

Testing of Natural Products and Synthetic Molecules Aiming at New Antimalarials

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Abstract: The search for new antimalarials, which in the past relied on animal models, is now usually performed with cultures of *Plasmodium falciparum* (PF) blood parasites by evaluation of parasite growth inhibition. Field isolates of PF human malaria parasite, parasite strains and clones, well characterized for their susceptibility to chloroquine and other standard antimalarials are available for the *in vitro* tests. The simplest method to evaluate parasite growth is the determination of parasitemias in Giemsa stained blood smears through light microscopy. Other methodologies have proven to be more precise and allow mass screening of new compounds against PF blood stages, such as: (i) measuring the incorporation of radioactive hypoxanthine by the parasites; (ii) indirect colorimetric assays in which specific parasite enzyme activities, and histidine-rich protein II (HRP2) production are measured with the help of monoclonal antibodies; (iii) the β -haematin formation, and; (iv) assays using green fluorescent protein (GFP) in gene-expressing parasites. The advantages and disadvantages of the different *in vitro* screening methods, as well as the different *in vivo* models for antimalarial tests, are described in this review. Such tests can be used for the evaluation of medicinal plants, synthetic and hybrid molecules or drug combinations.

Key Words: Malaria, *P. falciparum*, antimalarials, experimental chemotherapy, *in vitro* tests, animal models, drug-resistance, hypoxanthine.

1. INTRODUCTION

Malaria constitutes one of the most impoverishing infectious diseases, affecting 200-500 million people worldwide and causing millions of deaths. The most affected populations are children and infants, pregnant women, as well as non-immune adults developing the first episode of acute malaria [1]. A fraction of the exposed population also seems to develop asymptomatic infections, i.e. to harbor circulating parasites without being ill. The infection is transmitted by inoculation of sporozoites during the bite of an infected *Anopheles* mosquito. There are no prophylactic curative antimalarials available in use to suppress this parasite cycle, as discussed below, so that prophylaxis is with compounds affecting the parasites already in the infected red blood cells (iRBC).

The symptoms of malaria (fever, sweating, chills and headache) appear as a consequence of the parasite multiplication in the iRBC, and can be mistaken with other acute diseases, such as influenza, virus or dengue. Four parasite species infect humans, *Plasmodium vivax*, *P. malariae*, *P. ovale* and *P. falciparum*, the later being highly lethal if not treated. Malaria treatment depends on the diagnosis of such

species, the level of parasitemia, type and level of drug resistance in the region where the infection was acquired, the stage of infection, and the availability of antimalarials, among other factors. There is increasing evidence that most *P. falciparum* isolates in the endemic areas have developed multi-resistance to most available antimalarials, especially to those in the aminoquinoline group (chloroquine, amodiaquine and mefloquine). At present, artemisinin derivatives are recommended in combination with other antimalarials to treat the disease caused by multidrug resistant parasites. The fact that resistance to artemisinin has also been reported [2] constitutes an enormous threat to the malaria control programs, making the development of new antimalarials urgent.

Several methods are used to test new antimalarials and most use *P. falciparum* iRBC, the stages responsible for the disease symptoms and death. The methods for antimalarial screening against the other stages of the parasites are more complex, e.g. to test prophylactic drugs which hampers the sporozoite development in the liver, or substitutes for primaquine, used to cure *P. vivax* late relapses. This parasite is highly prevalent in the world, accounting for almost 80% of the malaria cases in Latin America. Since *P. vivax* drug-resistance against chloroquine and primaquine has also been reported, there is also a need for new drugs to cure late relapses caused by this parasite species. These tests require the malaria sporogonic cycle in the mosquitos, the oocysts and sporozoites, which are more difficult to be produced than the cultures of *P. falciparum* iRBC [3-6]. In this review we discuss some advantages and limitations of the *in vitro* tests and of the animal models for antimalarials screening with more focus in the blood stage parasites, since they cause the dis-

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ease symptoms, and are the only malaria stages maintained in continuous cultures.

2. ANIMAL MODELS FOR TESTS OF BLOOD SCHIZONTICIDES

Various animal species are susceptible to *Plasmodium* infections, a few are used for antimalarial tests, as illustrated in Table 1. In the past, the avian malaria was extensively used. Monkeys from the Old and New World, that also bear malaria parasites and/or are susceptible to human malaria parasites, have also been extensively used. The sporozoite induced infections and prophylactic drug testing in monkeys are important for its similarity with human disease. But monkeys are expensive to maintain, thus only available in a few laboratories. Undoubtedly, the rodent malaria model in mice is the most extensively used, and ideal for the primary *in vivo* tests of new antimalarials. Mice are easy to raise, maintain, handle, and are small (20-30g), thus requiring little amount of drugs (molecules or plant extracts and fractions, for instance) to test, compared to chickens, rats or monkeys (Table 1). Mice are also susceptible to several species of malaria parasites isolated in other rodent species (i.e. *Thamnomis sp.*). Mice may develop acute fulminating diseases induced by blood stage parasites, or by sporozoites injected either by mosquito bites or intravenously, being excellent models for testing new drugs including those for malaria prophylaxis.

