Review Article

In vitro and *in vivo* models used for antimalarial activity: A brief review

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Abstract

In this review, the laboratory protocol commonly employed for the biological evaluation of antimalarial activity of new drug substances including natural products has been detailed in a generalized, but concise form. The evaluation strategy covers a systematic and standardized methodology starting from *in vitro* cell-based screening to the *in vivo* assay method using animal models. These assay methods primarily focus on blood/ erythrocytic stages of the *Plasmodium* parasite, either *in vitro* (*P. falciparum*) or *in vivo* (*P. yoelii*) since this particular stage of the parasite mainly causes symptoms, manifestations and associated pathogenesis of the disease. This is the only parasitic stage in malaria disease that can be maintained in continuous blood cultures and also remains to be the prime target for most of the antimalarial drug molecules. A general approach to the antimalarial screening (*in vitro* and *in vivo* assay methods) routinely used for the sensitivity testing of newly developed antimalarial compounds is described herein.

Keywords: Malaria, antimalarial evaluation, *P. falciparum*, drug resistance, *in vitro*, *in vivo*

Introduction

During the past few decades, the emergence of drug resistant strains of *Plasmodium falciparum* has become an increasingly serious concern in malaria control and prevention worldwide (Rudrapal and Chetia, 2016a; Rudrapal et al., 2018). Because of this emergence of multi-drug resistant strains of *P. falciparum*, the development of new and potent antimalarial drugs active against resistant malaria could be a key therapeutic strategy for the control and prevention of malaria (Patowary et al., 2019). New antimalarial drugs should possess desired therapeutic efficacy, minimal toxicity and low cost (Rudrapal and Chetia, 2011). The efficacy screening remains, therefore, an integral part in the development of new antimalarial drugs. Several *in vitro* and *in vivo* screening methods have been used to test the antimalarial efficacy of synthetic compounds, hybrid molecules, drug-combinations and natural compounds (Rudrapal and Chetia, 2016b). The evaluation for antimalarial activity needs a systematic and standardized protocol starting from cell culture-based *in vitro* screening to the *in vivo* study in animal models. The assay methods are primarily focused on the blood stage of parasites, since it causes the disease symptoms and related pathogenesis, and are the only malaria stages that can be maintained in continuous cultures (Fidock et al., 2004).

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In vitro screening of antimalarial activity

In vitro screening constitutes a key component for the preliminary evaluation of efficacy for antimalarial drugs. It requires continuous cultivation of culture of *P. falciparum* in human erythrocytes *in vitro* for maintaining stock culture of parasites as well as drug screening and long term assessment (Desjardins, 1984). Several human strains of *P. falciparum* parasites with diverse drug susceptibilities are available for *in vitro* screening of drugs. The most widely used are CQ-sensitive and CQ-resistant strains of *P. falciparum* (Desjardins, 1984; Fidock et al., 2004).

Traditionally, the blood stage parasites of *P. falciparum* are mainly used for *in vitro* screening of antimalarial drugs. *In vitro* tests allow a quantitative assessment of intrinsic drug sensitivity which is based on microscopic evaluation of parasitemia followed by determination of inhibitory concentration (MIC/IC_{50}) (Medhi et al., 2018). The activity of antimalarial drugs is evaluated by the inhibition of parasite growth in drug-exposed *P. falciparum* 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 to assess the efficacy of compounds in terms of IC_{50}. The assay procedure

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used in *in vitro* screening was originally adopted by Trager and Jensen (1976). There are different assay methods used to test *in vitro* activity of antimalarial drugs (Desjardins, 1984; Fidock et al., 2004).

Giemsa stained slide method (also known as MIC method) is a traditional method used widely for *in vitro* screening of antimalarial compounds. It is basically a microculture microscopic test used for testing small number of compounds (Rudrapal et al., 2013). In this method, parasites are incubated with test compounds and after incubation the parasitemia of control is compared with that of the test by counting Giemsa stained parasites in blood smears using light microscopy (Sharma et al., 2016). Comparison between parasitemia in the controls (considered as 100% growth) and that in test cultures allow evaluating the percent inhibition of parasite growth followed by determination of the inhibitory concentration of 50% of the parasite growth (IC_{50}) for test compounds. This is a simple measurement which is classically known as the Minimum Inhibitory Concentration (MIC) method (Rudrapal et al., 2013; Sharma et al., 2016, Rudrapal et al., 2017a; Rudrapal et al., 2017b). Alternatively, instead of counting all parasites in the blood smears, the number of schizonts is counted against the total number of parasites (after 24 h of incubation) in thick films prepared from the cellular layer of the cultured samples (Gogoi et al., 2016; Kashyap et al., 2016). 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 some data (Noedl et al., 2003; Fidock et al., 2004). Parasites growing from ring to late-trophozoite stages, yet do not reach the schizont stage within 24 h, contribute the same weight as parasites that do not show any development at all (Trager and Jensen, 1976; Lambros and Vanderberg, 1976). This micro-test method of assessing parasitemia used for the *in vitro*drug sensitivity assay of drugswas recommended by WHO and is, therefore, known as WHO Schizont Maturation Inhibition assay (Kalra et al., 2006; Antoniana et al., 2009; Matthews et al., 2013) .The diagrammatic representation of the basic mechanism involved in theSchizont Maturation Inhibition assay is given in figure 1.

