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A comprehensive study of the antiplasmodial effects of Artemisia spp. infusions against multiple parasite developmental stages including hypnozoite

Kutub Ashraf

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THÈSE

PRÉSENTÉE A

L'UNIVERSITÉ PIERRE ET MARIE CURIE-SORBONNE

Ecole Doctorale 515 - Complexité du vivant

Par kutub ASHRAF

POUR OBTENIR LE GRADE DE

DOCTEUR

SPECIALITÉ: Parasitologie

A comprehensive study of the antiplasmodial effects of *Artemisia spp.* infusions against multiple parasite developmental stages including hypnozoite.

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“Be less curious about people and more curious about ideas”

Madam Marie Curie (1867-1934)

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Abstract

My PhD thesis entitled ‘A comprehensive study of the antiplasmodial effects of *Artemisia spp.* infusions against multiple parasite developmental stages including hypnozoite’. The dormant liver resident parasite is called hypnozoite which can linger for weeks to months, and then relapse to cause recurrent blood stage infection. This hypnozoite reservoir is one of the critical barriers towards malaria eradication, mainly due to the lack of mass medication with a drug that can clear the hypnozoites in the liver. There is a dire need for the development of new hypnozoite-killing drugs but phenotypic screens are hindered by a lack of *in vitro* culture platforms. Under conventional culture conditions, hypnozoite cultures are often contaminated. This effect partially arises from infection with unpurified sporozoites that adversely affect the hepatocytes culture. Addressing this issue, in chapter 1, firstly I focused on the development of a short protocol with an antimicrobial cocktail, methyl paraben and penicillin streptomycin in a supplementation with sucrose solution that ensure yeast free sporozoite production in the salivary glands. In **chapter 2**, I tried to describe the research objectives, methodology and findings of the experiments. After developing a robust culture strategy, I moved on drug screening against the hypnozoite stage. We selected two *Artemisia sp.* *A. annua* and *A. afra*. Aqueous infusions prepared from them and were tested against the hepatic stage of all *Plasmodium sp.* The reasoning behind these plant selections have been described in detail in this manuscript. I found a strong antimalarial activity of *Artemisia* infusions that blocked the relapse causing hypnozoite formation and cleared the liver parasites. **In chapter 3**, I described the effect in more detail. We believe that this profound inhibitory activity of *Artemisia* infusion is partially mediated by the disruption of the biogenesis of apicoplast and mitochondria. **In chapter 4**, I showed various confocal images of apicoplast disruption and quantitative PCR data of apicoplast DNA confirm our observation. Drug resistance to *P. falciparum* is a growing problem in Southeast Asia. New drugs are required to solve the problem. **In chapter 5**, I described *in vitro* activity of *Artemisia* infusions against the drug resistant *P. falciparum* isolates. It can be noted that the *in vitro* antimalarial effect that I observed in the liver and blood stage of *Plasmodium* was not artemisinin dependent as *A. afra* contains negligible amount of artemisinin but showed potent inhibitory activity. This is the first indication that compounds other than 8 aminoquinolines and artemisinin could be effective against the relapsing malaria and overcome the antimalarial drug resistance.

Chapter 1

General Introduction

1. Etiology and Impact of malaria

Malaria is a life-threatening disease caused by the protozoan parasite of the genus *Plasmodium* which is transmitted to humans by the bite of infected female *Anopheles* mosquitoes¹. Till date, four *Plasmodium* species are historically categorized as accountable for human malaria disease: *Plasmodium falciparum*, *P. malariae*, *P. ovale* and *P. vivax* and they possess a distinct global distribution. Very rarely, humans can be infected either accidentally or naturally by simian malaria species for instance *P. cynomolgi*, *P. simiovale*, *P. brasilianum*, *P. schwetzi*, *P. inui* and *P. knowlesi* but human to human transmission has not been documented so far²⁻⁴. Zoonotic malaria *P. knowlesi* is now considered as a source for an increasing number of human infections particularly in the regions of South-East Asia implicating an emerging menace to human health⁵. Evolutionary, *Plasmodium* has been considered belonging to the phylum of *Apicomplexa*, and emerged from the coccidian stem that surrounded the subsequent acquisition of complex life cycles⁶. The successful survival of malaria parasites need a completion of life cycle in two evolutionarily distant species (*i.e.* human as host and the mosquito as vector).

Of all diseases, malaria is probably the one with the most subtle interplay with human history. It has long affected human beings, and has left its genetic mark on modern populations⁷. Unfortunately this ancient human tragedy is still thriving and according to WHO report (2020) there were an estimated 229 million malaria cases reported in 87 malaria endemic countries in 2019⁸. Although the global map of malaria is shrinking but children under age of 5 are still under vulnerable and one out of five children in Africa are dying because of malaria^{9, 10}. In addition, over 90% of malaria morbidity and mortality is restricted to Sub-Saharan Africa¹¹. Such morbidity and mortality, the social and economic burden of malaria, specifically in underdeveloped countries, remains enormous. In South-East Asian regions, *falciparum* malaria is still a major public health a problem because of its virulence and rapid drug resistance¹². Its pathogenicity is due to asexual reproduction rate in the host and an ability to sequester in small blood vessels. Following the infective bite by an *Anopheles* mosquito, a period of time (the “incubation period) goes by before the first symptoms appear. The incubation period in most cases varies from 10 to 14 days. The shorter periods are observed most frequently with *P. falciparum* and the longer ones with *P. malariae*. Some strains of *P. vivax* have a 3–6 month primary incubation period¹. On the other hand, *vivax* and *ovale* malaria are responsible for

malaria relapses which occur by the reactivation of hidden reservoir of dormant parasites in the host liver. Development of interventions against this stage was overlooked as the disease was considered 'benign'¹⁴. But wide distribution and accumulating evidences of severe and complicated cases of *vivax* malaria indicate that this disease has been more underestimated than benign^{15, 16}. Thus malaria forms one of the most dreadful global public health problems¹⁷ and ranked 8th as one of the most important life threatening infectious diseases in the WHO list¹⁸.

2. Malaria life cycle and Epidemiology

2.1 Life Cycle

The life cycle of malaria initiates with the bite of an infected female *Anopheles* mosquito to a human during probing for a blood meal. During the journey, a malaria parasite undergoes a complex series of morphological alterations in different phases. These changes happen in two distantly related different hosts: a vertebrate (intermediate) host and an insect (definitive) host, known as mosquito vector. Briefly, after mosquito bite, salivary gland forms of the parasite so called 'sporozoites' are deposited into the mammalian skin. The sporozoites glide around and the gliding of sporozoite can be as quick as on glass slides ($\sim 1-2 \mu\text{m}$ /second within the first 30 minutes) exhibiting tortuous movements with undetectable tactism although their speed gradually decreases with time¹⁷. However, some sporozoites although rapidly leave the injection site whereas some of them take hours to exit the site in a slow trickle and enter into the blood stream. 20% of the inoculum are transported in lymph vessels, draining lymph nodes or travel through the bloodstream to the liver. In the liver, they traverse across the sinusoidal barrier to reach their final destination inside a hepatocyte¹⁹. Investigation with rodent malaria implies that sporozoites can cross this barrier by multiple mechanisms, using kupffer cells or endothelial cells, either or not in combination with cell traversal. After crossing the barrier, sporozoites generally traverse several hepatocytes of host and settle into one where they transform into early exo-erythrocytic liver forms²⁰. However, it is not known how sporozoites specifically select a hepatocyte to be settled into of it. These forms rapidly proliferate via nuclear division to form new parasite, called schizont. Rodent parasites like *P. berghei* and *P. yoelii* complete their liver stage development within 48 h-55 h of post infection while in human malaria, the cycle last for $\sim 7-10$ days¹⁷. This hepatic phase traditionally viewed as clinically asymptomatic and no malaria complications have been detected and documented to date.

Nevertheless, a complex series of events can take place in the hepatic stage to initiate the immune response for example parasites can stimulate type I interferon (IFN) transcription program in the infected hepatocytes, observed *in vivo* in a rodent malaria model²¹.

Upon complete maturation, hepatic parasites egress from the liver cells and culminate into thousands of new parasitic forms known as merozoites. These merozoites rapidly invade the circulating red blood cells and initiate blood stage infection known as erythrocytic stage of malaria. Blood stage parasites also proceed through several rounds of asexual replication resulting in different morphological features (**Fig 1C**) and antigenic characteristics. However, this is the most invasive stage where each replication round results in the rupture of host red blood cells and invades new red blood cells, continue the cycle called 'exponential multiplication' over again²². Consequently an individual patient starts exhibiting clinical episodes of malaria ranging from uncomplicated malaria symptoms (e.g. headache, nausea, body ache, shivering and chill following with high fever and general malaise) to severe (e.g. anemia, cerebral malaria, acute kidney injury, hemoglobinuria, hypoglycemia, coma and occasionally death). *P. falciparum* malaria may cause severe disease in the mother leading to premature delivery or delivery of a low-birth-weight baby. On rare occasions *vivax* malaria can cause rupture of spleen.^{13, 14} A small subpopulation of blood stage parasites (less than 10%) leave the asexual stages and transform into sexual stages differentiating male and female gametocytes²³. These sexual parasites are needed to be transmitted from human to mosquito through a mosquito vector bite. Following a development cycle for 10-18 days inside the mosquito, the parasite (infectious sporozoites) reaches to the salivary glands and thereby becomes ready to introduce a new infection when the mosquito bites to another host²⁴. This life process of malaria is fully adapted by all malaria species except two other human malaria sp. *P. vivax* and *P. ovale* and primate malaria *P. cynomolgi* as they possess an additional unique biological phenomena that define them as relapsing malaria species. These relapsing species develop into non-proliferative dormant parasites inside the liver and remain present for prolonged periods of time, known as hypnozoites ('sleeping animals'). These hidden forms can activate after weeks, months or even years later through unknown triggering mechanisms to resume liver stage development causing relapse infection²⁵. On top, virtually nothing is known about the bio-physiology of hypnozoite stage and its interaction with host^{25, 26}.

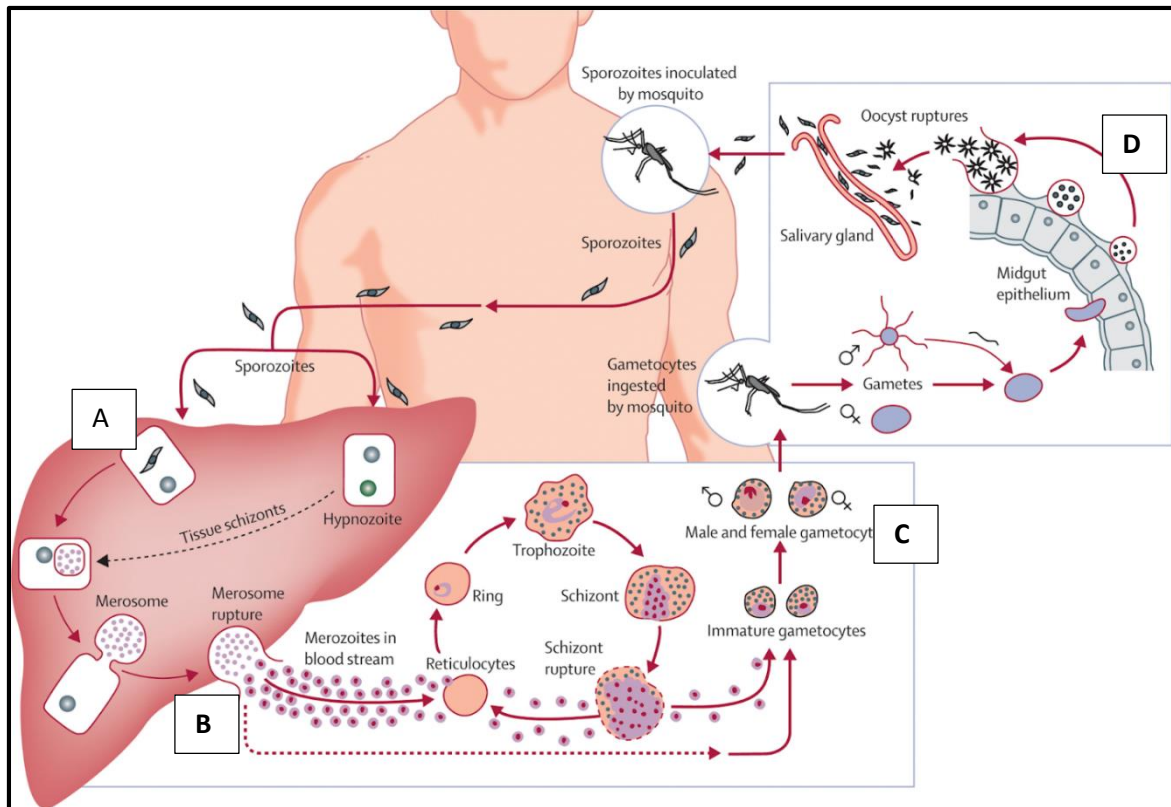


Figure 1: (adapted from²⁷) Life cycle of relapsing malaria parasites. Following the bite of an infected *Anopheles* mosquito, infective sporozoites reach the bloodstream and enter into hepatocytes to initiate liver stage infection **(A)** malaria parasites proliferate in the liver, develop into schizonts. A subset parasite population of relapsing malaria species (*P. vivax*, *P. ovale* and simian malaria species *P. cynomolgi*) alternatively can remain dormant in the liver (hypnozoite) for prolonged periods of time before activating and continuing development to initiate relapse infection **(B)** nearly 7-10 days later, matured schizonts rupture and release new forms of parasites into the bloodstream called as merozoites. **(C)** This is then followed by a cyclical development in blood by invading young, immature blood cells reticulocytes, during which some parasites develop into sexual stages, so-called gametocytes. Circulating gametocytes are round in shape. When taken up as blood meal by an *Anopheles* mosquito **(D)** they fertilize and develop into motile ookinete, subsequent days later they form into oocysts and multiply to generate new sporozoites in the infected mosquito salivary glands. Thus parasites undergo more than ten stages of cellular differentiation by invading four types of cells within two distinct hosts to complete full malaria life cycle.

2.2 Geographical distribution of *Plasmodium vivax*

Vivax malaria is a neglected tropical disease, considered as an intrinsically benign and self-limiting infection²⁸. The parasite is currently extensively restricted to tropical and subtropical latitudes in Asia, Oceania, and the Americas. A substantial attempt of malaria control has nearly halved the death rate and reduced the global incidence rate by more than 30%²⁹. *P. vivax* accounts for 42% of all reported cases of malaria outside Africa. The geographical range of *P. vivax* covers a widespread distribution putting 2.5 billion people at risk of infection.³⁰ Consequently, *P. vivax* presents a major challenge to the roadmap of malaria elimination program. To bring the global health community's ambition of eradicating malaria by 2040, it is important to understand of all *Plasmodium* species known to cause human malaria.

P. vivax was rarely studied across most of Sub-Saharan Africa. The overwhelming dominance of the Duffy-negative blood group was considered as a key determinant preventing the sustained endemic transmission. This malaria species although once viewed as a benign infection but now is being recognized as a cause of severe morbidity and mortality that result in a substantial negative effect on health³⁰. This is enabled by intracellular hidden parasite reservoir in the human liver. Some studies suggest that *vivax* infection might reduce the *P. falciparum* disease severity, in contrast others suggest that it might increase the risk of severe disease. In endemic areas where *P. vivax* co-habits with *falciparum* and in these regions mixed infections with the two distinct malaria species are also common incidents. Not only does *P. vivax* presents a barrier to elimination but it can be as debilitating as *P. falciparum* malaria, causing severe disease and death^{31,32}. India accounts for 77% of total malaria in Southeast Asia whereas Cambodia (58%) and Myanmar (31%) accounted for most of the cases of malaria in the Greater Mekong regions³⁰. The global prevalence of *P. vivax* was estimated low compared to *P. falciparum* in 1-to 99 years range (**Fig. 2**).

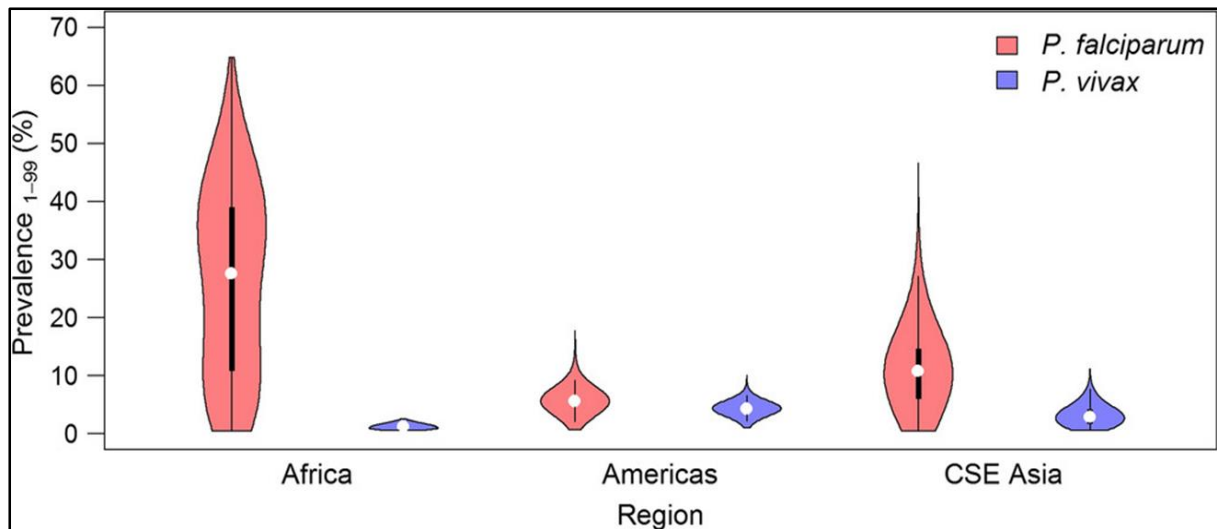


Figure 2: Adapted from³⁰: Graphical trends, *P. falciparum*: *P. vivax* ratio to compare the prevalence. Prevalence values in kernel density plot covering the malaria affecting regions from *P. falciparum* and *P. vivax* endemicity surfaces standardized to the 1- to 99-year age range. The black central bar depicts interquartile range whereas white circles point out the median values. *CSE Asia = Central and Southeast Asia.

2.3 Rising reports of *Plasmodium vivax* in sub-Saharan Africa: Barrier of malaria elimination agenda

It was thought that *P. vivax* parasites depend on Duffy antigen to internalize and establish within the erythrocytes. Hence erythrocytes lacking the antigen were refractory to this parasitic infection. Consequently it was thought that individuals with the Duffy-negative phenotype and their diaspora are resistant to *P. vivax* invasion. The recent substantial appearance of *vivax* cases (albeit low numbers) added a concern in the context of malaria epidemiology and local surveillance in the horns of Africa. This could be due to the utilization of very sensitive diagnostic tools (e.g. PCR) and substandard reagents, which might exacerbate the underestimation of detecting non-*falciparum* malaria in this sub-region. Consequently some of the countries e.g. Mali, Mauritania, Sudan, Cameroon, and Southwestern Nigeria have challenged this notion by exhibiting a low occurrence of *P. vivax* prevalence to the Duffy-negative phenotype^{33,34}. Therefore, now it is more widely accepted that Duffy positive and negative populations of these regions including diverse ethnic backgrounds are able to sustain the transmission of *P. vivax* parasites. Therefore the global expression of this antigen binding protein (*PvDBP*) is considered as a prime *P. vivax* vaccine target³⁵.

3. Historical perspective and unique biology of *Plasmodium* liver stage

3.1 Discovery of pre-erythrocytic malaria stage

Malaria has been known as a disease since prehistoric times that shaped and became a part of history and politics of mankind. In 1880, a French medical doctor Alphonse Laveran discovered *Plasmodium* parasites in an Algerian patient's blood although at that time it was raised skepticism to some medical pundits who thought malaria was originated by bacterial pathogens. In that time Golgi was persuaded about the concept of Laveran while he noticed that parasites divided simultaneously with the onset of fever³⁶. Later in 1897, Ross first demonstrated avian malaria parasites in the insect vector which was supported by other parasitologists³⁷. It was Manson and Fearnside who documented their own malaria relapse story after 8-9 months treatment by quinine³⁸. Relapse is a widely used clinical term that denotes the return of the clinical manifestations of a disease after its apparent cessations. In addition, the theoretical and cyclic aspects of relapse events can be described in various ways considering geographical variations.

The pioneering contribution by Raffaele and his colleagues in the 1930s brought light to the discovery of exo-erythrocytic schizonts in macrophages, spleen and reticuloendothelial cells in infected birds at the inoculation site in the skin³⁹. Shortt and his co-workers⁴⁰ observed these exo-erythrocytic schizonts in *P. cynomolgi* infected parenchymal tissue of rhesus monkey. However, later it was shown that infecting sporozoites settle in hepatocytes and undergo a single schizogony. An interesting phenomenon was the discovery of another parasite form in some mammalian species of malaria that can persist inside the hepatic cells for weeks to years. The breathtaking discovery was coined as hypnozoite by Krotoski ' and his colleagues⁴¹. Later it was assumed that at least some of the malaria recurrences and relapses was due to the hypnozoite origins.

The hepatic stage of rodent malaria was discovered in African rat *Grammomys surdaster* in 1948 and transmitted in laboratory rodents in 1965⁴⁰. Since then, *P. yoelii* and *P. berghei* became a robust and easily accessible model for studying the fundamental biology of malaria. Especially, these parasites, amenable to fast and simple genetic manipulation, made simpler the identification of the role of *Plasmodium* genes using inverse genetics.

3.2 Unique biology of *P. vivax*

The key distinguishing feature of *P. vivax* biology is its ability to form long-lasting liver stages, the hypnozoites and relapses. Stress and seasonal abundance of local mosquitoes in a *vivax* endemic region might play a part in the relapsing event^{42, 43}. Another undisputed biological characteristic of *P. vivax*, its preferential target of immature reticulocytes over normocytes in the blood of host erythrocytes. The reason for such preference is still not well addressed as understanding the molecular and cellular mechanisms underlying reticulocytes invasion is hindered due to lack of a long term *in vitro* blood stage culture⁴⁴. Moreover, *P. vivax* is difficult to diagnosis in the field as reticulocytes parasitemia appears to be low (reticulocytes comprise only 1-2% of erythrocytes). Subsequent destruction of reticulocytes lead to severe and chronic anemia which accelerates patient's morbidity⁴⁵. Morphologically, *vivax* parasites are more amoeboid than *P. falciparum*, produce cleft structures during its growth in the reticulocytes⁴⁶. Moreover, sexual stages of *vivax* malaria can be developed early and could be transmitted before the disease symptoms appear although this notion is greatly questioned.^{47, 48}. It is reported that *P. vivax* can be transmitted by approximately 71 *Anopheles* species and sporogony in mosquitoes can occur at a lower temperature compared to *P. falciparum*⁴⁹.

To date, *P. vivax* liver stage biology is poorly understood compared to other *Plasmodium* life stages, which has impeded our ability to target these parasites for *vivax* malaria prevention.

3.3 Hypnozoite discovery and its distinct features

Malaria relapse is not only a recent enigma but intrigued the malariologist and parasitologists over a century to understand its unique biology. Krotoski and his colleagues detected non merozoite like liver forms (nearly 5 µm in size) in the sections of liver biopsy from a monkey that had been infected 7 days before with *P. cynomolgi bastianelli* sporozoites⁵⁰. The remarkable discovery of the hypnozoite was literally a coincidence as it was identified during the quality assay of IFA reagents in hepatic stage. However, these small intrahepatic, singly occurring uninucleate, stages were then again re-appeared in liver biopsy at day 50 implicating that those forms could reside in the liver for an extended periods of time. These stages were

eventually coined as hypnozoites ('sleeping animalcules')⁵¹. The unique characteristic observed in *P. cynomolgi* was also observed in the case of *P. vivax* infections among human volunteers⁵². Human experiment findings of Helsinki failed to detect hypnozoite in humans but subsequent studies detected these latent forms in the liver biopsies of chimpanzees infected with *vivax* sporozoites⁵³. Apart from *P. vivax*, both *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* also produce hypnozoites in their life cycles. Furthermore hypnozoites have been observed in the life cycles of the relapsing primate malaria species *P. simiovale*.⁵⁴ Histological analyses of *P. vivax* from patients and experimental non-human primates revealed that asexual and sexual gametocytes of *P. vivax* can exist in the hematopoietic niche of bone marrow and probably also in the spleen⁵⁵.

Hypnozoite identification is not possible at the very early of sporozoite infection both *in vitro* and *in vivo* due closer morphological similarities of early parasite liver forms (trophozoite, schizont and hypnozoites)^{56, 57}. The size of hypnozoite was first estimated in liver biopsies of chimpanzees infected with *P. vivax* (approximately 4-5 μm in diameter) lying within the cytoplasm⁵⁸. Several *in vitro* and *in vivo* humanized mice model revealed that hypnozoites can be distinguished from dividing schizonts at day 4 of post infection based on their uninuclearity, smaller size distribution (approximately <10 μm in diameter) while schizonts begin to grow and increase their nuclear mass⁵⁶. Earlier, the hypnozoites detection was an assumptive basis; estimating the size by immunolabeling with a chaperon marker HSP70 but the chaperone protein equally mark the dividing schizont in the fixed tissue. Nowadays, UIS4 (Up-regulated Infectious sporozoites), a parasitophorous vacuole membrane marker (PVM) which is now widely used to mark schizont and hypnozoite in the culture based on PVM prominence⁵⁹. Hypnozoite was thought to be stagnant in the liver but steady growth of hypnozoites documented from day 3 of post infection as size increased from an average 4 μm to 7 μm at day 7. This observation indicated a slow metabolic and biochemical activity possibly occurred in the hypnozoite even to a lesser extent. The well known hallmarks of hypnozoites are smallest area distribution, uninucleated shape and persists for several weeks in the liver and activates later. In addition, hypnozoite identification in *in vitro* culture could be addressed with the drug sensitivity. Till date, hypnozoites only showed susceptibility to 8-aminoquinoline-derived drugs e.g primaquine and tafenoquine but not with other schizonticidal drugs atovaquone and phosphatidylinositol 4-kinase inhibitor (PI4K)^{60, 61}. *P. cynomolgi* is non-human

primate malaria, found in rhesus macaque monkey, genetically and phenotypically similar to *P. vivax* in the context of disease pathogenesis including relapsing phenomena. This is considered as an acknowledged experimental model of *P. vivax* infection.

3.4 Reactivation of hypnozoites and relapse pattern

It was reported that 80–90% of *P. vivax* blood stage infections identified in Thai patients were due to the hypnozoite mediated relapses and was not linked to newly acquired infections^{62, 63}. Thus the hidden reservoir inside the host greatly facilitates the *vivax* parasites to survive in regions where mosquitoes are only prevalent in a certain period of time in a year. Relapses variations are associated with different strains of parasites. For instance, *P. vivax* sporozoites from North Korean strain recorded long latency characteristic with a predominance of hypnozoites and strains that exhibited short prepatency and latency characteristics produced a mixture of parasite populations (schizonts and hypnozoites)^{64,65}. The *vivax* malaria in tropical regions capable of repeatedly induce new blood stage infections might compete with more virulent *P. falciparum*. Instead of a predetermined duration of dormancy, reactivation of hypnozoites might occur stochastically⁶⁶⁻⁶⁸. In addition, environmental cues might regulate the relapsing phenomena. The relapse frequency in temperate climatic conditions generally long exceeding to 6-9 months (the interval from primary infection to relapse) whereas it occurs frequently in the tropical regions at short intervals (3 to 4 weeks after malaria treatment without any anti relapsing drugs)⁶⁵. Hence, time-to-recurrence, particularly the interval time from treatment to the preceding clinical malaria episodes might offer valuable information to understand the relapse dynamics⁶⁹⁻⁷¹. The tropical Chesson strain from New Guinea-South Pacific exhibits a short-latency between early and late attacks whereas the temperate St. Elizabeth strain consistently displays a long-latency times⁶⁵. However, the molecular basis of underlying long latency is unknown⁷².

Due to various practical reasons, it is a herculean task to dissect the precise kinetics of relapse periodicity and hypnozoite activation from *in vivo* biopsies model. The probable kinetics, if could be determined, might allow to understand the critical aspects of a hypnozoite's behavior; for instance, how it enters and quits dormancy in a systematic manner.

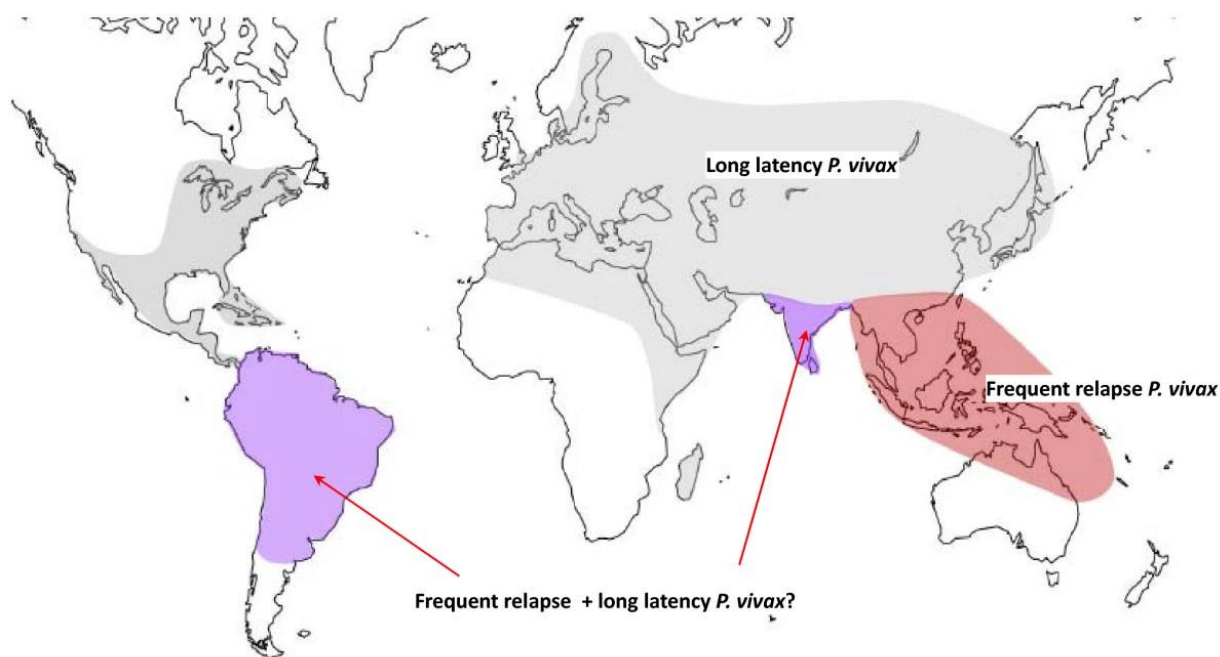


Figure 3: Adapted from³² A tentative latency phenotype based geographical distribution of *vivax*. Areas exhibit tropical "frequent relapse phenotypes" demonstrated in pink whereas frequent relapse and long-latency phenotypes have been pointed out in purple and prevalence of long-latency phenotypes shown in grey.

3.5 Mathematical concept of reactivation kinetics

Relapses, arising from liver resident hypnozoites, primarily responsible for most of the cases of *vivax* malaria. However, in endemic areas, a recurrent blood stage infection following treatment can be hypnozoite-derived (relapse), a treatment failure either by drug resistance that fails to eliminate complete blood stage parasites from the host (recrudescence), or a newly acquired infection by an infected mosquito bite (reinfection). Moreover, acute pathogenic infections are also reported to be involved in triggering relapses. To distinguish these patterns of infection is technically challenging as relapse causing parasites were found genetically different from those observed in the most recent blood-stage infection^{73,74}. For this reason, it is not possible to differentiate hypnozoites reactivation from new primary infection using

genotyping tools. In addition beyond the first relapse, multiple relapses could be directed by a single mosquito inoculation at any given time⁷¹.

Mathematical model named 'within-host model' was previously used to understand infectious disease patterns. The structure of this epidemiological 'microparasite' model could define an individual patient either uninfected or infected and susceptible or refractory to a disease infection⁷⁵. In *vivax* malaria, this model was described to predict hypnozoites mediated relapse dynamics and facilitated to quantify the corresponding contribution of new primary infection versus infection occurred due to the hypnozoites reactivation⁷⁴. Apart from this, another mathematical model identified how hypnozoite reservoirs contribute to *vivax* transmission. The model described that hypnozoites in the reservoir either die or are activated and enter into the bloodstream following a constant rate. This resembles how often relapses occur in patients. In general, hypnozoite reservoirs in many individuals can be over dispersed and people having more hypnozoites are vulnerable to more chances of malaria relapses. Therefore, the model suggests that *vivax* transmission could be substantially reduced from any *vivax* endemic regions, if patients are treated with radical cure drugs with an incorporation of a blood schizonticide. Although other confounding factors such as drug pharmacokinetics, susceptibility, parasite biomass and host immune response could also influence the estimated timing of relapse infections⁷³.

