doi: 10.5123/S2176-62232010000300015

Methods for assessment of antimalarial activity in the different phases of the *Plasmodium* life cycle

Métodos para avaliação da atividade antimalárica nas diferentes fases do ciclo de vida do *Plasmodium*

Métodos para evaluación de la actividad antimalárica en las diferentes fases del ciclo de vida del *Plasmodium*

Fátima Nogueira
Unidade de Ensino e Investigação da Malária, Centro de Malária e Outras Doenças Tropicais, Laboratório Associado, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, Portugal

Virgílio Estólio do Rosário
Unidade de Ensino e Investigação da Malária, Centro de Malária e Outras Doenças Tropicais, Laboratório Associado, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, Portugal

ABSTRACT

Malaria is a mosquito-borne disease caused by parasites of the genus *Plasmodium*. In humans, parasites multiply in the liver and then infect red blood cells. The *Plasmodium* life cycle consists of a sexual phase in the mosquito vector (sporogony) and an asexual phase in the vertebrate host (schizogony); both life cycle phases can be detected in assays. In general, *in vivo* and *in vitro* are the two basic approaches routinely used to evaluate the antimalarial activity of compounds. The antimalarial activity measured in an *in vivo* test results from a variety of factors associated with both the parasite and the host. Conversely, *in vitro* tests reflect more accurately the "isolated" effects of the compounds on parasite metabolism. *In vivo* assessment of antiplasmodial activity can be achieved using rodent models and by assessing transmission-blocking activity using mosquitoes. There are several *in vitro* tests for the assessment of antimalarial activity based upon observation of parasite development in blood cells through thick films, isotopic assays, quantification of parasite proteins and DNA dye intercalation assays. Besides antimalarial activity, a promising antimalarial compound should also lack toxicity to host cells; the degree of selectivity of a compound towards the malaria parasite includes such assessment. In this manuscript, we intend to summarize the most frequently used methods for assessing the antimalarial activity of compounds in the different stages of the *Plasmodium* life cycle.

Keywords: Malaria; Drug Resistance; Antimalarials; Cytotoxicity Tests, Immunologic; *Plasmodium*.

INTRODUCTION

Approximately 300 million people worldwide are affected by malaria and between 1 and 1.5 million people die from it every year. Previously extremely widespread, malaria is now mainly confined to Africa, Asia and Latin America. Malaria species predominate with different prevalence depending on geographic region; for example *Plasmodium vivax* is more prevalent than *P. falciparum* in Latin America. In general, the absence of a vaccine and the problem of drug resistance has hindered many malaria control programs.

Correspondência / Correspondence / Correspondencia:
Fátima Nogueira
Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa
Rua da Junqueira, nº 100
CEP: 1349-008 Lisboa-Portugal
Tel: 00351213652600
E-mail: fnogueira@ihmt.unl.pt

PLASMODIUM LIFE CYCLE

Malaria infects both a vertebrate and an invertebrate host. Mosquitoes ingest *Plasmodium* gametocytes in a blood meal from an infected vertebrate host, and the gametocytes then develop into infectious form (sporozoites) within the salivary glands of the mosquito. Sporogony in the mosquito takes approximately 10 - 20 days, and thereafter the mosquito remains infective for 1 - 2 months. The schizogonic phase starts with the inoculation of the parasite into the blood of the vertebrate host by the bite of a female *Anopheles* mosquito. In a short period of time, the sporozoites invade liver cells through a complex cycle[1] and initiate schizogony, which has two stages, the hepatic stage (pre-erythrocytic schizogony) and the erythrocytic stage (erythrocytic schizogony). Within hepatocytes, the trophozoites start their intracellular asexual division. At the completion of this stage, thousands of exo-erythrocytic merozoites are released from each infected hepatocyte. The time taken for the completion of the tissue phase is variable and depends on the infecting species: 8 - 25 days for *P. falciparum*, 8 - 27 days for *P. vivax*, 9 - 17 days for *P. ovale*, 15 - 30 days for *P. malariae* and 9 - 12 days for *P.*

knowlesi[2]. This interval is called the pre-patent period. In the case of *P. vivax* and *P. ovale*, some sporozoites may go into latency, also known as the cryptobiotic phase, where they are called hypnozoites. They can lie dormant for months or years, and upon reactivation they cause clinical relapse. The merozoites released from the hepatocytes enter red blood cells and start asexual division, developing through the stages of rings, trophozoites, early schizonts and mature schizonts. Each mature schizont consists of thousands of erythrocytic merozoites. These merozoites are then released by the lysis of the erythrocyte, and they immediately invade uninfected erythrocytes. This repetitive cycle of invasion – multiplication – release – invasion continues. It synchronously occurs as the merozoites are released at approximately the same time, resulting in the characteristic clinical manifestations of the disease. A small proportion of the merozoites in the erythrocyte undergo transformation into gametocytes, male and female, and the full cycle will be reinitiated if another female mosquito feeds on the infected vertebrate host.

ASSESSMENT OF ANTIMALARIAL ACTIVITY

In general, two basic approaches are routinely used to study or measure antimalarial activity of compounds: a) *in vivo* and b) *in vitro*. These can be complementary, as data obtained from *in vitro* assays require confirmation from the use of live models.

A – An *in vivo* test is associated with animal models and their compulsory ethical issues. In these assays, one measures mainly a) the clearance of parasites as detected by optical microscopy or other more sensitive methods (polymerase chain reaction – PCR), b) the time that elapses between last drug dose and clearance of parasitemia, and c) the drug dosage that clears parasites in a dose response manner.

When adjusted to humans, *in vivo* studies usually represent the following of a selected group of symptomatic and parasitemic individuals that underwent carefully controlled treatment with subsequent monitoring of the parasitological and/or clinical responses over time. Diminished therapeutic efficacy of a drug/compound can be masked by immune-mediated clearance of parasites among hosts with acquired immunity. When performed with human subjects, *in vivo* tests most closely reflect actual clinical or epidemiological situations; that is, the therapeutic response of currently circulating parasites infecting the actual population in which the drug will be used.