2.1. Malaria Parasites and the Antimalarial Tests in Mice

The *Plasmodium* species used in mice for chemotherapy studies (Table 1) are *P. yoelii*, *P. chabaudi*, *P. vinckei*, and *P. berghei*. Such parasites can be maintained in liquid nitrogen and/or by serial weekly passages of iRBC into mice, or in chronically infected Wistar rats. Some parasite strains and

clones have been selected for antimalarial drug-resistance, usually through drug pressure using sub-curative drug doses (Table 2) [7-10], and these parasites are available at MR4 (<http://www.malaria.atcc.org>).

Most laboratories use iRBC with *P. berghei* in the four day suppressive method, known as the Peters method [7], to test new antimalarials. In this method, the mice are inoculated by intraperitoneal route, treated daily during 4 days and then examined for the efficacy of the new compounds, by comparison of parasitemias on day 5 after parasite inoculation, between treated and control untreated mice. This protocol has been successfully used at our laboratory for decades, as recently reviewed [3], with slight modifications. In brief, it consists in using adult outbred Swiss Webster female mice, 20±2 g weight, which are inoculated with 1×10⁵ iRBC by intraperitoneal route (ip). The mice are kept together for at least 2h and up to 24h after inoculation, and then are randomly distributed, in groups of six mice per cage. For drug treatment, three mice are used per test drug, three for chloroquine (the standard antimalarial used in parallel); six as controls untreated in each test. Treatment is performed in the next 3 days with the test compounds, the control antimalarial, or with the drug vehicle alone (DMSO used for plant extracts) given by gavage (per os) or by subcutaneous route (sc) once a day; the ip route is usually reserved for parasite inoculation only but some researchers use ip route for treatment.

The drug solutions are prepared at the day of the experiment and diluted accordingly, so that each mouse receives a total volume of 200 µl. For the initial primary tests, daily doses of up to 1g/kg/day are advised in the case of crude plant extracts because the active compounds occur in low levels in natural products and activity may not be detected in lower doses [11]. For purified plant compounds, or for syn-

Table 1. *Plasmodium* Species and Animal Models Used in Antimalarial Tests

Parasite Species /Strains or Clones	Experimental Host	References
<i>P. berghei</i> / RC, NK65, K173, ANKA, NY, P, KFY	Rat (White, Brown Norway) Mouse, Hamster.	[7, 45-48]
<i>P. yoelii</i> (<i>nigeriense</i>)/N67, ART, 17X, MDR	Mouse	[48-50]
<i>P. vinckei</i> (<i>petteri</i>)/279BY	Mouse	[51-53]
<i>P. vinckei vinckei</i>	Mouse	[54, 55]
<i>P. chabaudi</i> /AS	Mouse	[48]
<i>P. cynomolgi</i> /B, M, Ro	Rhesus monkey	[17, 56, 57]
<i>P. knowlesi</i> / W1	Rhesus monkey	[24]
<i>P. fragile</i> / Ceylon	Rhesus monkey	[58]
<i>P. falciparum</i> /Vietnam Oak Knoll, Uganda Palo Alto, T24	<i>Aotus</i> and <i>Saimiri</i> monkeys Mouse	[14, 17, 20, 59]
<i>P. vivax</i> / Palo Alto, AMRU1	<i>Aotus</i> and <i>Saimiri</i> monkeys	[20]
<i>P. coatneyi</i>	Rhesus monkey	[60]
<i>P. gallinaceum</i> / IOC, 8A	Chicken	[6, 61]

Table 2. Some *Plasmodium* Species, Strains and Clones, Resistant to the Standard Antimalarials Chloroquine (CQ), Pyrimetamine (PYR), Mefloquine (MFQ), Artemisinin (ART), and Multi Drug-Resistant (MDR) Used in Antimalarial Tests in Animal Models

<i>Plasmodium</i> Species/Host	CQ	PYR	MFQ	ART	MDR
<i>P. berghei</i> / Mouse	NK65 [62], RC [7]	NPN [63], PYR [71], KFY [48]	N1100 [48], ANKA 25R/10 [64]	SANA [65]	G [72]
<i>P. yoelii</i> / Mouse	NS[66]			ART [66]	
<i>P. yoelii nigeriensis</i> / Mouse	N-67 [67, 68]				MDR [67]
<i>P. chabaudi</i> / Mouse	AS(30CQ) [69]	AS(0CQ) [9]	AS(15MF/3) [10]	AS-ATN, AS-ARTB [12]	
<i>P. cynomolgi</i> /Rhesus		Ro/PMV [57]			
<i>P. knowlesi</i> /* Rhesus			W1 [67]		
<i>P. vivax</i> / Aotus	AMRU-1 [70]				

* This monkey malaria species, morphologically similar to *P. malariae* parasites, is believed to be largely distributed and potentially life threatening, and has caused four human fatal death in Borneo Malaysia confirmed by nested PCR [73].

thesized molecules, 100mg/kg/day is sufficient. For the standard antimalarials the curative or sub-curative doses have to be determined (~10 mg/kg/day for chloroquine sensitive parasites).

To evaluate the activity of the compounds, blood smears are collected from the tail of all mice on days five and seven after parasite inoculation or for another week or more in the case of active compounds being re-evaluated in the primary screening. The dried blood smears are methanol-fixed, stained with Giemsa (one drop per ml of phosphate buffer saline, pH 6.8) for 20 min, dried, and kept for microscopic examination and parasitemia determination. The total number of RBC counted varies according to the level of parasitemia: in the case of negative smears, they should be up to 10,000 RBC (or up to 100 microscopic fields); for parasitemias lower than 5% up to 6,000 RBC; for parasitemias of 5 to 20% a total of 2,000 RBC; and for higher parasitemias a total of 500 RBC. Smears should be read double blind, and codified with the help of a second technician, so as to decrease the possibility of error by the observer and biases. Inhibition of parasite growth is then evaluated by comparison with the non-treated controls (taken as 100% parasite growth).

