and E) are schizont forms. In C) and F) a gametocyte is presented. From A)-C) light microscopy images. From D)-F) depolarization microscopy images where it is possible to distinguish bright white spots of depolarizing Hz crystals. Smears were stained with 10x Giemsa, amplification (1000x).

Consequently Hz can be considered an optimal parasite maturation indicator since its amount increases as the parasite matures (Figure 5).

Due to the fact that Hz is birefringent it rotates the plane of polarized light, a process called depolarization, which is possible to detect by flow cytometry. LASER light, commonly used as a light source in flow cytometers, has polarized light. Thus, by placing a polarization filter orthogonally (90° rotated) to the plane of the LASER light in front of a second Side-Scatter (SSC) detector, allows to detect depolarized light and consequently Hz (Figure 6) ^[54].

With a small modification of a CyFlow® Blue (Partec, Münster, Germany), creating two SSC detectors, using a 50%/50% beam splitter (Figure 6) it was possible to develop a drug sensitivity assay based on the detection of Hz depolarization. It allows the detection of cells with Hz relatively to a total number of cells, thus assessing the percentage of parasites that mature. Because the majority of the available antimalarial drugs act on the intra-erythrocytic stage of the malaria infection and, moreover, some of them act directly upon Hz formation it appeared possible to establish an accurate and reliable sensitivity assay based on the measurement of infected RBCs containing of Hz. Afterwards it was proved that the flow cytometric Hz detection allowed to determine the inhibitory effect of antimalarial drugs ^[49].

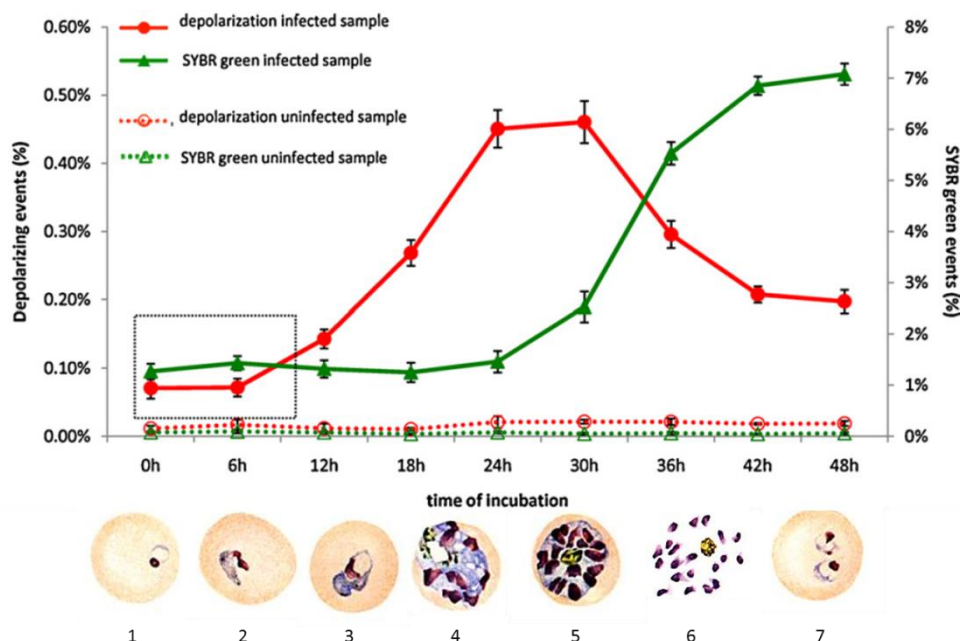


Figure 5 – Parasite growth and maturation detection. Red line: detection of depolarization during incubation time. Through the first 24 to 30 hours the percentage of depolarizing events increases because more and more parasite presents Hz within the food vacuole. Green line: detection of parasite DNA using SYBR green I stain. The initial parasitemia is maintained through the first 24 to 30 hours before the erythrocyte lysis and release of new merozoites increasing SYBR green detection due to reinvasion and increased parasitemia. Parasite intraerythrocytic maturation. 1 – Ring form; 2, 3 – Trophozoite form; 4, 5 – Schizont stage; 6 – Erythrocyte lysis and release of new merozoites; 7 – Ring form again in a red blood cell reinvasion by more than one merozoite/parasite. Graph adapted from: Rebelo, M. *et al.* 2012 ^[52]; parasite images adapted from CDC: Diagnostic findings (http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Malaria_il.htm last accessed 23.10.13)

1.6 Specific Gametocyte detection for drug testing

The development of drugs that also act against the sexual forms of the parasite and thus block transmission is of great importance, especially considering the new eradication agenda ^[55]. However, very few methods exist to test the drug sensitivity of gametocytes ^{[56], [57], [58]}, certainly as compared to the ones available for asexual intra-erythrocytic stages ^[36]. One problem may be the required culture methods to induce gametocytes. These culture methods imply selective pressure to the development of the sexual stages of the parasite, such as low hematocrit, a low parasitemia or drug treatment ^{[59], [60], [61]}. However, it is not straightforward to detect gametocytes in a mixed culture of sexual and asexual forms, a reason why assays often use enriched gametocyte cultures ^[57].

Gametocytes contain Hz, which is distributed in the form of many small crystals, contrary to the large, usually single crystal observed in schizonts (Figure 4). Thus it appears possible that the proposed flow cytometric assay for Hz detection might also allow the detection of gametocytes, based on the presence of a population with higher levels of depolarization than that observed in schizonts. Because Hz in gametocytes does not agglomerate and is dispersed (Figure 4 - F), it probably has a higher degree of side scatter than a single crystal, even if the total amount of Hz

was the same. Thus, if gametocytes could be recognized as a separate population, distinct from schizonts, this approach might open the way for the development of a novel drug sensitivity assay to assess drug effects against the sexual forms of the parasite.

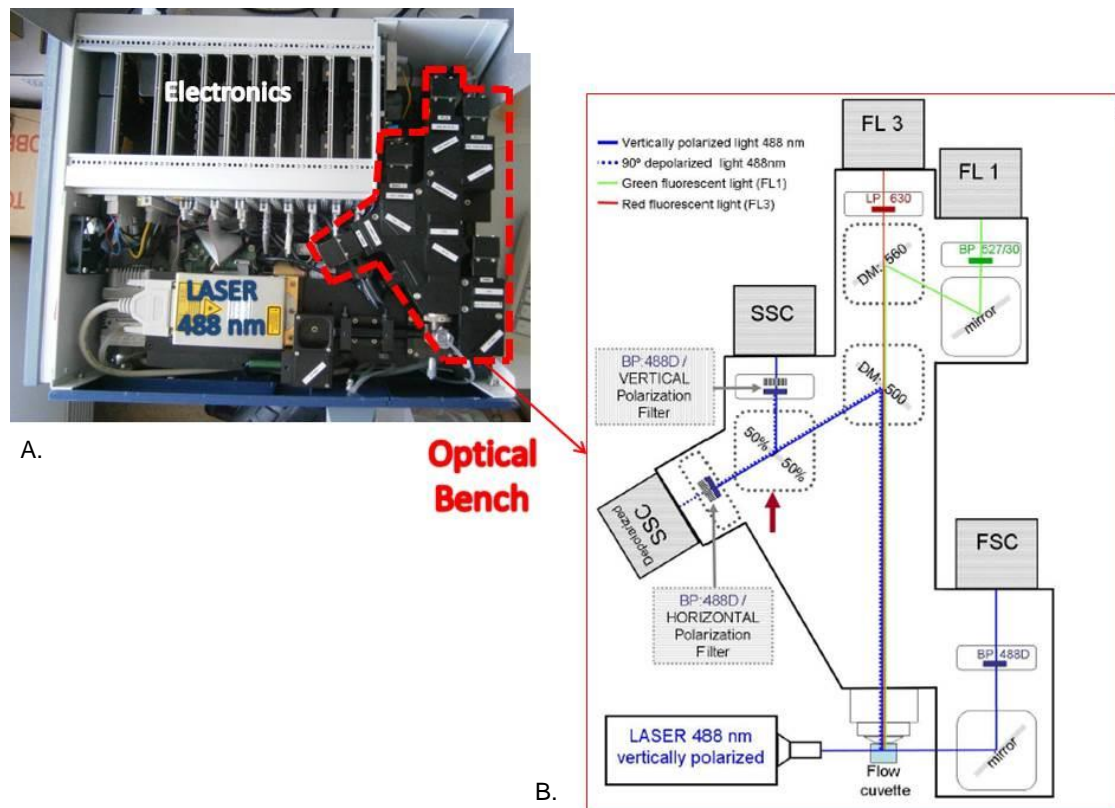


Figure 6 – Adapted flow cytometer which allows detection of depolarized side-scatter. A.: Five parameter CyFlow® flow cytometer with a blue laser (488nm) excitation, and detectors for forward scatter (FSC), side scatter (SSC), depolarized side scatter (dSSC), green fluorescence (FL1), and red fluorescence (FL3). B.: Two SSC detectors were created, with a 50%/50% beam splitter between them. A polarization filter was placed orthogonally (horizontal) to the polarization plane of the laser light (vertical), in front of one of the SSC detectors, allowing the detection of depolarized side scatter. Adapted from Frita, R. *et al.* 2011 ^[54].

2. Objectives

The primary objective of this study was to assess the potential of the novel flow cytometric method for Hz detection for the screening of new antimalarial compounds against *P. falciparum* blood stages. Newly synthesized compounds were screened and compared using two other methods.

The secondary aim was to evaluate if gametocyte cultures could be established and if gametocytes could be specifically detected using the same recently proposed flow cytometric method based on Hz detection.

3. Materials and Methods

3.1 Culture media, solutions and reagents

3.1.1 Malaria complete parasite medium (MCM): is composed by 500 mL of RPMI 1640 (1x, without L-glutamine, with NaHCO₃) (Gibco™, Life Technologies, Madrid, Spain) supplemented with 12 mL of HEPES Buffer Solution at 1 M (Gibco™, Life Technologies, Madrid, Spain), 500 µL of gentamicin at 50 mg/mL (Gibco™, Life Technologies, Madrid, Spain), 5 mL of L-glutamine at 200 nM and 50 mL of 10x AlbuMAX II®.

3.1.2 10x AlbuMAX II® solution: 25 g of Albumax II® was dissolved in 500 mL of an aqueous solution with 5.2 g RPMI 1640 (with L-glutamine, without NaHCO₃), supplemented with 500 µL of gentamicin (50 mg/mL), 2.98 g HEPES, 1.67 g of sodium bicarbonate, 1 g of glucose and 0.1 g of hypoxanthine, pH adjust to 7.2 – 7.4 and filtered 0.22 µm. All reagents were obtained from Life Technologies (Madrid, Spain) except HEPES which was purchased from VWR (Carnaxide, Portugal).

3.1.3 1X Phosphate-buffered saline (PBS 1x): 10x PBS pH 7,2 (Gibco™, Life Technologies, Madrid, Spain) was diluted 1:10 in filtered distilled water, obtained with Milli-Q Synthesis Q-Gard® water purification system (Millipore, Billerica MA, USA) or using ultrapure DNA/RNA free water from Gibco™, Life Technologies, Madrid, Spain.

3.1.4 Sorbitol 5%: 5 g of D-sorbitol (Sigma-Aldrich, Life science) dissolved in 100 mL of distilled water, then filtered 0.22µm.

3.1.5 Cryoprotective solution: constituted by 57% of glycerol, 16 g/L of sodium lactate (VWR, Carnaxide, Portugal), 300 mg/L of potassium chloride and 25 mM of sodium phosphate pH 6.8.

3.1.6 Chloroquine: stock solution of 12.8 µM prepared chloroquine (Sigma-Aldrich, Life science)

3.1.7 Paraformaldehyde (PFA) 2%: paraformaldehyde at 16% (Electron Microscopy Sciences) was diluted 1:8 in PBS 1.2 X, to obtain PFA at 2%.

3.2 *P. falciparum* in vitro cultures

3.2.1 *P. falciparum* continuous cultures maintenance

P. falciparum strains 3D7 and Dd2 were obtained from Malaria Research and Reference Reagent Resource Center (MR4; Manassas VA, USA). These strains were grown in continuous culture using uninfected red blood cells (RBCs) from healthy donors were isolated from buffy coats obtained from Instituto Português do Sangue. Buffy coats were washed 3x with RPMI 1640 (1x, without L-glutamine, with NaHCO₃) and centrifuged for 5 min at 1800 rpm without applying the brake, allowing the separation of RBCs from white blood cells and plasma. Isolated RBCs were stored at 4°C for a maximum of 2-3 weeks. Continuous *P. falciparum* cultures were incubated at 37°C in a 5% CO₂ atmosphere. Culture medium was changed on a daily basis and the parasitemia was kept at around 0.5% and the hematocrit at 5%.