4. Treatment of *vivax* malaria

4.1 8 aminoquinolines, the only anti-relapsing therapies for *vivax* malaria and limitations

For the last approximately 70 years, 8 aminoquinolines, e.g. primaquine can cure relapsing malaria, and almost nothing has been exactly known about the mode of the action of these drugs. However these drugs cannot be globally deployed as it renders a life threatening risk of hemolytic anemia among the G6PD deficient individuals (the prevalence is 8% in malaria endemic countries)^{76,77}. Even if it is prescribed, patient requires a long 14 days drug regimen treatment concurrently with chloroquine at a dose of 15 mg base per day (210 mg total dose) which is another serious limitation of PQ. Moreover cases are reported where the correct PQ treatment failed to block the relapse infections and consequently patients

experienced multiple attacks of *vivax* malaria and encountered clinical complications⁷¹. This could be explained from the notion that PQ acts as a pro drug whose therapeutic activity depends on its metabolization by host hepatic enzymes cytochrome P450s⁷⁸. Thus host cytochrome status is crucial and largely contributes to the efficacy of primaquine. PQ might offer potential risk for the non-immune individuals who do not respond adequately to PQ due to reduced CYP2D6 activity. The identity and source of the primaquine metabolite active against hypnozoites in humans remain unknown, but it has been reported that patient with intermediate or poor metabolizer CYP2D6 genotypes encountered therapeutic failure. Furthermore, PQ treatment is strictly contradicted in pregnant or lactating women and children under 6 months old because of safety issues⁷⁹ although no direct evidence has been shown till date. Moreover, alternative dosing of primaquine (7 days treatment plan) is under clinical evaluation but some of them received positive outcomes⁸⁰. Considering all health issue, clearly, there is a great need for well-tolerated new drugs (or chemotherapeutic strategies) to eliminate the hypnozoite reservoir as it is regarded as a major barrier of malaria eradication.

To overcome PQ liabilities, tafenoquine (TQ), analogue of PQ was discovered by Walter Reed Army institute, USA with collaboration of GlaxoSmithKline and MMV (Medicines for Malaria Venture). TQ has been recently registered for both prophylaxis and radical cure treatment of *P. vivax* and *P. ovale* after successful completion and safety evaluation in phase clinical trials and approved for medical use. Prospectively, clinical study of TQ demonstrated equal therapeutic effect like PQ after a single dose of 300 mg whereas PQ requires a 14 days of treatment. Although tafenoquine exerts a long terminal half-life (approximately 16 days) which offer advantages over PQ but TQ is still associated with hemolytic liabilities like PQ and thus need a point- of-care diagnostic on site to prescribe⁸¹⁻⁸⁴. Hence still there is room to optimize and modify tafenoquine to treat the mass people.

4.2 Proposed mode primaquine action

The first synthetic antimalarial 8-aminoquinoline primaquine has been widely used clinically⁸⁵. Years after, still primaquine is the only anti-hypnozoite drug against latent malaria. However, till date, the mode of action is elusive. An old study revealed that 48hours exposure to primaquine caused lesions and impaired the life process of the exo erythrocytic parasite *Plasmodium fallax*⁸⁶. Another avian malaria culture model showed that PQ exposed exo-

erythrocytic parasites induced abnormal phenotypes with swollen mitochondria⁸⁷⁻⁸⁸. It indicated that PQ is specific in its action by targeting the parasite mitochondria where host mitochondria remained unaffected. It means the genome architecture of parasite mitochondria and host mitochondria could be different. In addition, the 8 aminoquinolines also affect the gametocytes and thereby PQ administration in the patient could substantially reduce malaria transmission⁸⁹. However the mode of PQ action underlying significant anti-parasitic activity is still not clear for decades. But it was described that the drug has to be rapidly metabolized by liver enzymes to exert effect against hypnozoites⁹⁰. The major metabolites identified following PQ administration is carboxy primaquine that reached peak levels within 3-12h, tenfold higher than its parent molecule but without any proven antimalarial activity^{91, 92}. However, recent article described PQ mode of action on *P. falciparum* hepatic parasites and gametocytes. Authors reported that PQ activity in the liver stage depends on host CYP2D6 status, whilst its metabolite OH-PQm exhibited CYP2D6-independent activity against gametocytes. Thus, PQ effect on gametocytes delineating from hepatic metabolism of PQ⁹³. Such mechanism might be epitomized in the context of PQ activity against hypnozoites although a particular demonstration will be required. Undoubtedly, PQ can eliminate hypnozoite reservoirs in the patient as radical cure manner (a cure in which one will no longer relapse and no longer be able to infect others⁶¹) when the drug is administered with a blood schizonticide. It is not clear to what extent PQ as monotherapy is effective to prevent the relapsing malaria. Indeed, a radical cure phenomena of primaquine has not been attained *in vitro* against hypnozoite till now, particularly when the drug was added into *vivax* infected human hepatocytes culture from day 5 of post infection and continued the drugging until 2 weeks of infection⁵⁶. Camarda et al. postulated PQ could act in a two - step process⁹³.

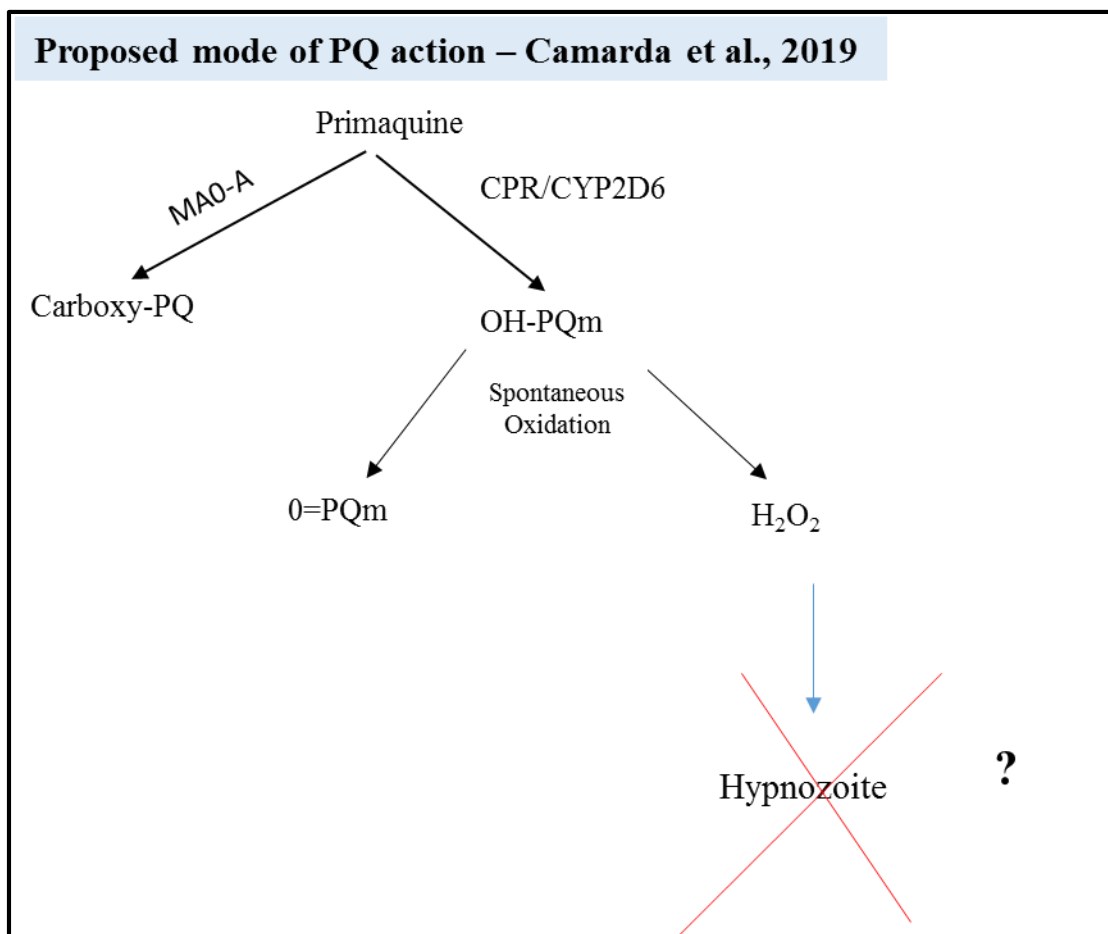


Figure 4: Adapted from⁹⁴: Primaquine is hydroxylated into hydroxyl-primaquine metabolites (OH-PQm) by the cytochrome P450 2D6, mediated by the complex NADPH cytochrome P450 oxidoreductase (CPR). The spontaneous oxidation of this metabolite yields quinoneimines (O=PQm) by producing successive cycles of H₂O₂. It is then, does H₂O₂ probably kill parasite?

5. Basic understanding of pre-erythrocytic biology

5.1 Sporozoite: factors contributing hepatocyte permissiveness

The invasion of suitable hepatocytes by infectious sporozoites is a bottleneck in the malaria parasite lifecycle and is an early step for successful parasite infection. Sporozoites entry to hepatocytes occurred via two distinct pathways depending on *Plasmodium* species: by

disruption of the plasma membrane followed by parasite migration through cells, or by the formation of a vacuole essential for further replication of the parasite. Two major proteins on the surface of sporozoites, circumsporozoite protein (CSP) and thrombospondin-related adhesion protein (TRAP), are essential for sporozoite motility and liver-stage infection⁹⁶. By the advent of reverse genetics several parasite proteins have been identified that are considered to play an essential role in cell traversal likely SPECT-1, Perforin like protein PLP-1, CelTOS etc. CelTOS additionally also contributes in sporozoite gliding⁹⁷. However sporozoites, lack of these proteins were unable to traverse through hepatic cells and impeded parasite migration. But eventually retain the normal invading capacity.

Other sporozoite protein family *Plasmodium* s48/45 for instance; P36 and P52 were documented to be involved in productive human hepatocytes invasion as their gene disruption resulted in growth-arrested parasites lacking a parasitophous vacuole^{98,99}. Moreover sporozoite protein family 6 cis family like P52 and P36 are crucial for hepatocytes invasion and PVM formation. Thereby it was suggested by authors that host receptors may energize sporozoite protein that can facilitate the invasion process¹⁰¹⁻¹⁰³.

5.2 Role of hepatocyte surface proteins in liver infection

Tetraspanin CD81, a putative receptor for hepatitis C virus 2 also contributes to *Plasmodium* infection¹⁰⁴. The role of human receptors during parasite entry and invasion inside the host is not well understood. However, human tetraspanin CD81 was recognized as one of the essential components for *P. falciparum* and *P. yoelii* sporozoite entry. Besides CD81, cholesterol-dependent tetraspanin-enriched microdomains (TEMs) situated on the hepatocytes surface are also crucial for this process. Surprisingly another rodent *Plasmodium* species *P. berghei* does not necessarily require CD81 which was observed by the infection of HepG2 cells that lacked CD81¹⁰⁵. It clearly hints that alternative pathways are being involved for the *P. berghei* permissiveness into hepatocytes^{104, 105}. Scavenger receptor BI (SRB1) is a lipoprotein receptor expressed in the hepatocyte membrane and Kupffer cells is also considered a co-receptor along with CD81. Gene knockout of SRB1 in mouse reduced liver infection load. However authors found an interesting link between CD81 and SRB1 where SRB1 deficiency reduced CD81 expression^{98, 107}.

5.3 Liver Host molecules accelerate *Plasmodium* exo-erythrocytic development

- Mucin-13

Study of host- parasite interactions in *Plasmodium* infection still can be an important strategy that can impact multi-faceted research goals of malaria elimination including drug discovery.

Dual RNA seq study revealed that human mucosal immunity gene, mucin -13 (Muc 13) could be a hallmark of liver stage infection. Authors found a strong upregulation of mucin-13 following *P. berghei* and *P. vivax* infection. The transcript of this gene was also found in both primary hepatocytes and hepatoma cell lines¹⁰⁸. Moreover, their IFA assay also revealed a clear distinguishable feature upon the expression of mucin 13 that can distinguish the infected cells adjacent from uninfected cells. This expression is in contrast with previously identified liver stage parasite marker UIS4 or HSP70¹⁰⁹.

- Aquaporins

Recently it has been demonstrated that rodent parasite *P. berghei* manipulated the human hepatocytes to produce a protein called aquaporin 3 (AQP3). Generally this protein is involved to transport the nutrients, water and other solutes into and out cells. Molecular studies identified that AQP3 of host hepatocytes was deliberately trafficked to the parasitophorous vacuole membrane of parasites after invasion. Thus hepatic parasites utilized host protein for their own favour in order to replicate inside of it¹¹⁰. The observation was further validated by genetic manipulation wherein a systematic depletion of host AQP3 curtailed significant *P. berghei* load in hepatocytes. To confirm the role of AQP3, auphen (an inhibitor to inactivate AQP3 function) was used in the *P. berghei* infected human hepatocytes culture. It reduced the parasite's ability to reproduce inside the host liver. Later, authors extended their study in the relapsing *vivax* malaria and observed the same phenomena that they identified in the previous rodent model. AQP3 was found to localize to the adjacent host-parasite interface PVM of both schizonts and hypnozoites within 5 days after *in vitro* infection. In addition, AQP3 inhibitor auphen, significantly reduced *in vitro* *P. vivax* liver hypnozoite and schizont load¹¹¹. These results imply a new mechanism by inhibiting the function of host molecules like aquaporin-3 to block the development of both schizonts and dormant hypnozoites and thus may prevent malaria before the clinical complications start.

6. Basic understanding of hypnozoite

Several well-recognized puzzles impeded in understanding relapsing malaria and remained unsolved for decades. Moreover there is no extended information on the biology of hypnozoites. It is still undetermined what determines sporozoite development and differentiation into liver schizonts versus hypnozoites and what triggers the hypnozoite to break its dormancy and reactivate thereby transform into multinucleated schizonts. It is not known how it copes with the host environment with its limited metabolic activity hides weeks, months and even a year without any clinical manifestations. In the context of anti hypnozoite drugs screening- how the dormant hypnozoites are insensitive to current antimalarials is a scientific question. Notably a few decades ago, Shute and colleagues managed human challenge trials, the results hinted that the hypnozoite differentiation process was genetically predetermined¹¹². Hence in depth understanding of these clinical cues might facilitate identifying new diagnostic markers that can be detected in hypnozoites carriers.

6.1 Few research cues of hypnozoites are mentioned below-

Mosquito stage

- Is Hypnozoite formation in liver stage pre-committed from mosquito stage? Does infected salivary glands really harbor two distinct types of relapsing and non-relapsing sporozoites (bradysporozoites, tachy sporozoites)? Any role of salivary gland components that facilitate hypnozoite formation during the probing for blood meal to the host?
- It is known that only limited number of inoculated sporozoites can ultimately develop into liver forms. What is the fate of residual sporozoites? How do sporozoites decide finally to settle in a hepatocyte after traversing a few?
- Does hypnozoite formation depend on sporozoite inoculum of mosquito bite and related to dormancy and disease severity?

Hypnozoite-host connection

- What's the interaction between host and hypnozoites?

- What are the dissimilarities between *in vitro* and *in vivo* parasites in terms of parasite-host interactions?
- How does the hypnozoite evade from the host immune response and to what extent do hypnozoites are benefitted from host liver metabolism?
- Why is the liver stage asymptomatic?
- How can a hypnozoite persist in the liver for a longer period of time (longer than the estimated lifespan of hepatocytes) with its steady metabolic activity?
- Which parasite/host molecules regulate hypnozoite dormancy?
- Why does long latency occur in temperate regions?
- How hypnozoites resistant to the all licensed schizonticidal drugs?
- What's the other niches of hypnozoite beyond host liver and bone marrow?
- What triggered hypnozoite reactivation? Any master regulator, host and parasite molecules, epigenetic events, stress and environment-seasonality, altitude, latitude? Is there any mechanical resemblance with other latency forming species (plant, bacteria, HIV virus and other *Apicomplexan* pathogens, *Toxoplasma sp.*)?
- Does subsequent mosquito bites trigger hypnozoite activation? Does hypnozoite activate upon the bodily reaction by the human on a bite by an *Anopheles* female mosquito?
- How timing of hypnozoite reactivation is determined either *in vitro* or *in vivo*? Is there any possibility to provoke hypnozoites to proliferate with interference molecules such as supplementation or starvation of nutrient media, abiotic stress, and with epigenetic molecules?

6.2 Transcriptomics of hypnozoites

To date hypnozoite basic biology, mechanistic information regarding hypnozoite formation, persistence and reactivation are not clearly defined. Understanding gene expressions through genetic manipulation-Omics profiling can largely contribute to understanding hypnozoite biology, identifying master regulators that are responsible for hypnozoite mediated quiescent state in the liver. To unravel the mystery, few research teams relentlessly attempted to the approach of preliminary transcriptomes of cultured *P. cynomolgi* and *P. vivax* liver-stage parasites utilizing their unique live liver culture protocols and with the advent of transcriptome tools and reagents. It can be noted that *in vitro* hepatic cultures of relapsing malarial model, infected with sporozoite species (*P. vivax* and *P. cynomolgi*) always exhibit two distinct liver

parasite populations (non replicating hypnozoite and replicating multinucleated schizont). However to perform the hypnozoite transcriptomics studies, sorting of those liver stage parasites is obligatory which is not easy to manipulate with the conventional culture setup. After subsequent attempts, three hypnozoite transcriptomics has been done so far, findings are available in literature and data are stored in GenBank.

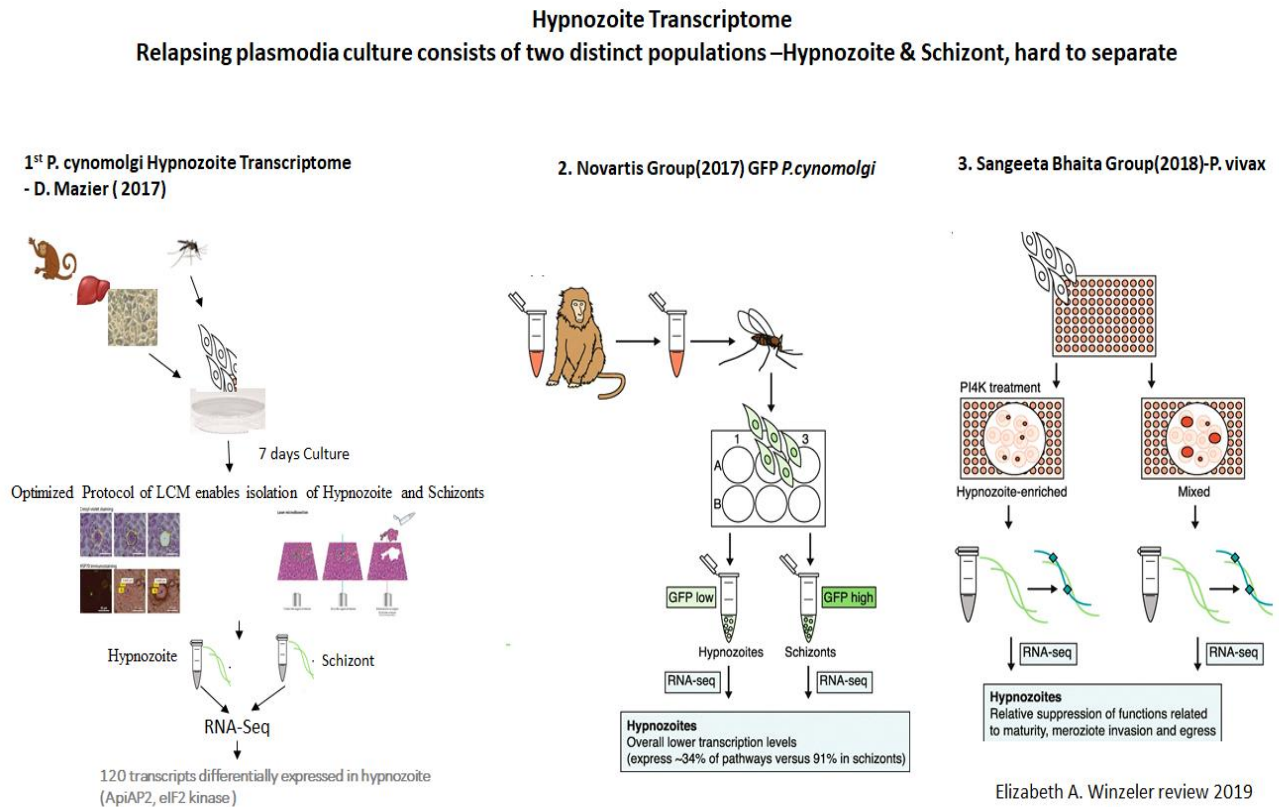


Figure 5: Adapted and modified from¹¹³. Short brief of hypnozoite transcriptomics

The first transcriptomic studies of hypnozoite came into light using relapsing malaria parasite *P. cynomolgi* by Dominique Mazier team¹¹⁴ (2017) and immediately after Novartis Group^{115, 116}(2017) and Sangheeta Bhatia team¹¹⁷ (2018) accelerated the understanding of hypnozoite transcriptomics. Using Laser capture microdissection (LCM) techniques, Mazier team succeeded to isolate of replicating and non-replicating hepatic forms from *P. cynomolgi* infected primary *M. fascicularis* hepatocytes *in vitro*. Upon successful sorting they succeeded to do RNA sequencing between these two forms and did a comparative transcriptomic profiling. According to their study, 120 transcripts were differentially expressed in hypnozoites whereas

ApiAP2 (coined as 'AP2-Q' PCYB_10239) was found transcriptionally upregulated in considerable manner compared to schizont and prospered as crucial mediator in hypnozoite formation. But it was also identified in non-hypnozoite producing malaria species for example *P. knowlesi* and *P. gallinaceum*. Subsequent transcriptomic studies with other protocols failed to identify such AP2-Q transcript *upregulation* in *P. cynomolgi* and *P. vivax* hepatic models. Interestingly Mazier team reported up-regulation of eIF2 kinase IK2 in hypnozoites compared to schizonts, implicating a possible translational repression activity associated in liver stage quiescence.

The development of GFP fluorescent *P. cynomolgi* facilitated Novartis group to understand the omics of hypnozoite in a border sense. Based on GFP intensity (GFP high schizont versus GFP low hypnozoite) of the parasites Novartis researchers executed transcriptomics of day 7 and day 10 cultured hypnozoite. The comparative analysis revealed a 10-fold reduction of gene expression during the transition of liver parasites into dormant state. Furthermore a limited set of 840 genes pinpointed from sorted hypnozoite samples indicating hypnozoite genes are also associated in maintaining metabolic housekeeping functions, genome integrity, limited energy metabolism process and altogether set the quiescence. Furthermore authors identified fully functional epigenetic events e.g. acetylation and methylation and chaperone-mediated nucleosome-histone interactions in hypnozoites and *alba* gene family members which takes part in DNA -RNA binding activity.

Bhatia's team previously developed micropatterned co-culture that facilitated hypnozoite culture and RNA-seq study. In their culture system, they extracted total RNA from the culture and purified the RNA samples using custom made baits that tilted the assembly of *P. vivax* P01 genome. Prior, as the relapsing *in vitro* culture consists of parasite mixtures of replicating and non-replicating forms so sorting of relapsing parasite (hypnozoite) was the main experimental goal to perform RNA sequencing. To achieve this goal, authors first treated the culture with a liver schizonticidal drug, PI4K inhibitor which selectively killed replicating forms by unaffected hypnozoites. Thereby a hypnozoite enriched culture was developed for RNA seq. Authors identified 40 % encoded proteins in hypnozoite with unknown functions considered as hypothetical proteins. Several genes were selected that are supposed to do important metabolic functions including catalytic activity. More specifically the Apetala2 member family proteins (ApiAP2) were upregulated in the hypnozoite enriched culture. Interestingly two previously identified drug targets PI4K and eEF2 demonstrated reduced expression in the samples

Altogether, all the previous transcriptome studies were not able to identify a hypnozoite specific biomarker or master regulator(s) but it revealed some interesting phenomena occurring in hypnozoite reservoir which warrants further critical understanding likely metabolic shutdown hypnozoites yet to continue several specific pathways involved in the maintenance of dormancy, including ATP homeostasis, genome integrity as well as chromatin maintenance.

6.3 Transcriptome of relapsing sporozoites

It is hypothesized that the decision of hypnozoite formation is pre-committed from the early onset of liver infection and or most probably preordained or pre-committed from mosquito stages -sporozoites. This conceptual issue is still under investigation and really a good clinical research questions for malariologists. Recently a few transcriptomics of relapsing sporozoites were carried out. One group performed a transcriptome of relapsing sporozoites and compared the data set with the proteomic analysis of *P. vivax* sporozoites^{118, 119}. Ultimately they observed that the transcripts that were identified in sporozoites but were not detectable in protein level. This could be due to the translational repression. However, authors identified a differential profiling of transcripts between sporozoites and asexual blood parasites including hypnozoite enriched liver parasites. Tightly programmed sporozoite transcriptomic profiling defines a broad spectrum of transcriptional activity but is translationally repressed in schizonts and hypnozoites¹¹⁸.

A groundbreaking step will be to eagerly see if single-cell RNA sequencing with relapsing sporozoites may unravel the long cherished scientific questions regarding the sporozoite role in the context of hepatic dormancy and mechanistic insights of stage specific parasite development inside the mosquito vector.

6.4 The association of hypnozoite tubulovesicular network with host water- solute channel

To date, very few organelles structures have been identified in hypnozoites. The previous immunofluorescence assay with cell marker antibodies revealed the presence of structures likely mitochondria, cytosol, apicoplast, parasitophorous vacuole membrane (PVM) in hypnozoite *in vitro* and also in liver sections of humanized mice.

It has been previously shown in rodent parasite *P. berghei*, host-parasite interaction in *Plasmodium* hepatic phase is connected with the structural alteration of PVM upon infection where parasite PVM is able to markedly expand towards the host hepatocytes in a dynamic membranous networking (TVN) with clusters, vesicles and tubules. Tubulovesicular network (TVN), mainly a PVM derived network, existed within the cytosol and previously identified in replicating hepatic schizonts^{120, 121}. However recently utilizing high resolution microscopy, an interesting phenomena was observed for the first time that connects the host water solute channel named aquaporin-3 regulating with tubulovesicular network (TVN) of *P. vivax* hypnozoites. Most surprisingly TVN features were found to be higher in non-replicating hypnozoites compared to schizonts that could implicate and map the differentiation of these two parasite population thorough further critical understanding how such host regulators like aquaporin contributing hypnozoite formation and playing role in its persistence and in its steady metabolomics¹²².

6.5 Hypnozoite re-activation

Nearly 80-90% malaria cases are reported due to hypnozoite mediated relapses. So understanding the relapse following the hypnozoite activation is a high clinical demand. Although the complete relapse picture is neither demonstrated well *in vitro* nor *in vivo* to date. *in vitro* hepatic cultivation of hypnozoites mimic the hypnozoite persistence where non hypnozoite parasite species e.g. schizonts generally egress from day 6 to day 10 post sporozoite infection. Despite this, we could get a hint of hypnozoite activation by consecutively estimating the ratio of hypnozoite versus schizonts at different time points of hepatic cultures. Such hypnozoite activating concept was described in study where a significant number of hypnozoites were decreased from day 6 (67%) to day 10 (19%) cultures indicating activation of hypnozoite¹²³. In addition, molecular characterization reported a high frequency of multiple-clone *P. vivax* (more than one genetically distinct parasite) carried by individuals where transmission level differed. Consequently the microsatellite genotyping epitomized that relapses might arise from the activation of parasite clones similar to those of the primary infections (e.g. homologous parasites) or genetically distinct clones (heterologous parasites)¹²⁴. Overall, processes that recapitulate and activate hypnozoite are yet elusive due to lack of proper biological tools although reactivation of hypnozoite is such an enticing hypothesis.

6.6 Relapse Pattern

After mosquito bite, the *Plasmodium* parasites travel organ to organs inside the human host undergoing remarkable morphological changes that enable them to thrive, survive and transmit to mosquito vectors along with demonstrable clinical manifestations. Relapse events are not only detrimental to the patient, but also render the risk of re-initiating the transmission cycle in previously malaria-free regions. The relapsing sporozoites transform into both dividing multinucleated schizonts and uninucleated, non-dividing hypnozoites. It is believed that in the case of relapsing malaria, hypnozoites are not produced immediately after inoculum of relapsing sporozoites but at least first 36-40 h following intravenous injection of sporozoites. Even *in vitro* generally hypnozoites can be differentiated from schizonts at day 4 of post infection.

However, the size of hypnozoite increases slightly over time *in vitro* culture with unknown mechanisms¹¹⁷ and thereby considered as metabolically active. Previous studies demonstrated that host metabolic pathways contribute to the growth and differentiation of liver stage parasites¹²⁵⁻¹²⁸ so it is necessary to decipher which pathways contribute to behind this hypnozoite steady growth. Current hypnozoite transcriptomics data also shed light on host metabolic interplay that are crucial for liver parasite development and thereby provides the information to develop new host based targeted therapies. The strategy can be accumulated by targeting the host cell in order to eliminate the persisters forms of parasites by activating the hypnozoites with a conjunction of current antimalarials. Almost nothing has been so far known about the host specificity either the environmental cues that regulate or trigger the relapse phenomena. However it has been known that people living in tropical regions generally yield rapid relapse patterns which range from weeks to months which is a prolonged pattern among the *Plasmodium* infected peoples in temperate regions. Moreover, subsequent *Plasmodium* infection and exposure to other parasitic infections predominantly by bacterial pathogens are also hypothesized to increase the relapse rates by reactivation of the cryptic hypnozoites¹²⁹.

This quiescent phenomenon is also displayed by *Toxoplasma gondii* and in comparison with bradyzoites, an environmental factor such as oxidative stress or any alteration in temperature might trigger the reactivation in hypnozoites. Although to what extent these comparisons are reliable need to be determined and understood^{130, 131}.

7. Hypnozoite biomarkers

7.1 UIS4 and LISP2 are critical markers of *Plasmodium* liver stage development: *in vitro*

. Clinically liver residing hypnozoite infection is silent, asymptomatic and undetectable in humans as hypnozoites exist in small numbers. To date, no definitive biomarkers have been detected in humans which made the invasive relapse diagnostic treatment absolutely challenging including the unknown prediction or timing of relapse periodicity.

- **UIS4:** (Upregulated in infective sporozoite genes 4) mainly positioned to the parasitophorous vacuole membrane, an important organelle between the host and parasite as it separates the parasite from the host cell cytoplasm. The UIS4 encodes a putative transmembrane acidic protein which is transcribed by all malaria species and synteny searching of *P. falciparum* genome database revealed 74% amino acid sequence identity with *P. berghei* and *P. yoelii*.^{132, 133}. However, UIS4 is not critically essential for sporozoite functions such as interaction with vector tissues, motility, gliding. Interestingly UIS4 lacking parasites are able to develop early liver forms that clearly indicates that function of UIS4 is not essential for the conversion of sporozoites to liver trophozoites. To understand whether systematic disruption of UIS4 could lead to blood stage infection. 1000 UIS4 mutant sporozoites were injected in mice who showed no symptoms of infection until 21 days but 59 % of mice were malaria positive when they were infected with high inoculum of 50000 UIS4 mutant sporozoites. The role of UIS4 in blood stage infections remain unknown¹³⁴. Nowadays, UIS4 is used as a critical marker to identify liver parasites in fixed liver tissue samples both *in vitro* and *in vivo*. Hypnozoite possesses a UIS4 prominence in the periphery of parasitophorous vacuole membrane (PVM) and thus possible to detect hypnozoite in culture. As PVM of replicating schizonts can also be marked with anti UIS4 hence UIS4 is not truly hypnozoite specific¹³⁵.
- **LISP 2:** Current identification of LISP2 (Liver specific protein 2) facilitates liver stage *Plasmodium* studies. Immunolabeling of LISP2 revealed an ability to distinguish non-replicating hypnozoites from early developing liver forms (trophozoites). LISP2 can be

a reliable marker for identification of the reactivating hypnozoites or trophozoites that were derived by the reactivation process of hypnozoite. Interestingly, LISP2 was found positive to early liver forms/trophozoites/reactivated hypno but donot express in hypnozoite which were positive by UIS4. So LISP2 showed a differential phenotypic expression in between hypno and reactivated hypno. They confirmed their study in *in vitro* model of *P. cynomolgi* and in *P. vivax* infected humanized mice. Using immunofluorescence assays with anti- *Pv*LISP2, authors observed a major fraction of non-replicating small parasites (hypnozoites) which did not express LISP2 signaling from day 3 to day 18. In contrast trophozoites and mature schizonts clearly expressed LISP2¹³⁶. Thus LISP2 was proposed as good marker to distinguish dormant hypnozoites from activated hypnozoites that differentiated to developing forms in relapse drug assays.

7.2 Hypnozoite biomarkers identified *in vivo*

Recently a proteomic study revealed the presence of proteins from hypnozoites that circulate in extracellular vesicles. The experimental approach was set up in a humanized mice model that engineered human liver cells inside the mouse liver with a view to mimic human *P. vivax* infection inside the rodent. Thereby authors succeeded to identify 17 different hypnozoite proteins by isolating human derived exosomes from that chimeric mice that might contain host- and parasite-derived proteins and nucleic acids, components of the endocytic pathway and translation machinery and few uncharacterized proteins. These sets of differential proteins can be a predictive marker of latent relapsing infection. However, a detailed investigation is warranted regarding the further biomarker discovery and whether these proteins are precisely specific for *Plasmodium vivax* liver stage infection rather than *P. falciparum*¹³⁷. This informative investigation could largely contribute to developing a non-invasive diagnostic treatment of relapsing malaria.