Compounds reducing parasitemia by 30% or more are considered active [11] and evaluated in a secondary screening when the drug concentrations inhibiting 50% and 90% of parasitemia (ED₅₀ and ED₉₀ values, respectively), in relation to controls, are calculated by plotting the doses tested against parasitemia. Mice with negative parasitemia, up to day 30 after inoculation, are considered cured. However, to ascertain the drug-induced cure, blood samples collected in anti-

coagulant (sodium citrate 3.8%), can be sub-inoculated into normal mice, which are then monitored for 15 consecutive days through blood smears examined by microscopy.

Some advantages of using rodent malaria model for *in vivo* antimalarial tests are: (i) mice are easy to handle; (ii) the rodent malaria parasites are not infectious to humans; (iii) *P. berghei* is highly virulent to mice allowing experiments of short duration; (iv) non-treated control mice die of malaria in two-three weeks; (v) experiments are concluded in up to 4 weeks; (vi) the iRBC with *P. berghei* parasites can be maintained in liquid nitrogen or in deep freezers, as well as in young rats in laboratories where such facilities do not exist. Rats develop a chronic disease and live for long periods, although they may cure the infection spontaneously.

The mortality of the animals daily monitored up to day 30 of infection may be useful, as well as repetition of the tests, in two to three independent experiments performed for each compound. The compounds found to be active at the highest dose are tested again in smaller doses to determine the dose which inhibits 50% of parasite growth (IC₅₀) and allow comparisons among different products. Details of this protocol vary from one laboratory to another, especially the number of iRBC used per mouse and days of treatment; the route of drug administration also depends on the drug solubility.

2.2. Drug Resistant Malaria Parasites in the Mouse Model

Several drug resistant parasites have been developed in mice models, especially using *P. berghei* which can be easily

selected by treating the infected mice with increasing doses of CQ followed by weekly blood transfers. *P. berghei* strains totally resistant to the maximal dose of CQ tolerated by the host are selected in a few weeks [7]. *P. berghei* parasites resistant to CQ induce parasitaemias significantly higher than the parental strain used for selecting resistance, in spite of the higher hematocrit and hemoglobin levels in the mice with CQ resistant parasites [8].

Using other rodent malaria parasites (*P. chabaudi*, *P. yoelli* and *P. vinckei*), resistance to AS derivatives and multi-drug resistance have been selected. Unlike the CQ resistant strains, the AS-resistance is stable in the absence of drug treatment [10]. In this case, the parasites were maintained through cycles in mosquitoes and mice, after being selected from a parasite strain already selected for quinine resistance [12] (Table 2).

2.3. Tests with *P. berghei* Green Fluorescent Protein (PbGFP)

High priority has been given to new assays that facilitate and accelerate the development of novel antimalarial compounds since the conventional *in vivo* mice model, using light microscopy, is an incompatible technique to test large numbers of drugs. The use of an autonomously fluorescent *P. berghei* (PbGFP) strain, stably transformed with the green fluorescent protein, has been shown to rapidly quantify parasite growth by flow cytometry [13]. One drop of parasite-infected blood, without further manipulation of the sample, will allow a quick and specific evaluation of parasitemia. The method in brief is, adult BALB/c mice are inoculated with 10^6 erythrocytes infected ip with PbGFP and then drug-treated by gavage as described for the four-day suppressive tests. The inhibition of parasite growth is determined by flow cytometry, in the treated and non-treated control groups, using a drop of blood collected directly into 3 ml of culture medium (RPMI-1640 or D-MEM plus 24 mM NaHCO₃). Fluorescence is read in a FACScalibur flow cytometer with a 488 nm argon laser and Cell Quest software. The iRBC are identified on the basis of their specific light-scattering properties in a total of 5000–10,000 events, which are counted for each sample. The results of drug-susceptibility assays by flow cytometry are comparable with those obtained by microscopic examination of Giemsa-stained slides, including a correspondence in the peaks of parasitemia.

2.4. *P. falciparum* Mice and Antimalarial Chemotherapy

A mouse model able to support *P. falciparum* infection has become recently available for chemotherapy studies. Thus, an immunodeficient (*bg/bg xid/xid nu/nu*) BXN laboratory mouse that lacks T cells and lymphokine activated killer cells, had the number of tissue macrophages reduced by administering dichloromethylene diphosphonate and the number of polymorphonuclear neutrophils controlled [14]. *P. falciparum* parasitized human red blood cells (PfhRBC) were then grafted into the mice using a sensitive NF54 strain, and a multiresistant T24 strain; both retained their usual drug susceptibility in the mice treated with chloroquine, quinine, mefloquine, or dihydroartemisinin. A parallelism was observed between previous susceptibility of the parasites in humans, since the mice parasitemia was resolved when clas-

sical antimalarial drugs were used to treat the mice infected with the sensitive strain, whereas mice infected with the multiresistant strain failed to be cured by chloroquine or quinine, but responded to dihydroartemisinin treatment. The speed of parasite clearance and the morphological alterations for each drug matched previously reported observations, hence stressing the relevance of the model.