The screening of antimalarial activity of new compounds follows standard procedures*. Non-human primates have provided experimental models for *P. falciparum* malaria[3]. Nevertheless, the use of this animal model has been difficult because of the economic and ethical considerations, a limited supply of monkeys, the narrow range of parasite lines that are adapted to primate infection and the different pathology that is seen in these models[4,5,6]. Consequently, most *in vivo* experimental studies on malaria have relied on different combinations of various murine strains and *Plasmodium* spp. of rodents[7,8]; however, the biological differences between parasite species must be taken into account[9].

External factors, such as host immunity, variations of drug absorption and metabolism, are reduced by the use of naive animals from well-known lines in the experiments. Also, potential misclassification of re-infection is not an issue, as assays use direct inoculation of blood stage parasites in well-protected animal house surroundings. However, antimalarial activity measured in an *in vivo* test does not always consider relevant side factors, such as metabolism and host genetics, which can affect the outcome of the experiment.

B – *In vitro* tests avoid some of the confounding factors listed above by removing parasites from the host and placing them into a controlled experimental environment. In the most frequently used procedure, *P. falciparum* parasites, obtained from culture or a human patient, are exposed to precisely known quantities of a drug or compound. Then the inhibition of parasite maturation is monitored to examine a dose dependent effect of the drug in preventing parasite development from ring into schizont. For assays related to sporozoite infectivity, *P. berghei* sporozoites in a HepG2 liver cell culture are usually used to study the drug dose effect on schizont maturation. The correlation of *in vitro* response data with clinical response in patients is not consistent, as clinical cure is affected by the patient's immune system. Another example of the limitation of *in vitro* assays is the use of prodrugs, such as proguanil, which requires host conversion into active metabolites. Although adaptation of erythrocytic forms of *P. vivax* to continuous culture has been achieved, their use in assays is complex and infeasible. In general, these tests are demanding and relatively expensive, but innovations are being implemented which may make them easier to adapt to routine field-work and make them more efficient as high-throughput tools for drug activity screens.

ASSESSMENT OF ANTIMALARIAL ACTIVITY OF COMPOUNDS DURING ERYTHROCYTIC STAGE

Antimalarial drug screening and validation is relatively time consuming and complicated. The first phase of drug screening is usually comprised of two steps. The first step involves the use of whole cell assays for the determination of the effect of the compound on the growth of the human parasite *P. falciparum in vitro*. The second step involves the testing of the *in vivo* efficacy of selected drugs in small animal models of malaria, mainly using the rodent parasites *P. berghei*, *P. yoelii* and *P. chabaudi* in laboratory mice.

PARASITE CULTURE

Most approaches to *in vitro* drug-sensitivity assays are based on the culturing of malaria parasites in erythrocytes, which was developed in the 1970s[10,11]. This development

* World Health Organization. Assessment of therapeutic efficacy of antimalarial drugs for uncomplicated falciparum malaria in areas with intense transmission. Geneva: World Health Organization; 1996. Available at: http://www.who.int/drugresistance/malaria/en/Assessment_malaria_96.pdf.

allowed for the removal of the parasite from the patient's bloodstream and its transfer into a highly controlled laboratory environment. It also allows research in non-endemic regions where there is no supply of fresh parasite samples. Short-term culture of *P. falciparum* parasites is fairly simple and requires little laboratory equipment, and it can be easily performed under field conditions. Although continuous culture poses considerably more technical and logistic challenges, it has nevertheless become an essential tool in laboratories working on malaria drug development and basic research. Together, with the capability to cryopreserve live parasites[12], culturing provides the basis for studies that require high parasite densities (proteomics) and for cloning. Although *P. falciparum* is asynchronous during *in vitro* culture, artificial synchronization (Figure 1) of erythrocytic stages of the parasite in culture is useful for studies searching for erythrocytic stage malaria vaccine candidates.

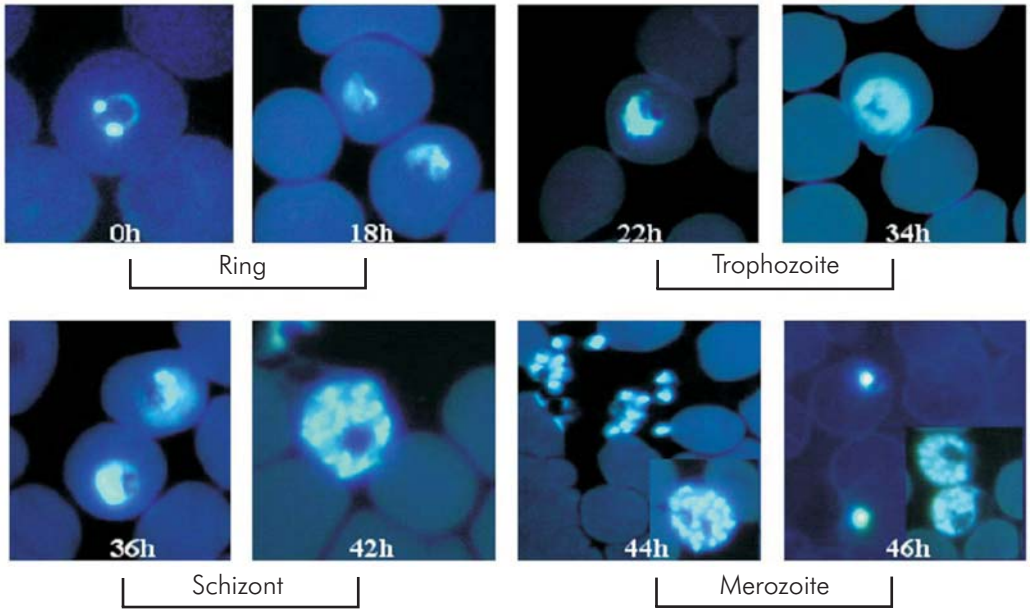


Figure 1 – *Plasmodium falciparum* intraerythrocytic life cycle: stages of development. *In vitro* sorbitol-synchronized culture parasites stained with DAPI (4',6-diamidino-2-phenylindole) and photographed under UV light in a fluorescence microscope (1,000×)