Recently a clinical cohort study executed in Thailand and Brazil where authors established a panel of serological exposure markers by measuring IgG antibody responses to 342 *Plasmodium vivax* proteins that capable of accurate prediction of the individuals with recent *P. vivax* infections that showed strong likelihood of bearing hypnozoites. The unbiased

identification was based on patients' immunogenicity and half-lives of the antibodies that could be targeted by liver stage antimalarials. In general *P. vivax* blood stage infections induce strong responses of IgG antibodies to a wide range of *P. vivax* proteins albeit low parasitic burden even after clearance of infection. However, the identified 8 candidate serological markers of exposure can validate their application in especially *P. vivax* endemic countries. Moreover the authors undertook mathematical modeling for further validation of such serological testing and treatment strategy could reduce *P. vivax* prevalence by 59–69% and eight antibody responses can serve as a biomarker, identifying individuals who should be targeted with anti-hypnozoite therapy¹³⁸. Altogether, further investigations of these antibodies are warranted e.g optimization, specificity, purification and testing of these antibodies in other expression systems, antigenicity prediction etc, as there is literature based evidence of cross reactivity of antibody between *P. vivax* and *P. falciparum*.

8. Progress of hypnozoite research and challenges

8.1 *in vitro* and *in vivo* models facilitates liver stage studies

The first *in vitro* demonstration of *P. vivax* and *P. falciparum* hepatic parasites were shown in a human hepatocytes model^{146, 147}. Subsequent attempts were taken to study the *Plasmodium* liver stage in other cellular models including cell lines^{117, 123, 148-150} and recently in *in vivo* humanized models^{151, 152}. Meanwhile, the toolbox of malaria liver stage has evolved. Despite, hypnozoite activating phenomena was not possible to demonstrate. In fact, studying the entire liver stage of *Plasmodium* cycle using human parasites under live or laboratory conditions is technically challenging. The use of model organisms, such as the rodent malaria parasite *P. berghei* is experimentally tractable and the genetic manipulation of this parasite is relatively easy which is already well established. Thus this model allows investigating the EEF development and filling gaps of knowledge that might also be relevant for human *Plasmodium* species. But genetic makeup of rodent models always does not mimic the human malaria. So as cellular model, cryopreserved primary human hepatocytes are required. Another hypnozoite research limitation is the lack of frequent supply of sporozoites that can be only obtained from

relapsing malaria species. Studying *P. cynomolgi* greatly facilitates in understanding *vivax* biology and anti-hypnozoite drug screening. Primaquine, the only radical cure drug was also validated with this primate monkey model¹⁵³. To improve the hepatic infection and for the long term culture maintenance, a sandwich culture was developed. In that approach, simian hepatocytes were seeded into pre-collagen coated plate and the addition of a matrigel cover (a solubilized basement membrane culture substrate, extracted from the Engelbreth-Holm-Swarm mouse sarcoma) greatly facilitated the development of *P. cynomolgi* exo-erythrocytic forms for over weeks (40 days) providing significant information of hypnozoite reactivation¹⁵⁴.

Cellular Models (<i>in vitro</i>)	Rodent Malaria		Mammalian malaria		
	<i>P.berghei</i>	<i>P.yoelii</i>	<i>P. falciparum</i>	<i>P. cynomolgi</i>	<i>P. vivax</i>
HepG2 ¹⁵⁰	✓		Poor infectivity		
HepG2 /CD81 ¹⁵⁰	✓	✓			
Huh7, Hela ¹⁴⁸	✓				
HCO4 ¹⁵⁰	n.d	n.d	✓ (0.066%)	n.d	✓ (0.041%)
Primary human hepatocytes ^{146,56,60}	✓	✓	✓	✓ (Poor)	✓
Primary monkey hepatocytes ¹⁵⁵	✓	✓	✓	✓	✓(Poor)
Microscale human liver ¹⁵⁶	n.d	n.d	✓	n.d	✓
Co-culture:Primary hepatocytes + cell line ¹⁵⁴	n.d	n.d	✓	✓	✓

Micropatterned Co culture: Human hepatocytes + murine embryonic fibroblast ⁵⁶	n.d	n.d	✓	✓
Human iPSC derived hepatocytes ¹⁵⁷	✓	✓	✓	n.d. ✓
Hepatic Spheroids ¹⁵⁹			n.d.	✓ ✓

Table 1: Cellular models used for the study of *Plasmodium* liver stages

8.2 Hepatic models for hypnozoite research: *in vitro*

Plasmodium liver schizogony can last nearly 7-15 days after sporozoite infection but hypnozoite remain dormant over weeks before their activation. So a robust hepatocyte model is prerequisite that can sustain liver physiology over weeks and recapitulate hypnozoite. Several hepatocyte culture strategies were developed but very few of them are effective to practice. However, co-culture method of hepatocytes with nonparenchymal cells (named as microscale of human liver), researchers succeeded to maintain the hepatocyte culture for a few weeks without any disturbance of liver-specific functions, interactions with neighboring cell types, extracellular matrices⁵⁶. In the microscale liver platform, hepatocytes were seeded in a micropatterned arrangement and maintained their phenotypic functions for several weeks. A similar coculturing strategy of rhesus monkey primary hepatocytes with another GFP-HepaRG cell line supported the development of hypnozoites of *P. cynomolgi*¹⁵⁴.

To date, *in vitro Plasmodium* liver stage models completely depend on the infection of hepatocarcinoma cell lines (HepG2, HepaRG, and Huh7) and primary cryopreserved monkey and human hepatocytes. As infectious agents, rodent infective *P. berghei* and *P. yoelii* are widely used for decades. One advent using these two rodent species are their accessibility and utility to rear in the insectarium on a regular basis and another usefulness is the development of

GFP reporter transgenic *P. berghei* lines. Systematically cell lines proliferate very well *in vitro*, support rodent parasite growth and the cost is relatively low. Altogether biochemical, genetic manipulation studies of exoerythrocytic biology largely rely on the rodent malaria species and cell lines. Recent high throughput drug screening utilizes both malaria species. However, one major limitation of aforementioned cell lines is that they don't support well to the human malaria (*P. vivax* and *P. falciparum*) infections. Another hepatoma cell line HC04 (an immortalized cell line, isolated from normal human hepatocyte) can be infected by the *P. vivax* and *P. falciparum* sporozoites but infection customarily between 0.13% and 0.7–1% for *P. falciparum*¹⁵⁰ in contrast, few studies reported the detachment of HCO4 cells from the culture within a few days of *Plasmodium* infection¹⁵⁹. Interestingly an improved invasion efficiency was acquired by a subcloned HC-04.J7 cell line that supported *P. falciparum* infection rates of 3-5%¹⁶⁰. Apart from this, another novel immortalized hepatocyte-like cell line (imHC), isolated from human bone marrow mesenchymal stem cells (hMSCs) was assessed for the *P. vivax* sporozoite infection¹⁵⁹. All the cell lines mentioned above do not entirely recapitulate liver stage infections of human malarias and also don't support for prolonged period for anti-hypnozoites drug screening approaches.

Undoubtedly, these newly established models will be critical for investigating hypnozoite formation, and the reactivation of these liver forms will be a critical validation for these models. While not currently suitable for high-throughput screening, the adaptation of these systems for economical and robust systematic searches for compounds with anti hypnozoite activity will be transformative for malaria drug discovery efforts.

8.3 Humanized mice *in vivo*

Mice have always been used as good *in-vivo* model to understand a diverse range of infectious diseases. Study of hypnozoite requires *in vivo* model that can recapitulate relapsing liver stage infection including hypnozoite formation and activation. To meet this need, recently human liver-chimeric mice (Fah^{-/-}Rag2^{-/-}IL2rg^{-/-} mice transplanted with primary human hepatocytes (FRG KO huHep) have been developed and opened a new scope for hypnozoite study. This engineered FRG KO huHep liver-humanized mice strongly supported *P. vivax*, *P. falciparum* and *P. ovale* sporozoite infection, growth and maturation of hepatic parasite and most importantly the development of small non-replicating form hypnozoite^{144, 151}. The presence of hypnozoite was confirmed at 5-8 day post sporozoite infection by the immunofluorescence assay using liver stage parasite antibody marker such PvUIS4, endoplasmic reticulum marker BiP, PvHSP60 (mitochondria marker) and Apicoplast marker ACP from the liver section of FRG KO huHep mouse¹⁵¹. Apart from the identification of subcellular localization, this engrafted model also responded well to the anti-hypnozoite drug screening assays. Additionally this model supported the culmination of additional blood stage proceedings e.g release of infectious liver merozoites and consequently the sequestered *P. vivax* parasites were able to mimic the blood stage infection when reticulocytes were injected intravenously to the humanized mice at day 9-10 post sporozoite infection. Thus *vivax* liver stage maturation and release of reticulocyte-infectious hepatic merozoites can be modeled and routinely used in the study of hypnozoite activation using FRG KO huHep mice. The role of liver architecture and its physiology in hypnozoite formation, establishment and activation is unknown. Animal models engrafted with human hepatocytes offer excellent *Plasmodium* LS development but intrinsically high costs and low-throughput hinders the regular use of this model for drug discovery approaches.

8.4 Hepatic organoid model convenient to study relapsing malaria

In vitro 2D liver culture model still facilitates the understanding of hepatic malaria since decades however this cellular model is not without limitations. In parallel 3D culture model has gained popularity nowadays to study infectious diseases and recently 3D spheroid- culture model of primary human and monkey hepatocytes has been reported with a view to understanding the relapsing *P. vivax* and *P. cynomolgi* malaria. Compared to 2D, authors

claimed that this disc shaped 3D cellusponge model superficially enhanced sporozoite permissiveness without deteriorating the hepatocyte physiology and thereby supported the full life cycle of liver parasites including dormant hypnozoite¹⁵⁸. This adapted model may further facilitate developing a radical cure model for drug screening as relapsing events in this model of *P. cynomolgi* was monitored *in vitro* until 20 days post infection which was not possible by the conventional 2D. In addition such models may curtail the utilization of biological resources (hepatic cells, sporozoite requirement, culture medium etc.) and thus may minimize the research cost significantly. Organoid (3D spheroid) models are relatively new so there is still room for optimization and improvisation that can retail the hepatocytes physiology over weeks and that undoubtedly will leverage a gap in current hepatic malaria research including drug assay against relapsing malaria.

8.5 Development of reporter lines facilitate understanding hypnozoite features

The genetic manipulation of *P. berghei* GFP parasites allows the live liver visualization of hepatic parasites that largely contributes towards the high throughput drug screening including fundamental research. In comparison most of the current *in vitro* relapsing models utilize non genetically modified parasites where non replicating small forms and replicating forms are only differentiated based on the morphological observations mainly size estimation in fixed hepatic cells which precludes the direct monitoring of individual parasitic growth over time in live cell culture. Therefore the actual transition of a hypnozoite into a schizont (reactivation phenomenon) was not demonstrated before neither *in vitro* nor *in vivo*. Previously, through episomal transfection, the first transgenic *P. cynomolgi* parasite was developed that expressed fluorescent markers. Then fluorescent activated cell sorting was done where hypnozoites were isolated based on their GFP-fluorescence intensity. Later hypnozoite transcriptomics, molecular characterization were done using these GFP-tagged parasites. Moreover, recently a dual transporter line of a *P. cynomolgi* M strain (constitutively expressed GFP and mcherry) has been established that selectively differentiated hypnozoites (green fluorescent protein, GFP positive and mCherry negative) from developing schizonts (both GFP and mCherry positive). Interestingly for the first time, this parasite line facilitated the direct demonstration of reactivating (dormant hypnozoite to schizont) phenomena *in vitro* indicating the activation process was not frequent (nearly one event per 400 hypnozoites) in culture which requires more understanding¹⁶¹. Altogether development of such valuable models will largely contribute to understanding the enigmatic hypnozoite activation and also facilitate the discovery of

such drugs that could potentially trigger the hypnozoite awakening and thereby readily be treated with established schizonticides.

8.6 Challenges associated with anti--hypnozoite drug discovery

- Difficulty in establishment of continuous relapsing blood stage propagation *in vitro*: limiting liver stage malaria research and current progress

in vitro culture of *P. falciparum* blood stage was developed in 1976 and still the strategy vastly contributes to the accessibility of *P. falciparum* parasites. Unfortunately to date, there is no equivalent long term culture method established for relapsing malaria mainly for *P. vivax* except short term culture. The difficulty with culturing *P. vivax* blood stage originally stems from its preference to invade red blood cells particularly the immature reticulocyte which constitute a minute percentage (0.5%- 2.5%) of all red blood cells among adults at any given time. In addition, reticulocytes quickly transform into mature erythrocytes at 37°C.^{162, 163} Apart from this, the half-life of reticulocytes is generally shorter than the asexual developmental stages of blood parasites. Reticulocytes enrichment can be attained by applying density gradient purification steps and magnetic beats but yet not routinely observed *in vitro*.

Thereby due to clinical importance a routine culture of pathogenic *P. vivax* parasites would largely allow frequent access of parasites which will facilitate greater understanding of hypnozoite biology and thereby it will be easier for the implementation of various functional assays and treatments. Most importantly this culturing step is crucial in the current attempt of anti-relapsing drug screening as the current anti relapsing drug screening requires frequent supply infectious sporozoites that can be derived from mosquitoes that are fed with patients' blood gametocytes. So, the current *P. vivax* research mainly relies on the endemic country research settings which is not always convenient. In addition there are ethical limitations associated with shipment of infectious mosquitoes from *vivax* endemic countries to western countries that could accelerate hypnozoite research. *P. cynomolgi* is considered a favoured model of *P. vivax* because of its genetic similarity disease pathogenicity and hypnozoite mediated relapsing capability. However to circumvent this, very recently an attempt of culturing *P. cynomolgi* Berok K4 strains of blood parasites has been documented with positive outcomes. The continuously cultured parasites were transmitted into *M. mulatta* monkey and day's later, female *A. stephensi* mosquitoes were fed with infected blood with a view to

sporozoite production. Following oocyst development and subsequent salivary gland dissection, Berok K4 sporozoites were used to infect *in vitro* cryopreserved monkey hepatocytes. Hepatocytes infection revealed the emergence and growth of relapsing (multinucleated schizonts) and non-relapsing (uninucleate hypnozoites) hepatic parasites. Author's successful investigation regarding the continuous adaptability of those cultured parasites *in vitro*, transmission capability into mosquito vectors and feasibility of sporozoite production can be a landmark in the arena of high throughput drug screening against hypnozoites¹⁶⁴. In addition it has also provided glimmers of hope to expand the use of this model species for fundamental and translational research on relapsing malaria.

- **Lack of frequent supply of relapsing sporozoites**

Frequent anti-hypnozoite drug screening relies on the secured supply of relapsing sporozoites. As human malaria is mostly prevalent in poorer income countries (occasionally few cases among travellers in developed countries) so from that practical point of view adequate research with advanced facilities is somehow restricted and has to depend on resource settings in the endemic countries. Moreover, sporozoites are naturally produced in mosquito salivary glands by feeding infected patient blood. In addition, there are no methods and reagents identified that can retain the freshly isolated sporozoites motility, viability and infectiousness for certain days and thereby be able to culture the thawed sporozoites axenically. *P. cynomolgi* parasites are rarely accessible to liver stage researchers and the hepatocytes infectivity rates range from 0.1%-1%. Fundamental biology of hypnozoites could be understood by this model and current hypnozoite transcriptomics of *P. cynomolgi* have provided insights that assisted a lot to understand the relapsing disease.

- **Difficulty in maintaining *in vitro* hepatic culture for long term: culture contaminations**

Hypnozoites remain dormant in hepatocytes culture for certain periods of time. On the other hand, hepatocyte culture starts to lose their tight junctions, morphology and physiology within a few days of infection. Even with the efficient cellular models and strategies, cultures are somehow contaminated with diverse range of unwanted microbial pathogens in spite of adding sufficient amount of growth favouring supplementations and cocktail of antimicrobials. In fact

most of the antibiotic cocktails used in the hepatocytes culture showed decreased sensitivity to pathogens and a few of them demonstrated anti-parasitic activity^{165, 167}. Generally, cryopreserved human hepatocytes are the automatic 1st choice for relapsing malaria studies. They have to be seeded in culture plate at least 3- 4 days prior of infection as they require time to settle and reveal their cellular functions. The source of hepatic contamination mainly originates from mosquito stages. Mosquitoes' bodies always harbor diverse pathogens in insectarium and breeding site¹⁶⁶. Till date, the only method of sporozoite isolation from the salivary glands of mosquitoes is hand dissection. Hence unpurified sporozoites extracted from mosquito salivary glands could be responsible for cell culture contamination. To overcome this issue, it is important to purify the sporozoites before hepatocyte infection. Different sporozoite purification methods have been developed but the sporozoite recovery rate is low due to frequent centrifugations steps. Alternatively, the emergence of unwanted pathogens could be prevented at mosquito stage and this could be gained by changing the mosquito feeding menu with a supplementation of antimicrobials. An antimicrobial agent methyl paraben was added in mosquito feeding diet that increased *P. falciparum* oocyst development and longevity of mosquitoes. However, information regarding the cleanliness of mosquito and effectiveness in *Plasmodium* liver stage is still missing^{165, 167}.

9. Pre-erythrocytic stage drug discovery

9.1 Drugs identified active against malaria liver stage

Most of the current anti malarial drugs are stage specific. For example, artemisinin, methylene blue which are active against blood stage parasites but liver parasites are poorly susceptible to artemisinin. This differential activity is not surprising in the context of distinct morphological evolution, function, location and clinical consequences of a *Plasmodium* parasite in its complex life cycle. The most conventional way to develop new antiparasitic drug like compounds consists of blind screening of a large number of compounds or extracts enriched with natural bioactive substances against certain pathogens. This traditional approach is directed without having prior knowledge about the mode of action of the drugs e.g. the molecular target(s) within the pathogen. Fortunately a significant number of antiparasitic compounds including antimalarial are either a direct outcome of such strategy or subsequent evolution of the drug development industries. However, nowadays the most contemporary and rational drug discovery strategy utilizes high throughput screening (HTS) with a bulk amount of compounds

and focuses on the identification of molecular targets within the parasite through translational research. Systematically, targets are identified based on metabolic and biochemical pathways integral for parasite survival. In the case of malaria, an efficient prophylaxis and urgent therapies are needed to prevent the disease. However, the development of vaccines against *vivax* malaria seems remote in future. Hence, anti-relapsing drug discovery is the current goal in vivax malaria research. The selectivity of an anti relapsing compound must depend upon its mode of specific disruption of parasite quiescence mechanism leaving the host functions unaffected.

Earlier liver stage drug screening was largely dependent on the smaller libraries compounds that were commercially purchasable with a known function against the blood stage parasites. But recent technological advancements have largely facilitated the arena of drug discovery. Advances in robotic automation and liquid handling, coupled with the ever-shrinking scale at which these assays are performed, have facilitated ultra-HITS of very large compound libraries¹⁶⁸. Furthermore, the continued development of cheminformatics, which encompasses the ability to analyze, filter, and interpret the large datasets generated from these screens, has greatly contributed to the establishment of HTS. A crucial shortcoming is that all high-throughput liver stage based drug screening largely depend on rodent malaria parasite *P. berghei* or *P. yoelii*. Therefore false positives and false negatives results may appear because genetic make-up of rodent parasites are not solely similar to human malaria and thus with a given compound, may not exhibit the same clinical response as human parasites to the treatment.

- Antiviral compounds

Malaria and HIV co-infection are common in Sub-Saharan Africa and geographical overlap between these two prevalent diseases is a worrying public health problem as both infections increase each other's fatality mainly by *P. falciparum*. In fact it has been shown that malaria is associated with intensified HIV replication and transmission load along with a lower CD4+ T cell count¹⁶⁹. Moreover like relapsing malaria, HIV relapse also poses a great therapeutic challenge. Hence discovery of novel drugs that can tackle both the infectious disease would largely upgrade the health system in Africa. Several existing antiretroviral drugs (AR) were screened *in vitro* before against exo-erythrocytic malaria which are as follows: -

AR drugs IC50 hepatic stage	Rodent species <i>in vitro</i>	Human malaria <i>P. falciparum</i>	References
Saquinavir(35.2nM),ritonavir (34.2nM), indinavir (500nM), lopinavir	<i>P. yoelli</i> and <i>P. berghei</i>	LPV, SQV and nevirapine	170,171
Non-nucleoside reverse transcriptase inhibitors - Efavirenz (17.2 μM) + Etravirine(4.4μM) and NVP50	<i>P. berghei</i>	n.d	172

Table 2: List of identified antiviral drugs active against *Plasmodium* liver stage.

It can be noted that neither the HIV protease inhibitor lopinavir-ritonavir (LPV-RTV) nor the antimicrobial trimethoprim-sulfamethoxazole (TMP-SMX) showed efficacy against malaria relapses observed in *P. cynomolgi* infecting nonhuman primates but both drugs were found effective against the dividing forms of hepatic stage of rodent and *P. falciparum* malaria¹⁷³.

- Epigenetic Inhibitors

Epigenetic events are associated with the gene regulation throughout the *Plasmodium* life cycle; hence modulation of such epigenetic events offers an attractive drug target in the context of antimalarial drug discovery. In fact epigenetic inhibitors such as malarial histone deacetylases (*Pf*HDACs) showed anti-parasitic activity and currently validated as epi-drugs.

However the relatively poor selectivity, unfavorable pharmacokinetics, pharmacodynamics and host toxicity offer a major challenge. Thus identification of drugs with nanomolar inhibitory activity which differentially target the epigenetic events of *Plasmodium* are of considerable interest.

Recently, epigenetic events in late stage hypnozoites of *P. cynomolgi* have been reported¹¹⁶ and for the first time, a possibility for an epigenetic basis of hypnozoite dormancy has been investigated¹⁵⁴. Beyond this, no extensive epigenetic study in relapsing liver stage has been documented. So screening of potential HDACi with low activity against hHDAC is likely to be a good starting point. Moreover epigenetic therapeutics ('epi-drugs') evidently hold a great promise as targets for various diseases. However a limited handful *in vitro* experimental drug screen was assayed with HDAC inhibitors in particular against *Plasmodium* liver stage. Using human hepatoma cell line, SB939 significantly inhibited the development of *P. berghei* ANKA mouse malaria parasites (IC₅₀ 150 nM). Although the mechanistic action of this compound is not known yet. Apart this SAHA another HDAC inhibitor which is known to abort the recombinant *Pf*HDAC1 activity profile and the encoded protein by this gene also transcribed in *P. falciparum* hepatic stage including asexual and gametocytes stage. However expression profiling of either *P. berghei* ANKA homologue (*Pb*HDAC1) or *Pf*HDAC1 hepatic stage still not elucidated but these two proteins share 94% amino acid sequence similarity which implies that HDAC inhibitors will target this protein in rodent malaria parasites. However SAHA has also been shown similar inhibitory activity against rodent parasites with IC₅₀ 164.8 nM (\pm 18.3 nM)¹⁷⁴. In addition, a recombinant epigenetic molecule named methyltransferase *Pf*SET7 was purified in large quantities with enzymatic characterization that localizes to cytoplasmic foci near to the nucleus in liver and blood stage parasites and also throughout the cytoplasm of salivary gland sporozoites. Next, this recombinant *Pf*SET7 was identified as a punctate pattern in hepatic schizonts where distinct dots were observed adjacent to the nucleus of each forming merozoite¹⁷⁵.

- **Kinase inhibitors and host regulators**

Humans possess approximately 518 kinases that are involved in extensive ranges of cellular processes whereas *P. falciparum* kinome encodes 86 to 99 kinases genes and found to be highly conserved among all *Plasmodium* species. Recently a predictive computational modeling ‘Kinase Regression (KiR)’ identified 47 host kinases and many of the kinase inhibitors were FDA approved that were not screened *in vitro* before in order to assess antimalarial efficacy. The predicted kinase inhibitors were experimentally validated and found to maintain *P. yoelii* liver stage infection¹⁷⁶. Thus, further mining of these kinases inhibitors may fulfil the criterion of candidate drugs against human malaria. A considerable progress was made in the discovery of kinase inhibitors where *Plasmodium* specific phosphatidylinositol 4-kinases (PI4K) activity were found essential for survival of parasite. Compound 34 (KDU691) was also identified from the serie of imidazopyridines /pyrazines /pyridazines which was found to be active *in vitro* against liver stage of rodent parasites most strikingly against *P. cynomolgi* hypnozoites in prophylactic manner¹⁷⁷. Thus one of the most persuasive reason to target *Plasmodium* kinases being key regulators involved in the key signaling pathways at multiple life stages of parasites. In addition, it has been reported that small molecule like takinib (a potent human TAK1 inhibitor) interacted with K63 linked ubiquitination in the hepatic stage *P. falciparum* protein kinase 9 (*Pf*PK9) which led to the parasitic death¹⁷⁸.

Host-based targeted therapies to fight against infectious disease are not unique to malaria. Previously host cell phosphatidylcholine was demonstrated as one of key mediators for the survival of liver stage rodent parasites. It was shown that the host phosphatidylcholine was procured by both *P. berghei* and *P. falciparum* malaria parasites during hepatic infection. Alternatively it was also shown that lack of de novo synthesis of a single phospholipid in the host cell strongly perturbs the life process of hepatic parasites which reflects on the lipid protein composition of the PVM and thus modulated PVM integrity¹⁷⁹. So targeting crucial host hepatocytes proteins may greatly extend the repertoire of prophylactic drugs against hepatic malaria. Indeed several biochemical studies also demonstrated that malaria parasites intrinsically manipulate few key hepatocyte factors that contribute to cell survival during hepatic infection. Parasites deliberately halt the tumor suppressor gene P53 which plays a considerable role in apoptosis as well as cell cycle arrest. Consequently malaria parasites modulate the mitochondrial apoptotic cascade by increasing levels of the prosurvival Bcl-2.

Hence study showed that both Bcl-2 inhibitors and P53 agonists strikingly decrease the liver stage load *in vitro* and *in vivo*. Bcl-2 inhibitors are known to induce apoptosis in infected hepatocytes whereas P53 agonists kill parasites in an apoptosis-independent manner. However the combinatorial administration of Bcl 2 inhibitor and P53 agonists act synergistically which delay the onset of blood stage infection and in some cases prevention was observed in humanized mice model infected with *P. falciparum*¹⁸⁰.

- **Antibiotic screening against hepatic *Plasmodium* parasites**

The use of antibiotics as antimalarial is not new. Various classes of antibiotics mainly macrolides, quinolones sulfones, and sulfonamide and tetracyclines are widely investigated in the context of repurposing drug discovery. The broad-spectrum tetracyclines are documented to be effective against *Plasmodium* liver stage. Besides demeclocycline treatment expanded the prepatent period of *Plasmodium cynomolgi* infection observed in *in vivo* studies with rhesus monkeys. Furthermore, minocycline and demeclocycline were also reported having LS inhibitory activity on *P. cynomolgi in vivo* and/or the liver stage of *P. berghei in vitro*¹⁸¹,¹⁸². A group of antibiotic panel quinolones- fluoroquinolones were also screened *in vitro* against hepatic infection of *P. yoelii* where a few were found active that can be considered as starting point of prophylactic drug validation after further subsequent investigation¹⁸³. Another broad spectrum antibiotic doxycycline was tested *in vitro* against the hepatic stage of rodent malaria. Screening revealed a significant reduction of parasite load in the liver and completely arrested the proceeding blood parasitemia in the mouse model. Furthermore, prophylactic efficacy of doxycycline had been investigated in various clinical trials however ended with negative outcomes mainly due to the insufficient drug dosage which led to poor patients' compliance¹⁸³. Another macrolide antibiotic azithromycin was proposed as prophylactic drugs based on the complete protection discerned *in vivo* against *P. yoelii* infection that led to further clinical investigations against *P. falciparum* and *P. vivax*. Notably clinical data suggested that azithromycin was less active than doxycycline against *P. falciparum* infections but both compounds were found to be effective in *P. vivax*-endemic regions.

Interestingly a clinical data has been documented where 7 days administration of AZT along with intravenous artesunate plus 3 days of an ACT prevented *P. vivax* relapse for until 330

days in a patient who frequently encountered relapsing malaria episodes with the treatments of other antimalarials including primaquine¹⁸⁴. The potential role of antibiotics confirmed in residents of malaria-endemic areas, periodic prophylaxis with safe and affordable antibiotics may offer a powerful shortcut toward a needle-free surrogate malaria immunization strategy.

- **Drug like compounds targeting multiple stages of malaria**

Current antimalarials are curative against asexual blood stage parasites but they cannot be used prophylactically and moreover they do not efficiently block malaria transmission¹⁸⁵. Drug-like compounds that are potential enough to target different, or preferably multiple parasitic life stages are on clinical demand. Indeed, synthesis of a compound from a chemical entity with a pre-defined multistage activity is quite challenging. Imidazolopiperazines were proposed as a new series of antimalarial by Novartis and their phenotypic screening revealed KAF156 as a lead compound from these chemotypes. KAF156 showed efficacy against the liver and *P. falciparum* drug sensitive and resistant strains and mature gametocytes *in vitro* at 5-50 nM concentration¹⁸⁶. It can be mentioned that it is one of the rare multistage inhibitors that showed prophylactic liver stage inhibitory activity by affecting sporozoite motility. Recently a drug like compound DDD107498 has been identified from Dundee protein kinase library with improved pharmacokinetic and good safety profiles that showed a novel spectrum multistage antimalarial activity by single dose treatment. Undoubtedly identification of such lead compounds potentially address the variety of clinical needs and contribute malaria elimination. Furthermore authors identified the molecular target of DDD107498 which targets the protein synthesis via translation elongation factor 2 (eFF2)¹⁸⁷.

- **MMV's drug like compounds**

Over decades non-profitable organization MMV's logistic support and scientific contribution towards malaria elimination policy, helmed by malaria and other parasitic researcher communities, especially the attempt of their research driven discovery for introducing early next generation antimalarials, is remarkable. Some of their candidate drugs are under pre-clinical developmental stages and 13 antimalarial agents are in clinical development and few have gained and passed satisfactory subsequent phase clinical trials so far¹³⁹. Not only this, MMVs

resource sharing - materials and reagents also accelerate malaria research in malaria endemic countries by locals. These drugs ideally should fulfil more than one target compound profile (TCPs) to kill *Plasmodium* parasite at different stages of its lifecycle and thus potentially relieve symptoms, protect, avoid relapse, and/or block transmission. Hence it can be hoped that new and safer anti-hypnozoite therapies will embark an enormous stride forward in eradicating *vivax* malaria.

- **Target based drug discovery**

Over the past three decades, target-based drug identification approach is the starting point and is a defined molecular target that is hypothesized to have an important role in disease — has been the elegant approach to drug discovery in the pharmaceutical industry, driven by advances in molecular biology and genomics. Moreover rational drug designing for any diseases wherein chemists and pharmacists estimate which changes are required for the drug candidate to make the lead more potent concerning the safety issue. Nowadays computational tools help to predict the interaction between a selected compound and its target which directs the structure activity relationship (SAR) that facilitates to recrystallize the drug like compound structures. Thus target-based drug screening even without knowing structure largely facilitates drug discovery portfolio. It also assists to counter screen against the human homolog to refine drug selectivity and optimizations. However, target based drug discovery approach is not a new arena in the context of malaria drug discovery. Several drugs have been identified active by disrupting the parasites organelles. Compounds active against these parasite targets alleviates clinical symptoms of malaria for example- Atovaquone, decoquinate, and tetracyclic benzothiazepine, ELQ300, GSK 932121 and BRD 6323 which target the mitochondrial cytochrome bc1 complex of the parasites¹⁸⁸.

Target identification in asexual blood stage malaria faced challenges which are even more complicated in liver stage. Apart from, these antibiotics targets the apicoplast of the parasites that presumably causes a delayed death phenotype. In liver stage whether this hallmark of antibiotic delayed death is relevant or yet to be investigated.

Drug Targets	Inhibitor(s)	Asexual Stage	Liver Stage

Dihydroorotate dehydrogenase	DSM265, DSM421, Genz-666136, BRD7539	✓	n.d.
Mitochondrion <ul style="list-style-type: none"> • Cytochrome bc1 complex • Pyrimidine synthesis • Folate synthesis 	Atovaquone, decoquinatone, tetracyclic benzothiazepine, ELQ300, GSK932121 and BRD6323 Primaquine	✓ Ref no.189	✓ Ref.190
Lysyl -tRNA synthetase	Cladosporin	✓	✓ Ref. no. 191,192
Apicoplast <ul style="list-style-type: none"> • Isoprenoid biosynthesis • Protein trafficking • Protein farnesylation 	Doxycycline, clindamycin, and azithromycin	✓ Ref. no. 193, 224	✓ Ref. 194

*n.d= not determined

Table 3: Drugs target liver and asexual blood parasites

- *Plasmodium* chaperons

Heat shock protein 90 (Hsp90) is an ATP-dependent molecular chaperone which is a highly conserved and ubiquitously expressed stress protein in pathogens. This chaperon family are considered as integral protein for the survival of asexual *Plasmodium* parasites but the functioning details in the hepatic stage is elusive. Recently a target-based screening identified compounds that selectively interacted with *P. falciparum* chaperons and revealed new mechanistic insights into the development of chemotypes exhibiting species selective binding. Using cell-based assay and time course inhibition study, authors demonstrated that the

identified Hsp90-binding compounds were active against both exoerythrocytic and erythrocytic stages of malaria. In addition a synergism was observed with the HSP90 inhibitor (SNX-0723) and a phosphatidylinositol 3-kinase-related kinase (PIKK) where both combination treatment reduced the hepatic stage parasite burden in non-relapsing *P. falciparum* malaria¹⁹⁵. In the case of relapsing malaria and hypnozoite stages such chaperon functionality has never been investigated in detail. However a recent transcriptomics study of day 9 cultured hypnozoite predicted a probable chaperon mediated modulation involving nucleosome- histone interaction in the maintenance of hypnozoite quiescence^{115, 116}. This hypothesis merits further investigation with a view to inferring the chaperon functions to develop an effective anti-relapsing malaria treatment.

