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Cell based assays for anti-*Plasmodium* activity evaluation

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ABSTRACT

Malaria remains one of the most common and deadly infectious diseases worldwide. The severity of this global public health challenge is reflected by the approximately 198 million people, who were reportedly infected in 2013 and by the more than 584,000 related deaths in that same year. The rising emergence of drug resistance towards the once effective artemisinin combination therapies (ACTs) has become a serious concern and warrants more robust drug development strategies, with the objective of eradicating malaria infections. The intricate biology and life cycle of *Plasmodium* parasites complicate the understanding of the disease in such a way that would enhance the development of more effective chemotherapies that would achieve radical clinical cure and that would prevent disease relapse. Phenotypic cell based assays have for long been a valuable approach and involve the screening and analysis of diverse compounds with regards to their activities towards whole *Plasmodium* parasites *in vitro*. To achieve the Millennium Development Goal (MDG) of malaria eradication by 2020, new generation drugs that are active against all parasite stages (erythrocytic (blood), exo-erythrocytic (liver stages and gametocytes)) are needed. Significant advances are being made in assay development to overcome some of the practical challenges of assessing drug efficacy, particularly in the liver and transmission stage *Plasmodium* models. This review discusses primary screening models and the fundamental progress being made in whole cell based efficacy screens of anti-malarial activity. Ongoing challenges and some opportunities for improvements in assay development that would assist in the discovery of effective, safe and affordable drugs for malaria treatments are also discussed.

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1. Introduction

An urgent need for the identification and development of new drugs for the treatment of malaria infections exists. Artemisinin, the current cornerstone in malaria treatments, had first been isolated in 1972 by a Chinese researcher, Tu Youyou, who was recently recognized with the Nobel prize in Physiology or Medicine, for the novel discovery (Su and Miller, 2015). The alarming increase in reports of artemisinin resistant *Plasmodium* parasite strains in Thailand and Cambodia presents a significant problem to effective control and mandates that better programs and strategies be implemented for the development of new drugs that

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would help to control the disease (WHO, 2014). Very few new anti-malarials have been successfully developed in the last quarter of the 20th century. The delay, or prevention of the emergence of multi-drug resistant strains requires the development of new and better drugs with new modes of action and longer pharmacological half-lives. The primary discovery of novel anti-parasitic drugs largely relies upon the development of effective, highly predictive *in vitro* assays for efficacy assessments.

Whole cell based screening is an invaluable tool in the early stage, anti-malarial drug discovery process and for the identification of novel lead compounds. Phenotypic cell based screening assays facilitate the parallel evaluation of drug targets, whilst simultaneously addressing compound cell permeability and bio-availability properties. Although phenotypic screens do not reveal the mode of action of the active compound, such approaches increase the quality of decisions regarding the test drug, by providing information about the pharmacokinetics and the safety of candidate drugs. Assay development for *Plasmodium* parasites is particularly complicated, due to the complex life cycle of the parasite. The emergence of artemisinin resistance has mandated that ongoing research programs intensify their search for identifying new anti-malarial chemical entities.

Key priorities in the transition from the control to the eradication of malaria, are to expand the drug pipeline with a new generation of compounds that would target the various life cycle stages of the parasite, *i.e.* the asexual blood stage drug resistant strains, the late stage gametocytes, the sexual exo-erythrocytic forms and the dormant hypnozoites that cause relapses in *Plasmodium* vivax (*P. vivax*) infections. Currently, the specific stages of the life cycle of *P. vivax* malaria parasites that remain critical to *in vitro* culture development, are the development of the sporozoites, the pre-erythrocytic liver stage and the continuous blood stage culture. Recent advances have resulted in new methods of replacing traditional assays and *in vitro* culture systems that encompass the various stages of the *Plasmodium falciparum* life cycle. These methods have in recent years been employed in the screening of large compound libraries, which yielded some promising new compounds (Gamo et al., 2010; Guiguemde et al., 2010; Rottmann et al., 2010).

2. The life cycle of malaria parasites

Plasmodium parasites develop through asexual replication in the human host and through sexual differentiation in the Anopheles mosquitos. Infection and parasite development begin when a female mosquito transmits approximately 10–100 sporozoites into the skin of the human host during a blood meal. Infective sporozoites transverse the skin, enter the blood circulation and eventually infect hepatocytes, and then undergo schizogony to produce thousands of merozoites in a period of about 50–70 h (Meis and Verhave, 1988). Infective sporozoites typically circulate in the blood stream for up to 45 min, before invading the hepatocytes that lead to the exo-erythrocytic form (EEF) of the parasite. Studies, using the rodent species of malaria, *P. berghei*, have shown that during a bite by an infected mosquito, approximately 40% of the transmitted sporozoites remain at the inoculation site in the skin epithelial dermis, for up to 6 h. Thereafter, 70% of those parasites in the skin will enter the blood circulation to infect the erythrocytes, whilst the remainder (30%) is drained by the lymph nodes (Fig. 1A) (Amino et al., 2006). It is, however, still unknown whether the *Plasmodium* species that infect humans are also able to develop in the host's skin. Nonetheless, drug discovery research efforts have primarily focused on 60% of the sporozoites that instantaneously infect the hepatocytes, following a mosquito bite, as well as on the parasite stages that develop in the liver cells.