3.2.2 Giemsa staining of blood smears

To assess the parasitemia of the cultures, blood smears were prepared every day and stained with Giemsa. The culture medium was removed and a small volume of culture blood was used to do a thin smear. The smear was fixed in absolute methanol, (Merk, Lisbon, Portugal) by dipping the slide for 30 s in a 50 mL falcon with absolute methanol, air-dried and stained with Giemsa at 10x (previously diluted 1:10 in PBS 1x) (Merk, Lisbon, Portugal) for 20 min. Then the slides were rinsed off with tap water and air-dried before observed by bright field microscopy under oil immersion. The parasitemia was estimated by counting the total RBCs and infected RBCs on eight different fields at a 1000x magnification/amplification. If the parasitemia was higher than ~2%, it would be lowered by adding a volume of uninfected RBCs equal to the amount of RBCs discarded from the culture.

3.2.3 Culture synchronization

Cultures were synchronized to obtain parasites at the same developmental stage. Cultures with a majority of ring-form parasites and a parasitemia higher than 2% were used. A volume of 10 mL of sorbitol 5% was added to the RBCs, after removing the medium, and then transferred to a 50 mL falcon and incubated for 10 min at room temperature after which it was centrifuged at 1800 rpm, for 5 min without brake, also at room temperature. After that, the supernatant was discarded and the RBCs pellet was washed two times in 35 mL of RPMI 1640 (1x, without L-glutamine, with NaHCO_3) by centrifugation (1800 rpm, 4 min, without brake, room temperature). In the end, in a culture flask, pelleted RBCs were diluted to 5% hematocrit in MCM and incubated at 37°C and 5% CO_2 atmosphere.

3.2.4 Frozen stocks of *P. falciparum*

When necessary, *P. falciparum* cultures were frozen in a cryoprotective solution and stored at -80°C. From a highly parasitized culture (> 2% parasitemia), with the majority of the parasites in ring form, the medium was removed by centrifugation (6 min at 1800 rpm). To the remaining volume of pellet cells, 0.33 and then 1.33 volumes of cryoprotective solution was added slowly, drop by drop, while mixing the tube gently.

To reuse cryoprotected-frozen vials, after thawing them for a few minutes at room temperature, the content was then transferred to a 50 mL falcon tube. To this volume, 0.1x volumes of 12% sodium chloride (NaCl) was added, followed by 10x volumes of 1.6% NaCl slowly, dropwise while shaking the tube gently. Then the tube was centrifuged for 3 min at 1800 rpm at 20°C. The supernatant was removed and the pellet was resuspended in 10x the volume of RPMI 1640 (without L-glutamine, with NaHCO_3) and centrifuged again for 3 min at 1800 rpm. In the end, the pelleted RBCs was resuspended in MCM and transferred to a culture flask. Uninfected RBCs were added if needed to obtain a 5% hematocrit and the culture flask was incubated at 37°C in a 5% CO_2 atmosphere.

3.3 Antimalarial drugs sensitivity assays

3.3.1 Inhibition of Chloroquine (IC₅₀ determination) – Influence of Oxygen

The influence of oxygen (O₂) on parasite's growth was assessed by determining the chloroquine 50% inhibitory concentration (IC₅₀) against *P. falciparum* 3D7 strain in different atmospheres.

To determine the IC₅₀, two plates with serial dilutions (6, 12, 25, 50 and 100 nM) of chloroquine were prepared. The plates followed the procedure established in point 3.3.2 to analyse the inhibitory effect. However, one plate was incubated in a low O₂ atmosphere incubator (Galaxy® 14 S, Eppendorf line of New Brunswick™) with 5% CO₂, 5% O₂ and 90% N₂, and the other was incubated in a standard CO₂ incubator (Thermo Scientific, Heraeus® HERAcCell®), with 5% CO₂ and the O₂ levels around 21%, as present in Earth's atmosphere.

3.3.2 Flow Cytometric Assays – General protocol

For this assay two controls were used: a growth control (without compound – drug free control) and a non-infected control (erythrocytes without parasite – uninfected control). In the screenings of compounds from Faculdade de Farmácia da Universidade de Lisboa (FFUL) chloroquine at a concentration of 100 nM (2x the determined IC₅₀) was also used as a control.

Ring-stage synchronized cultures were used. Compounds were prepared in MCM 2x concentrated to the concentration to be tested. In a 96 well-plate 100 µL of compound and a blood suspension, previously prepared from the synchronized culture at 1% parasitemia and a 5% hematocrit, were added to the respective wells. Thus, each well had a final hematocrit of 2.5%, with 1% parasitemia and the compound concentration to test (Figure 7). MCM was added to the drug free and uninfected controls. The assay was performed in triplicates.

Plates were incubated for 48 h, at 37°C in a 5% CO₂ atmosphere, and flow cytometric measurements were performed at 0 h, 24 h and 48 h of incubation. At each time point, the percentage of depolarizing events and SYBR green positive events (parasitemia) was assessed. To measure parasitemia, samples were stained with SYBR® Green I (Sigma) at 1x (previously diluted 1:10000 in PBS 1x) in the proportion of 1:10 (5 µL of the sample from each well plus 50 µL of SYBR green 1x). Then incubated for 20 min in dark and room temperature. After this period of incubation 900 µL of PBS 1x were add to each sample tube before analysis in the CYFlow® (Partec®, Münster, Germany) where a total of 100 000 events were acquired.

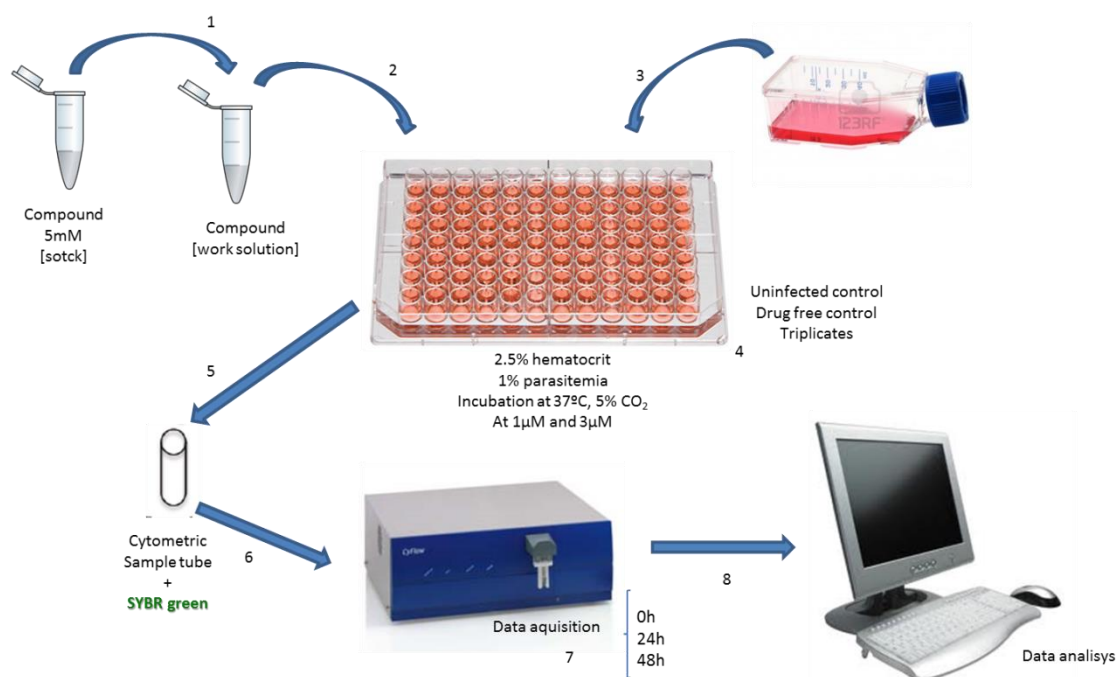
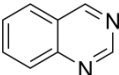
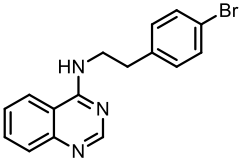
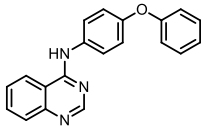
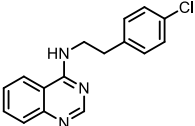
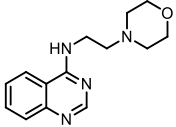
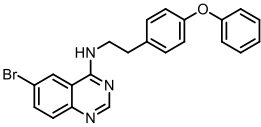
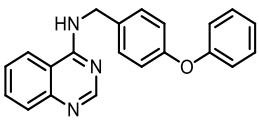
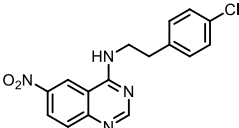
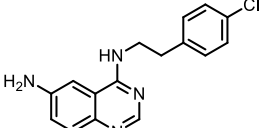
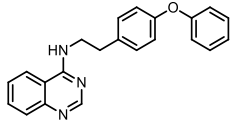
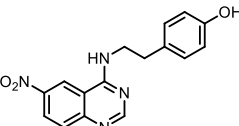
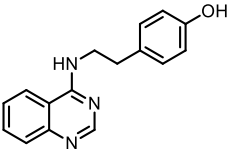
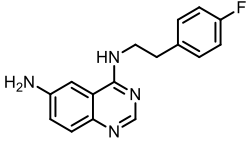
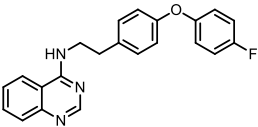
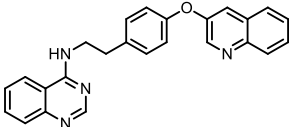
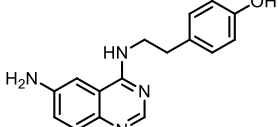
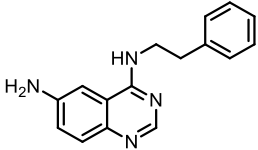
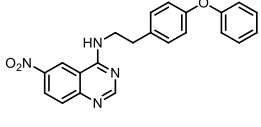
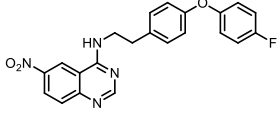
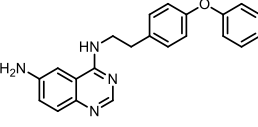
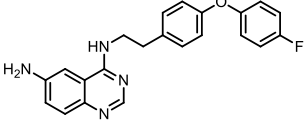
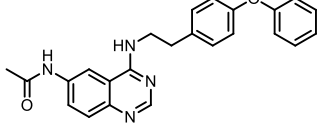


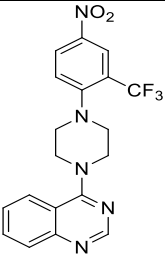
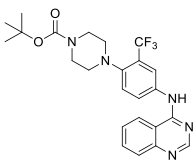
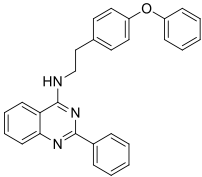
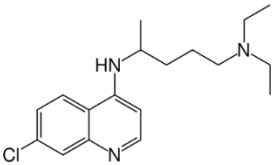
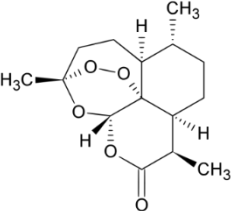
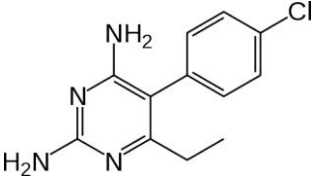
Figure 7 – Flow cytometric assay preparation. Work solutions were prepared from a 5 mM stock compound (1) and distributed in a 96 well plate (2). Blood suspension in 1:1 proportion, was added to the wells (3). Uninfected and drug free controls were also included. The 96 well plate was set with a final 2.5% hematocrit and 1 % parasitemia was incubated at 37°C in a 5 % CO₂ atmosphere (4). Samples from each well stained with SYBR green I (5) before and analysed by flow cytometry (6). Sample analysis was performed at 0 h, 24 h and 48 h of incubation (7). Data was analysed using the FlowJo software (8).

3.3.3 Screening novel compounds from Faculdade de Farmácia

The inhibitory potential of 24 new compounds obtained from FFUL (Professor Rui Moreira's group) was investigated. The compounds are quinazoline derivatives (Table 5). The action of these compounds against *P. falciparum* 3D7 and Dd2 strains was determined. The screening was performed using cut-off concentrations of 1 µM and 3 µM. Inhibitory activities of the compounds was assessed by flow cytometric and HRP2-ELISA assays, described in 3.3.2 and 3.3.6. Chloroquine at 100 nM, twice the determined IC₅₀ concentration, was used as an internal control.

Table 5 – Chemical structure of the quinazoline derivative screened compounds as well as from chloroquine, artemisinin and pyrimethamine as comparison.