- **Inhibition of protein synthesis**

Recently a drug like compound DDD107498 has been developed from the phenotypic screening program of Dundee kinase library. Authors identified a novel mode of action exerted by this compound. DDD107498 inhibited protein synthesis through *PfeEF2* which is expressed in multiple stages of parasites¹⁹⁶. This observation provided a new mechanistic support highlighting intervention of protein synthesis as a novel drug target in malaria.

- **PVM targeting drugs**

It is known that in the host cell, a second membrane delineates the developing parasite beyond the parasite plasma membrane which architecturally provides a distinct cellular compartment named as parasitophorous vacuole (PV). The PV membrane (PVM) is composed of the parasite host cell interface to regulate nutrient acquisition, environmental sensing, protecting from innate defense and thereby central to host parasite remodeling. A good number of parasite-encoded PVM proteins have been discovered over the past decades. The identification covers a list of multigene families, protein complexes for example early transcribed membrane proteins (ETRAMPs) and *Plasmodium* translocons exported proteins (PTEX) family. Almost all *Plasmodium* PVM machineries are restricted to this genus and exhibit a transient and stage-specific expression revealed in several studies. In the hepatic stage of *Plasmodium*, parasite and host transgenesis study identified few PVM proteins such as UIS3 and UIS4, mainly involved

in nutrient acquisition, are encoded by genes belonging to the up-regulated in infective sporozoites (UIS) as these proteins are highly transcribed in the salivary glands of mosquito. Genetic manipulation revealed that the deletion of either UIS3 or UIS4 gene resulted in the arrest of early liver stage development which implicates their crucial role in the hepatic stage of *Plasmodium*¹²¹. Particularly it has been shown that *UIS3* interacts with liver fatty acid protein (L-FABP) and supports hepatic parasite growth inside the host. Another two PVM resident proteins e.g LISP1 which is involved in PVM breakdown and facilitates subsequent merozoite release from the host hepatocytes whereas LISP2 is localized to the PV and PVM and gene deletion studies revealed parasites lacking either two proteins don't grow well in hepatocytes¹⁹⁷. Recently, LISP2 has been claimed as an early molecular marker of liver stage parasite development in both relapsing malaria species *P. vivax* and *P. cynomolgi* that can substantially differentiate dormant hypnozoites and early developing parasite forms¹³⁶. A hepatic stage based drug assay targeting PVM documented that torin (a tricyclic benzonaphthyridinone) strongly blocked trafficking of liver stage parasite proteins to the physical host–parasite interface PVM and thereby inhibited liver stage development¹⁹⁸. Therefore, such biochemical, experimental genetics and drug screening data suggest that some PVM proteins are potential targets for the novel antimalarial intervention strategies for the treatment of *vivax* malaria.

- **Apicoplast and mitochondria in *Plasmodium* liver stage : drug targets**

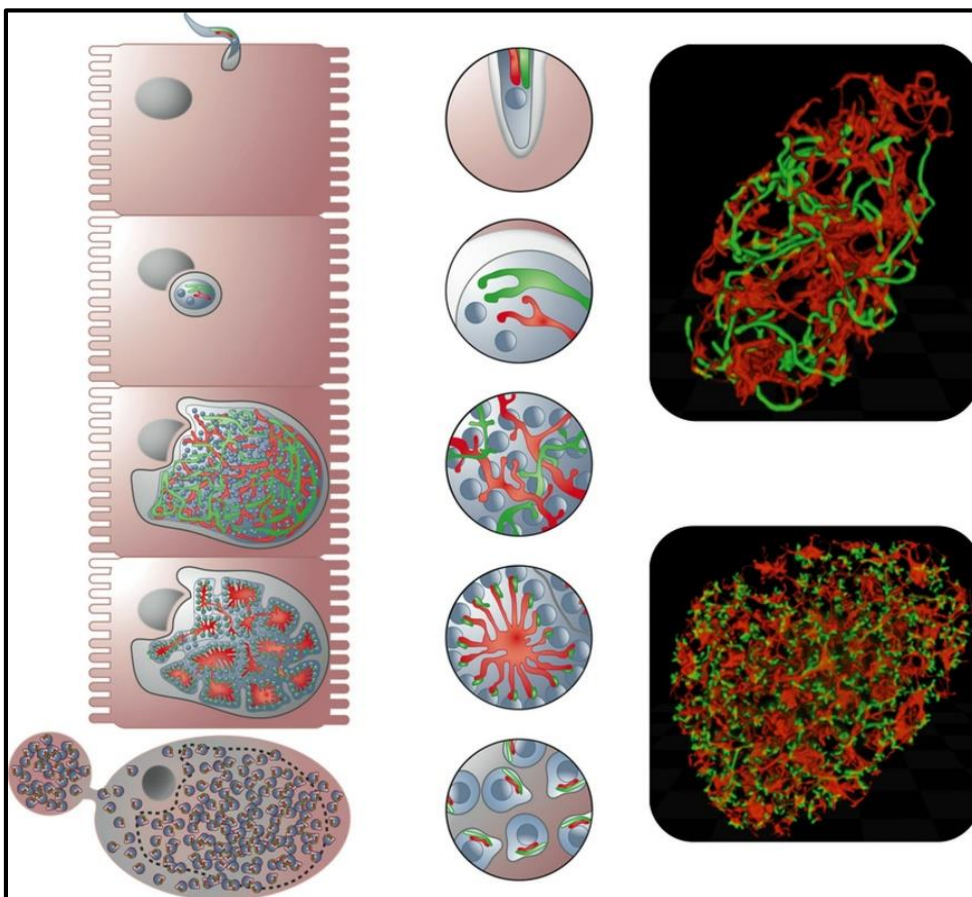
The malaria parasite and other apicomplexans possess an unusual and self replicating organelle called apicoplast. This vestigial, relict plastid was acquired via secondary symbiosis. Although evolutionarily apicoplast lost its photosynthetic ability its still retained its critical metabolic pathways¹⁹⁹.

Organelle evolution of apicoplast and mitochondria in *Plasmodium* rodent liver stage

Ultrastructure studies revealed that apicoplast and mitochondria are naturally close to each other in the human malaria parasite *P. falciparum*^{200, 201}. In malaria liver stage, *P. berghei* sporozoites contain a single nucleus, a single punctate /dot form apicoplast and a mitochondrion. After hepatocytes infection, the dot apicoplast stretches out to form an elongated structure. At 24-48h post infection, rodent liver parasites undergo a rapid hepatic schizogony while parasite grows from having a single nucleus to multiple nuclei. Thereby following the karyokinetic

proliferation, both mitochondria and apicoplast become increasingly branched or form a network in a manner of fission by segregating into multiple daughter organelles²⁰². It was reported that apicoplast division occurs always in late schizogony but before mitochondrial division. More specifically it was observed a divided apicoplast with elongated mitochondria but never divided mitochondria with branched or elongated apicoplast^{201, 203} (Fig.7). However in hypnozoite, the presence of apicoplast and mitochondria were identified through specific antibody staining but their organization and function have not been revealed yet.

Figure 7: Adapted from²⁰³; Organelle development and distribution into daughter cells in rodent model: invading sporozoites contain a mitochondrion (red) and apicoplast (green) and a nucleus (cyan). During schizogony, the nucleus divides but the mitochondrion and the apicoplast continue to grow and branch extensively. After egress, each merozoites contain a single apicoplast, nucleus and



mitochondria.

Plasmodium apicoplast contains a small genome (35 kb) and prokaryotic machinery required for housekeeping and metabolic functions. Four biosynthetic pathways are identified in *Plasmodium* apicoplast e.g heme synthesis, Iron-sulphur complex synthesis, fatty acid

biosynthesis and isoprenoid precursor synthesis^{204, 205}. Metabolic pathways like fatty acid biosynthesis was found essential in *Plasmodium* liver stage²⁰⁶. Our understanding about apicoplast role in liver stage has been heightened from blood stage studies where genetical and pharmacological experiments demonstrated that apicoplast is indispensable for parasite²⁰⁷⁻²⁰⁹. Parasite specific processes that are essential for their survival but absent in the host are instinctively a drug target. Most of the apicoplast perturbing drugs showed a ‘delayed death’ phenomena where parasite growth was unaffected during the first lytic cycle of antibiotic treatment but was severely inhibited in the second lytic cycle²⁰⁷.

Drugs	Targeted pathway/ Effect type	<i>Plasmodium sp.</i> <i>Blood stage</i>	Liver stage
Doxycycline	Translation / delayed and intermediate effect	Ref. 210	n.d
Clindamycin, Azithromycin and chloramphenicol	Inhibit prokaryotic DNA gyrase	Ref. 211	n.d
Triclosan	FASII Biosynthesis and unknown targets / immediate effect	Ref. 212	Ref. 216
Fosmidomycin	Isoprenoid biosynthesis, FASII pathway/ immediate effect	Ref. 213	Ref. 217
Actinonin	prokaryote-like post-translational modification	Ref. 214	n.d.
Rifampicin	Transcription	Ref. 211,215	n.d

Table 4: Examples of Apicoplast-targeting drugs

Mitochondria is considered one of the essential druggable attractive target for antiparasitic drug discovery especially in the blood stage where parasites in this stages can be selectively targeted by anti mitochondrial drugs as mature erythrocytes do not contain mitochondria ^{218, 219}.

- **Repurposed drugs against hepatic malaria**

Drug repurposing has successfully identified a list of promising candidate drugs, active substances that have created new therapeutic avenues to counteract against various diseases including malaria. In addition in the case of infectious disease treatment, synergistic drug combinations utilizing clinically approved safety drugs identified from drug repurposing screening may be a highly useful alternative which may enhance the activity of parent drugs by synergistic effects. Historically, accomplished malaria control interventions accumulate vector control policy, transmission blocking, and well tolerated antiparasitic drugs that prevent disease and reduced death tolls. However, existing tools and drug banks are really precarious because of frequent emergence of drug resistance so there is an urgent call for the development of novel drugs or the repurposing of old drug formulations as valuable intervention tools that can quickly prevent malaria infection. One of the fascinating features about repurposed drugs are their known pharmacokinetic, pharmacodynamic and toxicity profiles which greatly reduce research time, cost, and investment including the risk of failure. Importantly there is room to optimize the drug moiety. Considering this, anti-*Plasmodium* drug banks also benefited from drug repurposing such as tetracycline, doxycycline which affects both pre-erythrocytic and erythrocytic *Plasmodium* parasites²²⁰⁻²²⁴. Indeed, doxycycline is presently recommended by the Centers for Disease Control and Prevention (CDC) as a chemoprophylaxis treatment plan mainly for endemic countries travelers, and also against the uncomplicated malaria in combination with other antimalarial drugs²²⁵. In those contexts different antiretroviral drugs were screened against hepatic infection that were previously employed against HIV infection mitigation and assessed for their antimalarial activity against *malaria hepatic infection vitro* and *in vivo*.

- **Ivermectin**

Ivermectin, a Nobel winning FDA approved drug which is widely used in veterinary medicine and also shown effective in controlling malaria because of its insecticidal effect, potentiality to abort the *P. falciparum* sporogony, blood stage inhibitory activity by various studies. However recently ivermectin was screened against hepatic stage of rodent malaria and *in vitro* results demonstrated that this macrocyclic lactone was found active against *P. berghei* infection of human hepatoma cells. In addition, 1–10 mg/kg administration of Ivermectin significantly decreased the *P. berghei* load *in vivo* mouse model which showed equivalent inhibitory activity

like controlled drug primaquine. Following this, an extensive pre-erythrocytic screening was tested against rhesus malaria model *P. cynomolgi* both *in vitro* and macaque model *in vivo*. *in vitro* data showed the inhibitory concentration of *P. cynomolgi* schizonts ($IC_{50} = 10.42 \mu M$) and hypnozoites ($IC_{50} = 29.24 \mu M$) when added in high-dose prophylactically in primary monkey hepatocytes culture. In contrast neither *in vitro* nor *in vivo* *P. cynomolgi* models offered a radical cure phenomena by Ivermectin. However co-administration of ivermectin (0.6 or 1.2 mg/kg) and chloroquine was found safe and well-tolerated that improved ivermectin or chloroquine pharmacokinetics²²⁶. Interestingly there is a rising interest that the activity of ivermectin is not restricted to the parent compound alone and that ivermectin metabolites may be active as well. In fact, when the ivermectin metabolites from humans was compared to its parent compound mixed in human blood, the metabolites effect was 20- to 35-fold more lethal to mosquito species e.g *Anopheles dirus* and *Anopheles minimus* compared to parent compound²²⁷⁻²³⁰. In addition, metabolites exerted 5-fold more potent sporontocidal effect against *P. vivax* development²²⁸. Hence further identification of ivermectin metabolites warrants a screening against hepatic malaria infection.

- **Metformin**

Metformin, another widely used antidiabetic drug purportedly screened against hepatic *Plasmodium* infection *in vitro* and *in vivo*. Administration of metformin impaired liver stage burden of both rodent-infecting malaria human-infecting *P. falciparum* parasites. Prophylactic metformin treatment perturbed the intracellular development of hepatic parasites *in vitro*. In addition, a decreased number of infected hepatocytes were observed in the mice model. Moreover, metformin efficacy can be accelerated by combining low dosed available antimalarials to reduce their dose associated side effects which was observed in mice where single suboptimal dose primaquine (15 mg/kg) and metformin (500 mg/kg/d) remarkably reduced the *P. berghei liver* infection and substantially lowered the rodent malaria severity in mice²³¹. Altogether the findings indicate the utility of metformin as a repurposed drug as a prophylactic that may facilitates malaria chemoprevention.

- **Methylene blue**

Methylene blue (MB), phenothiazinium salt is the ancient synthetic antimalarial drug. However there is a growing interest to use this drug purportedly after the discovery of *P. falciparum* glutathione reductase as a new drug target. Field and lab based data of methylene blue exhibited

potential *in vitro* activity against *P. falciparum* when combined with artemisinin and related endoperoxides but it also demonstrated unexpected antagonistic effects with chloroquine and other known quinolines²³²⁻²³⁵. Recently a differential antimalarial activity by methylene blue demonstrated in different life cycles of *Plasmodium* parasites. The investigation revealed MB was found not active *in vitro* against *P. cynomolgi* liver stage, including hypnozoites. Moreover further *in vivo* studies found a moderate effect on *P. yoelii* hepatic development but in contrast it showed strong inhibitory effect against blood stage. Moreover, MB exhibited excellent transmission blocking by impairing the gametocytes and thus aborted parasite transmission from mice to mosquitoes²³⁵.

- **Pipeline drug candidate against relapsing malaria**

The search for a well-tolerated anti hypnozoite molecule is still ongoing in high demand. Various pre-clinical studies based on phenotypic screening revealed few lead compounds that showed efficacy against hypnozoite. The selected compounds need further investigation combining *in vivo* studies and consequently clinical trial studies^{236, 237}. History taught us most of the identified antimalarial drugs are stage specific. It means that one drug is not largely effective against all life stages of a parasite including artemisinin resistant parasites. Although a significant number of multistage inhibitors were identified by several studies which are still in rudimentary stage, most of the experiments were done utilizing rodent parasites and against *P. falciparum* hepatic stage. Imidazolopiperazines were proposed as new series of antimalarial by Novartis and phenotypic screening revealed KAF156 as a lead compound from these chemical entities that showed efficacy against the liver and blood stage parasites. Recently phase 2 clinical trial in Thailand and Vietnam has been completed against acute *P. vivax* and *P. falciparum* malaria^{186, 238}.

9.2 Quantitative high throughput screening facilitates liver stage drug discovery

Earlier liver stage drug screening was largely dependent on the smaller libraries compounds or chemists' sources that were commercially purchasable with a known function against the blood stage parasites. However the rapid technological advancements since a few decades, the liver staged based drug assay has been accelerated mainly due to support from robotic automation

facilities and development of faster data processing/control software, liquid handling tools and sensitive detectors have facilitated to identify hits from very large compound libraries. An adapted high throughput screening facilitates the whole drug discovery process e.g. a series of compound preparation, incubation, and analysis of many micro plates simultaneously within a short time. Furthermore the HTS system can detect the chemical compounds which are related to cell toxicity. Apart from this, the ongoing development of cheminformatics has also played a role to refine and improvise the chemical moieties of the selected hit compounds and interpret the large datasets generated from these screens.

Previously all high-throughput anti malarial drug screening efforts have emphasized against the asexual blood stage parasites. However human liver, being a bottleneck of the malaria disease and hypnozoite reservoir, it's highly imperative to find drugs against liver stage that can block the preceding blood stage infection, malaria relapse and thereby led to the interruption of transmission cycle. To fulfil these unmet needs, a high-throughput phenotypic liver stage based drug screening was operated with diverse chemical scaffolds. The screening recapitulated the murine liver-stage infection successfully by adapting in 384 well plates. Prior to infection, luciferase-expressing *P. berghei* parasites were extracted from the salivary glands from infected mosquitoes and inoculated into confluent human liver cell line HepG2

propagation *in vitro* Finally HTS screening was optimized based on the luciferase intensity after a suitable incubation period (0, 3, 8 and 24 h post sporozoite infection). However, the screening identified 37 liver-stage malaria inhibitors from 5,375 known bioactive compounds and also from Food and Drug Administration (FDA) drug banks with different modes of action which further warrants to evaluate the efficacy against relapsing species. The identified compounds demonstrated nanomolar to picomolar potency *in vitro* by salinomycin, monensin, lasalocid A, ATP synthase/proton pump inhibitors e.g. esomeprazole, omeprazole, tenatoprazole and telmisartan. In addition one of the hits salinomycin was tested *in vivo* against murine malaria model where mice received a single salinomycin treatment (30 mg per kg of body weight). A reduced liver parasite burden of 98% compared to control was observed ²⁴³. Thus open-access high-throughput screening may provide a rich resource in the search for new antimalarials.

It can be noted that the larger scale drug screening against relapsing parasites *P. vivax* and *P. ovale* systematically require frequent supply of infectious sporozoites in bulk and that must be incubated with larger quantities of cryopreserved primary hepatocytes. Pragmatically, such experimental set up is too ambitious in terms of cost management and reagents utilizations and other ethical logistics problems e.g availability and shipment of infectious mosquitoes are associated with it. Therefore high-throughput screening utilizes rodent parasites *P. berghei* because of its easier transmission adaptability to mosquitoes in the insectarium. So the initially identified compounds from such screening may not directly hypnozoitocidal compounds however provide information such as a filter to narrow-down the compounds considering toxicity profile thus may enter the next phase of the screening cascade to assess its anti hypnozoite property in relapsing malaria models.

Recently researchers extracted rodent *Plasmodium* sporozoites from over one million mosquitos through hand dissection over a 18 month period and subsequently tested over 500,000 chemical compounds from Charles River compound libraries that showed potency to shut down the parasite life in *in vitro* hepatic cellular models. The massive experiment was designed with 384 drug assay plates, *P. berghei* luciferase parasites, *P. yoelii* and HepG2-CD81 as cellular models. Further a single point high content imaging screen was performed against *P. vivax* infected primary human hepatocytes. After three round refinement and toxicity evaluation, the team was able to narrow down their list to just 631 compounds that retain the scope for the further development of chemoprophylaxis drugs²⁴⁴. The active series of drugs namely imidazopyrazine group (phosphatidylinositol-4-OH kinase -PI4K) and MMV drug like compounds. It can be expected that such diverse drug scaffolds prove a ‘treasure trove’ that can represent a valuable starting points for the development of new generation antimalarial agents for chemoprophylaxis, disease transmission blocking and can reveal novel mechanisms of action, identification of new targets on host and parasites as the drug targets of these candidate profile are already identified

In addition, another robust ‘quantitative high throughput screening’ approach was reported with 450,000 compounds. Ultimately after initial screening on *P. falciparum* blood stage the active compounds were further filtered and subsequently further tested against *P. berghei* hepatic infections which expressed a GFP-luciferase reporter with a view to identifying dual targeting novel chemotypes²⁴⁵.

10. Anti-hypnozoite drug screening scope

10.1 Previous drug screening failed to find a radical cure drug

Radical cure denotes an efficient clearing of the dormant parasite (hypnozoite) from the host liver. Malaria elimination is completely restricted due to the scarce of true anti-relapsing compounds although having taken enough collaborative drug discovery approaches. Moreover most of the identified drugs showed only prophylactic phenomena *in vitro*. So the current anti-hypnozoite drugs screening failed to find a suitable drug like compounds that could efficiently kill the late staged hypnozoites which are considered as bonafide persister.



Figure 8: Previous drug screening on relapsing malaria failed to find a radical cure drug.

Rather the identified compounds likely PI4k kinase- KDU691²³⁹, KAI407²⁴⁰, Ionophores^{60, 241}, MMV390048²⁴² showed *in vitro* and *in vivo* chemo preventive activity by preventing hypnozoite formation when the drugs are administered prophylactically at the time of sporozoite. Matured or already established hypnozoites were not sensitive to these drugs when they are added few days after sporozoite infections. Clearly, it is imperative to identify new drugs that can act on mature hypnozoites to block the malaria relapses.

10.3 Necessity of anti-hypnozoite drugs

In spite of taking massive efforts and international collaborative approaches ‘Global Malaria Elimination’ strategies are hampered. Undoubtedly spurred by major steps to eliminate malaria developments in the last decades have vastly reduced the burden of this disease. Nevertheless the treatment of malaria largely depends also on prevention by antimalarial drugs beside protection by mosquito nets or insecticides. These selective drugs mostly target the blood stage parasites and latent hypnozoites are insensitive to these drugs. In addition there are now strains of blood parasites that are resistant to these licensed drugs. Moreover, the current antimalarial drug regimen fails to eradicate the relapse problem. Apart from this, given the limited availability of sporozoites from relapsing *Plasmodium*, many studies have solely focused on drug identification against blood stage wherein liver stage based drug screening was underestimated. It can be noted that prospectively over the last 15 years, antimalarial drug screening has been rapidly accelerated using phenotypic screening wherein more than 7 million compounds were screened against asexual blood stage malaria and over 500000 compounds were recently screened against rodent liver stage and 681 validated hits were identified with micro molar activity^{139, 140}. Hence there is a great room and scope to select those murine liver hits testing against relapsing models.

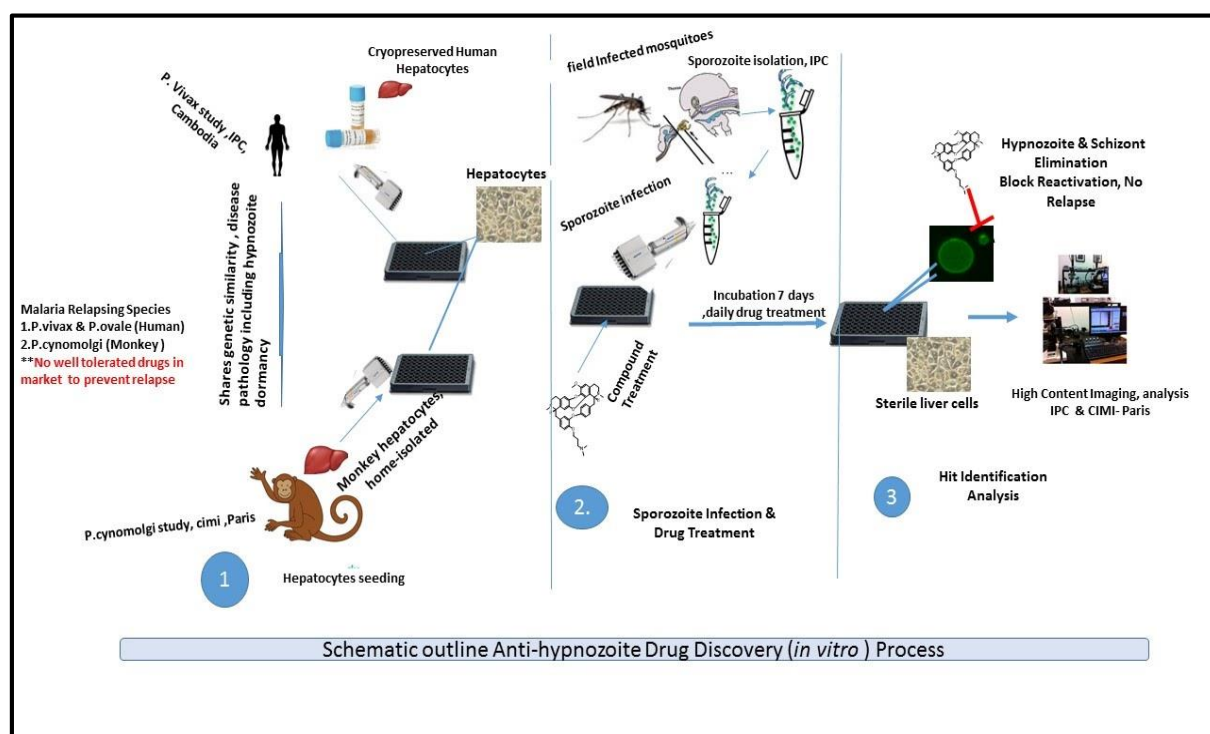


Figure 6: Schematic model of anti-hypnozoite drug discovery in collaboration with *vivax* endemic country 1) hepatocytes seeding either home-isolated or commercial cryopreserved Human hepatocytes 2) sporozoite availability on malaria site from field insectarium setting and infection, drug screening 3) High throughput screening platform to assay the antimalarial like compounds, toxicity evaluation and hits identification

Presently, there is an acute dearth of well tolerated *P. vivax* relapsing therapies highlighting the necessity to find urgent anti-relapsing drug-like compounds (hypnozoitocidal or hypnozoite-activating drugs). This formidable challenge is hindered by the scarce of appropriate biological tools that support to recapitulate liver stage and screening assays including logistic challenges regarding the availability of human tissue and sporozoites. In addition, limited understanding of the molecular pathways involved in hypnozoite formation and quiescence is also a limiting factor. Reactivation of dormant hypnozoite possibly leads to malaria relapse, increases the risk of patient morbidity and favors opportunistic malaria transmission which perturbs the health care policy of least developing Asian and American endemic countries¹⁴¹. The problem exacerbates due to the scarcity of globally deployable drugs that can kill the relapse contributing hypnozoites. *Vivax* malaria harbors relatively low parasitemia^{142, 143}. Therefore, the clinical outcome of *P. vivax* can be anything lethal but as benign and need to be considered as equal to

P. falciparum in terms of research priorities. In addition primary and secondary attacks alike often stimulate a debilitating febrile illness that offer life threatening complications. In that case, a prompt and accurate diagnosis can differentiate the malaria species carrying the patient's blood and therefore prescribe effective schizontocidal blood therapies can be crucial in anti-relapse treatments. But unfortunately the current standard antimalarials ACTs except 8-aminoquinolines- primaquine and tafenoquine are not effective against dormant hypnozoites which may awake with or without any mosquito bite days to months to years after the final bout of malaria with unknown triggering mechanisms¹²³. Clinical data revealed that if hypnozoites become stagnant inside host, then the subsequent blood stage infections in early relapses (< 2 months) possible to prevent by the administration of a slow acting blood staged anti-malarials (chloroquine, artemisinin combination treatments), but they are not usually removed and provoke the risk of recurrent infections¹⁴⁴.

10.4. Inability of current malaria diagnostic tools to detect hypnozoite mediated relapses from recrudescence and reinfection

There is no effective diagnostic tools that can detect the presence of hypnozoite reservoir from a patient as liver stage malaria is asymptomatic. Moreover current understanding of relapse patterns of *vivax* strains are mainly restricted to the clinical studies on soldiers working on malaria endemic countries, experimental challenges and barley from migrant workers returning from endemic countries¹⁴⁵. The timing of relapse varies with different isolates of the parasite based on disease endemicity, geographical and climatic variations which make it more unpredictable. It is imperative to understand the epidemiology of *P. vivax*. To accelerate this, efficient tools or assays are required that can distinguish between recrudescence, new infection, and relapse.

Discovery of such chemotherapeutic interventions, undoubtedly will facilitate to evaluate the current drug efficacy against *P. vivax* and more remarkably will enable to identify phenotypic characteristics of short- and long term latency within disease endemic regions. It will also help to estimate the total disease burden due to relapse which is greatly underestimated before. So development of genotyping methods will allow to decipher the discrimination of the primary attack parasites from the different relapse populations. However current genotyping strategies for understanding recurrent *vivax* infections mostly depend on microsatellite markers. Through

these markers it has been known that relapses are often multiclonal and generally derived from the activation of heterologous hypnozoites (displaying a different genotype compared with the preceding blood-stage infection in the same individual)¹²⁴.

Therefore application of next-generation sequencing technologies may shed more light understanding the genetic signatures of relapse (e.g., that they are meiotic siblings) that may be exploited to develop better genotyping methods.

10.5 Reactivating drugs: New avenue for relapsing therapy

The concept of provoking the dormant forms to become a reactivated one was previously applied in the treatment of HIV latency which was dubbed as ‘shock and kill’. A process involves activating the dormant virus within the immune cells where it generally hides by a suitable drug²⁸¹. In parallel to the discovery of radical cure drugs, there is also a promising scope to identify reactivating drugs that can interfere with hypnozoite’s quiescence and provoke the hypnozoite to transform into activating schizont which thereby can be killed with available schizonticidal drugs. This concept is termed as ‘wake and kill’, described by Dembele and Franetich et al.. In a robust hepatic culture model, authors identified an epigenetic histone methylation inhibitor TM2-115 which increased the rate of hypnozoite activation and accelerated the transformation of hypnozoites into schizonts¹⁵⁴. This result expanded another avenue of further understanding of epigenetic role in maintaining hypnozoite quiescence and a scope of epigenetic based drug discovery for the treatment of relapsing malaria. The recent hypnozoite transcriptomic studies also supported the hypothesis as histone acetylation and methylation was found fully functional in the cultured hypnozoites.

11. The contribution of natural products in antimalarial drug discovery: advances and opportunities

11.1 Historical perspective

Since prehistoric times malaria chemotherapy has largely benefitted from natural products and its derived metabolites. For thousands of years, plant-based natural drugs have been used as a good source of drug-like products that alleviated disease-like symptoms including malaria. The

complexity of the malaria life-cycle and its numerous biochemical and physiological interactions associated with the human host and *Anopheles* mosquito vector implicates a multitude of chemotherapeutic agents development for disease prevention and intervening the parasite transmission. Consequently the then earliest drug hunters used to quest drug like sources and continue their empirical research focusing on natural plant based remedies. Still this history is befitted for worth reviewing and exploring, not least because two of the crucial drug like products discovered (quinine as well as artemisinin) from plants which greatly contributed to disclose an Achilles' heel of the malaria parasite that continues to be primely exploited as a therapeutic solution to date^{246, 247}. Although most of the ancient herbal remedies have lost their efficacy to varying degrees over time due to the development of resistance, there is still a strong desire shown by a part of African malaria endemic population to extrapolate their ethnopharmacological heritage with a view to identifying new medicines to fight against malaria.

Timeliness is another advantage, particularly during emergencies. Once a herbal decoction or component is found to be effective, it can be immediately used for treating patients, its safety already established. Ironically, compared to chemically synthesized drugs, plant natural products are less understood mechanistically²⁴⁸.

Quinine, the first antimalarial drug still exists in today's modern world, originally extracted from cinchona tree bark (a tree native to South America). According to the legends, from the seventeenth century, infusions prepared from Cinchona bark were the only rapid treatment solution for diseases like malaria until its active component quinine was isolated by two French chemists and purified in 1820. Consequently this plant derived quinine has become popular as an important and effective ailment of malaria in spite of its sporadic resistance observed in a few corners of the world²⁴⁹. Previously WHO recommended the use of quinine plus antibiotic clindamycin for the treatment of malaria during the first trimester of pregnancy, as the safety issue of ACTs (Artemisinin combination therapy) during this period was not known. Later quinine's prolonged treatment course and tolerance problems led to poor patient compliance and in this aspect, ACT took advantage over quinine since it is administered once or twice daily over three days. However the frequent effort to synthesize quinine chemically was not possible but potentially led to the discovery of new synthetic antimalarials based on structural elements of quinine by pioneering contribution of a German scientist in the early twentieth century. From this synthesizing concept, the classical 4-aminoquinolines series as well as amino-alcohols, for

instance chloroquine, amodiaquine and mefloquine supported many years and some of them are still considered as mainstay of malarial treatment²⁵⁰.

Chloroquine: Discovery of resochin (synthetic drug like compound which is chemically related to the quinine and named as chloroquine in 1946) showed potency against both uncomplicated and severe cases of malaria. This superior activity of chloroquine expelled quinine. Comparatively chloroquine was one of the most successful antimalarial agents and saved millions of lives until drug resistance was reported in a scattering manner but still holds strong antimalarial medications against relapsing malaria *P. vivax* in few countries including densely populated endemic India. Moreover, the natural product naphthoquinone lapichol reported as an active component in tree bark and facilitated to direct the selection of lapinone which laid the foundation of the discovery of atovaquone, a integral drug component of Malarone®, still recommended by WHO as malaria prophylaxis for travellers intended to visit malaria endemic countries^{251, 252}.

Artemisinin annua L.