EEFs multiply and undergo maturation to generate a large intracellular schizont (Fig. 1B). The schizont continues to grow in size inside the hepatocytes over a period of 5–6 days and eventually bursts and releases merozoites into the blood stream, which invade circulating erythrocytes and initiate blood stage infection (Sturm et al., 2006). The *Plasmodium* species, *P. vivax* and *Plasmodium ovale* sporozoites are known to develop into the non-dividing hepatic forms (hypnozoites), which may remain dormant in the liver for long periods, before growth is activated and is this thought to lead to the relapse of malaria, months, or even years after the initial infection (Fig. 1C) (Markus, 2015). Factors that lead to dormancy and the re-activation of hypnozoites to activate infection, are still unknown. Currently, the only drugs with significant activity against proliferating EEFs and hypnozoites are the 8-aminoquinolines, such as primaquine, bulaquine and tafenoquine (Sweeney et al., 2004) Fig. 2. However, only primaquine has to date been approved by the World Health Organization (WHO). 8-Aminoquinolines are toxic and cause haemolysis in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiencies and is this condition prevalent in some endemic countries (Baird, 2015). This emphasizes the need for having to continue the search for new, non-toxic drugs that target the liver stages of the malaria parasites. The targeting of the liver stages will yield compounds that would prevent infection (prophylactic) and cure the disease (radical cure), therefore making these compounds essential for malaria eradication strategies.

Many of the existing anti-malarial drugs were developed for action against the intra-erythrocytic stage of the life cycle to target the circulating merozoite infected erythrocytes. During the 48 h cycle in the red blood cells, the intra-erythrocytic merozoites develop within a parasitophorous vacuole to form a ring stage, which persists for the initial 24 h, before developing into trophozoites. Each trophozoite undergoes rapid nuclear division (schizogony) to grow in size and progresses to form a heme, crystal containing schizont stage during the last 24 h of the cycle. Upon maturation of the schizont, the parasitized red blood cells rupture and release 8 to 32 merozoites each that rapidly infect fresh, uninfected erythrocytes to continue the cycle (Fig. 1D). The asexual erythrocytic cycle and the continuous rupture of the merozoites from the infected erythrocytes are responsible for the clinical manifestations of malaria.

Following the invasion of the erythrocytes, some of the merozoites leave the cycle of asexual replication to undergo gametocytogenesis, a process whereby male and female gametocytes develop into sexual forms of the parasite, which then circulate in the blood stream (Fig. 1E). A critical discovery recently identified the gene/protein Apetala 2 (AP2), that plays a central role in inactivating a set of genes that initiate the development and maturation of the gametocytes (Sinha et al., 2014). This discovery will have major implications for future strategies to evaluate the inhibitors of gametocyte development. The formation and maturation of gametocytes occur in five morphologically recognizable stages (I–V). Early stage gametocytes (I–IV) are sequestered in deep tissue, micro-vasculature places, whereas only the mature stage V gametocytes circulate in the peripheral blood and are they detectable in the blood stream on days 7 to 15, following the initial wave of asexual parasites from which they are derived (Day et al., 1998; Eichner et al., 2001). After the invasion of the sexually committed gametocytes into erythrocytes, the parasites differentiate into mature gametocytes (V) over a period of 8–10 days. A mosquito becomes infected during a blood meal from an infected human host. Once the mature gametocytes are ingested by the Anopheles mosquitoes during their blood meal, they become active and prepare for fertilization during a process, called gametogenesis, during which they can be ingested by mosquitoes to continue the infectious cycle. Most malaria drugs exhibit partial activity against the early stage *P. falciparum* gametocytes, but are they ineffective against the late stage gametocytes (Adjalley et al., 2011; Bousema and Drakeley, 2011). In fact, some drugs may even enhance gametocytogenesis, such as sulfadoxine-pyrimethamine, which had reportedly been associated with an increase in gametocyte carriage after treatment (Govere et al., 2003). Although atovaquone blocks transmission, it has no effect on the mature gametocytes (Butcher, 1997; Lelievre et al., 2012). Indeed, it had demonstrated the inhibition of transmission by blocking the development of the parasites from ookinetes (*Plasmodium* parasite stage, following the fusion of the male and female