 Quinazoline		
 ABJ1	 AD3	 AD6
 AD20	 AD26	 AD34
 ASR 252	 ASR 256	 ASR 291
 ASR 292	 ASR 294	 ASR 295
 ASR 299	 ASR 300	 ASR 304
 ASR 305	 ASR 306	 ASR 317
 ASR 321	 ASR 322	 ASR 323

 <p>ASR 327</p>	 <p>ASR 338</p>	 <p>ASR 341</p>
Example of antimalarial drugs structure:		
 <p>Chloroquine</p>	 <p>Artemisinin</p>	 <p>Pyrimethamine</p>

3.3.4 Flow cytometer

The flow cytometer, the CyFlow® (Partec®, Münster, Germany), used in the assay is a five parameter flow cytometer with a blue laser (488 nm), adapted to detect depolarizing events. The optical bench include detectors for: forward scatter (FSC), side scatter (SSC), green (FL1 - band pass 535/45 nm filter) and red fluorescence (FL3 - long pass 610 nm filter), as well as a detector for depolarized side scatter, used to detect infected red blood cells containing Hz (Figure 6).

Flow cytometry enables the counting and 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 different angles. Light scattered in the forward direction (0° angle), is quantified in the FCS detector and usually a measure of particle size. Light that is scattered to the side (at 90° angle) indicates the granularity and structural complexity inside the cell. The depolarized side scatter detects light which has been scattered at 90° and depolarized. For this, a polarization filter, perpendicularly positioned to the incident polarized laser beam is placed in front of the detector blocking out scattered light which remains in its original polarization plane.

3.3.5 Flow cytometric assays - results analysis

Flow cytometric data was analysed by using FlowJo (version 9.6.2) software. The gating strategy was established by comparing the uninfected RBCs and *P. falciparum* infected RBCs populations at each time point (Figure 8). Drug effects were assessed by comparing the percentage of depolarizing events or percentage of SYBR green positive events between the drug free control and the drug treated samples. Depolarization assay results were assessed at 24 h, when most of the parasites are schizonts, which contain Hz. The SYBR green assay results were determined at 48 h, the time point when reinvasion of uninfected RBCs already occurred and the parasitemia has increased.

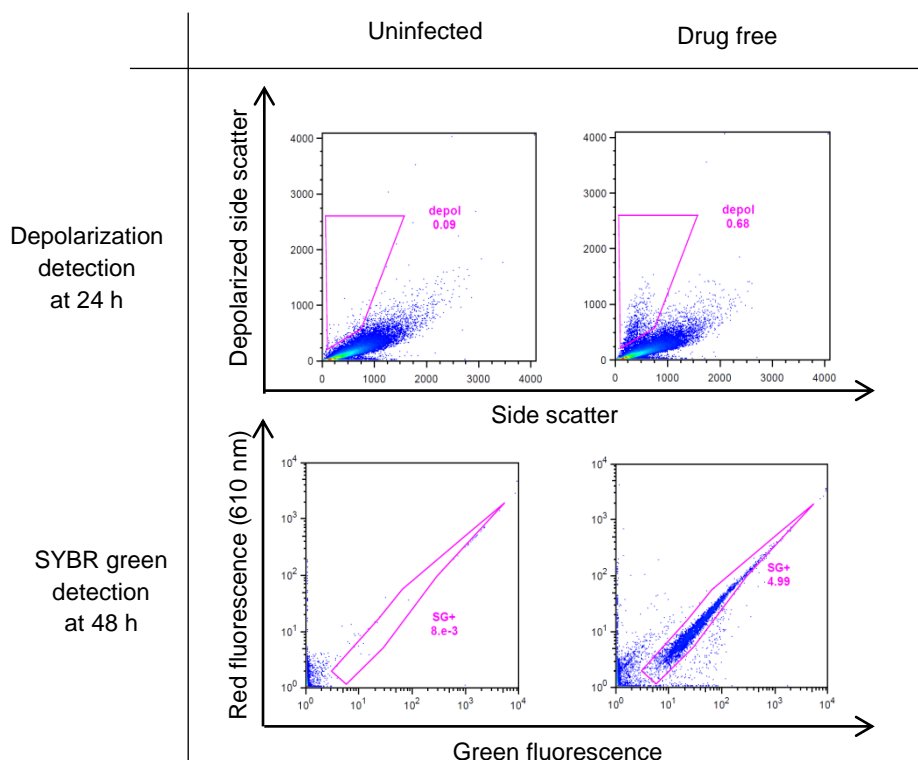


Figure 8 - Gating strategy. At 24 h of incubation, depolarization is measured and a gate was established according to the existing population of events that depolarize in the drug free control and that are absent in the uninfected control. At 48 h of incubation, the SYBR green positive (SG+) events were assessed by establishing a gate around the detected population in the drug free control that is absent in the uninfected control. After setting the gates they were applied to the data acquired for the tested compounds.

3.3.6 Histidine-rich protein-2 sensitivity assay

The histidine-rich protein 2 (HRP2) ELISA was performed to corroborate the flow cytometric results using the *P. falciparum* strain 3D7. For this assay a growth control (without compound) was used as well as an effective concentration of chloroquine at 100 nM as a control for inhibition.

3.3.2.1 Pre coating protocol:

Primary IgM antibody (MPFM-55A, Immunology Consultants Laboratories, Inc, Newberg, OR, USA) was diluted to 1 µg/mL concentration in PBS 1x, and 100 µL was transferred to each well of 96 flat-bottom well-plates. The plates were sealed and incubated at 4°C overnight. After this, plates were pad-dried and 200 µL of blocking solution (2% bovine serum albumin (Sigma, CAS No. 9048-46-8) solution in PBS 1x) was added to each well and the plates were incubated for 2 h, at room temperature. Then, plates were washed 3x with washing solution (PBS/Tween 20 [0.05%]) and pad-dried. Finally, the plates were sealed airtight and frozen at -20°C.

3.3.7 HRP2-ELISA Sample analysis

From a synchronized culture, a blood suspension with a final hematocrit of 1.5% and a parasitemia of 0.05% was prepared. The blood suspension was distributed in a 96 well plate, together with the compound at the concentration to be tested. The plate was incubated at 37°C in a 5% CO₂ atmosphere for 72 h. At the end of incubation plates were frozen at -20°C until the HRP2 ELISA was performed.

To perform the ELISA, plates were freeze and thawed twice and 100 µL of the lysed sample was transferred to a 96 well-plate pre-coated with MPFM-55A antibody (Immunology Consultants Laboratories, Portland, USA) and incubated for one hour at room temperature. Plates were washed three times with washing solution (Tween 20 at 0.05%), then 100 µL of the secondary antibody at 0.1 µg/mL, MPFG-55P (Immunology Consultants Laboratories, Portland, USA) was added and plates were incubated for another hour. Plates were washed again and incubated with the 100 µL of chromogen, TMB One (Biotrend, Köln, Germany) for 5 to 10 min, at room temperature, in the dark. The reaction was stopped by adding 50 µL of sulphuric acid at 1 M (Merck, Darmstadt, Germany). Finally, the absorbance at a wavelength of 450 nm was immediately determined using the Infinite M200 plate reader (Tecan, Mannedorf, Switzerland).

3.3.8 50% inhibitory concentration determination of selected compounds

The 50% inhibitory concentration (IC₅₀) was determined for chloroquine in both atmospheres, and for three of the FF compounds.

The IC₅₀ values for chloroquine were calculated based on 2-fold serial dilutions (6, 12, 25, 50 and 100 nM). The inhibitory effect was assessed by the flow cytometric detection of Hz and SYBR green, as explain above in 3.3.2.

IC₅₀ values were determined for the compound 321, which had been previously reported to have an inhibitory activity at 3 µM, and for two other compounds that were randomly selected (256 and 291). Concentrations of 1, 2, 4, 6, 8 µM were used for all three compounds. An additional concentration of 10 µM was used for compounds 256 and 291.

The SigmaPlot software from Systat Software Inc. (SSI) (San Jose, California) was used to trace the dose-response curves of the acquired data, in order to calculate the IC₅₀ values.

3.4 Gametocyte cultures

Gametocytogenesis is only induced in *in vitro* cultures when the parasites present in the culture are “stressed”. For that, culture conditions were modified to induce stress in the asexual parasites, and two methods were experimented (A) and (B):

3.4.1 Culture Method A

Starting with a 5% hematocrit, culture medium was changed every day until a 2% - 3% parasitemia was reached. At this point the hematocrit was decreased to 2.5%, representing the “stressing factor” to induce the gametocyte culture. Every day until day ten, medium was changed

daily and cultures were monitored by flow cytometry, as well as by microscopic observation of thin blood smears.

3.4.2 Culture Method B

In a recently synchronized culture, the hematocrit was reduced to 2.5% and the parasitemia to 0.7%, corresponding to day one. Additionally to this “stressing factor”, the medium was not changed until day four. On this day, culture medium was changed and replaced by a suspension of lysed RBCs that corresponded to twenty percent of the final culture volume. This suspension consisted of RPMI 1640 with lysed RBCs which were frozen and thawed at least twice, in 1:1 proportion. The medium was changed on days six and eight and thereafter on a daily basis. Flow cytometric acquisitions as well as blood smear were done every day until day ten.

3.4.3 Erythrocytes fixation using paraformaldehyde

Cells were centrifuged to remove the culture medium. A suspension of *P. falciparum* infected RBCs with a 50% hematocrit was prepared. A volume of 5.5 mL of paraformaldehyde (PFA) 2% (previously diluted 1:8 in PBS 1.2x) was added to 250 µL of the infected RBC suspension. The same procedure was performed using uninfected erythrocytes. Samples were incubated for 2 h at 37°C in a water bath. Subsequently, they were centrifuged for 3-5 min at 1800 rpm. The supernatant was discarded and the pellet was re-suspended in PBS 1x.

3.4.4 Sorting Gametocyte cultures

The MoFlo (Beckman Coulter, Fort Collins, USA) cytometer is a high speed sorter with an open architecture. This enables many different configurations based on the modularity which meet the diverse sorting applications. For example, it is easy to include polarizing filters in any light-path and thus create depolarized SSC detectors.

After fixing the erythrocytes, the gametocytes were sorted in a MoFlo cytometer according to its SYBR green fluorescence and depolarization degree. First, the SYBR green positive population was selected and its depolarization was analysed. In the depolarization plot three gates based on the depolarization degree were established: (i) non-depolarizing events, (ii) medium depolarizing events and (iii) high depolarizing events (Figure 9). Cells within these gates were sorted into separate tubes. The purity of sorted cells was assessed by reanalysing each sorted population in the MoFlo.

Sorted cells were left to sediment overnight. Then, after removal of most of the supernatant, a small volume of cells were transferred onto a slide and analysed immediately by microscopy. Pictures of each population were taken using light microscopy and depolarizing microscopy.

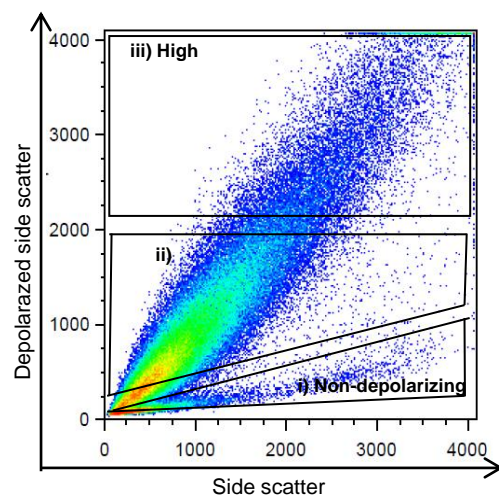


Figure 9 – Sorting gating strategy.

SYBR green positive population was plotted based on their depolarized side scatter and side scatter. Three gates were established according to the depolarization degree: i) non-depolarizing events, ii) medium depolarizing events and iii) high depolarizing events. Cells within these gates were sorted using a MoFlo (Beckman Coulter, Fort Collins, USA).

4. Results

4.1. Baseline Chloroquine IC₅₀ determination for screening

The chloroquine (CQ) concentration at which 50% of the parasite growth is inhibited was determined. The obtained value was used to choose the concentration of the CQ would be used as a control in the screening assays of the new compounds.

CQ's IC₅₀ was determined using the *P. falciparum* 3D7 strain growing under two different atmosphere: both containing 5% CO₂, one at a high oxygen percentage (~21% O₂) and the other at a low O₂ percentage (5% O₂).

After the flow cytometric measurements it was possible to determine the CQ's IC₅₀ for both atmospheres. Results obtained according to the depolarization detection at 24 h of incubation, and the SYBR green stain detection method at 48 h are shown in Table 6.

Table 6 – Chloroquine IC₅₀ (nM)

	Depolarization	SYBR green
High oxygen incubator (~21% O ₂)	53	65
Low oxygen incubator (5% O ₂)	41	41

The results showed that the IC₅₀ was higher when the culture was incubated in the standard CO₂ incubator with ambient atmosphere (~21% O₂). Despite these observed differences, the magnitude was considered to be sufficiently small, that a concentration of 100 nM CQ was chosen for the subsequent experiments, constituting about 2x IC₅₀. Furthermore, the differences were considered to be within a range which would allow to perform all further experiments in the standard CO₂ incubator (~21% O₂). It is possible to find in Table 7, various CQ IC₅₀ values were reported by different authors using different methods in the nM range.