Later, the urge of microorganism resistance in 1967 towards the classical *N*-containing antimalarial drugs prompted Chinese government officials to initiate a systematic drug screening program (historically known project-523) which was led by Dr Tu Youyou and her team from the ancient medicinal manuscripts of Chinese herbs in order to save dying people. Being imbued by this spirit, Tu Youyou and her colleagues identified more than 2,000 Chinese herb preparations and systematically identified 640 hits that were predicted having antimalarial potentials. More than 380 extracts were evaluated against a murine malaria model. However, the experimental progress was uneven and no positive findings emerged immediately. The turning point came when an *Artemisia annua* L. extract demonstrated a significant degree of inhibition against malaria parasite growth and development. As the cure rates were not reproducible in the subsequent repeated experiments so they again went through the recorded literature where it was mentioned “A handful of qinghao was allowed to soak within two liters of water, wring out the juice and drink it all” From the quote stated in the manuscript they were convinced that their conventional extraction process mainly heating at high temperatures might probably destroy the active component(s). Thus switching into lower temperature and subsequent extraction enabled them to extract a neutral, non toxic molecule crystallized as a colorless crystalline substance (compound summary : molecular formula: C₁₅H₂₂O₅ , molecular

weight: 282 Da and melting point: 156-157°C). Tu Youyou found a better antimalarial activity with this *A. annua* extract which was further validated into *P. cynomolgi* infected monkey model. Thus historical- remarkable attempts led the discovery of *Qinghaosu* (now called Artemisinin, ART) from the plant *Artemisia annua* (Sweet wormwood) which was helmed by the world and Tu Youyou endowed with Nobel Prize. At initial clinical trials, ART demonstrated a promising therapeutic activity that saved life of 2099 patients suffering from *P. vivax* and *P. falciparum* where all patients were treated with either oral ART tablets (2.5-3.2 g) or intramuscular injection 0.5-1.2 g doses of ART daily for 3 days. Surprisingly, within 40 hours of administration, the ART-tablet completely cleared the parasitemia from patient's blood²⁵³⁻²⁵⁵. However during ART evaluation they also found that ART derivative- dihydroartemisinin (DHA) was more stable and ten times more efficient than parent artemisinin with less disease recurrence evidence. So addition of a hydroxyl group to the ART molecule also paved the way to develop new artemisinin derivatives through esterification that can render improved oral bioavailability compared with that of ART.

Artemisinin derivatives were designated first-line antimalarial drugs by the WHO in 2002 and since then almost all malaria-endemic countries have rapidly switched to artemisinin (ART) based combination therapies (ACTs) that combines an ART semisynthetic derivative having a short half-life with faster killing activity and with a longer-lasting partner drug as first-line treatment of *P. falciparum* malaria. Although the mechanism of action of ART-based drugs has not entirely determined yet however there are few and yet to be identified pieces of affirmation. A proposed mode of action suggested an activation of peroxide bond in the *Plasmodium parasite* vacuole when the endoperoxide linkage interacts with heme iron²⁵⁶⁻²⁵⁷.

Although ACTs are globally deployed against all human malaria and that contributed largely for the treatment of the disease however in the context of *P. vivax* treatment and eradication, hypnozoite mediated relapse is a concern and the worrying issue is that those relapsing forms of parasites are not sensitive to ART and its partner drug. So total annihilation of liver resident exo-erythrocytic parasites including the dormant hypnozoite is obligatory and ironically no other current antimalarials offer anti hypnozoite activity except primaquine and tafenoquine. In the meanwhile ACTs treatment encountered *Plasmodium* parasite resistance within immediate drug deployment in Southeast Asia and most strikingly there are few substantial reports of the

emergence of drug resistance in Africa where *P. falciparum* malaria is the most prevalent^{258, 259}.

To combat all of this and future uncertainties we desperately need novel antimalarial drug like compounds from different reliable sources e.g. exploration of ancient pharmacopoeia, back to the root-drug repurposing, development of synthetic and semi-synthetic compounds that are devoid of toxicities and well tolerated to the malaria patients.

11.2 *Artemisia* infusions: Traditional panacea and future antimalarial drugs discovery aspects

For many decades, the folk medicine either water (infusions or decoctions) or hydro-alcoholic (tinctures) extracts are frequently practiced to benefit from the naturally healing properties offered by plants. Considering the incredible health benefits from such botanical extracts, medicinal plants can offer a wide range of bioactive components (e.g. polyphenols) and can be explored to treat infectious diseases. *Artemisia* infusions from which the frontline antimalarial artemisinin (ART) is extracted have been traditionally used to treat malaria like symptoms since centuries. Such traditional practices are still included in Chinese pharmacopoeia and also widely popular in a part of Africa subcontinent. The ethnobotanical practice includes the use of whole plant decoction for the treatment of malaria, cough, chill like fever and catarrh. Thus there has been a growing interest in the possible benefits of *Artemisia* infusions in particular areas where conventional drug therapies are not readily accessible or are too expensive to purchase. In fact the whole flowering plant is documented as anthelmintic, antipyretic, antispasmodic, antiseptic, tonic as well as stomachic²⁶⁰. As mentioned above, *A. annua* contains artemisinin, which provides a structural chemical base for combinatorial treatment therapy for worldwide antimalarial programs. Moreover research studies also reported that artemisinin is effective for killing human breast cancer cells. Therefore, isolation and characterization of artemisinin has increased the interest in *A. annua* worldwide. Several ethnobotanical uses in Africa claim that the *A. annua* tea is also effective against human immunodeficiency virus (HIV). In fact *Artemisia* infusions are reported effective against human parasitic diseases²⁶¹.

Artemisia afra, another species of *Artemisia* family and indigenous to Africa which is reported containing negligible amount of artemisinin contents (even no trace amount) theoretically but phytochemical screening revealed a rich amount of essential phytochemical constituents, high

concentration of flavonoids (160 individual flavonoid components) and phenols. Hence, the phytochemical research of this plant is very important in terms of discovering bioactive profiles that may have therapeutic importance against malaria. Moreover it is reported that *Afra* samples collected from different geographical localizations and dissolved into various solvents demonstrated different antioxidant properties leading to higher antimalarial activities²²⁴. The IC₅₀, derived from hexane extract of *A. afra* treatment against *P. falciparum* strains D6 (CQ sensitive) and W2 (CQ resistant) are 3.18 µg/ml and 0.71 µg/ml respectively²⁶². Recently, gametocidal activity of *Artemisia* infusion observed *in vitro* in *P. falciparum* model²⁶³.

The rapid emergence of frontline antimalarial drug resistance mainly in Southeast Asian regions is a current concern as it is primarily associated with treatment failure. Thus overcoming the development of resistance and tackling of it is therefore a key research goal. Different *in vitro* studies tested against chloroquine resistant strains of asexual *P. falciparum* parasites which showed good inhibitory activity by *Artemisia* infusion treatment. In addition, a *in vivo* study performed into two rodent malaria models, mice infected with artemisinin-resistant strain of *P. yoelii* to assess whether *A. annua* potentially overcome drug resistance and another *P. chabaudi* to track the development of resistance as it closely resembles to *Plasmodium falciparum*. The result revealed a reduced parasitemia observed in mice treated with dried whole plant *Artemisia annua* compared to the mice receiving pure artemisinin²⁶⁴. Furthermore it took at least three times longer for a second rodent malaria parasite to commence a stable resistance in the case of *A. annua* treatment compared to artemisinin. Hence it is thought that the whole plant treatment would have an advantage compared to ACTs because compounds other than artemisinin could have anti-parasitic activity or the total existing metabolites present in the plant may act synergistically thereby improving artemisinin efficacy and bioavailability which can exert more strong antimalarial effect. The administration of the plant would thus not be equivalent to a monotherapy containing only one active component against the parasite but an effective therapy composed of sufficient bio active compounds acting in a concerted way.

This aforementioned information greatly necessitates a systematic extraction-fractionation to identify other valuable non-pharmaceutical forms of artemisinin (ART) from these *Artemisia* plants that can overcome the resistance and thereby offering a longer effective lifespan in malaria treatment. Indeed, the genus *Artemisia* is well known to contain many bioactive compounds, phytochemicals, terpenes, flavonoids and some of which demonstrated better antimalarial efficacy both *in vitro* and *in vivo* as mentioned above. Extracts such as arteannuin

B, arteannuic acid and scopoletin isolated from *A. annua* have been proposed as new artemisinin-based natural combination therapy of *P. falciparum* malaria²⁶⁵.

11.3 Scope of anti-hypnozoite compounds fishing from natural resources

Preliminary screening of chosen plants or fractions is generally performed in *in vitro* models against rodent parasites. When results were obtained, they could be screened on human malaria especially against relapsing malaria. Surprisingly screening of plant derived extracts or metabolites against liver stage of relapsing malaria is totally unprecedented in literature database PubMed after a thorough search of the relevant scientific databases under these key words ‘plant metabolites *P. vivax*’, natural products, marine products *P. vivax*.etc. Yet a few plant derived compounds (morphinan) and secondary metabolites (triterpenoids, isolated from *Momordica balsamina*) were tested against rodent *Plasmodium* liver stage^{266, 267}. True anti-relapsing compound is very scarce in the current antimalarial pipeline. Only a bonafide radical cure drug (a drug able to kill the dormant hypnozoites inside the liver during the blood stage infection and treatment) can leverage the gap. Drug screening with natural resources largely conducted in *Plasmodium* blood stages but recently anti-hypnozoite drug screening has gained momentum and expanded well mainly due to the development of robust hepatic culture models that facilitate culture more than a week. Hence from that perspective, an idea to further explore natural resources for a specific effect (most strikingly for an anti hypnozoite effect) without any clue is not rational.

11.4 Other natural products

Looking at the current preclinical and clinical pipeline, one can be optimistic about the future of antimalarial chemotherapy. Buoyed by these successes, the field is looking ahead to future challenges such as blocking disease transmission, eliminating disease in specific geographic areas, and eventually eradicating the disease globally.

Recently a malaria growth inhibition assay (GIA) revealed identification of four compounds (berberine chloride, coptisine chloride, palmatine chloride, and dehydrocorydaline nitrate) and two refined extracts (*Phellodendri cortex* and *Coptidis rhizoma*) from natural product library demonstrated promising antimalarial activity with the equivalent IC₅₀ of chloroquine against *P. falciparum* drug resistant (Dd2) and sensitive strain(3D7)²⁶⁹. Salinipostin A, a natural marine

sediment product produced by a *Salinispora* sp. bacterium exhibited potent inhibitory activity against *Pf* parasites (EC₅₀ of 50 nM)²⁷⁰. In addition, a bioassay-guided fractionation and multigenomic comparative analysis revealed that romidepsin derived from natural product *Chromobacterium* sp. exerted a potent antimalarial activity

12. Malaria response in today's scenario of COVID 19 pandemic

COVID19: can provoke *Plasmodium vivax* relapse ?

The early symptoms of SARS-CoV-2 infection for example myalgia, fatigue and fever which lead to morbidity could be bewildered with the symptoms of malaria. These symptoms similarities may lead to arise confusion in the early diagnosis particularly malaria endemic regions²⁷¹.

Previous historical records indicate that *vivax* relapses could be provoked by other other febrile infectious diseases including viral infections. Malaria epidemics suggested that bacterial pathogens caused typhoid fever might activate *P. vivax* hypnozoites and cause relapsing malaria²⁷². However in the case of viral infections, such attributes were not documented till many decades except one rare finding where dengue infection was reported to trigger *P. ovale* relapses in a patient residing in Rio de Janeiro, Brazil²⁷³. Meanwhile, in the context of the current outbreak of CoVid 19, recently a patient has been identified with co infection of *P. vivax* malaria (parasitemia 1.2 %) and SARS COV2 infection²⁷⁴. Apart from this, a case of COVID-19 co-infection with *vivax* malaria reported in a 10 years old boy who previously had received primaquine to prevent *vivax* relapse infection²⁷⁵. The accurate mechanism of relapse is not known however previously it has been postulated that high plasmatic cytokine response with systemic illness may contribute to *vivax* relapse^{276,277}. Similarly it has been known that COVID infection also leads to cytokine storms making the disease more severe^{278, 279}. Hence it was hypothesized that COVID-19 infection with its cytokine response could probably induce *P. vivax* relapse in the aforementioned patient^{275, 280}. This scattering clinical data and circumstantial evidence can't be underestimated as it implicates a possible role of COVID-19 in the induction of malaria relapse.

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Chapter 2

Supplementation with a cocktail of methyl paraben and penicillin-streptomycin in mosquito diet prevents microbial contamination in hepatic *Plasmodium* culture

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Abstract

in vitro liver stage culture of relapsing malaria species and radical cure drug screening sometimes encountered with technical difficulties; associated with microbial contamination. To study relapse causing hypnozoites, hepatic cultures must be continued over weeks, after their infection with sporozoites obtained by hand dissection of salivary glands from infected mosquito.

In the insectarium, *Anopheles* mosquitoes are continuously exposed to a huge diversity of microbiota in their breeding site and potentially carry numerous microorganisms. Thus, microbial pathogens; mainly fungi, frequently emerges in culture which ultimately results in poor outcome of the assay. To avert such unexpected pathogenic contamination, various laboratory protocols have been developed; for example sporozoite purification steps of post salivary gland dissection, use of antibiotic cocktails in mosquito feeding meal, blood meal or axenic breeding of mosquitoes. In addition, a broad panel of antibiotics are also routinely used in hepatic *Plasmodium* culture to mitigate the possible presence of microbial agents. However, some of them showed anti-parasitic activity in *Plasmodium* liver stages and could generate false positive results.

Here, we focused on improvising mosquito feeding solution that could annihilate microbes in the mosquito stage. We chose MP-PS cocktail (a combination of methyl paraben, penicillin-streptomycin and para amino benzoic acid in sucrose solution) as a supplement in mosquito diet given from their adult emergence to until salivary gland dissection. The goal was to obtain aseptic sporozoites to facilitate long term; germ free hepatic culture.

MP-PS, in diet solution showed no drastic effect on mosquitos' survival, nor on *Plasmodium* infection. Oocysts prevalence and sporozoite development were not affected and importantly sporozoites remained microorganisms free, which was confirmed through microbial analysis. Hepatic cultures, inoculated with these sporozoites develop normal liver parasites and remained free from microbial contaminants until their fixation of two weeks later. Thus we employed a robust mosquito feeding strategy to prevent microbial contamination that could be ideal for germ free sporozoite production and long term maintenance of hepatic infection. We aim that such feeding protocol could be also useful for other scientific communities working with mosquitoes.

Introduction

A major challenge to study *Plasmodium* hypnozoite, is the difficulty to maintain infected hepatic cells for several weeks in culture with the hope to observe these dormant forms resuming their proliferation¹. Hence during this extended period, hepatocytes cultures inoculated with sporozoites of relapsing malaria parasites frequently encounter contamination of microbial pathogens. Indeed, infectious sporozoites are isolated from the salivary glands of laboratory infected mosquitoes by hand dissection, but *Anopheles* mosquitoes not only harbor *Plasmodium* sporozoites, but they possess their own microbiota depending on their breeding environment^{2,3}. It is well known that bacterial load and/or microbiome community composition can significantly influence *Anopheles spp.* mosquito susceptibility to *Plasmodium* infection^{4,5}. This situation is even worse when working in tropical areas conditions where the warm and wet climate could favor mold proliferation. Hence, under these conditions, obtaining microbes free sporozoites is technically almost impossible.

An aggravating factor is that liver stage experiments require huge amount of sporozoites⁶. This implies the dissection of a considerable number of infected mosquitoes, especially when working with mosquitoes infected with blood samples obtained in the field from patients with unstandardized parasitaemia/ gametocytaemia. It is well known that working with low infected mosquitoes (typically, with less than 10^4 to 10^5 sporozoites per mosquito) drastically increases the risk of contaminating the culture by adding numerous microorganisms coming with the inoculum. Although routine protocols of hepatocytes culture include antimicrobial agents like penicillin-streptomycin, gentamycin and fungizone^{7,8} but they are not always effective against the unwanted pathogens in culture. Bacterial, fungal, and yeast contaminants are generally not visible to the unaided eye, but may quickly proliferate and kill the hepatic cells either by nutrient depletion and chemico-physical culture media modification (generally acidification), or by releasing microbial toxins deleterious for the cells. Although few culturing aids such as collagen used to embed explant tissue on plates and matrigel are used to improve the hepatocytes culture but this collagen gel based cultures are also prone to fungal and bacterial infections⁹. Moreover, contaminated sporozoites could also affect *in vivo* studies of *Plasmodium*. Immunocompromised mice e.g humanized mice has been recently developed and facilitated understanding of human malaria; *P. vivax*, *P. ovale* and *P. falciparum*^{10,11}. Infection of these mice with unpurified sporozoites could introduce a high load of microbial burden which might affect the health status of mice by perturbing their innate immune response against the liver

parasite infection¹². Besides, biochemical and proteomics studies of those unpurified sporozoites revealed a higher mosquito debris mass compared to parasite mass yielding insignificant numbers of parasite derived peptide hits. Moreover, translational and fundamental research e.g. transcriptomics of sporozoite, single cell sequencing or anti-hypnozoite drug screening require sporozoites devoid of mosquito debris and microbial pathogens¹³. To overcome these contamination issues, different strategies have been developed:

Purification of the sporozoites

Since parasitologists started to study malaria liver stage, they had to isolate sporozoites from infected mosquitoes and face these high risks of contaminations. Addressing this issue, numerous protocols of sporozoites purification have been developed, leading to more or less clean parasite inoculum but with an always substantial loss of sporozoites.

These methods for sporozoite isolation have included centrifugation through glass wool, a protocol well known as Ozaki method¹⁴ that lead to highly contaminated sporozoites, even when combined with density gradients¹⁵⁻¹⁹. Whilst the addition of gradients increases sporozoite yield, the final output is still contaminated with mosquito debris²⁰⁻²¹. Other methods trialed for sporozoite isolation have included ion exchange chromatography^{20, 22} and later free-flow electrophoresis (FFE)²³. Another nuclepore membrane based filtration, a protocol that significantly prepared cleaner sporozoites compared to discontinuous gradient method²⁴. All of the above mentioned protocols although claimed to be mosquito debris free but add time consuming steps and led to inconsistent purity. Although authors of these techniques claimed to recover (55–75%) of viable sporozoites that retained infectivity and immunogenicity. But even with the most used purifications protocols, using centrifugation over a discontinuous gradient of percoll or accudenz^{15, 25} these yields are only reached when the best conditions are met, starting from heavily infected mosquitoes. Unfortunately, this turn out to be considerably reduced when it comes to purifying parasites from low infected mosquitoes such as mosquitoes infected from field isolates. Subsequent studies reported to the limitations of these gradient purification methods to prevent microbial contamination from purified sporozoite inoculum²⁶.

The considerable loss of sporozoite and complexity of such techniques limit their interest in routine experiments.

Breeding axenic mosquitoes

Isolation of sporozoites, devoid of mosquito debris and microbial pathogens is prerequisite to produce live attenuated sporozoite for human vaccination. Therefore, improving the quality and purity of sporozoites is essential. If sporozoites of different *Plasmodium* species, including the most lethal human parasite *P. falciparum*, were produced *in vitro* under laboratory conditions, these axenic parasites not only exhibited low infectivity, but the scale of these productions was far to reach the requirements of vaccine commercialization, which obviously implies manufacturing huge quantities of sporozoites²⁷⁻³⁰.

Germ free infectious sporozoites could only be obtained by rearing mosquitoes in an insectarium under aseptic conditions, including sterilized food. A previous study reported that development of axenic adult *Aedes aegypti* mosquitoes exhibited delayed development time and stunted their growth³¹. Complete axenic development of malaria mosquito stage was first reported with *P. berghei*, in axenic *Anopheles stephensi*³². However, carrying out malaria infection in aseptic mosquitoes was then exploited by Sanaria Inc., a company with the aim to produce a human whole sporozoite vaccine, and is a main part of the production process of a *P. falciparum* malaria vaccine meeting the cGMP (current Good Manufacturing practices) standards. Combined with purification steps to remove mosquito debris, producing these aseptic sporozoites allowed Sanaria to gain FDA approval for using this vaccine in humans in 2010³³.

Considering reality, rearing axenic mosquitoes in a complete germ free environment is not within the reach of most academic research laboratories, and in spite of having good promise by purification procedures but culture contamination could not be completely alleviated. Thus it could be more practical to abort the emergence of pathogens in the mosquito stage by improving mosquito diet with antimicrobial agents. Beside, animal blood, female mosquito also requires floral nectar and plant fluids for their energy provision. Sugar is the core component of floral nectars and a vital factor that shapes mosquito fitness. Hence mosquito rearing protocol in insectarium always include sugar solution, generally supplemented with para-aminobenzoic acid (PABA) as mosquito diet.

Methyl paraben (MP), an antibacterial and antifungal agent, had been described before as an attractive mosquito feeding additive which increased the development of oocyst of *P. cynomolgi* and *P. falciparum* infected mosquitoes and improved the longevity of *A. gambiae*³¹. In contrast, another study found the mosquito survival impact as inconsistent³⁴. Indeed various

studies reported that a wide variety of bacteria and microbes could be inhibited by paraben esters³⁵. However, no study demonstrated about the possible MP effect against the microbial communities in mosquito stages. Addressing this, we developed a MP based mosquito feeding protocol, together with penicillin-streptomycin (PS) antibiotics, aiming to prevent fungi and bacterial contamination in hepatic culture.

RESULTS

MP-PS reduces microbial burden in feeding solutions exposed for 2 days in the cages of mosquitoes

The feeding solutions supplemented or not in MP/ PS exposed to the mosquitoes throughout their feeding during a period of 2 days. Feeding pots were changed in every two days and separated, processed for microbial analysis. Dilutions (10^{-2} to 10^{-8}) of the feeding solution were inoculated to agar plates.

Microbial load in feeding solution supplemented with MP+PS, whatever the concentration of PS (0.2 or 1%); it remained free of germs whereas regular feeding diet contained various species of microorganisms. The microorganisms found in the control sucrose solutions were mainly bacteria (10^5 to 10^6 CFU / ml vs 10^2 to 10^4 yeasts). These microorganisms were identified as bacterial species of *Elizabethkingia miricola*, a gram- bacillus type bacteria, and the yeasts *Hyphophichia burtonii* and *Kodamaea ohmeri* (**Table 1**)

N°	Méthylparaben	Penicilline-Streptomycine	Concentration of microorganisms (CFU/ml)
1	0%	0%	4.04x10 ⁵ ● 1,88x10 ³ ◇ 8.6x10 ² □
2	0.2%	0.2%	0
3	0.2%	1%	0

● *Elizabethkingia miricola*
◇ *Hyphophichia burtonii*
□ *Kodamaea ohmeri*

Table 1: Estimation of microbial load in feeding solution

Impact of MP-PS on oocyst abundance and mosquito longevity

We next examined whether the MP-PS cocktail added in the mosquitoes' diet could impact oocyst development within mosquito midguts. We found an average number of oocyst in control, MP + 0.2% PS and MP + 1% PS groups of respectively 572.33 +/- s.d, 490.53 +/- s.d and 773.8 +/- sd. Compared to control, average number of oocyst was slightly increased when feeding menu contained 1% PS (**Fig. 1A**). Penicillin and streptomycin (PS) are commonly used in mosquito and parasite research and no negative effect was previously reported against mosquito life nor on parasite development. Moreover, studies showed that PS exposure to mosquitoes significantly affected gut microbiota and increased the susceptibility of *Plasmodium* infection and prolonged their survival³⁶. Next, we monitored MP-PS feeding effect on mosquitoes' survival. At day 21 post infective blood meal, we counted alive mosquitoes in the cages. No detrimental effect on mosquito life was observed with MP-PS feeding solution compared to routine sucrose solution (**Fig 1B**). As MP-PS supplementation in mosquito diet did not affect the normal oocyst development nor mosquito survival, we moved forward to analyze the sporozoite infectivity.

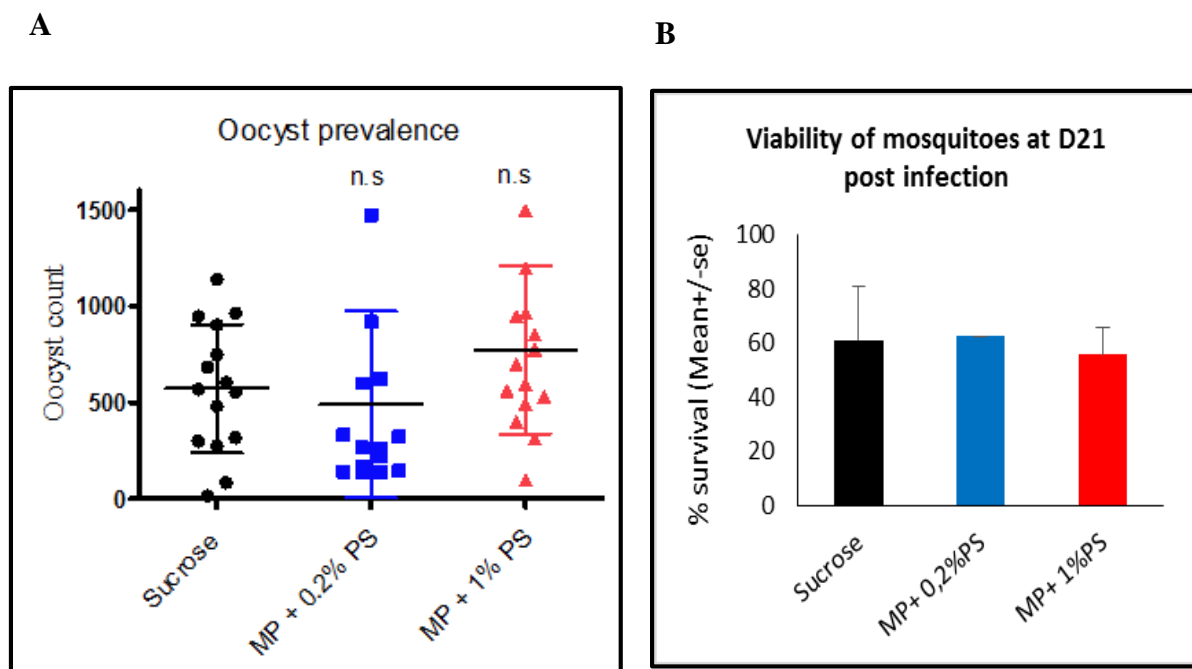


Figure 1: MP impact on oocyst abundance and mosquito survival **A)** at D7 post infective blood meal, 15 *PbGFP* infected mosquitoes were taken from each group. Midguts were dissected to check the presence of oocysts under epifluorescent microscope. Results show one experiment representative of two individual experiments. **B)** at D21 of post infective blood

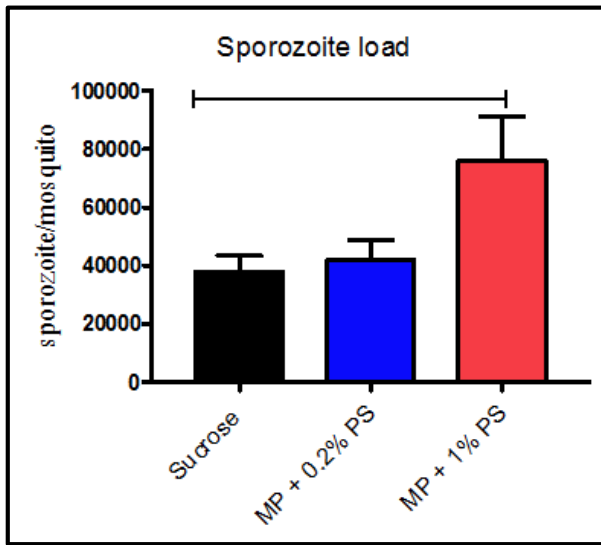
feeding, mortality of MP treated mosquitoes was monitored (mean of three independent experiments, except for the MP+0,2%PS with one experiment only).

Impact of MP-PS on sporozoite development and infectivity

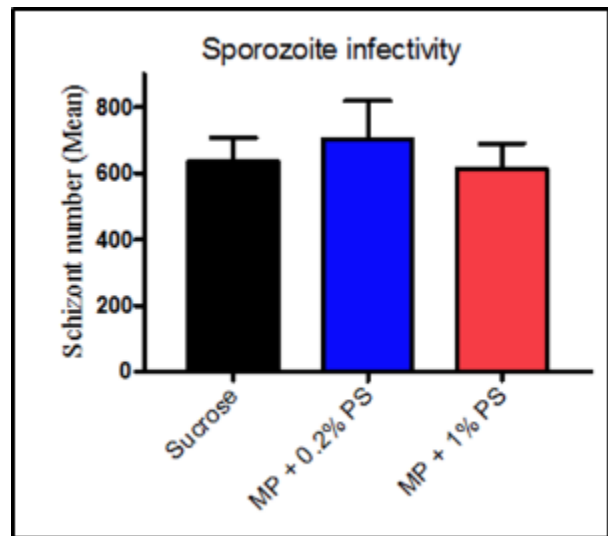
Next we checked whether treatment of MP-PS feeding solution could affect the sporozoite development. Previous study, using *P. falciparum* infected *An. stephensi* mosquitoes reported that both gram negative and gram positive bacteria could affect the sporogenic development³⁷⁻³⁸. Therefore, microbe free aseptic mosquitoes could display an increased susceptibility to *Plasmodium* infection³⁹. In our hand, mosquito group fed with (MP +1% PS) yielded approximately same sporozoite number or even more compared to control group (depending on the experiments), but never less (**Fig 2A**).

To assess sporozoite infectivity to hepatocytes, we infected monkey hepatocytes with the sporozoites isolated from different diet groups of mosquitoes. No remarkable differences were observed in the development of liver parasite forms with MP-PS feeding group versus control. Nearly equal number of hepatic schizonts were developed in all experimental conditions(**Fig 2B**). To go further, we wondered whether any anti-fungal agent could be required in the hepatic culture medium after feeding mosquitoes with antimicrobial agents. We then tested the effect of fungizone (1 / 500e) addition into the culture medium. In absence of fungizone, parasites were well developed in the MP-PS feeding group (**Fig 2C**). To check the contamination, we continued the hepatic cultures over 7 days. MP-PS feeding group hold a sterile liver culture condition but in contrast, turbidity was observed in infected culture where no antimicrobial agents were added neither in culture medium nor in feeding medium (**Fig 2D**).

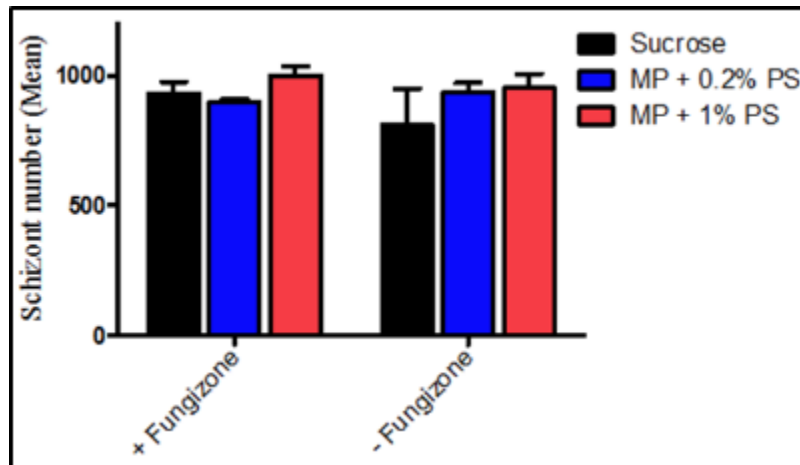
A



B



C



D

Hepatocytes culture (14 dpi)

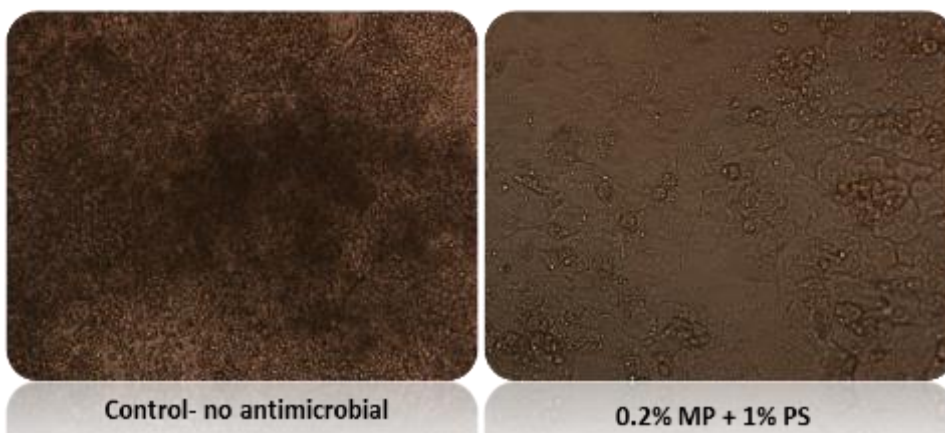


Figure 2: Analysis of MP effect on sporozoite development and hepatocytes infectivity

A) at day 21 post infective blood meal, *P. berghei* sporozoites were isolated through salivary gland dissection. Following dissection, sporozoites number from each group was counted. Statistical analysis was done using graph pad version 5.0 and significance was measured with nonparametric Mann -Whitney test (P value =0.05) **B)** isolated sporozoites were allowed to infect pre-seeded hepatocytes. 3h post sporozoite invasion, culture media was replaced with fresh culture media containing 1% penicillin-streptomycin. At 48 h of post infection (hpi), cultures were fixed and the number of *Pb* GFP schizonts was counted using the Cell Insight HCS platform. **C)** In additional assay, infected cultures were maintained with or without addition of fungizone in the culture medium and cultures were fixed at 48 hpi. Number of schizonts from each condition is shown. **D)** In separate cultures, sporozoites derived from different feeding solutions were allowed to infect hepatocytes and infected culture were kept for two weeks. Images were taken at 2 weeks of post infection showing hepatocyte culture condition: sucrose (control) versus sucrose + MP-PS inoculated cultures.