Some shortcomings to the immunosuppressed mouse model are the requirement of intraperitoneal infection, a high rate of failure or a limited reproducibility of the infection outcome, variable kinetics of parasitemia, and the use of toxic reagents which might interact in unknown ways with antimalarials. In order to circumvent these disadvantages, the authors [15] developed a new murine model of *P. falciparum* malaria, a non-myelodepleted mice (NOD^{scid/β2m^{-/-}} mice) defective in serum complement levels, NK cell activity and macrophage activation. Through serial passages of *P. falciparum* by intravenous route in these mice engrafted with human erythrocytes, a persistent malaria infection occurred in 100% of the animals. The response of the infected mice to chloroquine, pyrimethamine and artesunate, orally administered, validated the model proposed, since all three compounds were active against the parasites when administered orally once a day for 4 consecutive days, with a clear dose-response effect [15].

2.5. The Avian Malaria Models

Avian malaria models, before the rodent malaria models were available, allowed screening of thousands of new compounds as antimalarials in different birds, such as chicken, canaries, pigeon and ducks, but infection of chickens with *P. gallinaceum*, is by far the most used one. Considering the convenient use of mice, the avian malaria model is used for tests of prophylactic curative drugs [6].

The model is especially important in places where there are no *Anopheles* mosquitoes raised indoors available to infect with mammalian malaria, as recently discussed [3]. The life cycle of avian malaria is more complex since the exo-erythrocytic forms may originate from sporozoites, from iRBC, or from pre-erythrocytic tissue derived merozoites. The *gallinaceum* avian malaria model has allowed many studies on sporozoite evolution, characterization of the circumsporozoite protein, the immune response anti-sporozoites in the natural hosts, and a successful evaluation of a medicinal plant used in the Amazon to prevent the human disease [16].

2.6. The Non-Human Primates Used as Malaria Models

The non-human primate models of interest should be susceptible to the human malaria parasites, *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, as well as to parasites naturally infective to monkeys and apes [17]. The species and subspecies of the *Aotus* monkeys susceptible to infections with the human malaria parasites are *Aotus nancymai* and *A. vociferans* from Peru, *A. lemurinus lemurinus* and *A. lemurinus griseimembra* from Panama and Colombia, and *A. azarae boliviensis* from Bolivia (Table 1).

The *Saimiri boliviensis* and *S. peruviansis* from Bolivia and Peru and *S. sciureus* from Guyana, Columbia, and Pan-

ama, have been used for many studies with *P. falciparum* and *P. vivax* [17]. In order to adapt *P. falciparum* to the *Saimiri* monkeys and obtain reproducible high parasitemias, the animals need to be splenectomized [18].

Some of the species of monkeys that have been used for experimental chemotherapy include rhesus (*Macacca mulatta*), owl (*Aotus* species) and squirrel (*Saimiri* species) monkeys [19-24]. The choice of parasite species depends on the type of study to be carried out, for example, *Plasmodium knowlesi* in rhesus monkeys is a recognized model for severe and complicated malaria [21].

In order to increase the number of asexual parasites and production of infective gametocytes, the monkeys are frequently splenectomized, before infection or soon after the infection is patent, in the case of sporozoite-induced infections [17]. The route of infection varies within laboratories, either through intravenous injection of iRBC, or inoculation of sporozoites, in which case the mosquitoes salivary glands are dissected in phosphate-buffered saline (pH 7.2) supplemented with 20% fetal bovine serum, then crushed under a coverslip. The sporozoite suspension is washed from the slide and an aliquot examined in a counting chamber to determine the number of sporozoites, which should then be immediately injected intravenously into the animals [25, 26].

One parasite commonly used for the simian malaria model is *Plasmodium cynomolgi* (Table 1), a species of interest because it is closely related to the human parasite *P. vivax*. Several strains are known and infect rhesus monkeys inoculated iv with iRBC. For infections transmitted by sporozoites, mosquitoes are allowed to feed directly on a tranquilized animal, or the parasites are isolated from dissected salivary glands of infected mosquitoes and injected intravenously. Beginning 1 day after the injection of iRBC or 7 days after sporozoite inoculation, thick and thin blood films are taken, stained with Giemsa, and examined microscopically for counts of parasitemia, recorded per microliter of blood.

3. TESTING OF PROPHYLATIC COMPOUNDS

The development of specific monoclonal antibodies to malaria sporozoites, allowed *in vitro* studies of these developmental forms in cultures, the activity of protective antibodies against sporozoites, and, the activity of compounds able to inhibit sporozoite development. A successful demonstration of anti-sporozoite activity was recently described with a medicinal plant used for malaria prophylaxis in Madagascar (*Strychnopsis throuasi*) [27]. Sporozoites growth of *P. falciparum* in cell cultures, and of *P. yoelii* in cultures and in mice, was inhibited in the presence of plant extracts and molecules. The assays led to the development of a novel very active anti-sporozoite compound, a morphinan alkaloid named tazopsine. Similar tests performed with a Brazilian medicinal plant species (*Ampelozizyphus amazonicus*), named 'Indian beer' used to prevent malaria in the Amazon region of Brazil, demonstrated its prophylactic activity. The plant extract was extensively investigated in the past being inactive against the iRBC of *P. falciparum* in cultures, *P. berghei* in mice and *P. gallinaceum* in chicken. When tested against sporozoite-induced infections in the avian model the plant

extract inhibited the sporozoite development *in vivo* [6], data later confirmed in the *P. berghei* sporozoite mice model [16].