The 100 nM value was chosen, because the parasite would be sufficiently inhibited (> 50%), however, the inhibition would not be complete and, as such, allow to observe a slight growth. In fact, the 90% inhibitory concentrations (IC₉₀) obtained in this experiment were of 85 and 100 nM, for the depolarization and SYBR green detection assays, respectively.

Table 7 – Chloroquine IC₅₀s against *P. falciparum* 3D7 strain by different *in vitro* sensitivity assays

[3]-Hypoxanthine incorporation	WHO schizont maturation test	SYBR green I plate assay	HRP2 assay
15.7 nM	14.79 nM	8.1 nM	7.5 nM
6 nM		22.2 nM	9.7 nM
9.7 nM		16 nM	
11.3 nM		11.54 nM	
18.7 nM			
9.6 nM			
29.6 nM			

Adapted from: Rebelo, M., *et al.* 2013 ^[49] supplementary info

4.2 Compounds screening at 1 μ M against *P. falciparum* strain Dd2

Some of the compounds from the FFUL had been screened elsewhere using the CQ resistant Dd2 strain and had shown no effect at the screening concentration used (1 μ M). Thus, to confirm these results, eighteen compounds from FFUL were tested at the same screening concentration of 1 μ M against the *P. falciparum* Dd2 strain. The results were normalised in relation to the drug free control, which was considered to be 100% of parasite growth, as shown in Figure 10.

As expected, chloroquine at 100 nM showed no inhibitory effect because the strain is chloroquine resistant.

Results obtained with the depolarization assay showed that compounds ABJ1, AD6, AD26, 338 and 341 had a slight inhibition effect, however, none of the compounds exhibited an inhibition of parasite growth greater than 23%. Contrary to this, the SYBR green detection assay indicated that none of the tested compounds had inhibitory effects against the Dd2 strain at 1 μ M. Overall, both assays (depolarization and SYBR green detection) allowed to confirm that none of the screened compounds had any relevant inhibitory action against *P. falciparum* Dd2 at 1 μ M concentration.

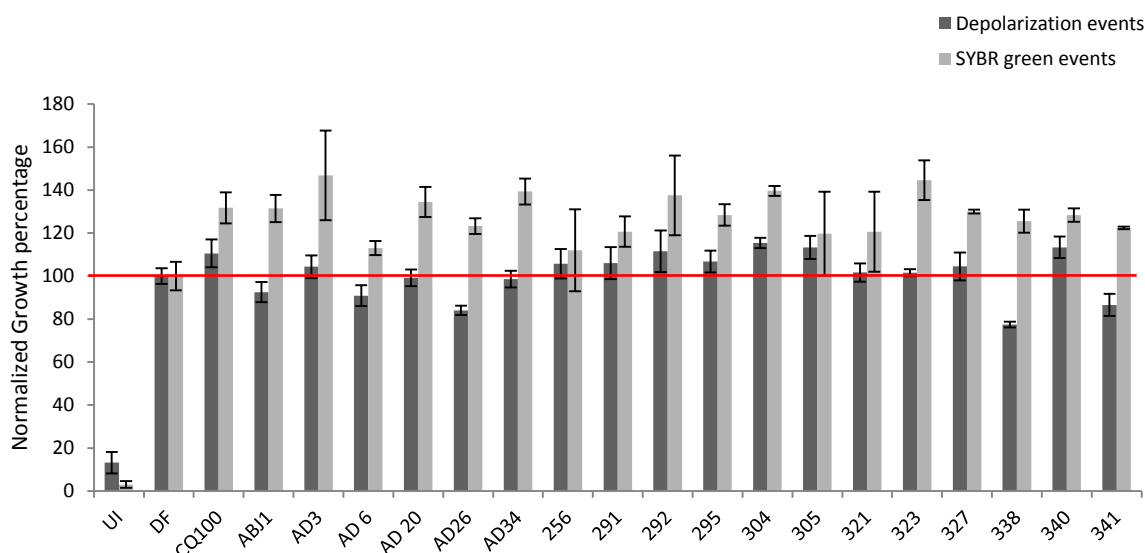


Figure 10 – Compounds activity tested at 1 μ M against *P. falciparum* Dd2.

Results from depolarization detection measured at 24 h, and SYBR green detection measured at 48 h. The results are expressed as normalized percentage, relative to the drug free control considered to represents 100% maturation or complete replication. Legend: UI – Uninfected; DF – Drug free; CQ100 – Chloroquine at 100 nM.

4.3 Compounds screening at 1 μ M against *P. falciparum* strain 3D7

The completely sensitive strain 3D7 was investigated to rule out the hypothesis that inhibitory effects may not be detected when using a CQ resistant strain.

The same compounds which had been tested against Dd2 and some further novel compounds, also obtained from FFUL, were tested against this strain. In total, 24 compounds were tested at 1 μ M against the 3D7 strain. Furthermore, the already validated HRP2-ELISA *in vitro*

sensitivity assay was used to validate the results obtained by the Hz depolarization and the SYBR green detection assays.

According to the results shown in Figure 11, none of the tested compounds had an inhibitory effect in any of the three assays as compared to the CQ control, which inhibited more than 50% of the parasite's growth. In general, parasite growth was at least 80% or more for all tested compounds, in comparison to the drug free control. Although, similar trends for inhibition were observed between the three performed methods, there were some variations in the detected growth percentage between methods. For example, in the HRP2-ELISA, the growth percentage observed for all the compounds is slightly lower, as compared to the other methods. On the other hand, this assay seemed to have a more coherent result (less variability) between the compounds.

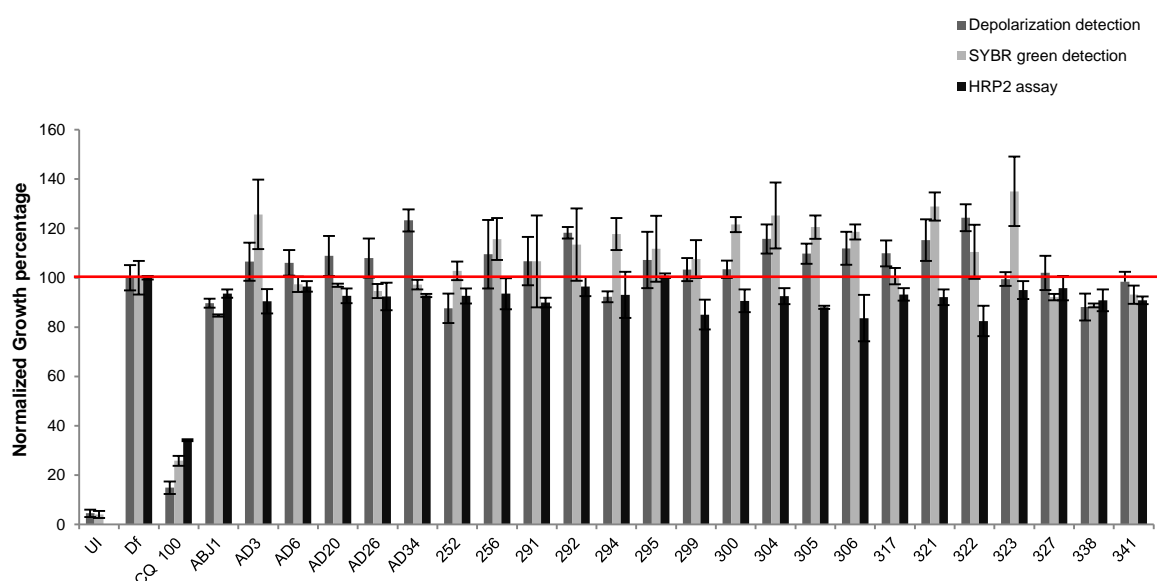


Figure 11 – Compounds activity tested at 1 μ M against *P. falciparum* 3D7.

Results from depolarization detection measured at 24 h, SYBR green detection measured at 48 h and the HRP2 assay measured at 72 h of incubation. The results are expressed as normalized percentage, relative to the drug free control considered to represent 100% maturation or complete replication. Legend: UI – Uninfected; DF – Drug free; CQ 100 – Chloroquine at 100 nM.

4.4 Compounds screening at 3 μ M against *P. falciparum* strain 3D7

To exclude some compounds that might have an inhibitory effect just above 1 μ M, another set of experiments were conducted, where all 24 compounds were tested at a higher concentration of 3 μ M against the *P. falciparum* 3D7 strain. The three sensitivity assays were performed.

Results presented in Figure 12, shows that most of the compounds even at 3 μ M have no inhibitory effect, regardless of the assay performed. The exceptions were the compounds 321 and 322. However, only compound 321 appeared to have an inhibition greater than 50%. To investigate this further, this compound (321) and two others were formally investigated to determine the IC_{50} value (next section – see point 4.5).

However, as already observed at 1 μM , there were some variations in the percentage of growth between methods. For instance, the compound 292 had a higher growth percentage in the depolarization assay than what was observed in the other two methods. Contrary to this, the compound 321, the inhibition effect appears to be less accentuated when using the HRP2 assay.

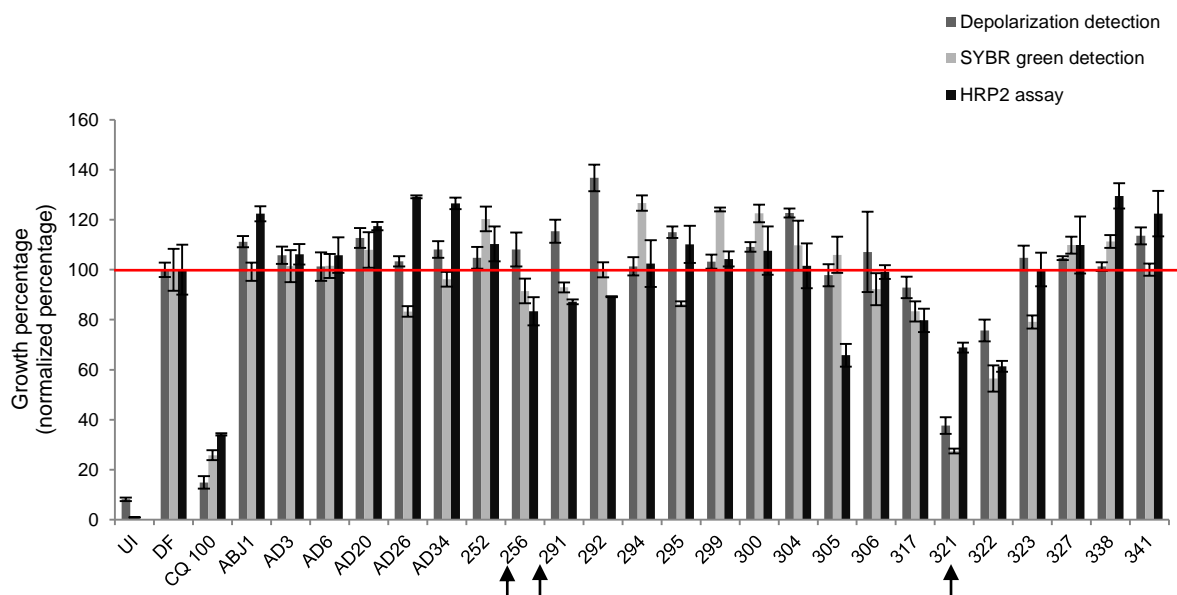


Figure 12 – Compounds activity tested at 3 μM against *P. falciparum* 3D7.

Results from depolarization detection measured at 24 h, SYBR green detection measured at 48 h and the HRP2 assay after 72 h of incubation. The results are expressed as normalized percentage, relative to the drug free control considered to represent 100% maturation or complete replication. Legend: UI – Uninfected; DF – Drug free; CQ100 – Chloroquine at 100nM. Arrows designate compounds for which an IC_{50} value was determined in subsequent experiments.

4.5 Inhibitory concentration (IC_{50}) values of compounds: 256, 291 and 321

Further experiments were performed to determine the IC_{50} values of three compounds, in particular to determine the IC_{50} of compound 321, which showed more than 50% inhibition in the depolarization assay at 3 μM (Figure 12). Apart from compound 321, two other compounds, which had not shown activity, were randomly chosen (256 and 291). The IC_{50} values are shown in Table 8.

Table 8 - IC_{50} values from 256, 291 and 321 compounds

IC_{50} (μM)	Depolarization detection assay	SYBR green detection assay	HRP2-ELISA
291	> 10	> 10	9.4
256	5	4.9	3.7
321	2	1.9	2.8

Legend: IC_{50} – 50% inhibitory concentration

HRP2 – Histidine Rich Protein 2

Compound 291 had the highest IC₅₀ value of >10 µM in the flow cytometry methods and just below 10 µM in the HRP2-ELISA. Compound 256 showed an IC₅₀ value of approximately 5 µM in the flow cytometry assays, and once again the value was slightly lower when determined by the HRP2-ELISA. Compound 321 which had shown inhibition at the 3 µM screening but not at the 1 µM screening had the lowest IC₅₀ values that ranged from 1.96 to 2 µM in the flow cytometric assays and, was interestingly slightly higher, with 2.8 µM, in the HRP2-ELISA.