MP-PS effect on microbial burden in salivary gland extracts

Salivary glands are involved in food acquisition and digestion⁴⁰. So if mosquito feeding diet become contaminated with microbes hence naturally microbes could transmit into salivary glands and could affect parasite development. Considering this, we enumerated microbial loads in salivary gland extracts immediately after grinding of salivary glands to check for microbial contamination. Salivary gland extracts were inoculated to agar plates and incubated.

Neither MP alone nor the MP-PS cocktail in the feeding diet could impact the bacterial burden in the mosquito salivary glands. Surprisingly, in one experiment, no yeast was grown on the agar plates inoculated with control salivary glands extracts (**Fig 3A**), whereas hepatocytes cultures inoculated with these salivary gland extracts demonstrated obvious fungi contaminations within the days following sporozoite inoculation (**Fig 2D**). In another set of experiments, we observed growth of yeast in control salivary gland extracts when they were inoculated into the LB agar medium (**Fig 3B**).

A

N°	Methyparaben	Penicilline-Streptomycine	Concentration of microorganismes (CFU/ml)
1	0%	0%	$2,4 \times 10^6$ Elizabethkingia miricola
2	0.2%	0.2%	$2,6 \times 10^6$ Elizabethkingia miricola
3	0.2%	1%	$2,9 \times 10^6$ Elizabethkingia miricola

B

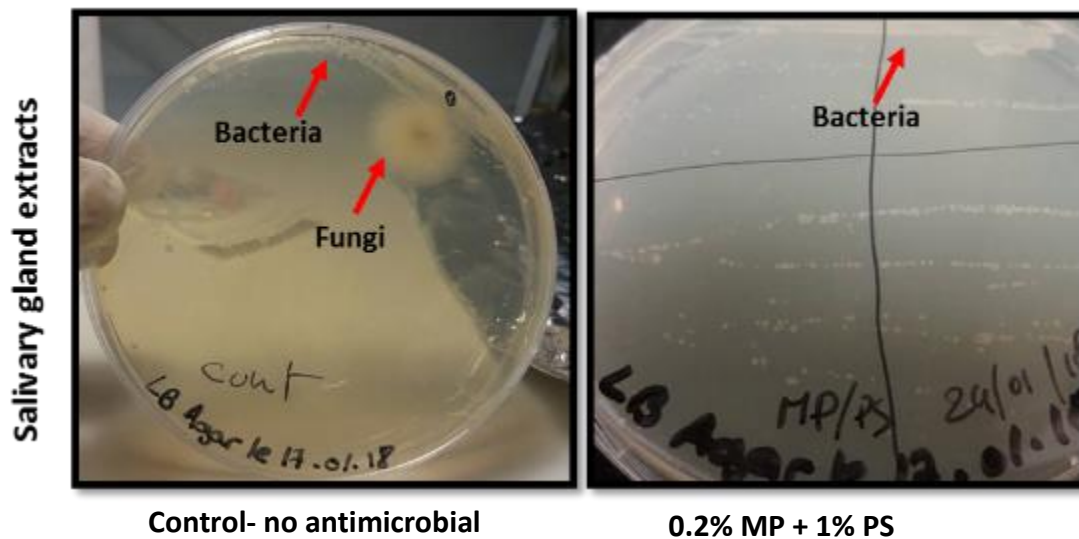


Figure 3: Characterization of microbial pathogens in mosquito salivary gland extracts

A) Salivary gland extracts were isolated from different groups of mosquito feeding conditions and number of viable microorganismes were estimated and species were identified. B) Freshly dissected sporozoite suspensions from each treatment group were also processed for microbial analysis inoculating in LB agar plate.

MP and MP+PS in the mosquito diet prevent fungi contamination in hepatocytes cultures inoculated with SG extracts

	Diet	parasite	Contamination
Exp 1	sucrose	<i>P. berghei</i>	YES (few wells contaminated with yeasts) *
	MP	<i>P. berghei</i>	NO
Exp 2	sucrose	<i>P. berghei</i>	YES (few wells contaminated with yeasts) *
	MP	<i>P. berghei</i>	NO
Exp 3	sucrose	<i>P. berghei</i>	YES (few wells contaminated with yeasts) *
	MP	<i>P. berghei</i>	NO
Exp 4	sucrose	<i>P. berghei</i>	NO
	MP+PS1%	<i>P. berghei</i>	NO
Exp 5	sucrose	<i>P. berghei</i>	Yes (massive contamination with yeasts) *
	MP+PS1%	<i>P. berghei</i>	NO
Exp 6	sucrose	<i>P. cynomolgi</i>	YES (1/3 of the culture contaminated with yeasts) *
	MP+PS1%	<i>P. cynomolgi</i>	NO
Exp 7	sucrose	<i>P. cynomolgi</i>	YES (1/3 of the culture contaminated with yeasts) *
	MP+PS1%	<i>P. cynomolgi</i>	NO

Table 2: Monitoring of fungi contamination in hepatic culture.

On the 7 sets of infected cultures carried out and monitored for a period of 2 weeks, 6 cultures inoculated with sporozoites isolated from mosquitoes fed with normal simple sucrose diet exhibited contaminated wells. All the contaminations happened during the first week of culture. In contrast, none of the cultures inoculated with sporozoites from mosquitoes fed on MP or MP+PS diet became contaminated until the end of the monitoring period at D14 of post infection. The complete culture media was supplemented with 1% PS like in routine experiments but did not contain other antimicrobial agent.

Our results demonstrate that MP is a potent antimicrobial agent, well tolerated by mosquitoes and do not impact parasite development nor sporozoite infectivity for liver cells. The cocktail prevented probable fungi contamination in liver stage cultures over a long period of 2 weeks when used as an additive to adult infected mosquito diet all along the sporogony of *Plasmodium*.

Discussion

In this study we show that by modulating regular feeding solution of mosquitoes with MP-PS solution we could prevent microbial contaminations in *Plasmodium* liver stage cultures, without

affecting parasite development all along the mosquito stages. Liver stage *Plasmodium* studies are extensively conducted using rodent parasites (e.g. *P. berghei* & *P. yoelii*) due to its ease of tractability while human malaria infection study requires specific biological and logistic tools (BSL2-BSL3) including ethical considerations. Moreover, human malaria infections can only be carried out in primary human hepatocytes as cell lines poorly support these infections⁴¹. These malaria species have an extended liver schizogony (approximately 7 ~ 14 days) compared to rodent ones (48 hpi-55h)⁴². In addition, hypnozoites remain dormant state in the culture for over weeks. So to deal with hypnozoites and their activation, hepatic cultures should sustain for longer durations. But worsening condition of cellular physiology and emergence of microbial pathogens contaminating the culture curtail the hypnozoite research. To date, the usual method to isolate sporozoites is hand dissection of salivary glands from infected mosquitoes⁴³. Unfortunately; intricate community of bacteria, fungi, viruses or different kinds of microorganisms always accompany of it²⁶. Current hepatocytes culture protocols includes antimicrobial cocktails but some of them showed opposing effect on parasite development and are poorly efficient to prevent fungal contamination..

Recently a standardized culture medium with supplementation of antibiotic and antifungal agents was described. This media contained a cocktail of antibiotics e.g. neomycin (100 μ M) and gentamicin (21 μ M) that effectively inhibited bacterial growth in *P. vivax* liver culture over 9 days, but yeast resisted to this cocktail R. However, a combine treatment of antifungal agent's 5-fluorocytosine (12 μ M) and posaconazole (0.5 μ M) acted synergistically and completely inhibited yeast in the culture²⁶. Clearly obtaining a sterile culture condition requires a huge combination of antimicrobial agents. However such cocktails could potentially affect parasite replication as some of the antimicrobial agents in its composition were reported as anti-parasitic on liver stage^{26,44}. To avoid hepatic contamination, infection with purified sporozoites could be good alternative. In fact, elimination of fungi at mosquito stage is highly important as fungi do a considerable havoc in culture and very often results can not be restored unless the fungal contamination is detected early during the duration of culture. Moreover bacteria expand in much slower speed than that of fungi in culture⁴⁵.

Sporozoite purification process such as percoll, accu-denz density gradient and free flow electrophoresis although effectively eliminates mosquito debris but not all the microorganism from mosquito carcasses and in the salivary glands. On top, these purification steps require higher number of sporozoites and lead to the loss of large fraction of the parasites. That's why most of research teams working on *Plasmodium* liver stage don't use these purifications steps

as regular basis. As an alternative means, we chose MP-PS cocktail in order to limit them at their origin by impeding the growth of microorganisms in the sugar meals and thus limiting the chances of contaminations in the mosquito stage.

The oocyst is the only parasite developmental phase that grows extracellularly and this stage is the preparatory stage of sporozoite formation⁴⁶. Presence of diverse range of microorganisms in this stage could induce parasite loss⁴⁷. Compared to transtadial passage, midgut bacteria are acquired more efficiently through the sugar diet. It was showed that when mosquitoes were exposed to a bacteria /sugar suspension, midgut bacterial counts were increased. The augmented bacterial load significantly reduced oocyst infection rates and densities⁴⁸.

We found that MP-PS strategy did not affect normal growth of parasites. Previously, streptomycin was found potential to inhibit yeast growth⁴⁹⁻⁵⁰. It can be noted that MP-PS feeding did not eliminate the *Elizabethkingia miricola* load in sporozoite whereas other bacteria like *Cedecea neteri* were eliminated with MP treatment. However presence of bacteria in sporozoite suspension was not a serious concern in our hepatic *Plasmodium* assay as hepatocytes culture medium always includes a minimal dosage of routinely used antibiotics that seemed to protect efficiently the culture from bacterial proliferation. Recently it has been showed that *Elizabethkingia miricola* could be easily prevented from hepatic culture by adding an antibiotic vancomycin⁵¹. Moreover, addition of broad spectrum antibiotics or preservatives in the mosquito feeding solution (sugar meal or blood meal is already documented to understand the interaction of *Plasmodium* with mosquito microbiota but they didn't mention microbiota cleanliness from mosquito stage.

Herein we report that MP-PS feeding diet was effective in ablating fungi contamination from liver stage cultures without affecting oocyst abundance, longevity and sporozoite load of infected mosquitoes. Furthermore such strategy will favour to isolate sporozoites from clean mosquitoes which will facilitate maintaining a long term sterilized hypnozoite culture without disturbance of microbial pathogens.

Methods

Mosquito rearing

Larvae of *Anopheles stephensi* mosquitoes (Sind-Kasur strain) were reared at 27 °C in trays containing 400ml deionized water for stages 1 and 2 and 2L for stages 3 and 4 with an approximately number of 300-400 larvae/tray. Larvae stages 1-2 were fed with Liquifry n°1 (Interpet) and Tetramin Baby E while stages 3-4 received only Tetramin Baby E twice a day. After emergence, adults were stored at 21°C and ~70–80% relative humidity (RH) and were fed on 10% sucrose supplemented with 0.05% para-aminobenzoic acid (PABA). Mosquitoes between 3 and 5 days post emergence were used for feeding on gametocyaemic blood.

Antimicrobial cocktail preparation

The antimicrobial cocktail was prepared with methyl paraben (Sigma) and 10,000 UI Penicillin/ml & 10 mg/ml Streptomycin (Gibco). 10X MP Stock solution was prepared by adding 2 g of MP in 100 ml of 10% Sucrose and heating in microwave to dissolve the powder. Finally, mosquito diet was prepared with 0.2% MP (10 fold dilution of stock) in combination with 1% PS and 10% sucrose including 0.05% PABA. Control mosquito groups were fed with 10% sucrose and 0.05% PABA.

Introduction of antimicrobials through sugar meal

Equal number of adult mosquitoes were distributed into three different cages and different concentrations of antibiotic cocktails were added into the regular feeding solution (sucrose + PABA 0.05 %). Feeding pots were changed every consecutive two days. 1st microbial analysis was done on the rest of the feeding solution after 2 days of mosquito feeding. At day 7 post infective blood meal, oocyst prevalence was checked and at day 14-21, sporozoites were isolated.

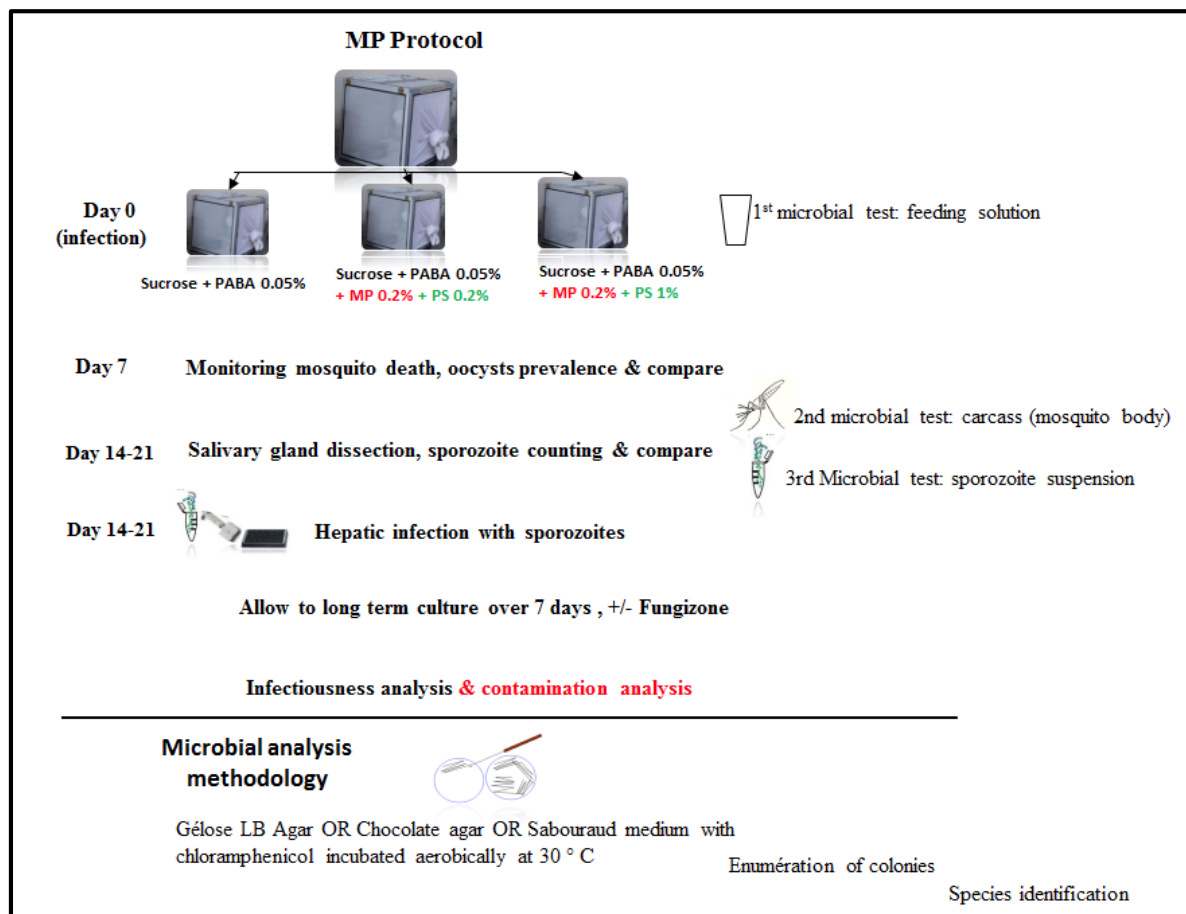


Figure 4: MP-PS mosquito feeding protocol

Microbial contaminations were checked from the sporozoite suspension. Dissected sporozoites were allowed to infect pre-seeded hepatocytes and culture was continued over 7 days to evaluate MP efficacy against the unwanted pathogens in absence of additional fungicidal agents in culture. Microbial analysis, was carried out for quantification of unwanted microbial pathogens by inoculating 2 chocolate agar plates (one incubated at 30°C and the other at 37°C) for quantification/identification of bacteria, and one Saboureaud-chloramphenicol agar plate for quantification/identification of fungi. All plates where incubated under normal oxygen pressure. Species were identified and classified using mass spectrometry approach.

***Plasmodium* infection assays**

In this study simian hepatocytes were used as host cells. Simian (*Macaca fascicularis*) hepatocytes were isolated from liver tissue of healthy animals (Kept at CEA or ICM) using collagenase perfusion as previously described. Cells were seeded in 96-well plates (Falcon by Becton–Dickinson Labware Europe, France) coated with collagen I (BD Bioscience, USA), at a density that a single cell layer homogenously covers each well (80-100 000 cells/well). Cryopreserved primary monkey hepatocytes were seeded generally 1 day before infection. Monkey hepatocytes were maintained at 37 °C in 5% CO₂ in William's E medium (Gibco) supplemented with 10% of fetal clone III serum (FCS, Hyclone), 1% penicillin–streptomycin (Gibco), 5×10^{-3} g/l human insulin (Sigma Aldrich, USA), 5×10^{-5} M hydrocortisone (Upjohn Laboratories SERB, France) and 1/70 matrigel (Corning, Ref. 354234) until infection with sporozoites. Infected salivary glands were isolated by hand dissection and crushed in a potter allowing sporozoite extraction. Upon sporozoite inoculation, the infected plate was centrifuged at 1600 rpm for 6 min at RT. Then the plates were incubated at 37°C for 3 hour. The cell culture medium was refreshed at 3 h post infection for *P. berghei* infections and then every 2 days until fixation of cells for primate parasite infection. Additional infected cultures were kept for 2 weeks without any media replacement for microbial contamination monitoring.

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Chapter 3

**Artemisinin-independent inhibitory activity
of *Artemisia* sp. infusions against different
Plasmodium stages including relapse-
causing hypnozoites**

Malaria, the ancient human tragedy is still thriving as sustainable control and elimination of malaria largely relies on effective treatment, most importantly drugs able to eliminate the dormant forms (hypnozoites) which are responsible for the disease relapses of the widely distributed *P. vivax*.¹ Artemisinin-based combination therapies (ACT), are currently the only globally effective frontline anti-malarial treatments. The emergence and spread of ACT-resistant *P. falciparum* lines in South-East Asia, is of major concern, and mounting evidences of similar emergence in Africa may have catastrophic consequences². So far, elimination of hypnozoites can only be achieved using 8-aminoquinolines (primaquine or tafenoquine), but their global deployment is highly restricted because of their deleterious side effects in individual patient with glucose-6-phosphate dehydrogenase deficiency³. In that scenario, novel antimalarials are urgently needed to leverage the gap.

Till date, artemisinin, extracted from an antipyretic herb called *Artemisia annua* (sweet wormwood), or its derivatives are considered to be only the bioactive compounds which are solely responsible for the potent activity against the parasite's blood-stages⁴. In fact, this plant had been used for hundred years in the forms of infusions to alleviate intermittent fevers, diseases like symptoms of malaria and still enlisted in Chinese pharmacopoeia⁵. However this classical and self-reliant treatment types have always become a heated debate and raised controversies against the western supported drug based treatment policy. Because unregulated use of this plant, treatment with variable and potentially sub-optimal artemisinin content may provoke drug resistance which can jeopardise the current malarial elimination plan⁶. However, it can't be denied that *Artemisia* plant based home remedy and quinine (natural cinchona alkaloid originated from Cinchona bark) was the first choice of malaria for many decades that cured and saved many lives before the discovery of artemisinin⁷. However, currently most of the malaria endemic countries especially the South-east Asian regions have shifted to drug resistance against all antimalarials particularly artemisinin based drugs within few years of deployment⁸. It has been described that *Artemisia* plant enriched with various phytochemicals and the family of *Artemisia* includes many species where few members of the family doesn't contain artemisinin for example *Artemisia afra*⁹. Nevertheless, further investigation and discovery of another lead compounds from this *Artemisia* family was underestimated except the commercial extraction of pure artemisinin and modification endoperoxide structures, ethers

and ozonides in artemisinin¹⁰⁻¹¹. However, nowadays, there are still ongoing endeavors in delineating the mode of action of artemisinin and anti-malaria activities of *A. annua*⁴. In the meantime, *A. annua* has been investigated in various diseases, ranging from inflammatory, and cancers to viral, bacterial and parasitic infections (*Plasmodium*, *Toxoplasma gondii*¹⁴, *Leishmania*¹³, HIV¹⁵, Tuberculosis¹⁷, and SARS-CoV-2¹²)

In a *Plasmodium* study, the whole *Artemisia* plant demonstrated advantages over ARTs which resulted in faster blood parasites killing compared to pure artemisinin and took longer time for the development of resistance compared to ART monotherapy in the rodent model *P. chabaudi*. Hence, it has been suggested that the efficacy also rests with additional plant compounds that can synergise to enhance parasite killing¹⁶. Moreover, *Artemisia afra*, is yet another species in the *Artemisia* family that lacks the major chemical constituent artemisinin but considered as a panacea to malaria in southern and eastern parts of Africa¹⁸. In some laboratory based studies, few *in vitro* blood stage efficacy by nonpolar extracts of *A. afra* against chloroquine resistant strain have been documented¹⁹. However, WHO critically discourages such plant based treatment approach because of the presence of low level of artemisinin in *Artemisia* infusion. Apart from this, probable injudicious and rampant plant consumption, inconsistent clinical study might offer a risk of worsening health situation with a high rate of recrudescence⁶. It is also true that this dispute cannot be resolved without organized therapeutic trials highlighting extensive inspections including a robust research methodology along with good clinical practices. Phytochemical analyses from non-pharmaceutical *Artemisia* infusion has been reported about the presence of anti-parasitic components and it has been claimed that flavonoids, terpenes, phenolic compounds may act in concert in the infusion and can enhance the bioavailability of artemisinin rendering *in vitro* synergistic benefits over ART monotherapy²⁰⁻²².

For the drug discovery perspective, lab based investigation is considered as initial step to for the pre-clinical validation of drug like compound. Being fully aware of *Artemisia* safety issues and inconsistencies of previous research analyses, we designed our drug screening with these two pyretic herbs *A. annua* and *A. afra* along with our standardized infusion recipe. With the scientific reasoning to choose these ancient herbal plants based on the assumption that, the presence of numerous phytochemicals in these plants with proven evidence on *Plasmodium* blood stage activity in comparison with commercially available ART drugs. Moreover in the

hepatic stage, no study with *Artemisia* infusion has been documented so far. Hence, the idea to screen this treasured plant for a specific effect (most strikingly for an anti hypnozoite effect) without any clue is not rational.

(ARTICLE 1 : Manuscript under revision in BIORXIV)

Artemisinin-independent inhibitory activity of *Artemisia* sp. infusions against different *Plasmodium* stages including relapse-causing hypnozoites

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Introductory paragraph (174 words)

Artemisinin-based combination therapies are the only globally efficient treatments against *falciparum* malaria. Recently the use of traditional infusions from *Artemisia annua* (from which artemisinin is obtained) or *A. afra* (lacking artemisinin) has been controversially advocated. Such unregulated plant-based remedies are strongly discouraged as they might constitute sub-optimal therapies and promote drug resistance. Here, we conducted the first comparative study of the anti-malarial effects of both plant infusions *in vitro* against the asexual erythrocytic stages of *P. falciparum* and the pre-erythrocytic (i. e., liver) stages of various *Plasmodium* species. Low concentrations of either infusions accounted for significant inhibitory activities across every parasite species and stage studied. We show that these antiplasmodial effects were essentially artemisinin-independent and were associated with damage to the parasite apicoplast and mitochondrion. In particular, the infusions significantly incapacitated sporozoites, and for *P. vivax* and *P. cynomolgi*, disrupted the hypnozoites. This provides the first indication that compounds other than 8-aminoquinolines could be effective as antimalarials against relapsing parasites. These observations advocate for further screening to uncover urgently needed novel antimalarial lead compounds.

Text (1529 words w/o figure legends)

Sustainable control and elimination of malaria mainly relies on effective treatment, as well as the ability to eliminate the dormant forms (hypnozoites) responsible for the relapses of the widely distributed *P. vivax*¹. Artemisinin-based combination therapies (ACT), are currently the only globally effective frontline anti-malarial treatments². The recent emergence and spread of ACT-resistant *P. falciparum* strains in South-East Asia, is of major concern, and a similar emergence in Africa will have catastrophic consequences³⁻⁵. Elimination of hypnozoites, the dormant hepatic forms responsible for *P. vivax* relapses, can only be achieved using 8-aminoquinolines (primaquine or tafenoquine), but their use is restricted by their deleterious

effects in persons with glucose-6-phosphate dehydrogenase deficiency¹. Thus, novel antimalarials are urgently needed.

Artemisinin present in *Artemisia annua* (sweet wormwood) is considered to be solely responsible for the plant's potent activity against the parasite's blood-stages^{6,7}. Recently decoctions of *Artemisia* dried leaves have been controversially advocated as cheaper traditional plant-based treatments for malaria. Such self-administered, and unregulated use of, treatments with variable and potentially sub-optimal artemisinin content is strongly discouraged, not least as this would promote the emergence of drug resistance⁸. It has been suggested that the efficacy of such remedies also rests with additional plant compounds that can synergise to enhance parasite killing. This claim is supported by the purported anti-malarial traditional herbal teas based on the African wormwood *A. afra*; a species devoid of artemisinin⁹. We have exploited this difference to explore this issue through comparative and extensive *in vitro* assessment of the inhibitory activity of infusions from both plants on *Plasmodium*.

Exposure of synchronised *P. falciparum* rings to infusions from either plant for 72 h inhibited their growth in a dose-dependent manner (**Fig. 1a**), with that of *A. annua* active at slightly lower doses. The exposed parasites appeared arrested at the early stages of the cycle (**Supplementary Data Fig. 1a**), and detailed morphological examination revealed that apicoplast biogenesis was disrupted. Thus, in *A. annua*- or *A. afra*-exposed parasites the apicoplasts failed to elongate and divide at the schizont stage as they normally do in unexposed parasites (**Fig. 1b, Supplementary Data Fig. 1b**). The inhibitory activity of the infusions is not primarily due to a loss of the 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway (the only apicoplast function essential for *in vitro* growth of the erythrocytic parasites in lipid-rich medium^{10,11}), since similar inhibition by the infusions was observed in apicoplast-negative parasites (generated via chloramphenicol treatment) grown in media supplemented or not with isopentenyl pyrophosphate^{10,12} (IPP) (**Fig. 1c-f**). It also appears that both infusions exhibit a strong growth inhibition at low exposition time (6h), not rescued by the IPP addition (**Fig. 1e**).

Further observations also showed that the parasite's mitochondria were also affected by both infusions (**Fig. 1g**).

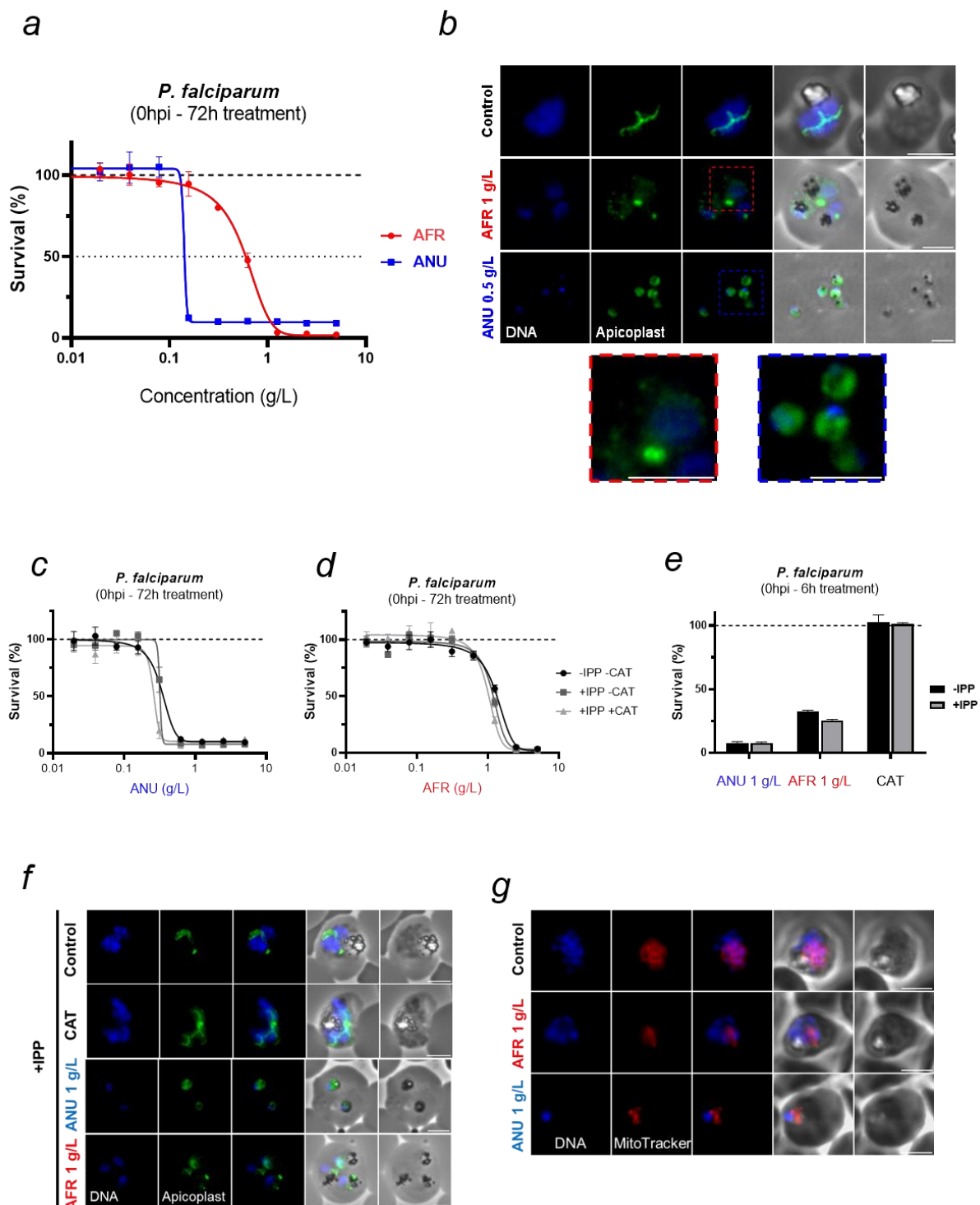


Figure 1. Activity of *Artemisia* infusions on the *P. falciparum* asexual blood stages. a, Survival of parasites cultivated 72 h in presence of increasing concentrations of *A. afra* or *A.*

annua infusions. **b**, Visualisation of apicoplasts by immunofluorescence in untreated control parasites (trophozoite stage) or those grown in the presence of the infusions *afra* (*A. afra*, trophozoite stage, red inset; *A. annua*, ring stage, blue inset). An anti-HA antibody was used to localize apicoplast in a *P. falciparum* cell line expressing a C-terminally HA tagged version of the outer membrane triose phosphate transporter (*PfoTPT*), a protein known to be located on the outer membrane of the apicoplast¹³. Parasite nuclei were detected by Hoechst 33342 staining. Scale bar is 3 μ m. **c**, **d**, Parasite survival after 72 h exposure to *A. annua* (**c**) or *A. afra* (**d**) infusions in normal cultures (-IPP/-CAT), apicoplast-free parasites (+CAT) in the presence or absence of IPP. **e**, **f**, Growth assay (**e**) and epifluorescence microscopy (**f**) of infusion, CAT treated (6 h treatment on ring stage + washes followed by 66 h growth) *P. falciparum* blood stage in the absence or presence of IPP. Scale bar, 3 μ m. **h**, Visualisation of parasite mitochondria with Mitotracker[®] Red in infusion-exposed and control cultures.

Given that apicoplast pathways are more important to the hepatic stages^{14,15}, the infusions were assessed on the pre-erythrocytic stages of diverse *Plasmodium* species. Exposure of primary simian hepatocytes from the moment of *P. berghei* sporozoites inoculation and for a further 48 h led to a significant dose-dependent reduction in the size and number of hepatic schizonts, with infusion of *A. afra* exerting their inhibitory effect at a slightly lower concentration (**Fig. 2a, b**). Similar observations were obtained with *P. falciparum*-infected human primary hepatocytes (**Fig. 2c, d**), a parasite with a longer hepatic development (5-6 days). Neither infusion had a cytotoxic effect on the hepatocytes (**Supplementary Data Fig. 2a-b**), nor did dihydroartemisinin (DHA, used as negative control) affect hepatic schizont numbers though their size was reduced (**Supplementary Data Fig. 2c**). We further demonstrated that exposure of sporozoites to the infusions for 1 h prior to their addition to the hepatocyte cultures, also lead to a significant reduction in the number of hepatic schizonts for both parasite species (**Supplementary Data Fig. 3**). Nonetheless, the viability of such exposed sporozoites, as assessed by vital staining and by their motility, was not altered (**Supplementary Data Fig. 4**).

The assays were then extended to two parasite species, *P. vivax* and *P. cynomolgi*¹⁶ that produce hypnozoites¹⁶⁻¹⁸. Prophylactic treatment (D0-D4) of *P. cynomolgi*-infected primary simian hepatocytes cultured in the presence of infusions significantly reduced the number of both schizonts and hypnozoites in a dose-dependent manner, at the highest concentrations by 85% for *A. annua*, and completely for *A. afra* (**Fig. 2e**). Due to restricted number of *P. vivax* sporozoites, only the 2.5 g/L *A. annua* infusion could be tested and this had a slightly higher inhibitory effect on both schizonts hypnozoites than that on those of *P. cynomolgi* (**Fig. 2f**). For both *P. vivax* and *P. cynomolgi*, the remaining surviving hepatic schizonts were significantly smaller than those in control cultures (**Fig. 2g-i**). Although, exposure of *P. vivax* in human hepatocytes on D5-D8 to the infusions did not lead to a significant reduction in the number of schizonts or hypnozoites (**Fig. 2j**), the schizonts showed a significant size reduction (**Fig. 2k, l**) that was similar to that observed in parasites treated with DHA (**Fig. 2j, k**).