IN VITRO TESTS

The main objective in drug-sensitivity testing is to evaluate or measure the parasite (*P. falciparum*) sensitivity to increasing drug dosages *in vitro*; data are presented as percentages of parasite growth compared to untreated cultures. This approach allows for an almost complete exclusion of interfering host-related factors, such as host immunity and host metabolism of the compound, thus offering a direct evaluation of drug impact. *In vitro* tests are basically without risk to the patient, are non-invasive (apart from the collection of a blood sample), and are crucial for drug development. *In vitro* tests for screening of antimalarial activity generally utilize the method of continuous culture developed by Trager and Jensen[11] and the microculture technique developed by Rieckmann et al[13]. The short-term *in vitro* culture of freshly collected field isolates of *P. vivax* and *P. ovale* has also been established[14,15], also allowing for the assessment of antimalarial activity of different drugs on these plasmodium species.

In terms of drug screening, several *in vitro* methods are commonly used for the assessment of *P. falciparum* growth inhibition: a) schizont counting on thick films, known as the World Health Organization (WHO) Schizont Maturation assay[16]; b) incorporation of a radiolabeled nucleic acid precursor (e.g., radiolabeled hypoxanthine) known as the isotopic assay[17,18]; c) the quantification of parasite proteins histidine-rich protein 2 (HRP2) and lactate dehydrogenase (LDH) by enzyme-linked immunosorbent assay (ELISA)[19,20]; and d) DNA dye intercalation assays[21,22,23,24].

Schizont counting on thick films

This drug sensitivity test is based on parasite counts following the evaluation of the morphology of parasite growth (rings into schizonts) by light microscopy. The WHO Mark III microtest assay (Table 1) is a low-cost alternative standardized by the WHO*. Experienced microscopists should carry out this test, as it is labor-intensive and prone to individual variability. It is based on the maturation of *P. falciparum* in a 24 to 36 h microculture, by microscopically counting the number of parasites that develop into schizonts (i.e. parasites with three or more chromatins) in Giemsa-stained thick films. Despite these drawbacks, the WHO assay is economical and simple to perform in the field. A number of published reports from Brazil have utilized this technique[25,26,27,28].

* World Health Organization. Drug resistance: malaria. Available at: http://www.who.int/drugresistance/malaria/en/.

Table 1 – Comparison of the most common assays for the measurement of the drug sensitivity of intra-erythrocytic stages of *Plasmodium falciparum* parasites *in vitro* (DELI, double-site enzyme-linked LDH immunodetection; HRP2, histidine-rich protein 2; pLDH, parasite lactate dehydrogenase; FACS, fluorescent-activated cell sorting). The values high, moderate, and low, indicate relative levels between the different assays)

	WHO Mark III microteste	Isotopic assay	pLDH enzymatic assay	pLDH DELI assay	HRP2 assay	DNA dye assay*
Sensitivity	high	moderate	low	high	high	high
Culture time	24 h	42 h (up to 72 h)	48 h	48 h	48 h - 72 h	72 h
Initial parasitemia	0.6 - 0.8%		0.5%	0.5%†	0.01 - 0.1%	0.05 - 0.8%‡
Assessment of growth inhibition	microscopy (schizont maturation)	incorporation radiolabeled precursors	enzymatic activity of pLDH	ELISA	ELISA	fluorescente
Demands on equipment	low (microscope)	high (scintillation counter)	moderate (plate reader)	moderate (plate reader)	moderate (plate reader)	moderate (fluorimeter)§
Labour involved	high	low	low	moderate	low	low

* Most used dyes: SYBR Green I, Pico green, DAPI and YOYO-1; † Parasite densities as low as 0.005% can be tested; ‡ In the case of YOYO1, detection is performed in a fluorescent-activated cell sorting (FACS) device which is quite expensive; § Depending on the authors and dye used.

Isotopic assays

The radiolabeled hypoxanthine incorporation assay (Table 1) has been used as the gold standard in *P. falciparum* drug susceptibility testing[18]. As an alternative to using radiolabeled hypoxanthine, Elabbadi et al[29] proposed ethanolamine, which has the major advantage that the culture medium can be supplemented with hypoxanthine to improve parasite growth. Several other precursors (e.g., palmitate, serine, choline, inositol and isoleucine) have also been suggested for use in isotopic assays[29]. However, regulations regarding the handling of radioactive materials have become considerably more restrictive, aiming to limit the application to domains where there is no alternative methodology available. Another limitation is the high purchase cost of the necessary equipment, such as liquid scintillation counters and harvesting machines. In addition, the relatively high parasite densities required for this test (approximately 0.5%) limits its application to the use of culture-adapted parasite strains or field samples with adequately high parasitemia. These limitations make it costly and difficult to routinely use in research and clinical settings, particularly in resource-limited environments.

Quantification of parasite proteins

A method that is simple to establish, highly reproducible, requires little technical equipment and could be applicable to a field laboratory is the ELISA. A few commercialized ELISA tests are already available, targeting *P. falciparum* lactate dehydrogenase (pLDH), pLDH activity or HRP2[30,19,31,32,33]. The principle behind the use of pLDH production as a measure of parasite growth comes from the different enzyme structure of pLDH compared to human LDHs[34] and the rapid decline of pLDH levels when the parasites die[35].

The double-site enzyme-linked pLDH immunodetection (DELI) assay (Table 1) has been used to assess *P. falciparum* antimalarial drug susceptibility[36,30,19]. It is highly sensitive, more sensitive than the detection of *P. falciparum* in blood smears and equally as sensitive as PCR[19]. This technique can be used to measure *in vitro* drug sensitivity of *P. falciparum* with greater ease, greater speed, and simpler equipment than that required for isotopic assays. In contrast with the latter, lower parasite densities can be tested in the DELI assay (as low as 0.005%), thereby extending the number of isolates that can be investigated.