4.6 *P. falciparum* 3D7 Gametocytes detection

Two different culture methodologies to obtain gametocytes were tested. Both were performed more than once, and analysed overtime by microscopic observation of culture blood smears and flow cytometry detection of depolarization and SYBR green.

4.6.1 Results Culture Method A

For each time point 100.000 events were acquired. The results presented in Figure 14 showed that the SYBR green detection, which corresponds to the parasitemia, increased until day four (9%), and then decreased on day five to 5%. In the following days, parasitemia started to progressively increase again until it reached 51% on day eleven. However, lysis of RBCs was observed from day four onwards, which may explain the observed decrease on day five, as well as the high parasitemia seen afterwards, as the total number of RBC was much reduced, as confirmed by FSC/SSC plots shown in Figure 13.

Concerning the detection of the depolarization, it started at 1.3% in the culture containing rings, trophozoites and schizonts, reaching a first peak at day four with 4%. On day five it decreased to 1.6%. In the following days the percentage of depolarization started to increase until 51% at day eleven. As already mentioned, on day eleven, there was evidence that the culture was mostly lysed (Figure 13).

In the depolarization plots (depolarized SSC/SSC) it is possible to observe, that events with a higher depolarization level started to appear over time (Figure 14 - B). Such degree of high depolarization is rarely observed in a standard continuous *in vitro* culture, even when there are mostly schizonts, Figure 14 - A. Thus, it appears to be a population with a higher degree of depolarization than schizonts which increases over time.

In blood smears it was also possible to confirm the presence of gametocytes in the culture, at day four and during the following days. In Figure 15, representative images of the blood smears show the depolarization of Hz within the parasites when polarization microscopy was used. By day nine some depolarizing agglomerates were observed as well as fewer red blood cells.

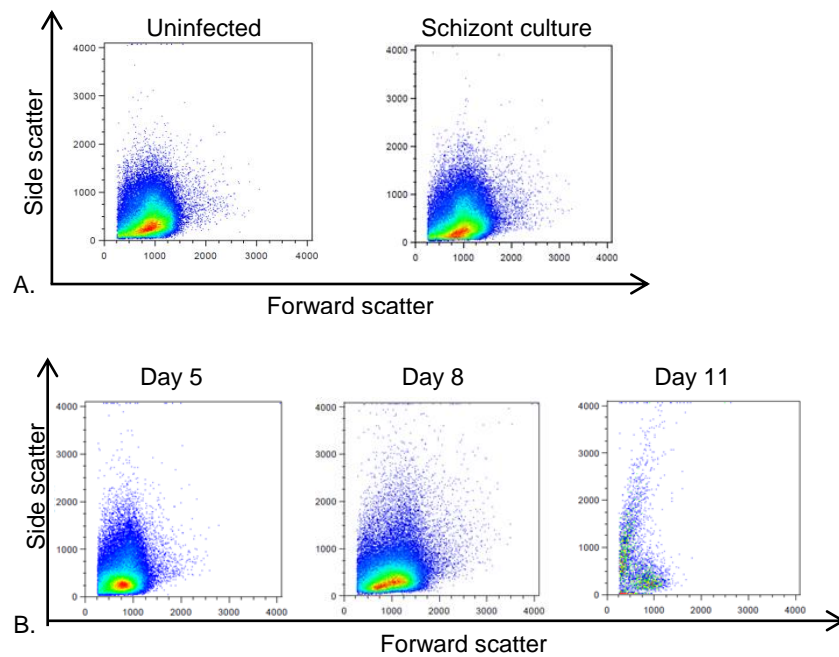


Figure 13 - Forward scatter/Side Scatter results of gametocyte cultures (method A).

A. Plots represents an uninfected RBCs sample and a continuous culture containing schizonts as comparison to B. the flow cytometric results of gametocyte cultures on days 5, 8 and 11. Forward scatter (x-axis) versus side scatter (y-axis). Note the disappearance of the main population on day 11, due to RBC lysis.

These results suggest that there are gametocytes in the culture, since they should have a higher level of depolarization.

However, to determine which cells are present in the higher depolarization gate, cells were sorted based on SYBR green fluorescence and according to their degree of depolarization. Results of this sorting experiment (Figure 16) show that gametocytes are indeed found exclusively in the upper depolarization gate. However, schizonts seem to appear in all three sorted populations, including those with the highest degree of depolarization where the gametocytes were also present. This was also corroborated by the fact that, observed in Figure 17, which shows the depolarization of each sorted population analysed in the Moflo, it is possible to find events from the medium and non-depolarizing gate in all sorted samples.

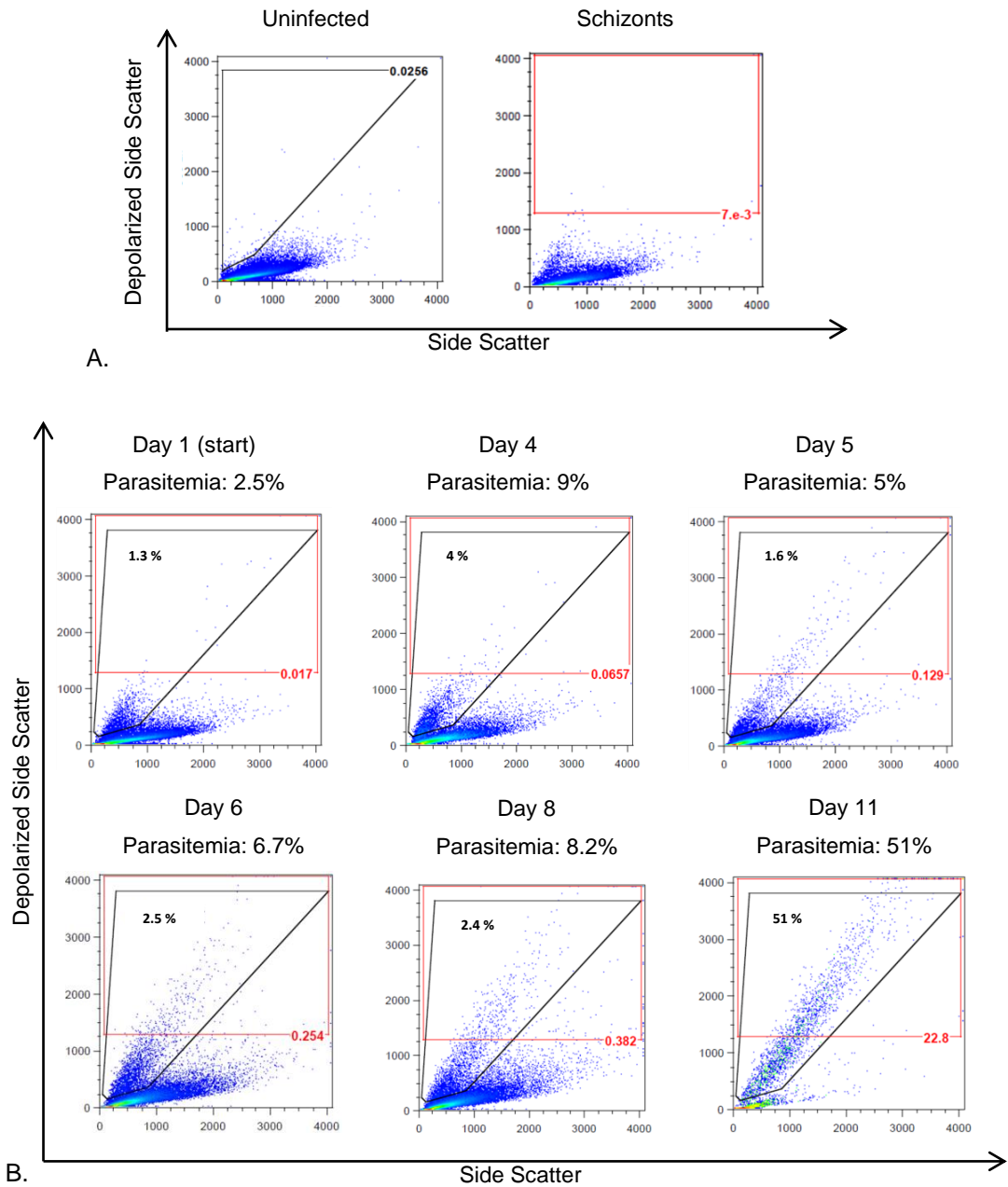


Figure 14 – Depolarizing events in a gametocyte culture over time (method A).

The black line gate selects for all depolarizing events and the red line gate represents events that depolarize at a higher level than a culture with schizonts.

A. Uninfected control and schizont containing culture to illustrate gating strategy. The uninfected sample was used to set the total depolarizing event gate. The synchronized schizont culture was used to define the gate where the higher depolarizing events would be present.

B. Representative plots of gametocyte cultures showing an increase of events in the highly depolarizing gate over time.

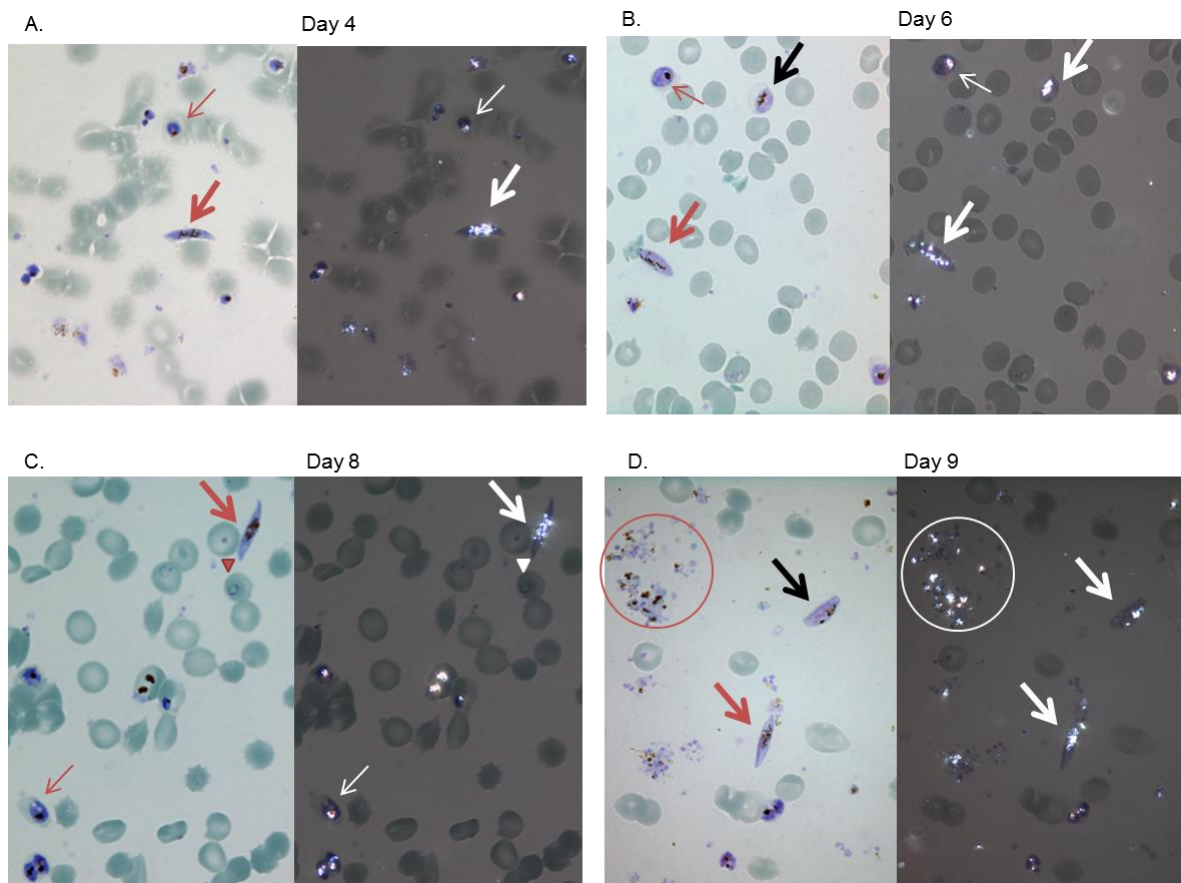


Figure 15 – Blood smears of a gametocyte culture (method A).

Representative images of a gametocyte culture on day 4, 6, 8 and 9. Ring forms (triangle arrows), schizonts (thin arrows) and gametocytes (thick arrows) are present as well as free Hz (within the circle). For each day a bright field microscopy image on the left side and a polarization microscopy image on the right are shown. Smears were stained with 10x Giemsa observed at an amplification of 1000x. In A. a gametocyte in stage III could be observed. In B. gametocytes in stage II (black arrow) and III (red arrow) could be detected. In C. a gametocyte in stage IV and in D. gametocytes in stage III (black arrow) and stage IV (red arrow) could be detected.