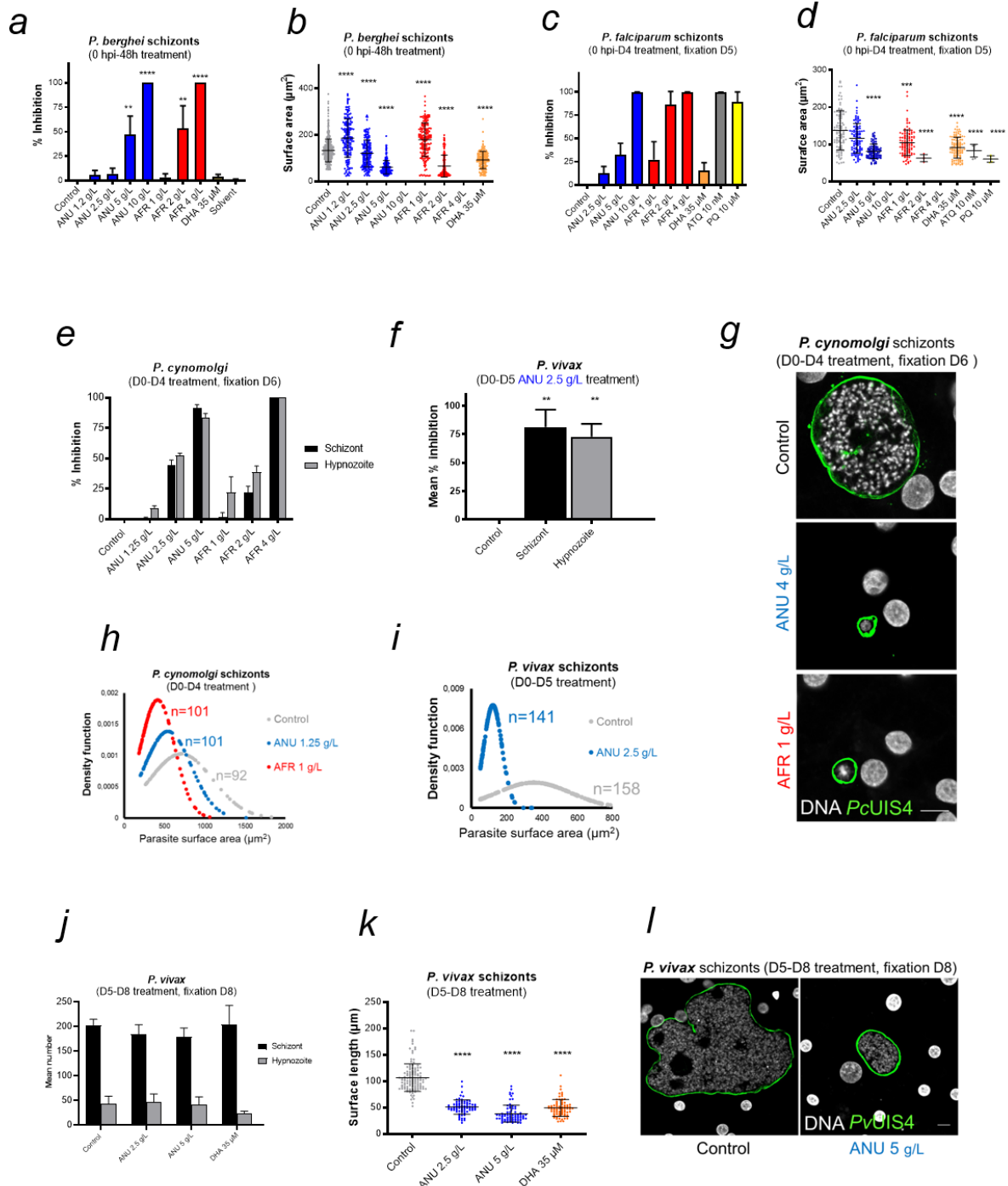


Figure 2. Activity of *Artemisia* infusions on the pre-erythrocytic *Plasmodium* parasites.

a, b, Quantification of intracellular *P. berghei*-GFP schizont numbers expressed as % of the mean numbers (536 per well) observed in control wells (**a**); size distribution of schizonts in simian primary hepatocytes treated with *Artemisia* infusion and DHA (**b**). **c, d**, Impact of the exposure (D0-D4) of *P. falciparum*-infected human primary hepatocytes on parasite numbers expressed as % inhibition compared to controls (mean of 732 per well) (**c**), and size expressed

as surface area (μm^2) (**d**). Results are representative of three independent experiments. **e, f**, Effect of exposure to infusions on the schizont and hypnozoite numbers in primary hepatocytes cultures of *P. cynomolgi* (**e**) and *P. vivax* (**f**) expressed as mean % inhibition. Control cultures had 120 (51 schizonts/69 hypnozoites) and 317 (205 schizonts/112 hypnozoites) parasites per well of a 96-well plate for *P. cynomolgi* and *P. vivax*, respectively. **g**, Confocal microscopy images of exposed *P. cynomolgi* schizonts. The parasitophorous vacuole of fixed parasites were immunolabelled with anti-*PcUIS4* antibody. Host and parasite DNA were visualized by 4',6-diamidino-2-phenylindole (DAPI) dye (Scale bar = 10 μm). **h, i**, Density function plots of *P. cynomolgi* (**h**) and *P. vivax* (**i**) schizonts size distribution ('n' represents the number of schizonts for which the size was measured); the peaks show where most of the population is concentrated. **j, k**, Quantification of *P. vivax* parasite numbers (**j**) and size (**k**) in human primary hepatocytes treated on D5-D8 pi with *A. annua* infusion. **l**, Confocal microscopic images of treated *P. vivax* schizonts fixed at D8 pi, using an anti-*PvUIS4* antibody as a parasite PVM marker and DAPI for the DNA marker (scale bar = 10 μm).

The apicoplast in the exposed sporozoites were affected in that the majority of the parasites displayed either the dot form or lacked an apicoplast ("void" category, **Fig. 3a**), in contrast to those in control sporozoites where some (19 %) presented as a single dot with the majority showing the mature elongated form (probably reflecting the presence in the salivary gland of sporozoites of different ages). For both *A. annua* and *A. afra* infusions, the shift to a high proportion of apicoplast-free sporozoites or with the dot form was dose-dependent (**Fig. 3a**), with no concomitant change to the size, shape or morphology of the parasite nuclei. The biogenesis of the apicoplast was also disrupted by the infusions in the hepatic stages of *P. falciparum* and *P. cynomolgi*, where an increasing fraction of parasites with "affected" apicoplasts was observed as infusion concentrations increased (**Fig. 3b, c**). The observed apicoplast loss is supported by a rapid loss with time of the apicoplast genome in infusion-treated cultures, which parallels that of doxycycline-treated positive control cultures (**Fig. 3d**,

e). Furthermore, quantification of mitochondrial DNA (**Fig. 3f**) during the 48-hour *P. berghei* hepatic cycle along with microscopic imaging (**Fig. 3g**) also revealed disruption of this organelle. The development of apicoplasts in hypnozoites has only been recorded once¹⁹⁻²¹, and consists of a single organelle elongating and then branching to form new discrete organelles that appear as a punctate pattern, which partition to the forming merozoites. We observed a similar pattern in the hypnozoites in the *P. cynomolgi* control cultures, while in those exposed to the infusions (D0-D4) we noted apicoplasts with a diffuse staining pattern, implying a disrupted biogenesis (**Fig. 4a**). Similar infusion-affected hypnozoites were also observed on D10 in *P. vivax* hepatic cultures exposed on D4-D10, though their proportion was less than for *P. cynomolgi* (**Fig. 4b**).

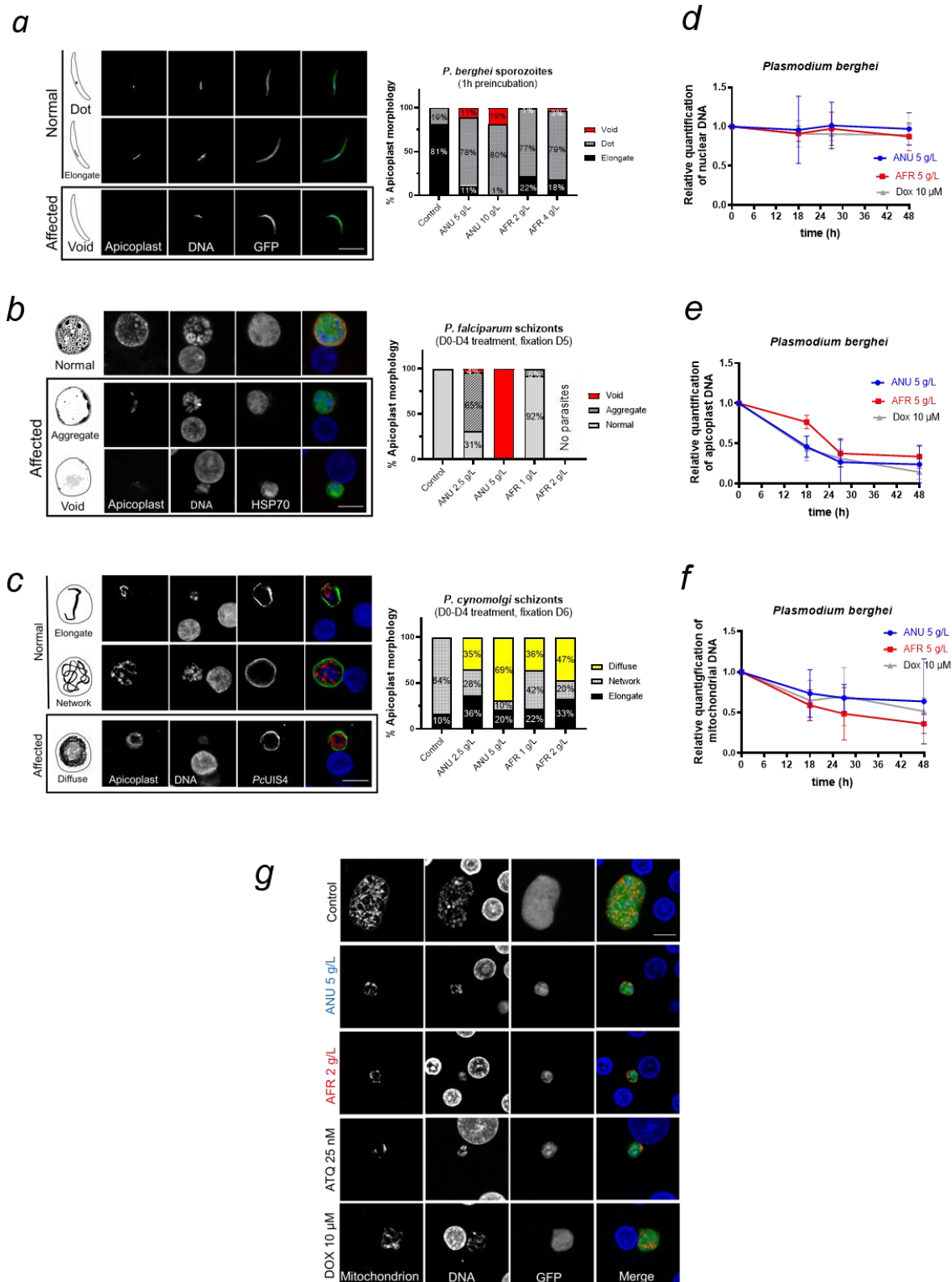


Figure 3. Disruption of apicoplast and mitochondrial integrity in *Plasmodium* pre-erythrocytic stages by *Artemisia* infusions. a, b, c, Schematic and confocal images of *P. berghei* sporozoites (a), *P. falciparum* schizonts (b) and *P. cynomolgi* schizonts (c) in control

and treated groups. The apicoplast morphologies presented in the “Affected” panels have only been observed in exposed parasites. The frequencies of the various morphologies are presented in the associated graphs. *P. falciparum* and *P. cynomolgi* parasites were detected by anti-HSP70 and *PcUIS4* antibodies, respectively. Apicoplasts were labelled by Anti-*PyACP* antibody (scale bar = 10 μ m). **d, e, f**, Quantification of nuclear (**d**), apicoplast (**e**), and mitochondrial (**f**) genomes by qPCR over the 48 h development of the *P. berghei* hepatic parasites exposed to infusion or doxycycline. **g**, Confocal microscopic images of treated and control *Pb*-GFP mitochondria in infected hepatocytes, as visualized by immunolabeling with anti-recombinant *PfHSP60* sera (scale bar = 10 μ m).

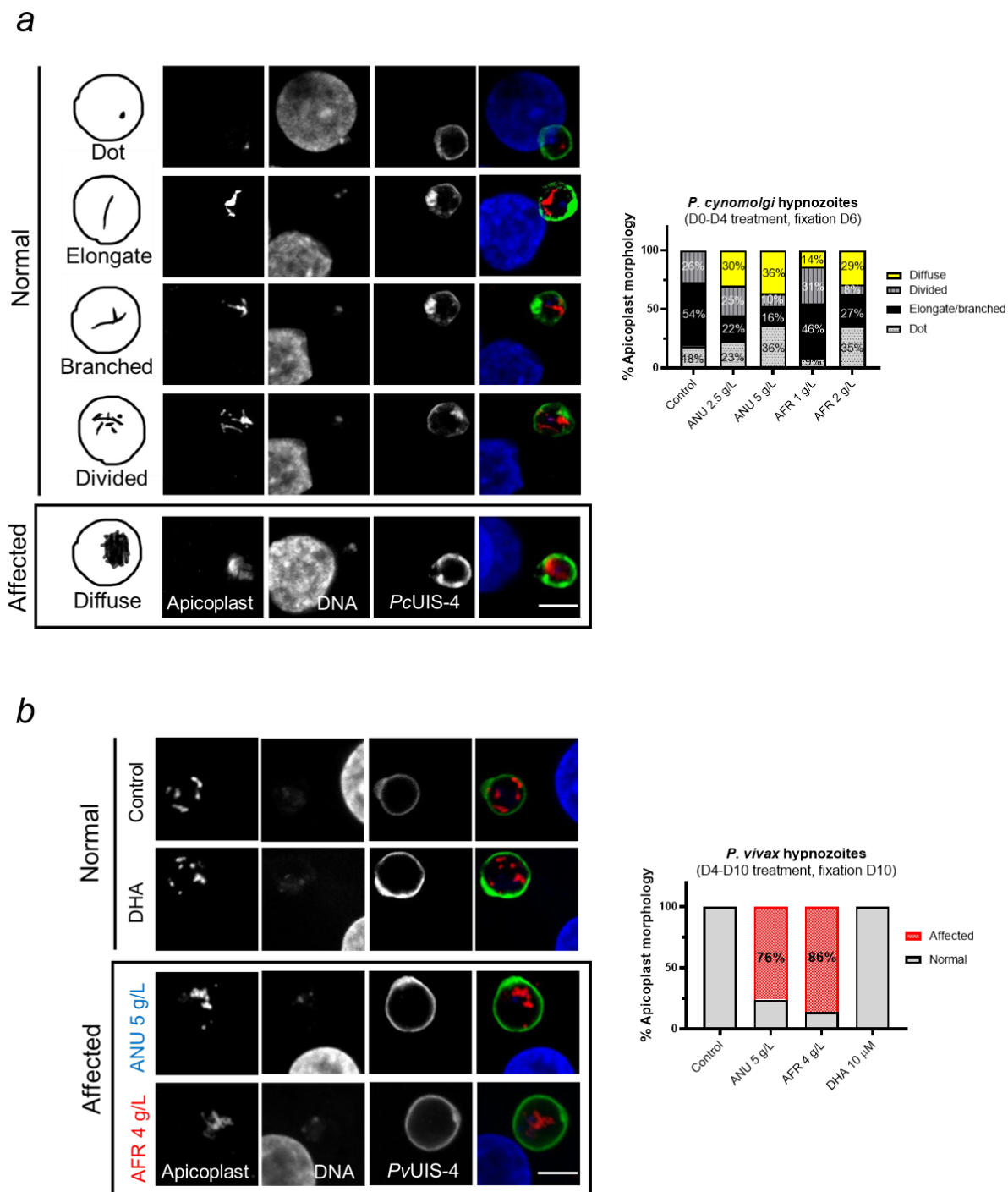


Figure 4. Disruption of hypnozoites' apicoplast by *Artemisia* infusions. **a**, Schematic (left panels) and confocal images of *P. cynomolgi* hypnozoites on D6 in cultures exposed to infusion during the early hepatic phase. In control cultures, three apicoplast morphologies could be observed, a dot shaped apicoplast in younger hypnozoites that evolves into an elongate organelle that then branches to show a divided morphology as the hypnozoites mature. In infusion-treated cultures (D0-D4), hypnozoites with a diffuse apicoplast signal were additionally

observed. **b**, Apicoplast morphology of *P. vivax* hypnozoites exposed to infusions from D4-D10 pi. The apicoplast morphologies presented in the “Affected” panels have only been observed in exposed parasites. Quantification of the various apicoplast morphologies in 100 and 50 parasites for **(a)** and **(b)**, respectively, are presented. *P. cynomolgi* and *P. vivax* were visualized by using an anti-*PcUIS4* and anti-*PvUIS4* antibodies, respectively (scale bar = 5 μm).

The discovery of artemisinin from Chinese traditional medicine has revived interest in *Artemisia* plants as a source of treatments for diverse infectious and non-infectious pathologies other than malaria^{9,22,23}. The investigations of the *in vitro* anti-malarial efficacy have been primarily conducted on infusions of either *A. annua* or of *A. afra*, and this principally on the erythrocytic stages^{6,24,25}. Their results are contradictory and difficult to interpret. Our data from the extensive comparative assays, clearly demonstrates that infusions from either plant species can fully inhibit the multiplication and the development of both erythrocytic and pre-erythrocytic *Plasmodium* stages *in vitro*. This inhibition is independent of their artemisinin content (see **Supplementary Data Table 1** for measurement of artemisinin in both infusions), with maximal activity occurring at broadly similar infusion concentrations, without observable cytotoxic effects against the host cells. Our observations are supported by a recent study investigating the same *Artemisia* infusions on the asexual and sexual *P. falciparum* erythrocytic stages⁷, that shows an inhibition of the asexual growth after 48 h exposure of both infusions with a significant stronger effect for that of *A. annua*. The higher inhibition levels we observed are probably due to the fact that we assessed parasite survival 72 hours post-exposure (as compared to 48 hours). This strongly suggests that the phenomenon of delayed-death, typical of apicoplast-targeting drugs^{26,27}, occurred in our exposed cultures. Of note, the inhibition of the hepatic stages appears to be mainly due to a detrimental effect on the ability of the sporozoite to achieve a productive hepatocyte infection, which significantly led to a substantial reduction in hypnozoite numbers. It is likely that the detrimental effects also observed on the

apicoplast in the developing *P. vivax* hypnozoite will adversely affect its capacity to reactivate, though this would require further *in vivo* testing. Our *in vitro* observations are clearly insufficient to support claims of *in vivo* prophylactic or curative efficacy for any traditional remedy from the two plants. They do however suggest the presence of hitherto unknown plant components with potent inhibitory activity. A comparative analysis of secondary metabolites present in both plants (see **Supplementary Data Information**) illustrates the complexity of the phytochemicals some of which are potentially responsible for the artemisinin-independent inhibitory activity. This warrants systematic bio-guided screening to identify which of these might be provide novel lead antimalarials, and in particular those that can safely prevent *P. vivax* relapses. Drugs that could supplant 8-aminoquinolines, the only drugs currently capable of exerting hypnozoitocidal activity¹, would significantly enhance the prospect of global malaria elimination.

Methods

Primary cryopreserved hepatocytes maintenance and infection

In this study, human and simian primary hepatocytes were used. Simian (*Macaca fascicularis*) hepatocytes were isolated from the liver of healthy animals using collagenase perfusion as previously described²⁸. Cryopreserved human primary hepatocyte vials were purchased from Biopredic (Lot no. LHuf17905A) or Lonza (Lot no. HUM182641). Cells were seeded in 96-well plates (Falcon by Becton–Dickinson Labware Europe, France) coated with collagen I (BD Bioscience, USA), such that a single cell layer homogenously covers each well. Cryopreserved primary human hepatocytes (Biopredic International and Lonza) were seeded generally 4 days before infection. Both human and monkey hepatocytes were maintained at 37 °C in 5% CO₂ in William's E medium (Gibco) supplemented with 10% fetal clone III serum (FCS, Hyclone), 1% penicillin–streptomycin (Gibco), 5 × 10⁻³ g/L human insulin (Sigma Aldrich, USA), 5 × 10⁻⁵ M hydrocortisone (Upjohn Laboratories SERB, France) and Matrigel (Corning, Ref. 354234). The sporozoites used to infect the cultures were purified from dissected salivary glands of

infected mosquito²⁸. During the infection matrigel cover was removed carefully before adding the sporozoite inoculum. The plates were then centrifuged at 900g for 6 min at room temperature before incubation at 37°C for 3 hours. Following a wash, matrigel was added to the culture and allowed to solidify at 37°C for 45 minutes. The cell culture medium was changed thereafter every 24h until cell fixation.

The list of different *Plasmodium* species used for infection of hepatocytes:

Parasites	Strains	Source
<i>P. berghei</i>	GFP ANKA	Centre d'Immunologie et des Maladies Infectieuses (CIMI), Paris, France
<i>P. falciparum</i>	NF54	Department of Medical Microbiology, University Medical Centre, St Radboud, Nijmegen, The Netherlands
<i>P. cynomolgi</i>	M	Primate center, Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA), Fontenay aux Roses, France
<i>P. vivax</i>	Unidentified	Symptomatic Patients, Pasteur Institute of Phnom Penh, Cambodia

Culturing *Plasmodium*-infected red blood cells

Plasmodium falciparum blood stage parasites were maintained at 2% hematocrit in 1640 RPMI-HEPES supplemented with 5% AlbuMAX II (GIBCO) and 0.25% gentamycin. Parasites were grown sealed Perspex chambers gassed with beta mix gas (1% O₂, 5% CO₂ and 94% N₂) at 37°C and maintained on 48-hour cycles. Cultures were tightly synchronized at ring stage using sorbitol treatment (%5 v/v) as previously described¹².

Production of *P. vivax* sporozoites

Sporozoites were obtained from mosquitoes infected by feeding on symptomatic patients presenting with *P. vivax* in Mondulhiri Province (eastern Cambodia) after obtaining their consent. The patients were managed by the medical staff based at these health facilities. Heparinized blood samples were collected by venepuncture prior to the initiation of treatment, and a diagnostic PCR assay was used to confirm that *P. vivax* was the only *Plasmodium* species present. The blood was centrifuged at 1174g at 37°C for 5 minutes, and the plasma was replaced by heat-inactivated naïve human AB serum. Batches of three hundred to six hundred 5-7 days old adult female mosquitoes (*Anopheles dirus*), starved overnight were fed via an artificial membrane attached to a water-jacketed glass feeder maintained at 37°C. Engorged mosquitoes were maintained at 26 °C and 80% relative humidity, and provided with a 10% sucrose plus 0.05% para-amino-benzoic acid solution on cotton pads. Salivary glands of mosquitoes were dissected 14-20 days post-blood meal in L15 medium supplemented with antibiotics and Amphotericin B (Gibco™).

Production of sporozoites (other species than *P. vivax*)

Plasmodium falciparum was transmitted to *Anopheles stephensi* mosquitoes using an artificial membrane feeding on *in vitro* cultivated gametocytes, in Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, The Netherlands. *P. berghei* and *P. cynomolgi* infections of *An. stephensi* mosquitoes were performed in Paris respectively by direct mosquito feeding on anesthetized *Pb-GFP*²⁹ infected mice, or by artificial membrane feeding on *P. cynomolgi* (strain M) infected blood from *Macaca fascicularis*. All monkey procedures (infection, monitoring and blood sampling) were done in CEA, Fontenay aux Rose before the infected blood samples were transferred to CIMI-Paris for mosquito infection. Two to three weeks post blood meal, *Plasmodium*-infected mosquitoes were killed with 70% ethanol. They were then washed once in Leibovitz's L-15 medium (Gibco™) containing 5% fetal calf serum, 100 UI Penicillin, 100 µg/ml streptomycin and 0.5 µg/ml amphotericin B, followed by another two washes in the same medium this time lacking serum.

The mosquitos were then hand dissected under stereomicroscope and the salivary glands were crushed in a 1.5 ml Eppendorf tube and passed through a 40 µm filter (Cell Strainer, BD biosciences, USA). Sporozoites were finally counted and after dilution adjustment, directly inoculated to the cultures.

Preparation of *Artemisia* infusion

Artemisia afra (PAR, voucher ID: LG0019528 Université de Liège) and *Artemisia annua* (LUX, voucher ID: MNHNL17733 Herbarium Luxembourg) were collected as leaves and twigs and were preserved at room temperature. Both products were protected from sunlight until infusion preparation.

The stock infusion was prepared as follows: 2.5 grams *Artemisia* dried leaves and twigs were added to 25 mL of pre-boiled 25 mL commercial drinkable water (Crystalline Company) and the mixture was then boiled while being stirred for 5 minutes. Following cooling over 10 minutes, the infusion was passed through a 40 µm cell strainer (Falcon, Corning Brand) in order to remove plant debris, and then centrifuged at 3000 rpm for 10 minutes to pellet any remaining fine solids, with a final filtration step over a 0.20 µm membrane filter (CA-Membrane) to obtain a fully clear solution. This stock infusion (100 g/L) was stored at 4°C (short term storage) or at -20°C (long term storage). For the *in vitro* drug assay, the decoction was diluted to the appropriate concentration (g/L) with William's E medium (along with supplements).

Frozen and freshly prepared *Artemisia* decoction samples were subjected to chemical analyses including artemisinin content quantification.

Quantification of artemisinin in infusion: UHPLC Analysis

The chromatographic apparatus consisted of Nexera X2 UHPLC system (Shimadzu, Marne la Vallée, France) equipped with a binary pump, solvent degasser, and thermostatted column compartment. A reversed-phase column was used for separation: Kinetex C18 (2,1 × 100 mm 1,7 µm from Phenomenex). Mobile phase A and B consisted of water (0,1% formic acid) and acetonitrile (0,1% formic acid) respectively. The 10 min linear gradient program used was 10–

100% B over 5 min, a plateau at 100% B for 2 min was used to wash the column, decreasing from 100%–10% B in 0,1 min, followed by a 2,9 min post-run isocratic step at 10% B to re-equilibrate the column. The flow rate was constant at 0.5 mL/min at 25 °C.

Quantification of artemisinin in infusion: MS/MS Detection

MS/MS experiments were carried out using the Shimadzu UHPLC system described above coupled to a Shimadzu LCMS-8050 triple quadrupole mass spectrometer using the multiple reaction monitoring (MRM) technique operating in positive ion mode. The following parameters were used for all experiments: electrospray interface voltage was 3 kV; heat block temperature of 350 °C; desolvation line temperature of 300 °C; interface temperature of 300 °C; drying gas flow gas was set at 5 L/min; and nebulizing gas flow at 3 L/min, heating gas flow rates were set at 15 L/min. Q1 and Q3 were set to unit resolution. The dwell time was set at 1 ms for each MRM transition; optimal CE values were chosen to obtain the most characteristic fragments (see below table).

Precursor (m/z)	Fragment ion (m/z)	Collision Energy (eV)
283.10	247.15	9
283.10	265.15	8
283.10	219.10	12

Drugs

Drugs were dissolved in different solvents according to manufacturer's instructions which are enlisted below: Atovaquone (Sigma-Aldrich, **A7986**), Primaquine bisphosphate (Sigma Aldrich, **160393**), DHA (Sigma-Aldrich, **1200520**), Isopentenyl pyrophosphate NH₄⁺ salt (Isoprenoids, LC Tampa, FL 33612), Chloramphenicol (Sigma-Aldrich, C0378-5G), Doxycycline (Sigma-Aldrich, D9891). Media with drugs were changed every 24 h during the assays.

Immunostaining

Cultured hepatocytes were fixed using 4% paraformaldehyde (PFA) for 10-15 minutes at room temperature. The fixed samples were subjected to immune labelling. The primary antibodies (see below table) were diluted in dilution buffer (1% w/v Bovine Serum Albumin and 0.3% v/v Triton X-100 in PBS) and incubated at room temperature. Primary antibody-stained cultures were washed thrice with PBS 1X and were incubated with secondary antibody (below table) along with DAPI (1:1000) in order to visualize the nuclei at room temperature for 1 hour. Mitotracker® (ThermoFischer Scientific) was used to visualise parasite mitochondria.

List of primary and secondary antibodies used in the study:

Antibody	Source	Working dilution
Recombinant mouse anti- <i>PvUIS4</i>	Noah Sather (Center for Infectious Disease Research, Seattle, USA)	1:20000
Mouse anti- <i>PcUIS4</i> , polyclonal	Laurent Rénia (Singapore Immunology Network, Agency for Science, Technology, and Research, Singapore)	1:500
Recombinant rabbit monoclonal antibody against <i>PyACP</i>	Scot E. Lindner (Huck Center for Malaria Research, Penn State, USA)	1:250
Mouse polyclonal serum raised against <i>PfHSP70</i>	Centre d'Immunologie et des Maladies Infectieuses (CIMI), Paris	1:1500
Mouse polyclonal anti- <i>PfHSP60</i>	Philippe Grellier (Muséum National d'Histoire Naturelle, Paris)	1:500
Rat IgG anti-HA	Roche	1:500

Alexa Fluor 488 goat anti-mouse IgG	Invitrogen	1:500
Alexa Fluor 594 goat anti-rabbit IgG	Life technologies	1:500
Alexa Fluor 488 goat anti-Rat IgG	Life technologies	1:1000

Confocal Microscopy and Image analysis

Confocal images of immunostained cultures were taken by Leica SP8 white laser microscope controlled by the Leica Application Suite (LAS) AF software at the QUANT microscopy platform (Institut du Cerveau et de la Moelle épinière, ICM) in Paris, France. Z-stack images were constructed and analysed by Fiji package of imageJ software. Final images were made by Microsoft Powerpoint. Schematic figures of *Plasmodium* apicoplast were designed by Procreate® application in Apple ipad pro.

Quantification of apicoplast morphologies

ACP immunolabeled parasites were quantified considering the distinguishable morphology in drug treated versus control by an individual with the aid of confocal (Leica SP8 white laser microscope) at 63X magnification. For each condition, 100 parasites were observed.

Parasite enumeration and toxicity assessment using high-content imaging

Upon fixation and immunostaining, cell culture plates were analyzed in order to determine the number and size of the parasites using a CellInsight High Content Screening platform equipped with the Studio HCS software (Thermo Fisher Scientific). Uninuclear hepatic parasite forms observed beyond D4 were considered to be putative hypnozoites, while multinucleate forms were classed as schizonts. The parasite size reduction was calculated on the average object

area using the total surface area of each selected object (μm^2). The high content imaging approach described previously³⁰. To assess cell toxicity of infusion for hepatic cultures, fixed plates were further scanned for the DAPI signal representing host nuclei. The analysis was based on counting of total cell nuclei.

qPCR to quantify apicoplast and mitochondrion

To perform relative quantification of apicoplast, mitochondrial and nuclear genomes we extracted genomic DNA from *Pb*-GFP infected hepatocytes treated or not with infusion using a NuceolSpin Tissue kit (Macherey-Nagel, Germany) according to manufacturer's manual. The amount and the purity of the extracted DNA were checked by a Nanodrop ND 1000 machine (Thermo Fischer scientific). The following gene-specific primers were designed to target genes found in each organelle or nuclear genomes:

*tufa*_ PBANKA_API00280 (apicoplast) 5'-GATATTGATTCATCTCCAGAAGAAA-3' / 5'-ATATCCATTTGTGTAGCACCAATAA-3',

Cytb3_PBANKA_MIT01900 (mitochondrion) 5'-AGATATATGCATGCTACTGG -3' / 5'-TCATTTGTCCCAAGGTAAAA -3'

Green fluorescent protein (GFP) 5'- GATGGAAGCGTTCAACTAGCAGACC -3' / 5'-AGCTGTTACAAACTCAAGAAGGACC -3',

and *CHT1*_PBANKA_0800500 (nuclear) 5'-AACAAAATAGAGGTGGATTT-3' / 5'-AATCCTACACCATCGGCTC-3'.

The quantification of each target gene was normalized to GFP, expressed as a transgene under the *P. berghei* elongation factor 1 α promoter³¹. All reactions were performed on an Applied Biosystems 7300 *Real-Time PCR* System using the Power SYBR Green PCR Master Mix kit (Applied Biosystems, France), according to the manufacturer's instructions. The cycling conditions were as follows: initial denaturation at 95°C for 5min, followed by 40 cycles at 94°C

for 30s, 56°C for 30s and 72°C for 30s. The experiments were performed in triplicate. Relative quantification of target genes was calculated according to the $2^{-\Delta\Delta Ct}$ method³².

Sporozoite viability assay

Freshly dissected *Plasmodium berghei*-GFP sporozoites were incubated with varying concentrations of *Artemisia* infusion for 1 hour. Then the infusion was removed by centrifugation and sporozoites resuspended in culture medium. One μL of propidium iodide (Gibco) was added to 15000 cells and incubated for 2-3 minutes. A 15 μL aliquot of sporozoite suspension were put on a KOVA[®] Glasstic microscopic slide (KOVA International) and visualized under a microscope. Finally, dead and viable sporozoites were quantified under epifluorescence microscope.