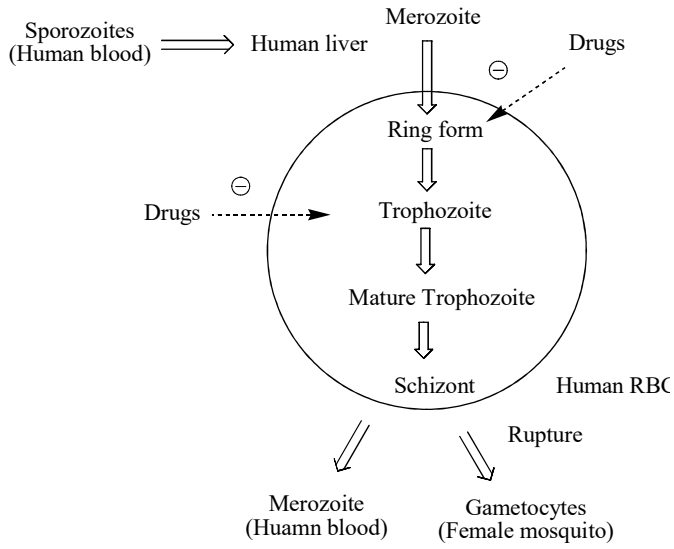

Figure 1. Schizont Maturation Inhibition assay (Desjardins, 1984)

This assay is relatively simple to perform and also requires little technical equipment. It usually requires only 24 h of incubation but, as in any test based on microscopy, is laborious and requires highly trained personnel to reduce individual variability in assessing the developmental stages of the parasites (Fidock et al., 2004).

Other *in vitro* methods used now-a-days for quantitative evaluation of parasite growth and drug′s sensitivity replace microscopic tests, include the [^{3}H] hypoxanthine incorporation method (microculture radioisotope technique), flow cytometry assay (SYBR Green I-based), colorimetric ELISA tests (*Pf* LDH assay, *Pf* HRP2 assay) and fluorescence assay *(Pf*GFP-based, SYBR Green I-based). These methods are relatively simple, require less tedious assay procedures and provide high throughput, but requires expensive equipments (Desjardins, 1984; Fidock et al., 2004; Kalra et al., 2006).

In vivo testing of antimalarial activity

Compounds effective in *in vitro* screening tests (i.e., those with $IC_{50} \leq 1\mu M$) are evaluated by *in vivo* screening methods. *Plasmodium* species that cause human disease are essentially unable to infect non-primate animal models. So, *in vivo* evaluation of antimalarial compounds begins with the use of rodent malaria parasites (Pandey et al., 2013). *Plasmodium berghei*, *P. yoelii*, *P. chabaudi*, and *P. vinckei* are the rodent parasites used extensively in *in vivo* screening (Nondo et al., 2016; Hilou et al., 2016)). Choice of rodent parasite species and mouse strains need to be carefully considered during experimental design and drug assay (Ager 1984, Noedl et al., 2003). The most widely used initial test, which uses infected erythrocytes with *P. berghei* or less frequently *P. chabaudi*, is a four-day suppressive test, known as the Peters′ method, to test new antimalarials (Peter et al., 1995; Abdulelah et al., 2011). In this method, the mice are inoculated by intraperitoneal route, treated daily for 4 days and then examined for the efficacy of the new compounds, by comparison of blood parasitemia on day 4 post-infection and mouse survival, between treated and untreated control mice. Compounds can be administered by several routes, including intraperitoneal,

intravenous, subcutaneous or oral (Fidock et al., 2004; Kalra et al., 2006).

Compounds identified as active in four-day assays can subsequently be processed through several secondary tests as described follows. In the dose ranging, four-day test, compounds are tested at a minimum of four different doses, by subcutaneous and/or oral routes, to determine ED_{50} and ED_{90} values (Matthews et al., 2013). This test also provides useful information on relative potency and oral bioavailability. In the onset of action/recrudescence test, mice are administered a single dose (by the subcutaneous or oral route) on day 3 post-infection and followed daily to monitor parasitemia. Results are expressed as the rapidity of onset of action (disappearance of parasitemia), time to onset of recrudescence, increase of parasitemia and survival time in number of days (Ager, 1984).