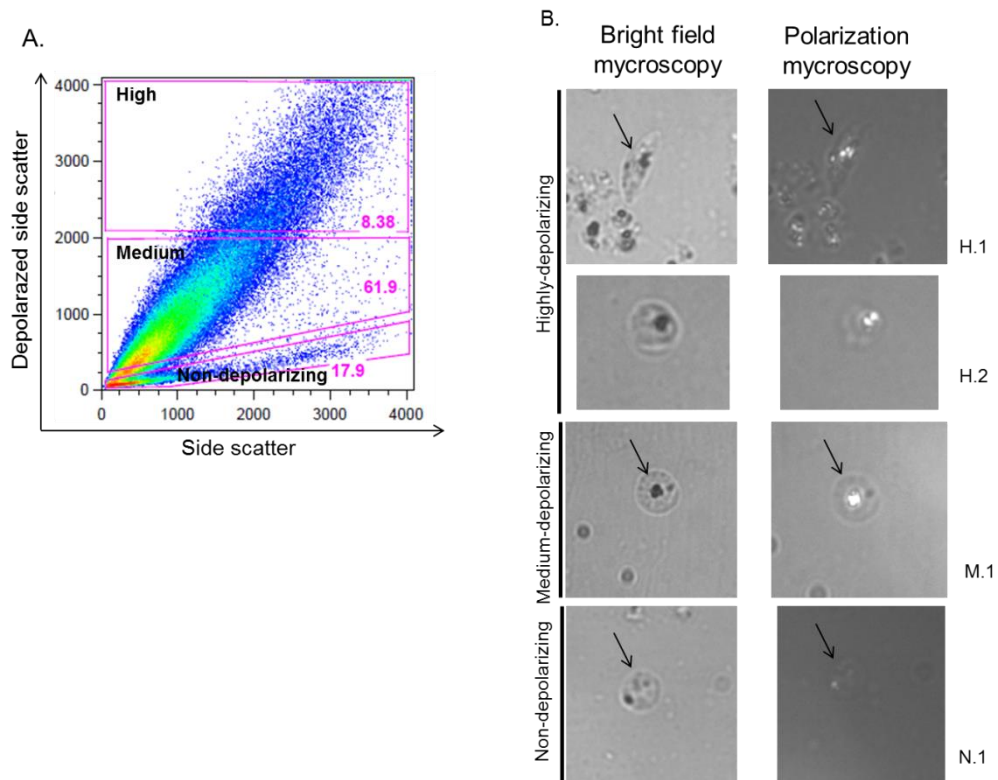


Figure 16 – Results after FACS sorting of gametocyte culture.

A. Side Scatter (x-axis) versus depolarized Side Scatter (y-axis). Gating strategy used for sorting different population based on their depolarizing degree: high (H), medium (M) and non-depolarizing (N) events. Cells were first selected for their positive SYBR green fluorescence signal.

B. Microscopy Images of parasites found in each sorted population. H.1 Gametocyte and H.2 schizont found in the high depolarizing population; M.1 Schizont found in the medium depolarizing population; N.1 Early schizont found among the non-depolarizing population.

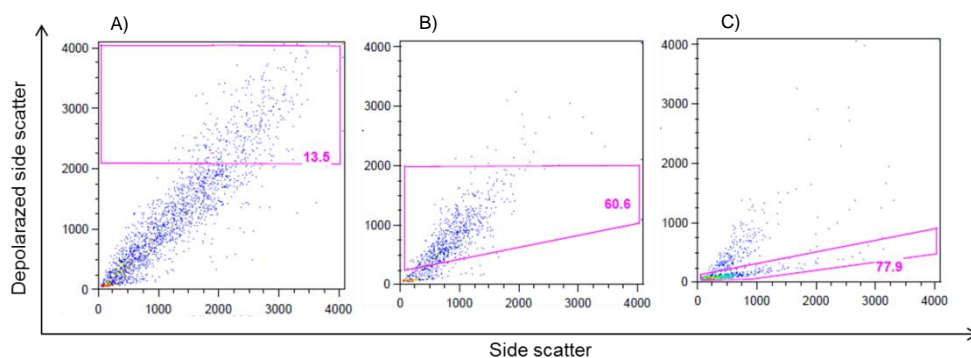


Figure 17 - Purity of sorted samples.

The sorted populations were immediately reanalysed using the same set-up and gating strategy.

A) Highly depolarizing events. B) Medium depolarizing events. C) Non-depolarizing events. The target population is present in the respective region; however, events with lower depolarization were also noted.

For example, highly depolarizing sorted sample also showed events with medium and no depolarization levels.

4.6.2 Results culture Method B

As an attempt to overcome the red blood cell lysis observed when using the method (A) described above, another gametocyte culture methodology was used. Daily flow cytometric assessment of the gametocyte culture was performed. Absolut counts were obtained to assess any decrease in the RBC population. Cultures were initiated after synchronization, representing day one. With this method we could not observe massive RBCs lysis until day thirteen (Figure 18). The flow cytometric evaluation of this gametocyte culture method (Figure 19) showed that at day four there were 1.6% depolarizing events. At the same day the culture reached a peak in parasitemia of 8 %. After day four both percentage of depolarization and parasitemia started to decrease.

However, it is possible to observe that on day five a small percentage of events with a higher depolarization than the schizonts appears and progressively starts to increase overtime.

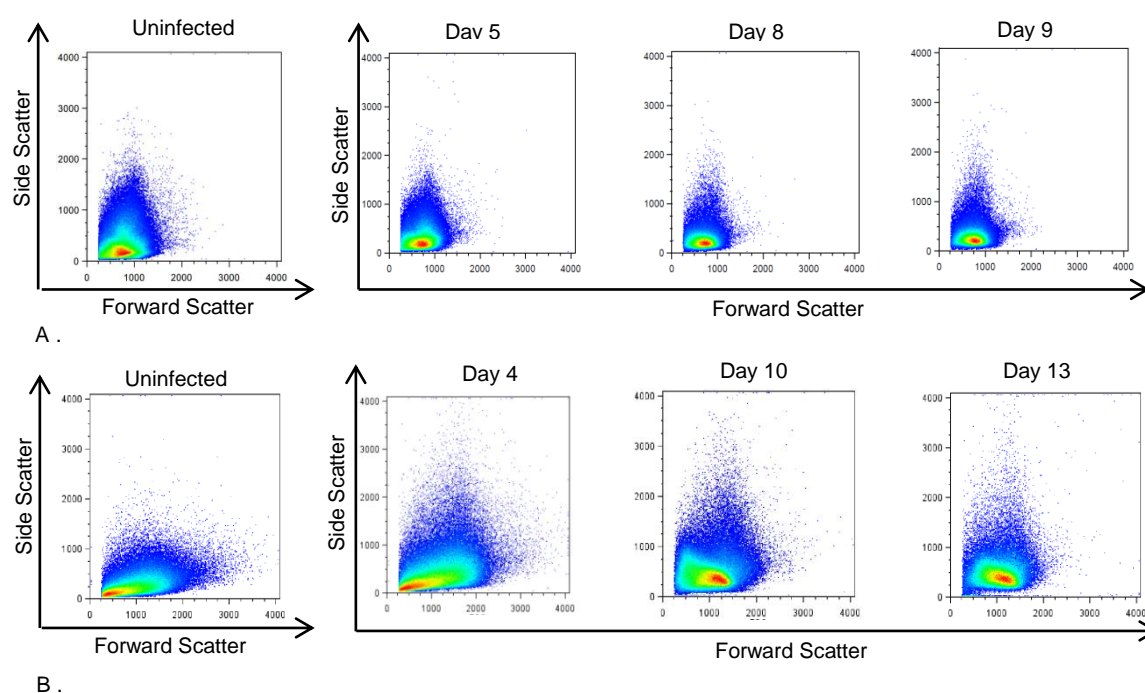


Figure 18 – Forward Scatter/Side Scatter results of gametocyte cultures (method B).

A. Forward Scatter (x-axis) versus Side Scatter (y-axis) results obtained until day nine, from the experiment which depolarization detection results are showed as well as the culture smears images. Flow cytometric results of gametocyte culture on days 5, 8 and 9. First plot on the left represents a population of uninfected RBCs assessed during the same experiment.

B. Forward Scatter (x-axis) versus Side Scatter (y-axis) results obtained until day thirteen, from other experiment. Flow cytometric results of gametocyte culture on days 4, 10 and 13. First plot on the left represents a population of uninfected RBCs assessed during the same experiment.

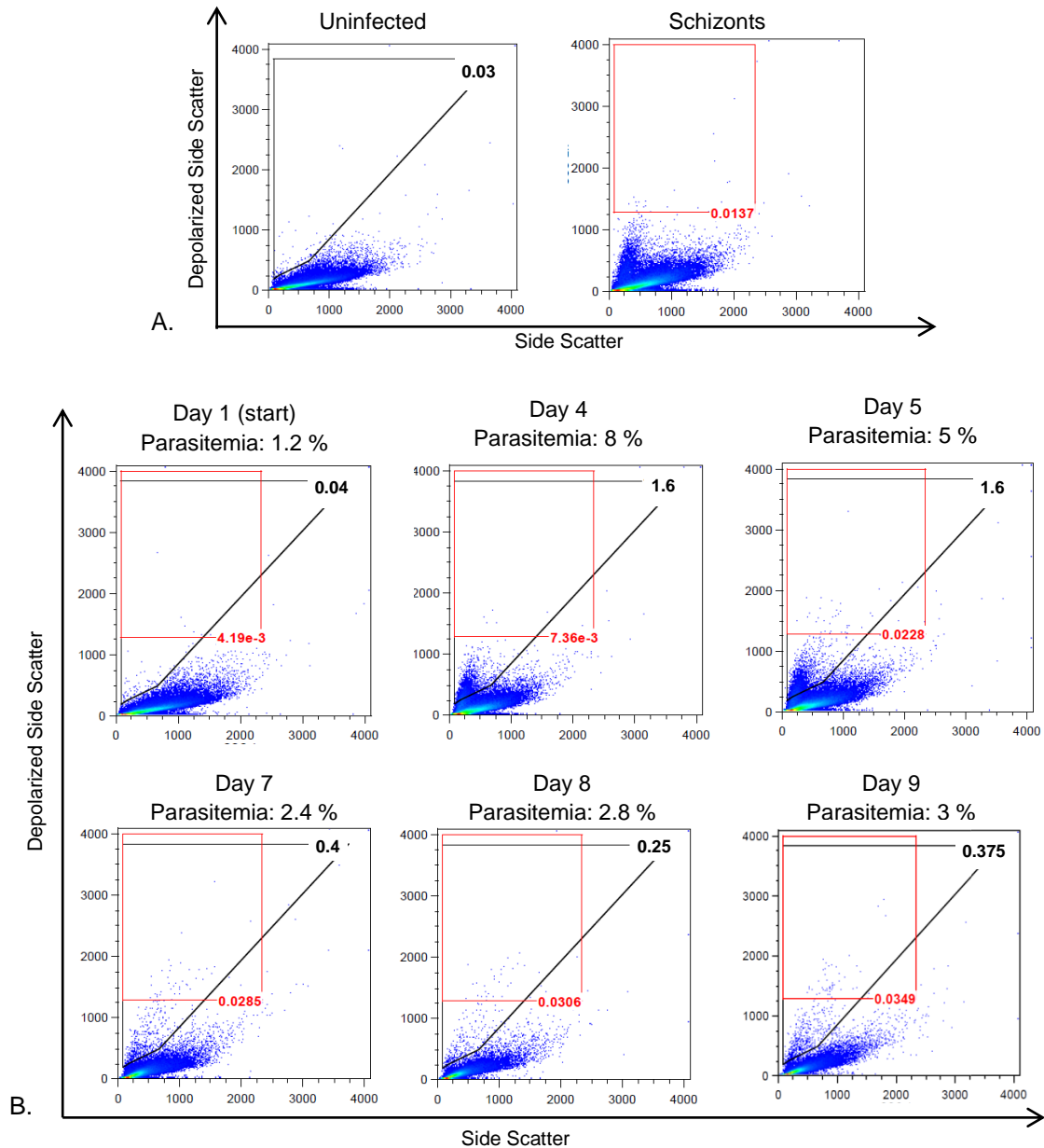


Figure 19 – Depolarizing events in a gametocyte culture over time (method B).

The black line gate selects for all depolarizing events and the red line gate represents events that depolarize at a higher level than a culture with schizonts.

A. Uninfected control and schizont containing culture to illustrate gating strategy. The uninfected sample was used to set the total depolarizing event gate. The synchronized schizont culture was used to define the gate where the higher depolarizing events would be present.

B. Representative plots of gametocyte cultures showing an increase of events in the highly depolarizing gate over time

Importantly, gametocyte culture method B did not present the depolarizing agglomerates that had been observed in method A. Probably, this was caused by the major red blood cell lysis in method (A), which possibly led to the release of Hz. Using method B there were not changes in appearance of the RBCs population up to day twelve, according to their light scatter properties and to what was observed by microscopy (Figure 20).