3.1. The Anti-Sporozoite Assay with *P. berghei* in Mice

The sporozoite assay, in brief, consists of treating the mice with up to 400 mg/kg of the plant extract, by oral route (gavage), for 6 consecutive days. After treatment, each animal, previously anesthetized is individually exposed to the bites of 2–12 infected mosquitoes (depending on the mosquitoes' infection rate). Treatment should be continued for another week or so. Two control groups are used in parallel, a placebo negative group treated with water plus the vehicle (2% Tween-20 or 1% DMSO in case of plant extracts), and a positive control-group treated orally with primaquine, as reference antimalarial. From day 5 after sporozoite inoculation onwards, in order to determine the malaria pre-patent period (PPP) and the course of the malaria infection, blood smears are prepared daily, fixed with methanol, stained with Giemsa, dried, then microscopically examined. Parasitemia is determined in the coded blood smears by randomly counting 1000–3000 erythrocytes or up to 100 microscopic fields (1000 x magnification) in the negative smear. Overall mortality should also be monitored daily in all groups for 30 days after sporozoite inoculation. In this model, mice treated with primaquine do not develop malaria, being therefore the best control for the prophylactic drug activity.

3.2. *P. gallinaceum* Sporozoites Model in Chickens

The model is very similar to the mice model, described above, requiring however one-day old chickens which are susceptible to the sporozoite infection and die of cerebral malaria caused by the exoerythrocytic stages. Chicks pre-treated with compounds presumably prophylactic, including medicinal plant extracts, are inoculated with sporozoites given by *Aedes* mosquito bites or subcutaneous inoculation, followed by blood examinations for parasitemia so that the malaria PPP is determined in treated and control animals. This is a way to measure the drug ability to hamper the parasite's initial development, thus the exoerythrocytic parasites. Soon as the malaria infection is detected in the blood of the non-treated control group, all the animals are killed under anesthesia, thick smears of the brain tissues are prepared from all animals, stained and examined. The number of capillaries blocked by the exoerythrocytic forms of development, in the chickens treated with the test or control compounds (primaquine or sulphadiazine block development of *P. gallinaceum* sporozoites *in vivo*) and in control non-treated chickens is counted by microscopy (400x magnification) [6].

4. INHIBITION OF *P. GALLINACEUM* SPOROGONY BY PRIMAQUINE-LIKE DRUGS IN *AEDES* MOSQUITOS

The *P. gallinaceum* parasites are useful in studying the activity of compounds aiming to cure the late relapse caused by *P. vivax*, like primaquine, which also blocks 100% oocyst formation in *Aedes* mosquitoes. This method for screening drugs with potential radical curative properties, like primaquine, was described by Gwadz *et al.* [28] and slightly modified [29]. It consists in evaluating the inhibition of the

sporogonic cycle in mosquitoes fed on *P. gallinaceum* infected chickens a few hours post-treatment. Compounds which directly affect gametocytes, and inhibit the sporogonic cycle, should have anti-hypnozoite activity. This method consists of infecting one to two weeks old chickens with $\sim 10^7$ *P. gallinaceum*-iRBC by intra-muscular or iv route, following the daily determination of parasitemia in Giemsa stained blood smears. Chickens with parasitemias between 0.9 to 3% are used to feed *Aedes* mosquitoes (time 0), and then are immediately treated with primaquine or the test compounds, given by oral route at the desired concentration. New groups of *Aedes* clean mosquitos are fed 2h and 4h after drug treatment in each animal. All unfed female mosquitoes should be discarded from the cages and seven days later, 20 mosquitoes from each group are dissected; their midguts are removed, stained with 2% mercurochrome and microscopically examined (100x magnification). The oocyst number is counted and the average number per midgut evaluated for each time point. Drug activity is evaluated by the percent reduction of the number of oocysts in relation to the control group of mosquitoes fed on the same infected chicken. In each experiment, one untreated chicken and one chicken treated with primaquine should be used as controls, knowing that primaquine blocks sporogony completely.

5. THE *IN VITRO* TESTS WITH *P. FALCIPARUM* CULTURED BLOOD PARASITES

The approaches to evaluate drug-sensitivity *in vitro* allow an almost complete exclusion of host-related factors, such as drug failure or host immunity. These tests provide a more objective insight into inherent drug sensitivity than the *in vivo* tests against the blood parasites, and allow studies on the mechanisms of drug activity *in vitro*. The blood parasites are maintained in continuous culture at 37°C using the candle jar method, as described by Trager and Jensen [30] or another controlled environment.

The effects of potential antimalarial drugs are evaluated by the inhibition of parasite growth in drug-exposed cultures, in relation to drug-free control cultures. The active compounds in the primary tests are then tested in serial drug dilution, and sigmoid dose-response curves are generated [31]. Several human strains of *P. falciparum* parasites with diverse drug susceptibilities are available for tests *in vitro*. The W2 clone, largely used, is chloroquine-resistant and mefloquine-sensitive; the BHz 26/86 strain, previously isolated from an imported case of malaria from the Amazon region [32], is also characterized as being chloroquine resistant and mefloquine sensitive [33].