The pLDH enzyme activity assay is based on the observation that the LDH enzyme of *P. falciparum* has the ability to rapidly use 3-acetyl pyridine NAD (APAD) as a coenzyme in the reaction, leading to the formation of pyruvate from lactate. In contrast, human red blood cell LDH carries out this reaction at a slow rate in the presence of APAD. The development of APADH is measured, and there is a correlation between levels of parasitemia and the activity of parasite LDH[37,38].

The HRP2 assay (Table 1) is based on the measurement of the increase in HRP2 concentrations in *P. falciparum* culture samples. There is a commercial kit available (Malaria Ag CELISA, Cellabs Pty. Ltd., Brookvale, NSW, Australia; http://www.cellabs.com.au), which takes only about 2.5 h to perform. If parasite growth is inhibited by antimalarial drugs, the inhibition is reflected in the increment of HRP2 levels and can therefore easily be quantified by antibody-mediated detection[39]. HRP2 has been reported to show extensive protein sequence diversity (mainly insertions) in *P. falciparum* isolates from geographically different areas[40], and some isolates from the Amazon region of Peru even the lack of *pfhrp2* gene[41]. Importantly, it has been demonstrated that the HRP2 protein diversity has an effect on the sensitivities of the HRP2 detection antibodies. For these reasons, there are growing concerns about its application for field studies. However, this method is widely used by the Amazon Network for the Surveillance of Antimalarial Drug Resistance (RAVREDA)* for drug susceptibility testing.

* Pan American Health Organization. RAVREDA-AMI: Amazon Network for the Surveillance of Antimalarial Drug Resistance (RAVREDA) / Amazon Malaria Initiative (AMI). Available at: http://www.paho.org/English/ad/dpc/cd/ravreda-ami.htm.

DNA dye intercalation assays

Several screening methods have been published that offer the potential for high-throughput screening of drugs against *P. falciparum*, and most are based on automated quantification of parasite growth after staining parasites with fluorescent DNA binding dyes[42,21,43,44,23,24]. The principle behind these assays is the contrast between host erythrocytes, which lack DNA and RNA, and the malaria parasites, which have both DNA and RNA; thus, parasites are readily stained with dyes that show enhanced fluorescence in the presence of nucleic acids.

DNA dye-based technologies (Table 1), such as those using DAPI (4, 6-diamidino-2-phenylindole), Pico green, YOYO-1, and SYBR Green I, have been shown to have comparable results to isotopic assays. In these assays, the fluorescence of the dyes is measured using either fluorescence microplate readers or fluorescence activated cell sorters (FACS). To date, only two of these dyes have been successfully used for high-throughput screening of existing libraries of compounds: YOYO-1 and DAPI[42,24]. The use of these assays has increased because they are relatively simple and inexpensive to run compared to their radioactive and ELISA counterparts.

IN VIVO ASSESSMENT OF ANTIPLASMODIAL ACTIVITY

Drug resistance in malaria has been defined as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended, but within the limits of tolerance of the subject". Another reason for failing to clear the parasite from the patient's body may be that not enough of the drug, or its active metabolite, reaches the parasite, an event that is termed therapeutic failure. Unlike drug resistance, therapeutic failure is a process directly related to the parasite and highly dependent on both the host and the properties of the drug.

Obviously, antimalarial drug discovery technologies are targeted to eliminate human disease. There is large heterogeneity in the behavior of patients and between different populations owing to gene polymorphisms, redundant targets, differently regulated pathways and environment challenges.

Follow-up of patients

In humans, the most traditional approach to determine *P. falciparum* susceptibility to antimalarial drugs is the assessment of the therapeutic (*in vivo*) response, which was originally defined by WHO in terms of parasite clearance [sensitive (S) and three increasing degrees of resistance (RI, RII, RIII)]. Because re-infection is difficult to exclude in areas with intense malaria transmission, WHO introduced a modified protocol based on clinical outcome (adequate clinical response, early treatment failure and late treatment failure) for areas with intense transmission, where parasitemia in the absence of clinical signs or symptoms is common*. Currently, the classification system extends the follow-up period to 28 days and includes different criteria for the definition of Early Treatment Failure, Late Clinical Failure and Late Parasitologic Failure, for areas with intense and low to moderate transmission†. The development of molecular tools has made it possible to distinguish incidents of recrudescence from re-infections[45]. All *in vivo* tests should be carried out with standard therapeutic doses and performed according to WHO guidelines‡. For *P. vivax*, criteria are different from those for *P. falciparum*†. The proliferation of antimalarial drug trials in the last ten years provides the opportunity to launch a concerted global surveillance effort to monitor antimalarial drug efficacy. The proposed World Antimalarial Resistance Network (WARN)§ aims to establish a comprehensive clinical database from which standardized estimates of antimalarial efficacy can be derived and monitored over time for diverse geographical and endemic regions[46]. The widespread adoption of such an approach will permit accurate and timely recognition of trends in drug efficacy, guiding the appropriate interventions to deal with established multidrug-resistant parasite strains and accelerate action when new strains of drug-resistant plasmodia first emerge.

Rodent models for *in vivo* screening of antimalarial activity

Four species of African rodent malaria parasites have been adapted for growth in laboratory mice: *P. berghei*, 1948[47]; *P. chabaudi*, 1965[48]; *P. vinckei*, 1952[49]; and *P. yoelii*, 1965[50] (Table 2). These model parasites have become widely used primarily due to the ease of handling and maintaining rats and mice in the laboratory; additionally, their life cycle can be maintained in the laboratory through cyclical transmission, using *Anopheles stephensi* and *A. gambiae*, which are also easily maintained in the laboratory.

Although direct extrapolation from rodent model biology to *P. falciparum* biology might not be applicable in all situations, each of the four rodent malaria species has similar characteristics to the four human malaria species, making them suitable for parallel study. For example, antigenic variation in *P. chabaudi*[51], *in vivo* drug testing with *P. berghei*[52], or *P. chabaudi*[53], cerebral malaria with *P. bergei* ANKA[54], pre-erythrocytic stage vaccines with *P. yoelii*[55] and chronobiology of *P. vinckei*[56].