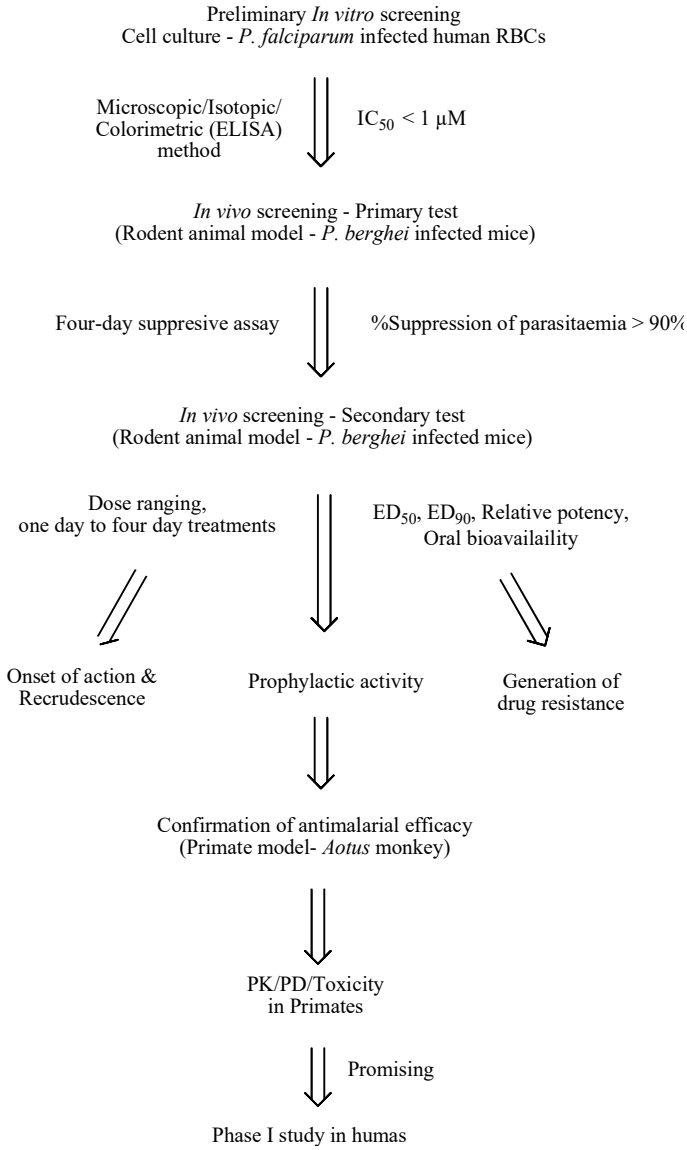

Figure 2. Protocol of efficacy screening for antimalarial compounds (Fidock et al., 2004)

Compounds can also be tested for prophylactic activity by administering the compound prior to infection, followed by daily examination of smears. The prophylactic activity is assessed in terms of suppression of parasitemia, and survival times (in days) (Fidock et al., 2004; Kalra et al., 2006).

Several drug resistant parasites developed in mice models, especially CQ-resistant *P. berghei* and CQ-resistant *P. yoelii* strains are used *in vivo* for the assay of drugs. These strains are intrinsically partially resistant to CQ, and are therefore a poor model for studying acquisition of CQ resistance to *P. falciparum* (Fidock et al., 2004).

The protocol for efficacy screening of new antimalarial compounds is depicted in figure 2.

Primate models also play an important role in preclinical development, by providing a final confirmationof the choice of a drug candidate (Fidock et al., 2004). Infection with certain strains of *P. falciparum* has been well characterized in both owl monkey (*Aotus trivirgatus*) and squirrel (*Saimiri sciureus*) monkeys. *Aotus* is one of the WHO recommended model for studies of malaria, and these are the only models which can sustain malarial infection caused by *P. falciparum* and *P. vivax* (Kalra et al., 2006). Primate models serve as reliable experimental models to investigate various complications associated with malaria, apart from testing drug′s efficacy. Primate models also provide a clearer prediction of human efficacyand pharmacokinetics than rodent models, providing alogical transition from pre-clinical to clinical studies (Ager, 1984).

Conclusion

To develop a new method for the biological evaluation of antimalarial effectiveness, it is inevitable to know about various parasitic molecular or enzymatic targets of drug action. The evaluation method would be successful if the proteins/ or the targets involved in antimalarial drug action of test molecules is known and studied as well. It is therefore necessary to follow up a standardized protocol for the evaluation or screening study and depending on which one can develop further a new method or optimize the existing method to achieve successful outcome in terms of good evaluation results with high accuracy.

Abbreviations

CQ: Chloroquine; ELISA: Enzyme-linked Immunosorbant Assay; ED_{50}: Half Maximal Effective Dose; HRP: Histidine-rich Proteins; IC_{50}: Half Maximal Inhibitory Concentration; LDH: Lactate Dehydrogenase; µM: Micromolar; MIC: Minimum Inhibitory Concentration; *Pf*: *Plasmodium falciparum*; WHO: World Health Organization.

Conflict of interest

Authors declare no conflict of interest.

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