5.1. Microscopic Evaluation of Parasitemia in Traditional Tests

The *P. falciparum* parasites maintained in continuous cultures in human erythrocytes are used in the search for new antimalarials in quantitative tests based on optical microscopy, here referred as the traditional test. In brief, a suspension of red blood cells with $\sim 1\%$ parasitemia previously synchronized [34] is distributed in 96-well microtiter plate (100 μ l/well) and cultured in complete medium (RPMI 1640 containing 10% human sera, 2% glutamine and 7.5% NaHCO_3). Two types of controls are run in parallel with the test com-

pounds: cultures without drugs and cultures containing chloroquine, or other reference antimalarials. The culture medium with or without drugs is replaced after 24 and 48h of incubation. After 72h in culture, blood smears are prepared, coded, stained with Giemsa and examined at 1000x magnification. Comparison between parasitemia in the controls (considered as 100% growth) and that in test cultures allow evaluating the percent inhibition of parasite growth and determination of the inhibitory concentrations of 50% of the parasite growth (IC_{50}) of each compound.

Alternatively, instead of counting all parasites in the blood smears, the number of schizonts is counted against the total number of parasites in thick films. This assay is relatively simple to perform and requires little technical equipment; it usually requires only 24h of incubation but, as in any test based on microscopy, is labor-intensive and requires highly trained personnel to limit individual variability in assessing the developmental stages of the parasites. The use of schizont maturation as the endpoint of parasite growth can overcome problems of background growth, since it excludes previous parasite stages; however, this might also result in a loss of data. Parasites growing from ring to late-trophozoite stages contribute the same weight as parasites that do not show any development at all [31].

Other assays have been developed to evaluate parasite growth and replace microscopic tests, and are chosen accordingly to the facilities of each laboratory. They include the hypoxanthine incorporation, colorimetric ELISA tests and GFP parasites, as further discussed.

5.2. The Hypoxanthine Incorporation Method

After the continuous culture of *P. falciparum* were available, another method was developed to replace microscopy for testing new compounds with antimalarial activity, based on the incorporation of tritiated hypoxanthine [35]. This semi-automatic test is more rapid, less tedious and more precise than microscopy for evaluation of the parasite growth, thus allowing mass screening of new compounds. The method is based on uptake of radiolabeled hypoxanthine (a nucleic acid precursor) by the parasite during short-term cultures. Since hypoxanthine is incorporated into both ribonucleic acid and deoxyribonucleic acid, it provides a reasonably broad index of parasite metabolism.

For the [^3H]-hypoxanthine incorporation assay, the ring-stages in sorbitol-synchronized blood [34] are cultured in the absence of hypoxanthine for at least 3 days. They are placed in 96-well plates after previous dilution in culture medium without hypoxanthine at 1% parasitemia and 1% hematocrit, then incubated with the test and control drugs in triplicates, at 37°C (in a candle jar or another controlled environment). After 24 h of incubating parasite-drugs, 25 μ l/well of medium containing [^3H]-hypoxanthine (0.5 μCi /well) is added, followed by 18 h incubation at 37°C. The plate is then frozen and thawed, the cells are harvested in glass fiber filters, which are placed in sample bags and immersed in scintillation fluid. The radioactive emissions are counted in a 1450 Microbeta reader and the inhibition of parasite growth evaluated after plotting the [^3H]-hypoxanthine incorporation levels to generate concentration-response curves. The half-maximal inhibitory response (IC_{50}) is compared to the drug-free con-

trols, estimated by a curve-fitting, using appropriated software. The data obtained in this technique shows good correlation with IC_{50} values determined by microscopic assessment [36].

All culture parameters should be carefully controlled in the hypoxanthine method. With initial parasitemias higher than 0.6%, radioisotope incorporation is often below that expected and varies in an unpredictable fashion. High concentrations of unlabeled hypoxanthine and other purine bases decrease incorporation of [3H]-hypoxanthine. Normal erythrocytes have a high concentration of many purines: they can act as an important purine source in the presence of large numbers of uninfected erythrocytes. Supplementing the medium with plasma causes a lower [3H]-hypoxanthine incorporation when compared with supplementation with serum: the release of ADP from platelets during clotting probably contributes to this [37].

The hypoxanthine method is more rapid than the traditional microscopy which requires parasitemia determination, very time consuming and subject to human mistakes. The [3H]-hypoxanthine incorporation by *P. falciparum* has less variability among replicates, and the automatic reading of the test reduces considerably the variability caused by human factors [37]. The main limitation of the technique is the use of isotopic material since the regulations regarding handling of radioactive restricts its use to cases where there are no other alternative tests [31]. Other important limitations of the hypoxanthine method are the high cost of the equipments and the high parasite densities required for the test, which limits its application to the use of culture-adapted parasite strains or field samples with high parasitemias.

5.3. The Lactate Dehydrogenase Immunodetection Assay (DELI)

The double-site enzyme-linked lactate dehydrogenase immunodetection assay (DELI) is a highly sensitive antigen-capture ELISA which requires simpler equipment than that needed for the isotopic microtest. It is easier, faster, and has no need for radioactive materials [38]. Two monoclonal antibodies (MAbs) are needed to develop DELI, the MAb 17E4, used as the capture antibody and specifically reacts with *P. falciparum* LDH and, the MAb 19G7, which is the detection antibody, reacts with both *P. falciparum* and *P. vivax* LDH. The advantages attributed to DELI [38] are: its high specificity for *P. falciparum*; the color reaction developed is read in a spectrophotometer; it is an indirect measure of parasite viability since only live parasites produce the enzyme; it is sensitive enough to precisely determine the IC_{50} values at a starting parasitemia of 0.005%; the degree of sensitivity reached after optimizing the method is as sensitive as the polymerase chain reaction (PCR), with a major advantage over this test, only DELI is quantitative. But hypoxanthine test can not be carried out at parasitemia lower than 0.2%. Finally, the results found at various initial parasitemias highly correlate with those obtained with the hypoxanthine method. The reproducibility is similar between both methods.