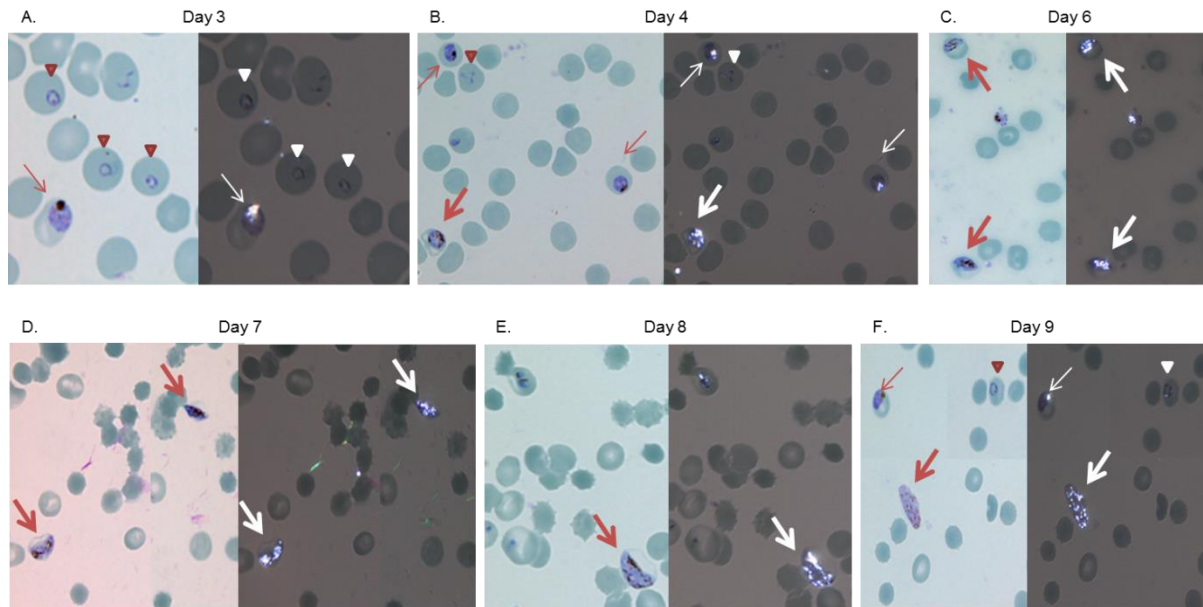


Figure 20 – Blood smears of a gametocyte culture (method B).

Representative images of a gametocyte culture show ring forms (arrows heads), schizonts (thin arrows) and gametocytes (thick arrows). For each day a bright field microscopy image (left side) and a polarization microscopy image (right side) are shown. Smears were stained with 10x Giemsa and observed at an amplification of 1000x. In B. a gametocyte in stage I could be observed. In C., D. and E. stage III gametocytes were detected, while in F. a mature gametocyte appeared.

5. Discussion

5.1 Drug sensitivity assays

Plasmodium falciparum has developed resistance to almost all antimalarial drugs, including to the artemisinins, which are the current first-line treatment of malaria. Thus, finding alternative compounds is most importance. According to the Malaria Eradication Research Agenda (malERA) ^[8] it is also important do discover/develop an antimalarial more suitable to our needs, meaning new drugs that prevent transmission and also relapse, but with better safety profiles than current ones.

To search for new antimalarial drugs, sensitivity tests are used to inquire/assess the effect of a compound in the parasite's growth. The available sensitivity tests used for *P. falciparum* include the WHO microtest, the hypoxanthine incorporation, the pLDH or HRP2 detection, and fluorescence detection of the stained parasite ^[36]. However, each of this assays have their limitations and/or drawbacks. Mostly, they take long incubation periods or require multiple incubation and washing steps. On the other hand, the microtest would not be suitable for compound screening since is very time consuming and it requires well-trained professionals. The hypoxanthine incorporation method implies to work with radioactive material. Thus, there is no ideal sensitivity test for *P. falciparum* yet.

Tough, a recent flow cytometric sensitivity assay has been proposed based on the detection of hemozoin (Hz) ^[49], due to its birefringent properties. Hz is the final bioproduct of heme digestion, and is accumulated inside the food vacuole of the parasite during its intra-erythrocytic maturation cycle ^[50]. The idea of using Hz for a sensitivity assay is not new and was described in the 1980s by Rieckmann, K. H. ^[62]. In 1999, a study using a flow cytometer based full-blood-count analyser, Cell Dyn (Abbott, Santa Clara, CA) ^[63], that could detected Hz within leucocytes was also reported. Other studies showed that the Cell-Dyn seemed to detect Hz inside parasitized red blood cells (pRBC) ^{[64], [65]}.

Due to the birefringent properties of Hz, which causes depolarization of light, it is possible to detect it inside red blood cells, using an adapted flow cytometer ^[54]. With this simple adaptation in one of the side scatters of the flow cytometer it is possible to detect the parasite within erythrocytes in a leucocyte depleted sample without using any stain or other reagent.

The recent Hz detection sensitivity assay was used as a screening tool for compounds provided by Prof. Dr. Rui Moreira, from Faculdade de Farmácia da Universidade de Lisboa (FFUL). The compounds come from a quinazoline derivative that inhibits nuclear factor kappaB (NF-κB). This quinazoline derivative was one of the screen hits of Derbyshire, E. R. *et al.* 2012 study ^[66]. Based on this quinazoline derivative, novel compounds were synthesized by Ana Ressurreição. These compounds had already been tested against liver stage parasites (*P. berghei*) and presented inhibitory activity, and some of them showed a low IC₅₀ against these forms. Therefore, they were tested against the blood stage *P. falciparum* parasites using the flow cytometric Hz detection assay.

5.1.1 *P. falciparum* growth in two different atmospheres (low and high oxygen)

Many factors like the initial parasitemia, the hematocrit, the time of incubation and the gas mixture, among other conditions, can influence the results of chemosusceptibility tests ^[67]. Often a more complex mixture, with low O₂ content is recommended. However, if results using a standard CO₂/ambient atmosphere incubator would give comparable results, this would facilitate any culture experiments. Thus, the impact of the atmosphere on *P. falciparum* cultures growth was evaluated.

The results showed that the obtained chloroquine (CQ) IC₅₀ values for each incubation atmosphere were not very discrepant from each other (Table 6). The IC₅₀ results were 53 and 65 nM for depolarization and SYBR green respectively in the higher oxygenated atmosphere and 41 nM for both methods in the low oxygen atmosphere. However, the low O₂ atmosphere showed lower values, although less than 1xIC₅₀ value. Interestingly, this is contrary to previous results. Briolant S. *et al* ^[68] observed no significant difference for the CQ IC₅₀ values for 3D7 under 5% O₂, 10% O₂ and 21% O₂ atmospheres, when using the hypoxanthine incorporation assay. Nonetheless, they observed that the parasites under 21% O₂ atmosphere increased the length of schizogony without lethal effect on the parasite. They also observed that the mature stages had a particular susceptibility to high O₂ concentration. In fact, the continuous culture used in our study was maintained in the high O₂ atmosphere (standard CO₂ incubator) and a subculture was transferred to the low O₂ atmosphere for the experiment. It is possible that this culture, not being adapted to the low O₂ may behave differently, because of the “stress” in the different atmosphere; and perhaps, if a culture that had been maintained in low O₂ the IC₅₀ results might be the same.

It is common for the IC₅₀ values to differ between assays, methods and laboratories, using the same strain (Table 7). However, all values are within the same order of magnitude, even though reported results differ from 6 nM up to 30 nM. The obtained CQ IC₅₀ values for both atmospheres were higher than the ones previously published when using other methods. However, because we used CQ only as a control to test that measurable inhibition would occur in the 3D7 strain, the difference may be less relevant. Likely, more meticulous attention to the preparation of CQ, constantly prepared fresh stock solutions, might have produced lower results.

After the determination of CQ's IC₅₀ we chose a concentration of around 2x the IC₅₀ = 100 nM, on the basis that this would produce a marked inhibition (> IC₅₀), yet not causing a complete inhibition (see Figures 11 and 12).

Because the results for the atmosphere were not highly discrepant, the compounds from FFUL were screened using a standard CO₂ incubator with a 21% O₂ atmosphere (ambient atmosphere).

5.1.2 Screening results

The compounds from FFUL were screened using two flow cytometry methods: the recent sensitivity flow cytometric assay for Hz detection and the SYBR green detection. To corroborate the obtained results with this novel method, compounds were also tested with the already validated HRP2 method (only for the 3D7 strain – see point 3.3.6).

It should be noted that currently used antimalarial drugs have IC₅₀ values in the low nM range, with CQ in the 10 - 30 nM, while drugs such as artemisinins may have IC₅₀s even below 1.0 nM [69], [70]. Having said this, a single concentration, like 1 μ M (=1000 nM), is often used as the screening concentration for novel compounds and it is rarely extended to 10 mM (value at which unspecific toxic effects may supplant specific antimalarial effects) [71]. As suggested by some researchers [72] [73], when a compound has activity at 1 μ M then the IC₅₀ should be determined, and ought to be ideally below 1 μ M. However, in our experiments none of the compounds had an effect at 1 μ M. This is not too unexpected, as the compounds were primarily designed to act against liver stages, yet they might have also showed inhibitory effects against the asexual blood stages. However, because some compounds had been screened before and shown some inhibition at 10 μ M, we proceeded to test all compounds also at 3 μ M concentration. Only one compound, 321, showed a major inhibitory effect at the higher concentration (see Figure 12 and Table 8).

5.1.3 *P. falciparum*: use of strains Dd2 and 3D7

The *P. falciparum* strains Dd2 and 3D7 were used for compound screening. There are a variety of *P. falciparum* isolated strains that can be used in laboratory research. They can be divided into chloroquine-sensitive, like 3D7, and chloroquine-resistant strains such as the Dd2 strain. Since different strains are genetically different, they can behave differently in the presence of antimalarial drugs, being relevant to test new compounds against more than one strain [74]. Certainly, one can argue to use the resistant Dd2 strain for screening to find compounds that would already act against *P. falciparum* forms, most of which are already resistant to CQ. On the other hand, using this strain may miss some compound leads which show activity only against CQ sensitive strains, the reason while many researchers use the 3D7 strain. In fact, because some compounds had been screened elsewhere before, using the Dd2 and had showed no activity, we decided to confirm these results first using the Dd2, and then repeating the screening process using the more sensitive 3D7 strain to make sure that no potential activity would be missed.

Therefore, two different strains were used to perform the screening assays, one chloroquine-sensitive (3D7) and the other chloroquine-resistant (Dd2).

5.1.4 Sensitivity assays: analysis of depolarization and SYBR green detection and HRP2-ELISA

The results for the Dd2 strain were obtained only by flow cytometric methods: the hemozoin depolarization detection and the SYBR green detection. In general, none of the tested compounds presented a marked inhibitory action against this strain. As it is possible to observe in the results, according to the SYBR green stain, which measures parasite replication, the parasite always grew more than 100 % in the presence of the compound. With the depolarization assay, which measures maturation, parasites also showed > 80% growth rate in the presence of the compound. Thus, with both assays it was possible to observe that the CQ control, at 100 nM, had no action against this Dd2 strain, which was expected because it is a CQ resistant strain, as mentioned above. However, one limitation of the Dd2 experiment is that an effective drug that

could inhibit Dd2 parasite growth, such as mefloquine, quinine or perhaps artemisinin should have been used as a positive drug control.

Then, all compounds were tested against the 3D7 *P. falciparum* strain. Overall, none of the compounds presented any marked inhibitory action at 1 μ M concentration with this strain, regardless of the used method. In fact, to validate the flow cytometry assay's performance, while testing the FFUL compounds against the 3D7 strain, the HRP2-ELISA was also performed. The parasite growth in the presence of the FF compounds was always above 80 %. According to the depolarization assay, in 18 tested compounds (75%) the parasite had a growth higher than 100%. With the SYBR green detection assay, parasite growth was also higher than 100% in 15 of the tested compounds (62.5%). However with the HRP2 method parasite growth was never higher than 100%. A possible reason for this larger variation on the Hz and SYBR green assay is, that both detect growth changes much earlier, and are more susceptible to small changes, while than the HRP2, which is based on the detection of HRP2 produced during a 72 h period. Thus, in the HRP2 assay variability may be less pronounced.

On the other hand, when comparing the results, it appears that the cytometric approaches may be more sensitive to detect small inhibitory effects. For example, the only evident active compound at 3 μ M (321) showed inhibition of 62% and 72% in the flow cytometric depolarization of Hz and SYBR green, respectively. However, in the HRP2 the detected inhibition was only of 39%. This could be explained by the fact that the background production of HRP2 over the long incubation period of 72h may mask some smaller inhibitory effects.

Thus, it appears that all three methods would have been useful to screen the compounds. Still the novel flow cytometric methods, despite a larger inter-compound variability may be more suitable to detect discrete inhibitory effects, which may be masked by assays with long incubation periods, such as the HRP2 assay ^[75].