* World Health Organization. Assessment of therapeutic efficacy of antimalarial drugs for uncomplicated falciparum malaria in areas with intense transmission [Internet]. Geneva: World Health Organization; 1996. Available at: http://www.who.int/drugresistance/malaria/en/Assessment_malaria_96.pdf.

† World Health Organization. Monitoring Antimalarial Drug Resistance, Report of a WHO Consultation [Internet]. Geneva: World Health Organization; 2001. Available at: http://www.who.int/drugresistance/publications/WHO_CDS_CSR_EPH_2002_17/en/index.html.

‡ World Health Organization. Drug resistance: malaria. Available at: http://www.who.int/drugresistance/malaria/en/.

§ WorldWide Antimalarial Resistance Network [Internet]. 2011. Available at: http://www.wwarn.org/.

Table 2 – Different characteristics of the four rodent malaria parasites and human parasites[57,58,59,60]

	P. berghei	*P. yoelii*	*P. chabaudi*	*P. vinckei*	Human parasite
Merozoites per schizonte	12 - 18	12 - 18	6 - 8	6 - 12	8 - 16 (32)
Synchronous blood infection	No	No	Yes	Yes	Yes/No
Optimum temperature mosquito transmission	19 - 21	23 - 26	24 - 26	24 - 26	> 26
Sporozoites in glands (days after infection)	13 - 14	9 - 11	11 - 13	10 - 13	dependent on temperature
Duration of pre-erythrocytic cycle (hours)	48 - 52	43 - 48	50 - 58	60 - 72	6 - 15 days
Duration of asexual blood stage cycle (hours)	22 - 24	18	24	24	48 - 72
Developmental time gametocytes (hours)	26 - 30	27	36?	27	48 h - 12 days

One of the most used *in vivo* dose-response tests for antimalarial activity is the four-day suppression test, originally designed by Peters[61]. Briefly, female mice are infected with rodent malaria parasites and the different treatment doses are given after infection on day 0 and repeated once daily for three days. On day 4 and day 7 post-infection, parasitemia is assessed. After day 7, no further manipulations are performed on the mice and they are monitored for survival.

Non-human primate, rodent or avian parasites have been used as surrogates of *P. falciparum*[62]. However, in spite of their value, there are significant biological differences between these species and the human parasites[63,64]. Recently, a reliable murine model of *P. falciparum* malaria has been established and is a valuable research tool, particularly in the field of drug discovery. The development of *P. falciparum*-infected humanized mice (HM) by successfully infecting *nude* or *scid* mice with *P. falciparum* parasites[65,66,67,68,69] has facilitated the harvesting of exo-erythrocytic, intra-erythrocytic, and cytoadherent intraerythrocytic stages of the parasite. It is possible to devise new *falciparum* murine models using HM immunodeficient mice expressing human adhesion molecules in the endothelium that also have specific defects in phagocyte activity/activation. These tools will hopefully open up additional ways to study the biology of the erythrocytic stages of *P. falciparum in vivo*; further, its molecular responses to selective pressures imposed by antimalarials or the human immune system reconstituted in mice could also be examined[70,71,65,72]. In fact, Angulo-Barturen and collaborators[73] succeeded in obtaining a dose-response relationship for antimalarials using a *P. falciparum* HM model.

SAFETY (CYTOTOXICITY) ASSAYS

A cytotoxicity assay is a rapid and cost-effective tool to help choose optimal candidates and to sort out likely failures before a compound is entered into the costly development process. Cytotoxicity primarily is considered as the potential of a compound to induce cell death. A reasonably good correlation has been found between basal cytotoxicity and acute toxicity in animals and humans[74]. Besides antimalarial activity, a promising antimalarial compound must also lack toxicity to host cells. The degree of selectivity a compound shows for the malaria parasite can be expressed by its selectivity index [SI = IC50cells/IC50 parasite (IC50, concentration that inhibits 50% of parasites)]. The higher the SI, the more promise a compound holds, due to its selectivity for the malaria parasite[75]. However, an SI below 2.0 indicates that a compound may possess strong antimalarial activity but may be a general toxin and should not be considered as a candidate. In practice, a drug is potentially interesting as an antimalarial if its IC50 is below 10 μM[76].

Though a number of organisms can be used, *in vitro* cell culture methods are generally accepted as an effective method for cytotoxicity screening of new compounds. The advantages of these systems over classical methods, such as long-term studies on experimental animals like rats, mice or even the less frequent brine shrimp lethality assay[77], include relatively well-controlled variables, decreased costs, a reduced time to completion, and reduced numbers of animals necessary to complete the study. There are six common targets of xenobiotic toxicity: liver, blood, kidney, nerve, lung and skin. There are many cell types available for each target: liver, HepG2[78] and BEL-7402; blood, HL60 and K160; kidney, Vero and HEK293; nerve, XF498 and NG108-15; lung, WI-38 and A549; skin, SK-MEL-2 and Lox. HepG2 is one of the most frequently used cell lines by pharmaceutical companies (GlaxoSmithKline[79]) to screen for cytotoxicity in libraries of compounds.

In works involving active anti-plasmodium compounds, the most frequently used cytotoxicity endpoints tested are based on a) the breakdown of the cellular permeability barrier (LDH leakage assay)[80], b) protein content[81], c) neutral red accumulation[82], and d) reduced mitochondrial function [MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide][83]. Each of these methods can be performed in microcultures, using multiple sample concentrations on 96-well or 384-well plates, thus allowing high throughput testing of large libraries of compounds.

a) The LDH leakage assay is based on the measurement of LDH activity in the extracellular medium. LDH activity is measured based on the diaphorase-mediated conversion of resazurin into the fluorescent resorufin product after LDH-catalyzed enzymatic conversion of lactate to pyruvate. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage. This assay is characterized by its reliability, speed and simple evaluation[80].

b) The protein content assay is an indirect measurement of the cellular protein content of adherent and suspension cultures. It works by coloration with sul-forhodamine B (SRB) and subsequent determination of optical density (490 nm) in a microtiter plate reader. The assay provides a colorimetric endpoint test, but SRB also fluoresces strongly with laser excitation at 488 nm and can be measured quantitatively at the single-cell level by static fluorescence cytometry[81].

c) The neutral red assay is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes[82]. The dye is then extracted and quantified by absorbance reading using a spectrophotometer.

d) Finally, the MTT assay has been used in different cell lines to assess cell viability. The assay involves conversion of the tetrazolium salt MTT, by mitochondrial dehydrogenases in viable proliferating cells, to an insoluble purple product formazan. Amounts of formazan are then rapidly quantitated using an automated spectrophotometric microplate reader[83].