For drug sensitivity studies, comparative results between DELI and isotopic microtests show that DELI is practical,

feasible and reliable, and the DELI microtests supply IC_{50} values similar to those obtained using the hypoxanthine microtest with practical implications [38].

5.4. The Histidine-Rich Protein II (HRP2) Method

The production of histidine-rich protein II (HRP2) by *P. falciparum* parasites is closely associated with their proliferation, thus reflects growth inhibition as a measure of drug susceptibility. The chemotherapy test quantifying this protein, presumably easier to establish and to perform, is as reliable as the traditional *in vitro* assays of schizont maturation and hypoxanthine incorporation [39]. Parasites are incubated for 48 to 72h on microculture plates at an initial level of parasitemia of 0.01 to 0.1% with the antimalarial drugs, after which the samples are freeze-thawed and transferred to ELISA plates. A commercial HRP2 ELISA kit (Malaria Ag CELISA; Cellabs Pty. Ltd., Brookvale, New South Wales, Australia) is available for the quantification of HRP2 in the culture samples. The optical density values correspond to the amount of HRP2 found in the culture samples and provide consistent indicators of parasite growth. The complete ELISA may easily be performed in less than 3h, and is largely independent of the number of samples to be tested.

Although this ELISA assay is very sensitive, it requires a minimum incubation time (up to 72 h) to obtain significant increases in HRP2 levels. Although 48h covers a full erythrocytic life cycle leading to an increased HRP2 concentration in parallel with parasitemia raise, 72h incubation is ideal for the successful testing of slowly acting drugs, even with slow growing parasite strains. The method does not involve particularly expensive equipments or the handling of radioactive substances [39]. Another advantage is the availability of commercial kits for the quantification of *P. falciparum* HRP2. Implementation of this assay is faster than that of any other *in vitro* test of antimalarials; numerous laboratories and companies produce monoclonal antibodies specific for HRP2 [39].

5.5. The *P. falciparum* Green Fluorescent Parasites use for Antimalarial Tests

The availability of a green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* allowed it to be constitutively expressed in *P. falciparum* parasites (*PfGFP*), making them particularly attractive for chemotherapy studies. *PfGFP* are highly stable and already used for setting up a protocol for antimalarial tests [40]. The activity of some standard antimalarials was quickly and specifically quantified by flow cytometry; the parasitaemia levels detected were statistically similar to those obtained by optical microscopy. The *PfGFP* parasites maintained in continuous culture for more than one year had no modification on the fluorescence profile, validating its use for chemotherapy studies. The protocol for using *PfGFP* is simple, and does not require previous preparation of the culture for the fluorescence reading. The method is rather specific (parasite fluorescent itself), it does not require radioactive reagents, and it is a simple step method, unlike other fluorimetric protocols. Thus, the *PfGFP* parasites seem useful to substitute the traditional method in experimental chemotherapy.

The *Pf*GFP parasite is available to the malaria research community, as well as the rodent malaria parasite *P. berghei* (*Pb*GFP), and may be free-shipped to endemic countries through MR4, (<http://www.malaria.atcc.org>). The *Pf*GFP-based method if adapted to be processed in a fluorimeter 96-wells plate reader, should be suitable for high-throughput, semi-automated or automated techniques.

5.6. The β -Haematin Formation (the Hemozoin Test)

Ferriprotoporphyrin IX (FP) biomineralisation is a *Plasmodium*-specific process in which FP derived from the digestion of ingested haemoglobin is converted to haemozoin (β -haematin). The inhibition of biomineralisation is valuable for the detection of compounds with potential antimalarial activity. A technique easily adaptable to high-throughput screening was described [41], based on the measurement of β -haematin as FP by optical density. The β -haematin remaining after contact with drugs at pH 5–5.2 is solubilised in 0.1 N NaOH to form an FP measurable spectrophotometrically. The method, in brief, consists of incubating, in flat bottom 96-well plate (37°C, 18–24 h) a mixture containing (50 μ l each) the test and control drug or the solvent (negative control) with 50 μ l haemin chloride freshly dissolved in dimethylsulphoxide (DMSO), plus 100 μ l of 0.5 M sodium acetate buffer pH 4.4; the final pH of the mixture is 5–5.2. It is important that the solutions be added to the plate in the following order: haemin chloride, the buffer, and the solvent or test-drug solution. After incubation, the samples are transferred into a 96-well Multi-Screen plate size (Millipore MAR4 N04 10) with the help of a multichannel pipette (sucking up and forcing back 10 times to assure an adequate transfer of all material). Vacuum is then applied to aspirate the solvent through the filter, and the wells are washed once with 200 μ l DMSO per well. The β -haematin adhering to the filters is then dissolved in 200 μ l of 0.1 N NaOH per well (the vacuum is turned off to avoid leaking of the solubilized FP through the fiber filters). Finally, 150 μ l from each well is transferred into a parallel well in a flat bottom 96-well plate and the absorbance read at 405 nm with a micro-ELISA reader. The data are expressed as percentage of inhibition of β -haematin formation calculated, i.e. % inhibition = $100 [(O.D. \text{ control} - O.D. \text{ drug}) / (O.D. \text{ control})]$.