Certainly according with the results, it is possible to conclude that none of the compounds would present an IC₅₀ value below 1 μ M, and consequently, none appears to be appropriate for further studies *in vivo* as an antimalarial drug. Though the compound 321 showed IC₅₀ values (Table 8) in the lower μ M range is too high to be therapeutically useful, but it may eventually allow pharmaceutical evaluation of this compound to investigate what makes this molecule more active than all the other ones.

Concerning the differences which are present between the used sensitivity assays further points have to be considered. First, the initial hematocrit was different between the flow cytometric approaches and the HRP2 assay as well as the initial parasitemia, which inherent due to the respective protocols. One consequence could be the inoculum effect ^{[49], [76], [77]}, due to different amounts of parasites being exposed to a specific drug concentration. Second, the parasite presence is measured at different times of its development in each method. In fact, Hz detection is performed during growth at 24 h, SYBR green measures replication at the end of the first cycle at 48 h, while HRP2 production is measured into the second cycle at 72 h only. Furthermore, in the flow cytometric methods, measurements are done on a single cell basis, and thus variation is much more influenced by the total number of cells which are analyzed (in our experiments 1000.000 cells

were analyzed per assay). On the other hand, with the HRP2 method, the parasite growth is measured on the whole population, because the detected HRP2 is present in the culture suspension. In fact, the number of parasites which are analyzed by each method can be calculated (Table 9). Doing this shows that the Hz detection assay only needs to analyze a fraction of the parasites to obtain results, although this also explains again, the higher variability which was observed.

Thus, flow cytometric methods are probably more favorable to subtle variations on the proportion of parasites detected/counted in each sample, relatively to the HRP2-ELISA. With the HRP2-ELISA, the total amount of protein produced by the parasites during the 72 h of incubation is detected, enabling a more global measurement of the parasite presence in each well.

Table 9 – Differences between methods

	Hemozoin detection	SYBR green detection	HRP2
Initial parasitemia	1 %	1 %	0.05 %
Hematocrit	2.5 %	2.5 %	1.5 %
Data acquisition	24 h	48 h	72 h
Detection	Single detection Cell by cell	Single detection Cell by cell	Whole population
Number of parasites contributed/analysed per assay*	~1000	4000-8000	~1.200.000

*calculation:

Hemozoin assay → 100.000 events analysed x ~1 % parasitemia = ~1000 parasites

SYBR green assay → 100.000 events analysed X 4-8 % parasitemia = 4000 – 8000 parasites

HRP2-ELISA →

Each well: { 1.5 % hematocrit ~ 120.000 cells/ μ L
200 μ L → ~24 million cells
0.05 % parasitemia → ~1.200.000 parasites
Note: 50 % hematocrit ~ 4 million cell/ μ L

Because the compounds are quinazoline derivatives that inhibit the NF- κ B activation, they are likely to interfere with DNA synthesis (transcription) ^[78]. The drugs that inhibit DNA synthesis are suitable to inhibit cells or microorganisms with a fast replication rate. However, *P. falciparum* has a rather slow replication rate, 48 h before a new cycle starts. Thus, it may be that the compounds exhibit no discernable action or only slight inhibitory action, as seen in this work.

Overall, the results from the screened compounds appear to agree between the used methods. Moreover, it is possible to affirm that none of the tested FFUL compounds have inhibitory action at a suitable antimalarial concentration, in the nM range. However, with the recently developed sensitivity assay, based on the flow cytometric detection of Hz, it was possible to obtain comparable results in 24 h, based on the analysis of just <1000 infected RBCs.

The Hz detection assay can detect inhibitory action of drugs quickly and early, specially those that inhibit Hz formation ^[49]. On the other hand, slow acting drugs effect, like pyrimethamine,

an antifolate drug that interferes with DNA synthesis, is usually only detected in the second generation^[49]. However, analyzing the results obtained with compound 321 and assuming that this action is also based on DNA inhibition, the Hz assay reliably detected this inhibitory effect already after 24 h of incubation, as compared with the already validated HRP2 assay.

However, potential drawbacks of this novel method is that it is not possible to use low starting parasitemias as the ones used the HRP2-ELISA, because the lower detection limit has been reported to be around 0.3 % parasitemia^[49]. Another downside of this method is related with the fact that, although it detects inhibitory action of novel compounds, it is not yet well suitable for the high-throughput screening of compounds since the flow cytometric acquisition of samples is time consuming. Commonly, antimalarial drugs screening is based on the IC₅₀ determination for each tested compound^{[72], [73]}. However, without an improvement in the way samples can be acquired in the flow cytometer, the determination of IC₅₀ values for a large scale screening would be difficult.

Nevertheless, flow cytometry offers the opportunity to provide more information about malaria parasite growth and development than most other currently available methods. The hypoxanthine incorporation, the ELISA based assays and the indicators of DNA quantity assays (fluorometric) all have the same shortfalls which are that they cannot detect easily stage specific effects^[44].

5.2 Gametocytes detection





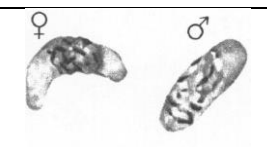
During the blood cycle of *P. falciparum*, a few merozoites differentiate into gametocytes. Environmental factors, normally a condition which constitute some kind of “stress”, influence the commitment to sexual development of the parasite. Factors like host immunity, anemia, drug treatment among others can be considered to trigger gametocytogenesis^[59]. In the gametocyte maturation there are five characteristic stages: I, II, III, IV and V, (Table 10)^[59]. During this maturation process Hz is also formed and accumulated inside gametocytes, however its distribution is rather different to the one observed in asexual forms (Figure 4).

Because gametocyte inhibitory drugs are needed, sensitivity drug tests for the screening of compounds against these forms are also required. Currently available assays are highly complex and difficult to perform. The gametocyte drug screening assays can be divided into four categories: those that assay gametocytogenesis^{[79], [80]}; those that focus on gametocyte development^[81]; those that assay for lethal activity against the mature gametocyte, either with an ATP production readout^{[82], [83]}, or stage-specific GFP expression^[81]; and finally those that assay the onward development of mature gametocytes^[84].

Because Hz pigment is not only abundant in gametocytes of *P. falciparum*, but it also has a different distribution compared with asexual forms, the assumption was that this novel sensitivity assay based on the Hz detection could also be applied to detect gametocytes. *P. falciparum* gametocytes particularly have the Hz crystal not organized into a single aggregate, as it is usually seen in mature schizonts, but rather in many smaller, dispersed fragments. Because side scatter is a measure of granularity/dispersion of particles, this would predict that the Hz crystals in

gametocytes would present a different profile in the depolarized side scatter as compared to other asexual forms. Due to this characteristic it was expected that gametocytes would be found in the depolarized side scatter showing a higher level of depolarization. However, before this detection it was necessary to test culture conditions which would produce sufficient number of gametocytes.

Table 10 - Gametocyte maturation stages: characteristics and images

Stage I	IA: Indistinguishable trophozoite. IB: Larger round shape, distinguished by granular distribution of pigment in food vacuole	
Stage II	IIA: elongates within the erythrocyte IIB: D shaped	
Stage III	D shaped, slightly distorted erythrocyte Pink/blue distinction of the male/female gametocytes.	
Stage IV	Elongated and thin parasite, distorted red cell. Male gametocyte: pigment tends to be scattered; in female gametocytes: pigment more dense	
Stage V	Sausage shaped parasite with rounded extremities. Male gametocyte: pigment scattered, pink aspect (Giemsa stain) Female gametocyte: dense pigment, light violet	

Based on Talman, A. M., *et al.* 2004^[59], and Carter, R. and Miller, L. H., 1979^[65]

5.2.1 Gametocytes cultures

Different techniques to culture gametocytes have been described^[86]. Two different ways to culture and obtain purified *P. falciparum* 3D7 gametocytes were used in this study. One based on the procedure described in Methods on Malaria Research^[87] (method A) and another based on Roncalés, M. *et al*^[86] research (method B). The cultures were followed on a daily basis using microscopy smears and flow cytometry evaluation.

Results showed that, although the described protocols affirm that usually the peak of mature gametocytes tend to appear only around days 14-15, it was possible to observe and detect them earlier. However, important differences can be observed between the methods used in this study. For instance, with the culture method A, massive RBC lysis occurred around day eleven, well before the time when the peak of mature gametocytes is supposed to be present. Even more, with this method, the cultured parasites seems to die, because at the end (day eleven) very few parasites and red blood cells were present (Figure 13). However, many free depolarizing agglomerates were observed in depolarization microscopy, and these tended to group together. Therefore, these clusters had the aspect of free Hz crystals and clustering would not be too surprising, because Hz crystals are known to have very strong adherent properties^[88] and thus, to stick together.

Consequently, due to the great amount of free Hz that accumulated in the culture over time, interference with the depolarized side scatter would be expected. In fact, great number of depolarizing events was detected in the depolarized side scatter.

Then, to improve the purity of gametocyte stages in a culture, another method was tried (method B). With this method it was possible to maintain the culture for a longer period without major RBC lysis, giving time for the gametocytes to mature through all the five stages. It was also possible to detect a higher population of events in the depolarizing side scatter which had never been observed in previous schizont cultures (Figures 14 and 19). However, with this culture method the amount of depolarizing events, especially in the highest region, is much less than the previous method. Still, this may confirm our notion that most events which were detected in the previous culture condition were in fact free Hz, as explained above.

The way gametocytes were cultured need to be improved or other methods have to be established so that it is possible to have more gametocytes in culture, before one can think about drugs that could be tested against the gametocytes stages. This relates to the fact that different compounds act in different stages of commitment of the gametocyte, and with different impact on the sex of gametocytes^[89]. Ways to improve gametocytes culture comprise the use of recently isolated strains that, according to several authors are more likely to form gametocytes than strains that have been in culture for long periods of time. Another way may be the use of *P. falciparum* NF54 strain, since different strains present different capacities to generate gametocytes^[86].

5.2.3 Detection of gametocytes using side scatter depolarization

Gametocytes were obtained and were present in culture using both methods. By day 6, a Fluorescence-activated cell sorting (FACS) was preform to investigate if the depolarizing characteristic of the gametocytes would be found in the highest depolarizing region. In fact, gametocyte stages, although in very low numbers, were only found in high depolarizing population. However, other parasite stages like schizonts were also found in the same population. Unexpectedly, since all events had been selected on their SYBR green positivity, agglomerates were also found in the sorted population, meaning that they should have DNA attached. In fact, it is known that Hz has strong absorptive properties and can bind DNA easily, so it is possible that these Hz agglomerates had free DNA bound to them^[88].

Schizonts were also found in the middle and non-depolarizing gates (Figure 16). This relates to the purity of each sorted population. As it is possible to find in the figure of population purity, the sorted upper population does not only depolarize in a restricted higher region but along the depolarization detection axis. In the sorted population from the non-depolarizing gate, schizont forms were also found, and once again, according to the purity data, a small group of events seem to depolarize.

The FACS sorting was performed using a Moflo flow cytometer due to the fact that it is one of the few flow cytometry sorters which can be set up to detect depolarized light scatter. Other sorters, like FACSAria do not detect depolarization, given that the light travels to the detectors through fiber-optical cables, and the polarization of the incident light beam is lost. However, to use

the MoFlo sorter, the gametocyte culture sample had to be fixed for safety reasons. Fixing a culture implies that red blood cells become autofluorescent, contrary to the unfixed RBCs^[44]. This could introduce additional difficulties when choosing the SYBR green positive population. However, this effect was negligible in our previous experiments.

It is also true that the ratio of parasites that commit to differentiation into gametocytes is very low^[90]. Thus it is not unlikely that the few depolarizing events which were observed are indeed gametocytes. However, only performing sorting after growing gametocytes in sufficient numbers and confirming that they are only present in the higher depolarizing population would definitely proof this concept. Of note, using nucleic acid double staining it might be possible to corroborate this hypothesis as well. Combining Hoechst 33342, a DNA stain, with a RNA selective stain like Pyronin Y, we could eventually detect the gametocytes nucleic acid profile, since in the gametocyte development, probably there is no DNA replication but RNA synthesis^{[61], [91]}.

Overall, in this first approach it was possible to confirm the basic hypothesis that gametocytes can be found in a specific region based on their depolarization side-scatter, which is a consequence of their particular Hz distribution.

6. Conclusion

Using the novel flow cytometric method based on the detection of infected red blood cells containing Hz, inhibitory effect of several new antimalarial drugs could be assessed as reliably as the two other performed assays (SYBR green staining and HRP2-ELISA). This screening allowed concluding that none of the compounds had an inhibitory activity below 1 μM concentration. Though, from the 24 screened compounds there was one (321) with an IC_{50} around 2 μM , once more in agreement with the three sensitivity assays performed.

Using this adapted flow cytometer to detect Hz also enabled to detect gametocytes, according to their depolarization properties. In fact, gametocytes had a higher depolarized side-scatter signal, since these forms have several, small and dispersed Hz crystals. However, additional work is required to improve the gametocytes culture method so higher numbers of gametocytes can be obtained. After this, gametocytes could be treated with different drugs, including newly synthesized compounds, thus establishing a novel *in vitro* sensitivity assay for transmission blocking drugs.

7. References

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