ASSESSMENT OF TRANSMISSION-BLOCKING ACTIVITY OF CANDIDATE DRUGS

The rapid emergence of resistance to antimalarial drugs from diverse chemical classes* is a major factor affecting the treatment and control of malaria[84]. The use of compounds capable of interrupting the transmission of malaria has been advocated as a way of preventing the development of drug resistance and limiting the spread of resistant parasites[85,86,87,88]. Gametocytocidal antimalarials, and the artemisinines in particular, might accelerate the process of malaria control as they reduce post-treatment transmission[89,90,91]. However, artemisinin derivatives do not completely prevent post-treatment transmission[92], and in view of emerging artemisinin combined therapy (ACT) resistance in South-East Asia[93,94,95], alternative drugs effective against gametocytes are urgently needed.

Transmission-blocking antimalarial drugs can affect the sexual stages of the parasite in the human host (gametocytocidal activity) or the development of the parasite in the mosquito (sporontocidal activity).

EFFECT ON MICROGAMETOGENESIS (EXFLAGELLATION ASSAY)

Exflagellation is the process in which an activated microgametocyte produces flagellar microgametes. The microgametes avidly adhere to neighboring erythrocytes to form multicellular exflagellation centers, which can be observed microscopically[96]. Gametocyte activation is triggered *in vitro* by simultaneous exposure to two stimuli: a drop in temperature of more than 5° C[97] and a pH shift from 7.5 to 8.0[98,99,100]. The standard method to monitor successful gametogenesis is by counting exflagellation centers under the microscope. However, as this method is labor-intensive, somewhat subjective, and cannot be automated, it is not suited for medium-throughput applications. DNA replication during microgametogenesis has been previously investigated, using DNA staining with various fluorescent dyes and subsequent analysis of individual cells by fluorescence microscopy or of cell populations by flow cytometry[101,102]. However, these methods are not easily adapted to higher throughput assays. Recently, an assay adapted to the 96-well format to monitor activation of gametocytes based on the incorporation of radioactive hypoxanthine into the newly synthesized DNA of microgametes has been developed[103].

EVALUATION OF THE IMPACT OF ANTIMALARIALS ON SPOROGONIC DEVELOPMENT

Post-treatment gametocyte carriage in humans can be examined[104], despite a number of ethical considerations. Alternatively, a rodent model where the animals are infected and then treated can be used. Experimental mice are infected by intraperitoneal inoculation of infected red blood cells, and five days after infection, the presence of mature gametocytes is verified by the presence of exflagellation observed microscopically in blood drops. Gametocytemic mice are then treated with the compound to be tested and placed over cages containing mosquitoes. Ten days after the blood meal, mosquitoes are dissected and their midguts are examined under the light microscope (400×) to assess for the presence or absence of oocysts[105,106]. Traditionally, oocysts were visualized by staining with 0.5% mercurochrome. The development of fluorescent protein (GFP) transformed rodent parasites has made the detection of oocysts in the midgut easier because GFP-generated fluorescence can be easily and rapidly detected in a fluorescent microscope (Figure 2).

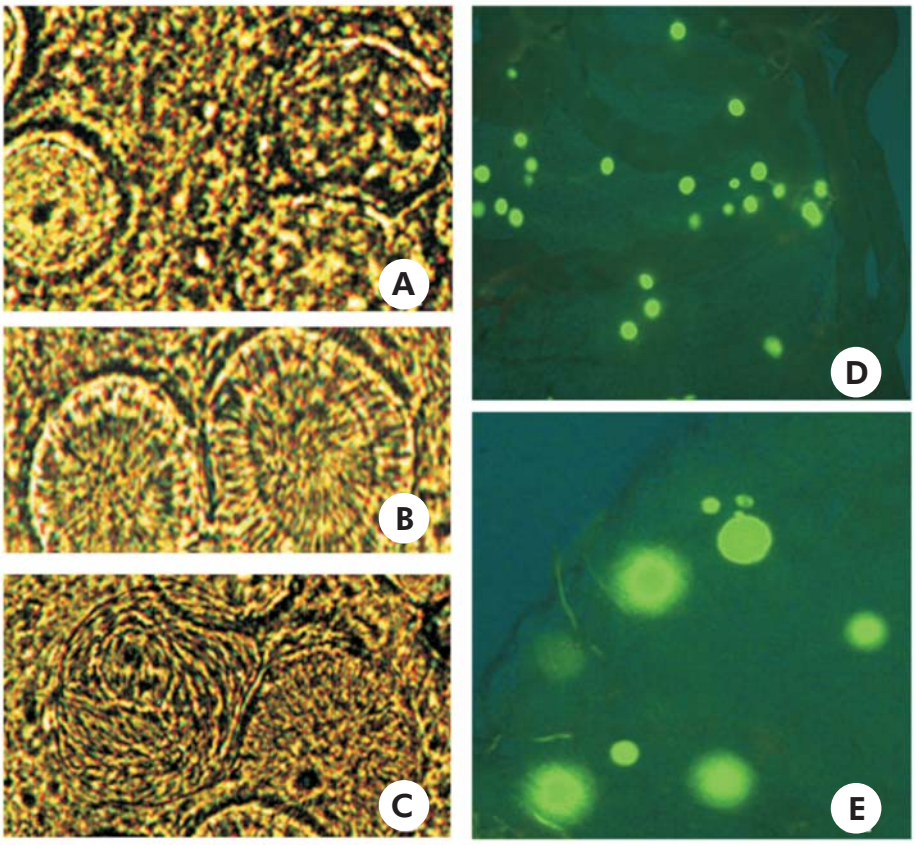
Evaluation of the impact on oocyst maturation can be established by observing the oocyst developmental status. Example categories of development include the following: a) immature oocyst, before the formation of sporoblasts; b) immature oocyst, with visible sporoblasts and budding sporozoites; and c) mature oocyst, containing fully developed sporozoites. Oocyst development can also be quantified by measuring oocyst diameter using an ocular micrometer.