6. THE *IN VITRO* CYTOTOXICITY TESTS

Before a new product undergoes clinical trials, it has to be evaluated for its toxicity in cell cultures *in vitro* as well as in non-human primates and in other animal species. If the new compound is not toxic and very active in the *in vitro* and *in vivo* tests in mice, it has to be tested in another malaria model, chickens, rats or monkeys. In mass screening, since the antimalarial tests are usually more expensive and more time consuming, it may be practical to test the new compounds for their cytotoxicities *in vitro*, before the antimalarial tests are performed, so that toxic compounds are discarded. The specific indexes (a ratio between drug activity and its toxicity) can be calculated and the criteria defined (go or not go to the next steps) for the promising molecules.

The techniques for *in vitro* studies of cytotoxicity, in brief, use one or more mammalian cell lines kept under conditions allowing them to undergo mitotic division. The rate

of cell multiplication and growth is measured indirectly by formation of color, the intensity of which is directly proportional to the number of cells present. A variety of experiments can be used to compare the rate of proliferation of a cancer cell line in the presence and absence of the test substance, usually after a specified time [42]. Some cytotoxicity tests use human cell lines, others use cell lines of lower mammals, or the animal cells directly (spleen cells for instance). Two main techniques used for cytotoxicity testing in antimalarial chemotherapy studies are the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT) assays.

Our experience is with the MTT assay using Hep G2 A16 hepatoma cells, kept at 37°C in RPMI supplemented with 5% fetal calf serum (complete medium), in a 5% CO₂ environment [43]. Cells from confluent monolayers are trypsinized, washed, counted, diluted in complete medium, distributed in 96-well microtiter plates (4×10⁴ cells/well), and then incubated for another 18 h at 37°C. The compounds to be tested (with or without the test drugs added to the cultures) are diluted in ethanol (final concentration of 0.02%), incubated for another 24 h at 37°C, followed by addition of 20 μ l of MTT solution (5 mg/ml in RPMI 1640) without phenol red to each well. After 4 h incubation at 37°C the supernatant is removed and 200 μ l of acidified isopropanol added to each well. The colors developed in the culture plates are read by spectrophotometer with a 570 nm filter and a background of 630 nm. The minimum lethal dose that kills 30% of the cells (MLD 30%) is determined; each assay should be performed two or three times using triplicates.

The XTT assay was designed to yield a suitably colored, aqueous-soluble, non-toxic formazan upon metabolic reduction by viable cells [44]. The assay is similar to the MTT assay but does not contain serum and uses cells in log-phase growth. When the cells in the control wells reach confluence, the tetrazolium salt XTT is added with phenazine methosulphate (an electron transfer reagent) to each well, the cells are incubated for another 2–4 h at 37°C. Absorbance is read at a dual wavelength of 450/630 nm in a plate reader.

CONCLUDING REMARKS

The worldwide spread of *P. falciparum* (*PF*) multi-drug resistant parasites in the last decades and, more recently, of *P. vivax* malaria parasites resistant to chloroquine and primaquine, has made the control of human malaria difficult. *PF* malaria is usually fatal in children, and non-immune adults experiencing the first malaria episode, and mortality often results from late diagnosis and/or inadequate treatment. Monotherapies are no longer efficient against malaria, thus the World Health Organization recommends treatment to be performed with antimalarial combinations, the most efficient one being the artemisinin combined therapies, or ACTs. But the choice of drug-combinations varies, and in each endemic area it is influenced by factors like the availability of the drugs, the level and type of resistance, economical restrictions, among others. The recent demonstration of *PF* resistance to the artemisinin derivatives makes the situation even more dramatic, requiring new targets to fight the multi-drug resistant parasites. Several researchers are involved in pri-

mary and secondary tests, aiming at new antimalarials, screening synthetic molecules, drug-combinations, hybrid molecules, and natural compounds especially those purified from medicinal plants used against fever and/or malaria by populations exposed to malaria endemic transmission. Considering that malaria affects mainly the poor populations of the world which are often exposed to high levels of the disease transmission, that vaccines are not yet available, that the disease prevalence is increasing, and that most *P. falciparum* parasites are multi-drug resistant, the search of more potent antimalarials aiming at malaria control is a high research priority and should therefore be reinforced. In the present review, the several methods described to test antimalarials *in vitro* and *in vivo* have been presented and their limitations and advantages discussed. Although most of them use simple protocols, they all require expertise, well trained personnel and laboratories with good facilities to keep the animals and the expensive equipments used in routine work. In addition, transforming the published data which describes the antimalarial activities of new compounds into final products, i.e. drugs to be used in humans for malaria treatment is even more complex and rather expensive. This goal can only be achieved with the involvement of the pharmaceutical industries.

ABBREVIATIONS

<i>i</i> RBC	=	Infected red blood cells
DELI	=	Double-site enzyme-linked lactate dehydrogenase enzyme immunodetection assay
DMSO	=	Dimethyl Sulfoxide
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
FP	=	Ferriprotoporphyrin IX
HRP2	=	Histidine-rich protein II
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PF	=	<i>Plasmodium falciparum</i>
PPP	=	Prepatent period
XTT	=	2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt

ACKNOWLEDGMENTS

To CNPQ, PAPES-FIOCRUZ and FAPEMIG for financial support; to Renata Cristina Paula for editorial help and to CNPq for fellowships to AUK and JOA.

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