Transmission-blocking activity is ultimately evaluated by removing mosquito salivary glands on day 21 post-infection and examining them for the presence or absence of sporozoites by phase-contrast microscopy[86,87,88], or by allowing these mosquitoes to feed on uninfected mice and then checking for parasitemia in the mice on subsequent days.

ASSESSMENT OF ANTIMALARIAL ACTIVITY OF COMPOUNDS DURING THE HEPATIC STAGE

This stage is a link between the extracellular sporozoite, inoculated by the mosquito vector, and the blood stage, which is responsible for producing disease.

* World Health Organization. Emergence and Spread of Antimicrobial Resistance. Geneva: World Health Organization. Available at: http://www.who.int/drugresistance/AMR_Emergence_Spread/en/index.html.



Light microscope images (400×): (A) immature oocyst with uniform content; (B) immature oocysts with sporoblasts and budding sporozoites; (C) mature oocyst, with fully developed sporozoites visible. GFP-expressing oocysts of the rodent malaria parasite *P. berghei*, fluorescence microscope images of (D) mature oocyst (100×) and (E) mature and immature oocysts with different stages of development (200×).

Figure 2 – *Plasmodium berghei* oocysts; developmental stages (day 10 after mosquito infection)

ANTIMALARIAL EXO-ERYTHROCYTIC SCHIZONTOCIDAL ASSAY

The development of pharmaceutical products inhibiting the growth of hepatic forms of *Plasmodium* is relevant for two main reasons. First, such compounds could be used as prophylactic agents by people exposed for a limited duration in an area where malaria is endemic (e.g., refugees and travelers) and, second, the emergence of drug-resistant strains is theoretically limited during the liver phase because of the lower parasitic load compared to the blood phase. Although hepatic stages provide attractive targets for antimalarial chemotherapy, the list of effective and widely available drugs is still limited. The only currently available prophylactic drugs are atovaquone and the two related drugs, primaquine and tafenoquine. Atovaquone has been demonstrated to be efficient in the inhibition of parasite development[107], however, its use is limited because of its high cost. Hematological toxicity restricts the use of primaquine, particularly in Africa because of the elevated frequency of Glucose-6-phosphate dehydrogenase (G6PD) deficiency in the human population[108]. The identification of new drugs is slowed down by the lack of a reliable and sensitive method that can be utilized in high-throughput screening.

Despite the significant advances, the exo-erythrocytic (EE) stages of mammalian malarial parasites remain the least understood of all stages of the parasite's life cycle. The earliest and most prolific cultures of EE stages were achieved using the avian parasites *P. fallax*, *P. gallinaceum* and *P. cathemerium*, cultured in chicken embryos or *in vitro*[109,110,111,112]. However, the biological disparity between the EE cycle in avian and mammalian malarial parasites, the differences in host immune systems, and the role of the hepatocyte greatly limit the use of the culture system as a model for human malaria. Some laboratories have pursued the use of primary hepatocytes from susceptible natural and laboratory hosts[113]. Primary hepatocytes have the advantage that they can be obtained from animals that are histocompatible to experimentally immunized hosts. In addition, they may allow for the testing of drugs requiring metabolic activation, although qualitative and quantitative inter-species differences commonly exist in the pathways of hepatic drug metabolism. However, primary hepatocytes also suffer from a number of major disadvantages: (1) the metabolic profile of the hepatocyte will vary during the period of culture; (2) the density of parasites obtained using these cells is relatively low; (3) for technical reasons, preparations will vary from experiment to experiment and between laboratories; and (4) the preparations will vary depending upon the nutritional status and other characteristics of the host.

Advances were made by the introduction of the hepatoma cell lines HepG2-A16 and Huh7[114]. However, in drug-sensitivity studies hepatoma cell lines may not faithfully reproduce drug metabolism by primary hepatocytes. For example, although HepG2 cells can perform cytochrome P-450 dependent mixed function oxidase and conjugation reactions, differences have been observed in the activities of some of the drug metabolizing enzymes as compared with freshly isolated primary human hepatocytes[115,116]. Primaquine, which is believed to require activation by the host cell, has a low activity against *P. berghei* EE forms cultured in HepG2 cells[117] compared to *P. yoelii* grown in primary *Thamnomys* hepatocytes[118]. This may reflect the inability of HepG2 to transform primaquine into its active state. To obtain a more complete picture of the activity of individual drugs, several different culture systems should ideally be used.

The *in vitro* cultivation of the EE forms of *P. berghei* (ANKA strain) or *P. yoelii* (265BY strain), by infection of the HepG2 cell line with sporozoites produced in *Anopheles stephensi* mosquitoes[119,78], constitutes one of the most used assays to assess the antimalarial activity of compounds during the hepatic stage.

In vitro drug sensitivity assays are largely based on counting the number of hepatic cells containing schizonts in sporozoite-infected cultures[120,113,114]. Although alternative methods have been proposed to detect exo-erythrocytic schizonts including the use of quantitative RT-PCR (qRT-PCR) methods[121,122,123], the use of ribosomal RNA probes[124], the use of infrared imaging system combined with a colony counter[125], and detection in culture and in live mice using real-time luminescence imaging[126], the number of infected cells is still largely measured by fluorescence microscopy analysis. These methods are not only prone to large variations between observers but are also time consuming given the low infection rates (generally less than 2%) observed in cultured hepatocytes[125]. Transgenic rodent malaria parasites (GFP-expressing parasites) expressing the fluorescent reporter protein luciferase have been developed[127,128]. The luminescence-based analysis of the effects of various drugs on *in vitro* hepatocyte infection using these parasites shows that these models can effectively be used for *in vitro* and *in vivo* screening of compounds targeting *Plasmodium* liver stages[129]. This strategy is a reproducible system in which a cloned parasite grows to maturity in a defined host cell, at the same rate and with the same morphology as that observed in a convenient laboratory host. Further, it represents a routine and reproducible assay for the growth of EE stages, which is both objective and statistically robust.

THE ORDEAL OF CHOICE

The abundance of available drug-sensitivity assays obviously leaves the researcher with the difficulty of choice. Both the WHO microtest and the isotopic assays have proven their reliability in their respective disciplines after more than two decades of use; the WHO assay in the field, the isotopic assay in well-equipped research laboratories. However, both assays are associated with serious drawbacks. The WHO assay is both subjective and labor-intensive, whereas the isotopic assay lacks sensitivity and involves the handling of radioactive material. The new colorimetric assays, the ELISA-based DELI and HRP2 assays and the DNA dye intercalation assays overcome many of these disadvantages and might currently be the methods of choice. They are sensitive, relatively simple and rapid, do not require highly specialized equipment, and have the potential to replace traditional assays. However, they are still a long way from the 'drug resistance dipstick'. As more information on the genetic background of resistance becomes available, and as new and improved tools (such as real-time PCR) are developed, molecular techniques might become an extremely valuable tool for the surveillance and early detection of antimalarial drug resistance in the future.

Drug assays are fundamental to malaria research and should always be complemented with clinical studies, host genetic studies related to drug metabolism, molecular markers for the quicker identification of presence of resistance in the field, and new diagnostic methods mainly applied to drug susceptibility characterization.

ACKNOWLEDGMENTS

The authors would like to acknowledge the many valuable suggestions made by Dr. Marcelo Silva (PhD), researcher at Centro de Malária e Outras Doenças Tropicais LA, Universidade Nova de Lisboa, Lisbon, Portugal.

Métodos para avaliação da atividade antimalárica nas diferentes fases do ciclo de vida do *Plasmodium*

RESUMO

A malária é uma doença transmitida por mosquitos e causada por parasitas do gênero *Plasmodium*. Em seres humanos, os parasitas multiplicam-se no fígado e, em seguida, infectam os eritrócitos. O ciclo de vida do *Plasmodium* consiste em uma fase sexuada no mosquito vetor (esporogonia) e uma fase assexuada no hospedeiro vertebrado (esquizogonia); ambas as fases podem ser detectadas por meio de testes. Em geral, a avaliação da atividade antimalárica de compostos é feita por testes *in vivo* e *in vitro*. A atividade antimalárica determinada por testes *in vivo* decorre de uma variedade de fatores associados tanto ao parasita quanto ao hospedeiro. Por outro lado, os testes *in vitro* mostram com mais precisão os efeitos "isolados" dos compostos sobre o metabolismo do parasita. A análise *in vivo* da atividade antiplasmódica pode ser realizada com o uso de modelos de roedores e pela avaliação da atividade bloqueadora do potencial vetor utilizando mosquitos. Há diversos testes *in vitro* para a avaliação da atividade antimalárica com base na observação do desenvolvimento do parasita em células sanguíneas por meio de gota espessa, ensaios isotópicos, quantificação de proteínas do parasita e testes de intercalação no DNA com o uso de corantes. Além da atividade antimalárica, um composto antimalárico promissor não deve apresentar toxicidade em relação às células hospedeiras; o grau de seletividade de um composto em relação ao parasita da malária envolve esta análise. Neste artigo, pretende-se resumir os métodos mais comumente utilizados para avaliar a atividade antimalárica de compostos durante os diferentes estágios do ciclo de vida do *Plasmodium*.

Palavras-chave: Malária; Resistência a Medicamentos; Antimaláricos; Testes Imunológicos de Citotoxicidade; *Plasmodium*.

Métodos para evaluación de la actividad antimalárica en las diferentes fases del ciclo de vida del *Plasmodium*

RESUMEN

La malaria es una enfermedad transmitida por mosquitos y causada por parásitos del género *Plasmodium*. En seres humanos, los parásitos se multiplican en el hígado y en seguida, infectan los eritrocitos. El ciclo de vida del *Plasmodium* consiste en una fase sexuada en el mosquito vector (esporogonia) y una fase asexuada en el huésped vertebrado (esquizogonia); ambas fases pueden detectarse por intermedio de pruebas. En general, la evaluación de la actividad antimalárica de compuestos se hace por ensayos *in vivo* e *in vitro*. La actividad antimalárica determinada por ensayo *in vivo* resulta de una variedad de factores asociados tanto al parásito como al huésped. Por otro lado, las pruebas *in vitro* muestran con más precisión los efectos "aislados" de los compuestos sobre el metabolismo del parásito. El análisis *in vivo* de la actividad antiplasmódica pode realizarse con el uso de modelos de roedores y por la evaluación de la actividad bloqueadora del potencial vector utilizando mosquitos. Existen diversos testes *in vitro* para la evaluación de la actividad antimalárica basados en la observación del desarrollo del parásito en células sanguíneas a través de gota espesa, ensayos isotópicos, cuantificación de proteínas del parásito y pruebas de intercalación en el ADN con el uso de colorantes. Además de la actividad antimalárica, un compuesto antimalárico prometedor no debe presentar toxicidad en relación a las células huéspedes; el grado de selectividad de un compuesto en relación al parásito de la malaria está involucrado en este análisis. En este artículo, se pretende resumir los métodos más comúnmente utilizados para evaluar la actividad antimalárica de compuestos durante las diferentes etapas del ciclo de vida del *Plasmodium*.

Palabras clave: Malaria; Resistencia a Medicamentos; Antimaláricos; Pruebas Inmunológicas de Citotoxicidad; *Plasmodium*.

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Recebido em / Received / Recibido en: 22/7/2010
Aceito em / Accepted / Aceito en: 28/9/2010