***in vitro* screening assay for sporozoite motility inhibition via video microscopy**

Salivary glands (SGs) from infected mosquitoes (17–24 days post infectious blood feeding on Swiss mice infected with *P. berghei*-GFP were isolated by hand dissection and placed into Leibovitz L15 media (Gibco 11415049) supplemented with 20,000 units penicillin- streptomycin (Gibco 15140122) and Amphotericin B. Sporozoites were then released by SG mechanical disruption, filtered through a 40 μm mesh (Cell Strainer, Falcon) to remove SG debris and diluted in activation medium (William's E medium supplemented with 10% of fetal clone III serum, 1% penicillin–streptomycin, 5×10^{-3} g/L human insulin and 5×10^{-5} M hydrocortisone) to a final concentration of 400000 sporozoites/ml and kept on ice. Aliquots of 50 μL were inoculated into wells of a 96-well plate (final amount of approximately 20000 sporozoites per well). An equal volume of *Artemisia* infusion dilutions was added to the sporozoites to give a final concentration of (*A. annua* 5%, *A. annua* 10% and *A. afra* 5%) and mixed by gentle pipetting. The plate was centrifuged for 6 min at 1600 rpm to maximize the sporozoite settlement and immediately placed at RT for 30 minutes. Then the motility of sporozoites was recorded under video microscopy Zeiss Axio Observer 7 at 40X objectives with the GFP excitation/emission filter and pictures were recorded under a frame rate of 1 frame per second

with a Hamamatsu Orca Flash 4.0 V3 camera and Zen software for 3 minutes. Notably, the experiments were performed at 37°C. Finally, the tracking of sporozoites were further analyzed with FIJI imageJ and the moving patterns characterized on maximum intensity z-projections. Thereby, sporozoites were classed as gliding if they moved with a circular pattern describing at least one complete circle loop during the 3 minutes acquisition. The percentage residual motile population was then calculated and compared to uninhibited controls (media containing an equivalent amount of water).

SYBR Green-I-based cell proliferation assay

Plasmodium blood stage parasites (regular or apicoplast-free lines) are incubated in 96 well flat bottom plates, 2% hematocrit in 1640 RPMI-HEPES supplemented with 5% AlbuMAX II (GIBCO) and 0.25% gentamycin complemented with appropriate *Artemisia* infusion dilutions and with or with IPP and CAT. Parasites were grown sealed Perspex chambers gassed with beta mix gas (1%O₂ 5%CO₂, 94% N₂) at 37°C for 72h. New 96 well black wall flat bottom plates are set up with 100 µL SYBR Green lysis buffer (20 mM Tris, pH 7.5; 5 mM EDTA; 0.008 % (w/v) saponin; 0.08% (v/v) Triton X-100) with freshly added SYBR Green I (1000X), 100 µL from the cultures are transferred and mixed with the SYBR Green lysis buffer and incubated 1 h at room temperature protected from the light. Fluorescence from each well is measured with TECAN infinite M200 plate reader (excitation: 485 nm, emission: 538 nm and integration time: 1000 µs).

Statistical Analysis

GraphPad Prism 5 (GraphPad. Software, San Diego, CA, USA) and Excel 2016 (Microsoft Office) were used in this study for the data analysis. All graph values are represented by means with standard deviations (s.d.).

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Author contributions

K.A., S.T., N.A., J.-F.F., C.S.A., G.C. and A.V. performed experiments.

K.A., S.T., N.A., J.-F.F., D.M., C.S.A. and C.Y.B. analysed data.

K.A., B.W., J.-F.F., C.Y.B., and D.M. designed the study.

J.-F.F., B.W., G.-J.G., G.S., T.B., V.S, J.C.B. and D.M. contributed essential materials.

S.T., K.A., C.Y.B., G.S., R.D. and D.M. wrote the manuscript. All authors read and approved the manuscript.

Competing Interests statement

The authors declare that there is no conflict of interest.

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Artemisinin-independent inhibitory activity of *Artemisia* sp. infusions against different *Plasmodium* stages including relapse-causing hypnozoites

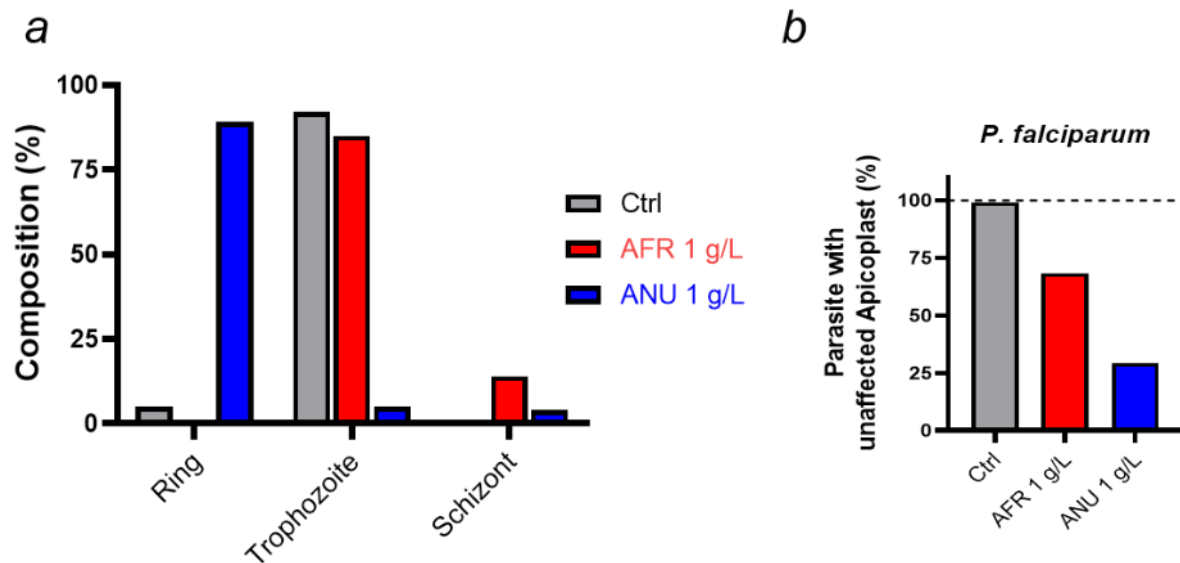
Kutub Ashraf, Shahin Tajeri, Christophe-Sébastien Arnold, Nadia Amanzougaghene, Jean-François Franetich, Amélie Vantaux, Valérie Soulard, Mallaury Bordessoulles, Guillaume Cazals, Teun Bousema Geert-Jan van Gemert, Roger Le Grand, Nathalie Dereuddre-Bosquet, Georges Snounou, Jean-Christophe Barale, Benoit Witkowski, Romain Duval, Cyrille Y. Botté, Dominique Mazier

SUPPLEMENTARY INFORMATION

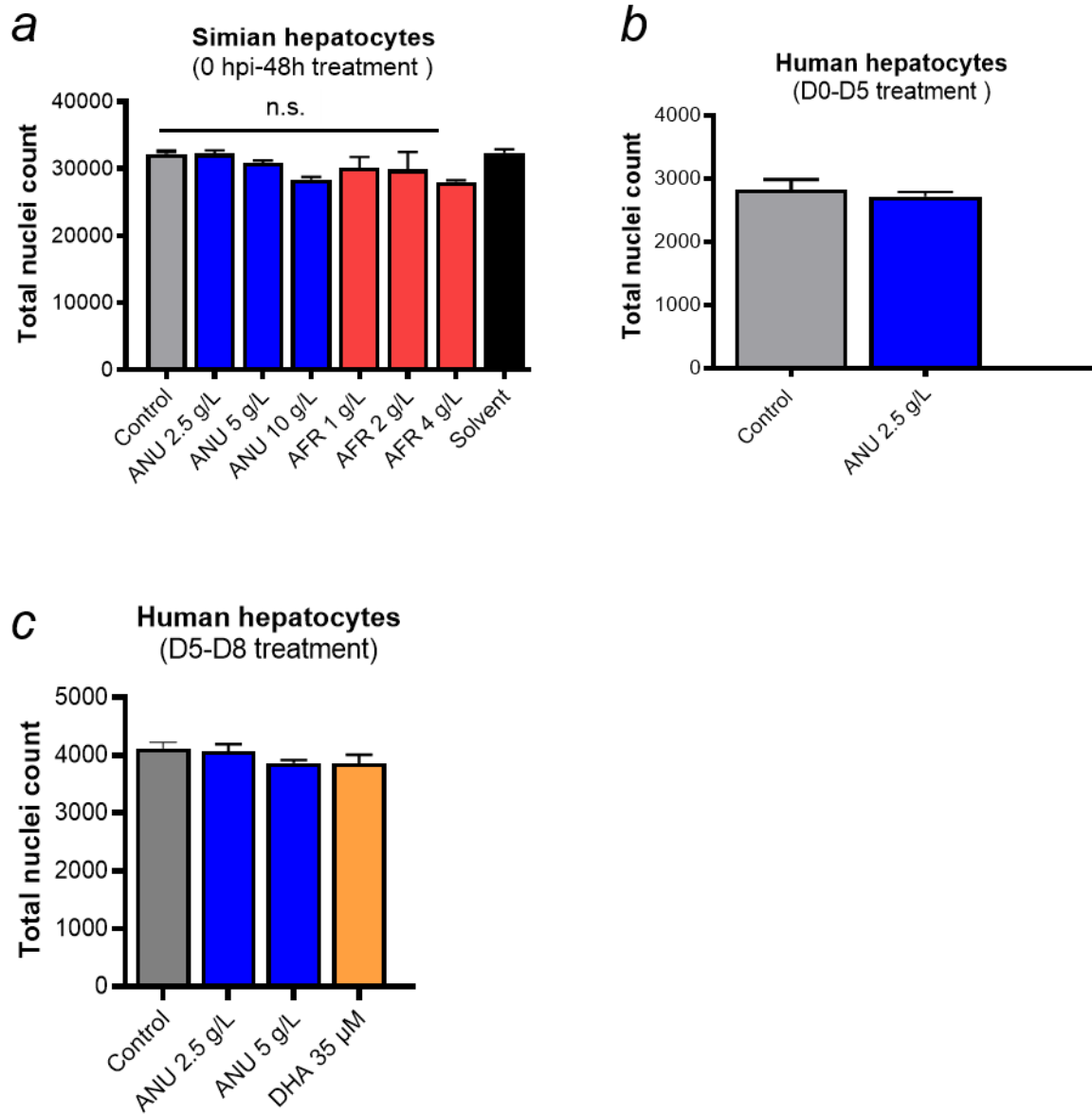
Supplementary Data Table 1. Quantification of artemisinin in *A. annua* and *A. afra* infusion by UHPLC-MS.

Stock <i>Artemisia</i> Infusion	Artemisinin concentration in stock infusion	Artemisinin concentration in the infusion used for hepatic cultures
100 g <i>A. annua</i> leaves and twigs/L	6.07 ± 0.01 µg/mL = 21.5 ± 0.03 µM	0.607 µg/mL (from a 10 g/L infusion) = 2.15 µM
50 g <i>A. afra</i> leaves and twigs/L	ca. 0.071 ± 0.007 µg/mL = 251 ± 25 nM ($<$ limit of quantification)	ca. 0.0028 µg/mL (from a 4 g/L infusion*) = 10 nM ($<$ limit of quantification)

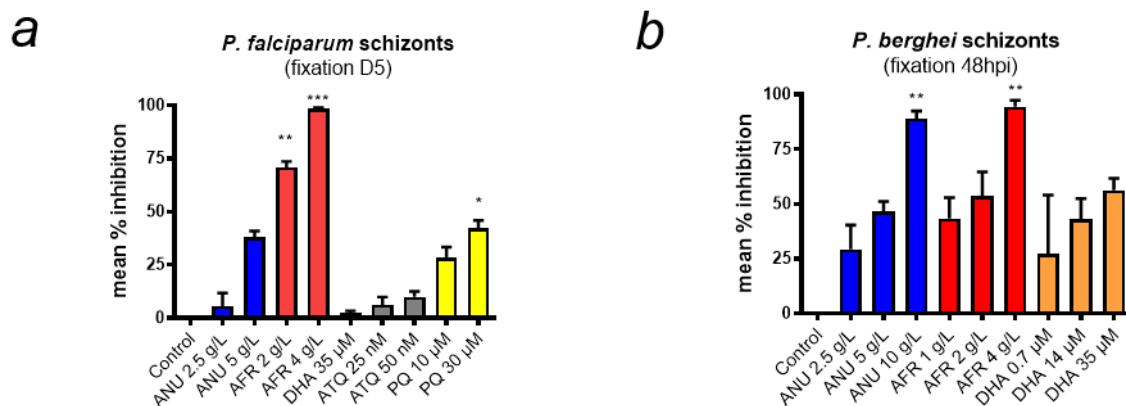
*4 g/L infusion was prepared from a 100 g/L stock *A. afra* infusion.



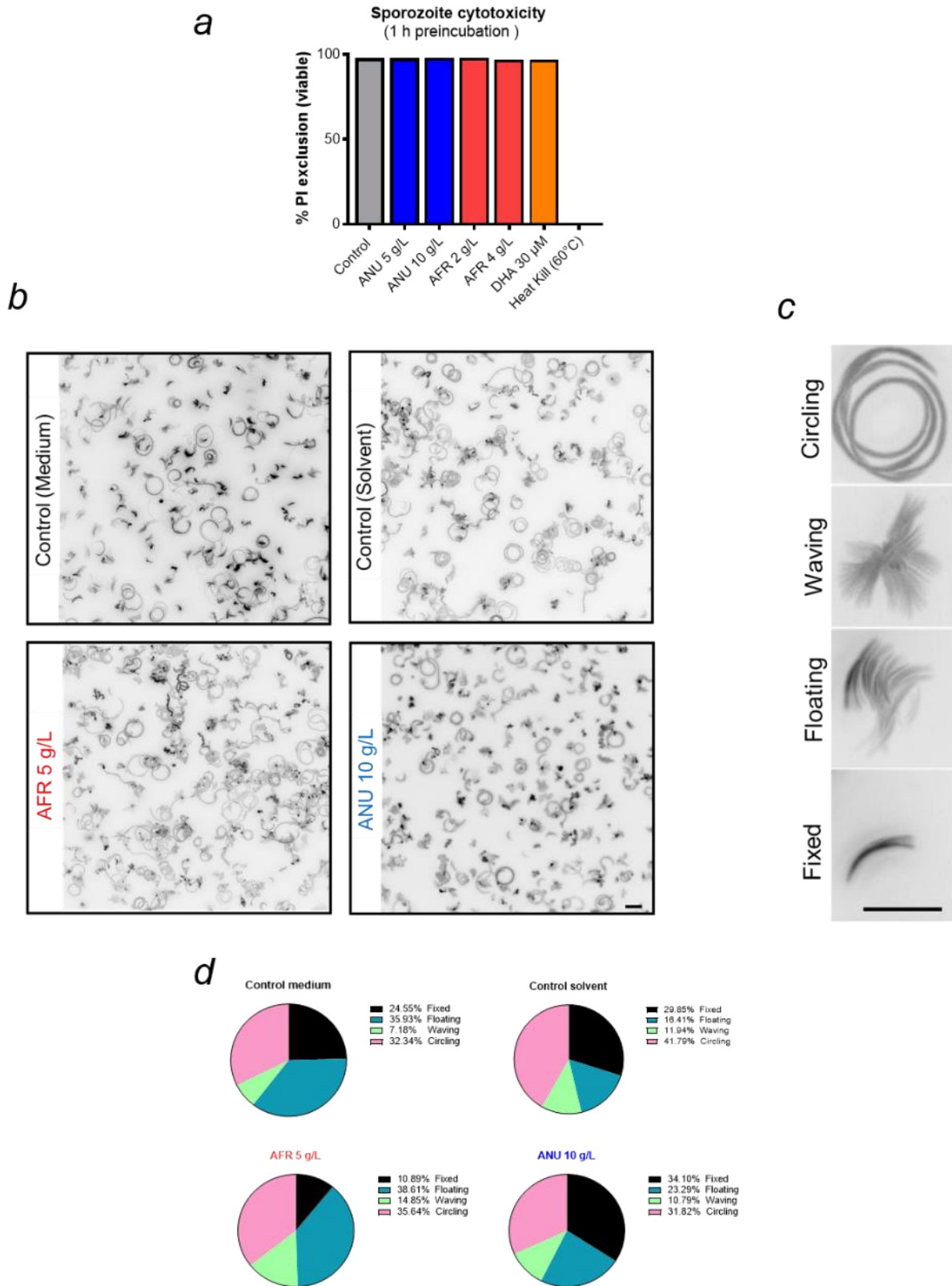
Supplementary Data Figure 1. Impact of *A. annua* and *A. afra* on the development of asexual blood stage of *P. falciparum* from tightly synchronized cultures at ring stage (1% total parasitaemia) followed by two life cycles of infusion treatments (0.5 g/L of infusion) or control (sub-culture at 48 h) (a) and quantification of parasites with affected apicoplasts in treated and control cultures (b).



Supplementary Data Figure 2. Toxicity of *Artemisia* infusions on simian (a) and human (b, c) primary hepatocytes. Hepatocyte nuclei are counted from an equal set number of microscopic fields of controls and infusion-treated cultures.



Supplementary Data Figure 3. Percent inhibition of *P. falciparum* (a) and *P. berghei* (b) hepatic schizont numbers observed in cultures inoculated by sporozoites incubated with *Artemisia* infusions or drugs for 1 hour prior to addition to the hepatocytes. The exposed sporozoites were washed free of infusion or drug before being allowed to infect hepatocytes. *P. falciparum*-infected human hepatocyte cultures and *P. berghei*-GFP infected simian hepatocytes were maintained until day 7 and day 2 pi, respectively, before parasite enumeration by computational scanning of the plates. *P. falciparum* schizonts were labelled with anti-HSP70 antibody before scanning. The results are representative of three independent experiments. Control wells had on average 552 parasites/well for *P. falciparum* and 185 parasites/well for *P. berghei*. Statistical significance was determined with GraphPad Prism 8 using a one-way ANOVA followed by Kruskal-Wallis Test (multiple comparisons to Control) where significance was evaluated by $P < 0.0001$ (****), $P < 0.0013$ (**) and $P < 0.05$ (*).



Supplementary Data Figure 4. The effect of infusion treatment on free *P. berghei*-GFP sporozoites motility and viability. **a**, Toxicity of infusions on freshly dissected *P. berghei* sporozoites exposed for one hour at room temperature. For each treatment condition, all the

movie frames recorded movie were stacked, and a single composite black and white image was prepared using ImageJ software. **b, c, d**, Four different patterns of sporozoite movement (**c**) were identified based on a previous publication¹ and the frequency of each pattern quantified (**d**). Treatment with infusion did not resulted in a significant shift from that of controls (scale bar = 10 μm).

Supplementary Information: Comparative analysis of *A. annua* and *A. afra* secondary metabolites with anti-plasmodial activity and their biological targets.

Terpenoids. Species of the Asteraceae plant family are rich in anti-plasmodial terpenoids other than artemisinin and congeners, including in those of the *Artemisia* genus.²⁻⁶ Due to their hydrophobicity, sesquiterpenes are virtually absent in *A. annua* aqueous infusions, with the exception of arteannuin B, dihydroartemisinic acid, and significantly, artemisinin.⁷⁻¹⁰ The terpenoid content of *A. afra* aqueous infusions, to the best of our knowledge, was never investigated except for the absence - or extremely low amount - of artemisinin.^{2,11-14} However, many of the volatile monoterpenes that constitute *Artemisia* essential oils (EO) can be predicted to be partly extractable by warm water, but can only be detected by GC-MS analysis.^{15,16} Among these, 1,8-cineole (*syn.* eucalyptol) and especially *Artemisia* ketone are consistently abundantly found in both *A. annua* and *A. afra* EO.¹⁷⁻²⁶ On the other hand, *A. afra* contains a high amount of thujones^{17,24,25,27} and 6,7-epoxylinool,²⁸ which are absent in *A. annua*.^{20,21,29} Minor specific compounds include limonene and linalool in *A. annua*,²⁹⁻³¹ which are extremely rare or absent in *A. afra*.²⁴ While the electrophilic 6,7-epoxylinool was never tested against malarial parasites, *Artemisia* ketone and thujones were shown to have very weak activity against intra-erythrocytic *P. falciparum* stages,²⁷ whereas eucalyptol,^{27,32} linalool³³ and limonene^{33,34} inhibited parasite growth at elevated (high micromolar to low millimolar) concentrations. Mechanistically, linalool and limonene exert anti-plasmodial effects on the parasite's blood stages by inhibiting enzymes downstream of the apicoplast DOXP/MEP pathway (i. e., prenyl synthases and protein prenyltransferases), presumably by competing

with their isoprenoid substrates.^{33,34} Stronger effects can be found in related non-*Artemisia* monoterpenes (e.g., perillyl alcohol) and sesquiterpenes (e. g., nerolidol). These inhibitors repress the biosynthesis of dolichols and prenylated proteins,³³⁻³⁶ as well as that of the octaprenyl chain of ubiquinone,³⁷ thus potentially affecting the mitochondrion.³⁸ In the case of *A. annua*, the presence of significant amount of artemisinin could partly contribute to a direct disruption of the parasite mitochondria.³⁹⁻⁴¹ Interestingly, no natural terpenoid has yet been found to inhibit the DOXP/MEP pathway in the apicoplast as does fosmidomycin.⁴² Due to the existence of established or putative anti-plasmodial terpenes in the rich EO of *A. annua* and *A. afra*, it appears important in the future to measure their quantity in *Artemisia* infusions, and also to test representative compounds (e. g., eucalyptol) against the parasite liver stages with a focus on anti-apicoplast and anti-mitochondrion effects.

Flavonoids and chlorogenic acids. Aqueous infusions of *A. annua* contain a major proportion of polar metabolites dominated by flavonoid aglycones (e. g., jaceidin, casticin), flavonoid glycosides (e. g., apigenin-6-C-glucopyranoside, *syn.* *iso*-vitexin, luteolin-7-O-glucopyranoside, *syn.* cynaroside) and quinic acid esters (*syn.* chlorogenic acids).^{7,8,19,43-45} *A. afra* aqueous extracts were never characterized in-depth except for the qualitative detection of flavonoids and phenolics.⁴⁶ However, this species is known to produce flavonoid glycosides common to *A. annua* (e. g., cynaroside) in addition to several flavonoid aglycones (e. g., apigenin, tamarixetin)^{2,47,48} which albeit distinct from those present in *A. annua* can be predicted to parallel or even surpass their hydrosolubility. *A. afra* also contains an important component of chlorogenic acids.^{13,49} These *A. afra* metabolites could thus be present in aqueous infusions of *A. afra*, similarly to those of *A. annua*. Most flavonoids and chlorogenic acids from both *Artemisia* species exert significant inhibition on blood-stages *Plasmodium* parasites, with activities ranging between low and high micromolar.^{2,7,13,43,47,50-55} The bulk contribution of these metabolites to the activity of *A. annua* infusions against *P. falciparum* erythrocytic stages, which is dominated by a both abundant and extremely potent artemisinin, was shown, however, to be negligible using plant strains deficient in either flavanone (Δ *CHI1*-

1) or amorpha-4,11-diene (SiAMS or $\Delta cyp71av1-1$) biosynthetic pathways.^{56,57} On the other hand, artemisinin activity falls by *ca.* three orders of magnitude against the parasite liver stages,^{58,59} consistent with their fewer endoperoxide-activating heme sources compared to those in actively-digesting blood parasites.^{41,60-62} In this context, most of the aforementioned compounds present in *A. annua* or *A. afra* - the latter species notably lacking artemisinin - may start to exhibit significant inhibition of *Plasmodium* hepatic stages. Mechanistically, certain anti-plasmodial flavonoids present in *A. annua* infusion (i. e., cynaroside) or abundant in *A. afra* (i. e., apigenin) were shown to inhibit the *P. falciparum* enoyl-acyl carrier protein reductase (PfENR, *syn.* FabI),^{53,54} an enzyme harboured by the parasite type II fatty acid synthase (FASII) machinery in the apicoplast.⁴² Other flavonoids from aqueous extracts of both *Artemisia sp.* could also be found to target the apicoplast, considering the low micromolar activity of various congeners against FabG, FabZ or FabI,⁵⁴ all part of plasmodial FASII complex.⁴² Regarding the anti-mitochondrial effects, many of the anti-plasmodial flavonoids present in *A. annua* infusions (i. e., *iso*-vitexin, vitexin and casticin) or present in *A. afra* (e. g., apigenin, tamarixetin) are known to disrupt the inner mitochondrial membrane potential, to generate reactive oxygen species (ROS) and to induce apoptosis in cancer cells.⁶³⁻⁷⁵ In plants, *iso*-vitexin was shown to disrupt mitochondrial respiratory chain by interacting with ubiquinone binding sites.⁷⁶ In *Leishmania* parasites, apigenin was responsible for ROS generation, mitochondria depolarization and swelling.⁷⁷ Similar mitochondrion-targeting mechanisms could underpin the action of these flavonoids in *Plasmodium* parasites, although this has never been demonstrated. On the other hand, certain anti-plasmodial flavonoids from both *Artemisia* species (i. e., cynaroside, chrysoeriol) possess ROS-scavenging capability, with mitoprotective and antiapoptotic effects,⁷⁸⁻⁸⁰ leading to tentatively exclude mitochondrion-disrupting mechanisms for these compounds in the parasite. The molecular targets of chlorogenic acids in *Plasmodium* are unknown, but the fact that chlorogenic acid itself was predicted to bind FabI *in silico*⁸¹ validates their investigation for anti-apicoplast effects related to FASII inhibition. Regarding possible mitochondrial effects, chlorogenic acid and congeners show cytotoxicity and induce mitochondrion-dependent apoptosis in cancer cells *via* ROS production and

collapse of the inner membrane potential, in addition to other effects (e. g., inhibition of the PI3K/AKT/mTOR pathway, activation of PKC).⁸²⁻⁸⁶ Interestingly, these effects seem to be inexistent in normal cells, where chlorogenic acid exerts SIRT1-dependent antioxidant, mitoprotective and antiapoptotic activities.^{84,87-90} Hence, similarly to *Artemisia* flavonoids, the possibility of beneficial or detrimental effects of chlorogenic acids makes it difficult to predict their action in *Plasmodium* mitochondria.

The artemisinin-independent anti-plasmodial effects exerted by *Artemisia annua* and *A. afra* infusions in the present study could well be explained by a concomitant, multi-target action of various moderately potent phytochemicals against *Plasmodium* parasites. However, the existence of novel, yet undetected minor compound(s) endowed with hyperactivity, constitutes a hypothesis worth being investigated in a drug discovery perspective.

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Chapter 4

***Artemisia* infusions perturbs the apicoplast biogenesis of liver resident *Plasmodium* parasites including hypnozoites**

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Introduction

In recent years, sophisticated and powerful cellular and phenotypic screening methods have identified drug candidates and hits that are validated against different stages of the parasite. The apicoplast, literally a plant-like Achilles's Heel of the parasite that is absent from the human host is considered to be an attractive drug target. The apicoplast (Apicomplexa plastid) is an essential organelle of Apicomplexa parasites, including *P. falciparum* that has been acquired by the secondary endosymbiosis of a red algal ancestor¹. Although apicoplast lost its photosynthetic ability it harbours all other essential metabolic pathways of a typical chloroplast: a prokaryotic type II fatty acid synthesis pathway, the isoprenoid precursor synthesis pathway, aka the DOXP/non-mevanolate pathway, the heme synthesis pathway split between apicoplast and mitochondrion, and the Fe-S cluster synthesis pathway². Apicomplexan parasites, including the *Plasmodium* spp., are thus part of the photosynthetic lineage algae, as well as all members of the Alveolata kingdom, including ciliates and dinoflagellates⁴⁻⁵. Of note, several organisms from this the lineage, some members have then lost their plastid in further evolutionary events. *Cryptosporidium* spp., responsible for cryptosporidiosis, is the only parasite in the Apicomplexa group known to lack the apicoplast³.

Like all plastids, apicoplast cannot be generated de novo and it must be vertically inherited⁴. The apicoplast genome (35 kb) is homologous to plant and algal plastid but is highly reduced. As a semi-autonomous organelle of endosymbiotic origin, similarly as the mitochondrion, the apicoplast possess a small circular genome of 35Kb that expresses a limited number of proteins (housekeeping genes for genome maintenance, ribosomal proteins, tRNAs and a handful of genes encoding for specific metabolic functions: tRNAs, rRNAs RNA polymerase, translational elongation factor (EF-Tu), ClpC chaperone and iron-sulphur cluster protein (SufB)^{7, 8}. However, the vast majority (~500) of the apicoplast proteins are nuclear encoded then transported into the Apicoplast (NEAT proteins). These NEAT proteins are imported via the conserved chloroplast-like translocons (TIC, TOC) and the ERAD system. The import system to reach the outermost membrane of the apicoplast is currently unsolved.

As mentioned since the apicoplast is absent the human host and essential to the parasite, it is considered an attractive drug target. Due to the cyanobacterial origin of algal plastid and the secondary endosymbiosis of that red algal ancestor that gave rise to the apicoplast, the latter is thus a reduced non-photosynthetic *Cyanobacterium* residing inside the parasite. As any bacteria,

cyanobacteria and hence apicoplasts are susceptible to antibiotics and herbicides. Malaria parasites are also sensitive to common anti-bacterials like doxycycline, clindamycin and ciprofloxacin. These antibiotics were used as prophylactics for the treatment of malaria⁹⁻¹⁰. After apicoplast discovery, the apicoplast was found to host the actual target pathways of some known antimalarial drugs, which motivated efforts for further research into its biological functions and biogenesis. Many apicoplast perturbing drugs were found to result in a 'delayed death' where parasites are dying at the second cycle following the addition of apicoplast-targeting drugs¹¹. The reason for delayed death is suspected to be linked to the IPP pathway^{11, 25} but not only. Full reasons currently remain unknown. However, such slow action provoked skepticism on the actual efficiency of such compounds as single molecule therapies. However, several antibiotics e.g. ciprofloxacin, rifampicin, and thiostrepton are not in line with the delayed death characteristics¹². Recent evidence of apicoplast inhibitors causing rapid killing would put the organelle as a potent target against malaria¹³ (or even as combination therapies with other fast acting drugs)

The organization, behavior and functions of apicoplast in *Plasmodium* liver stage are not fully deciphered particularly in human malaria species *P. falciparum* and *P. vivax*. Exhaustive imaging approaches have, however, revealed the complex and intricate way by which the apicoplast is generating, branching and being re-distributed to daughter cells during liver stage schizogony. The development of transgene apicoplast marker tagged with GFP parasites allowed direct live visualization of apicoplast⁷.

In mosquito stage, salivary gland sporozoites contain a single nucleus, a single apicoplast and a single mitochondrion. In the stage of sporozoite development, apicoplast looks like a punctate shape at early stage and then it transforms into a single elongated structure^{7,10}. Study showed that apicoplast-encoded genes (SufE) could play a role in maintaining sporozoite quiescence and also in early liver stage parasite forms¹¹.

Normal apicoplast development is required for exo-erythrocytic parasite growth. Examination of apicoplast in hepatic stage demonstrated similar morphological patterns to sporogony. It was described that *P. berghei* sporozoites, on the eve of hepatocyte invasion contain a single nucleus, a single apicoplast and a mitochondrion. According to literature, the first 24 h post infection, the parasite contains a single nuclei but mitochondria and apicoplast start their elongation. Parasite grows from a single nucleus to multiple nuclei, thereby following the

karyokinetic proliferation, apicoplast becomes increasingly branched (a huge organelle network). The branching does not seem to follow a specific or defined pattern due to the asynchronous development of exo- erythrocytic parasites. Prior to the merozoite formation, the apicoplast network starts to divide and becomes concertinaed. To form merozoites, the parasite undergoes cytokinesis and segregation into the forming daughter merozoites. Each daughter merozoite contains a single apicoplast, mitochondrion and nucleus is capable of invading erythrocytes¹⁴. In respect to the mechanism of apicoplast division, it is also unclear how individual sections of divided apicoplasts are segregated into forming daughter cells. The other liver resident parasite form is hypnozoite and apicoplast also exists inside of it which was demonstrated in immunofluorescence assays (IFA)^{15, 16}. Function of apicoplast in hypnozoite dormancy and linking to activation is unknown.

Artemisia plants and its extracts were documented having herbicide, antibacterial and anti-apicomplexan properties. Such diverse role playing of *Artemisia* plants indicate the existence of essential shared traits between plants, bacteria and apicomplexan parasites that could be targeted by the infusions¹⁷⁻¹⁹. Selected herbicides that particularly target plastid metabolisms might also perturb the apicoplast functions. Thus it creates a room to design or discover specific inhibitors that would perturb the apicoplast but have no side effect. We found *Artemisia* infusions affected the development of liver resident parasites including dormant hypnozoites. In this study, we investigated apicoplast morphology following treatment of *Artemisia* infusions and we showed that this anti-plasmodial effect of *Artemisia* infusions partially comes from the disruption of the apicomplexan specific organelle, the apicoplast.

Results

1. *Artemisia* infusion exposed sporozoites display defective apicoplast development

In previous experiments (Chapter 3), pre-infection exposure of sporozoites with infusions strongly affected to the development of hepatic forms when hepatocytes were infected with infusion exposed sporozoites. Subsequent toxicity and videomicroscopy analysis revealed that infusion-exposed sporozoites were viable, motile but FACS sorting confirmed a reduced rate of hepatocytes infection with those infusion exposed sporozoites. Therefore we envisage one

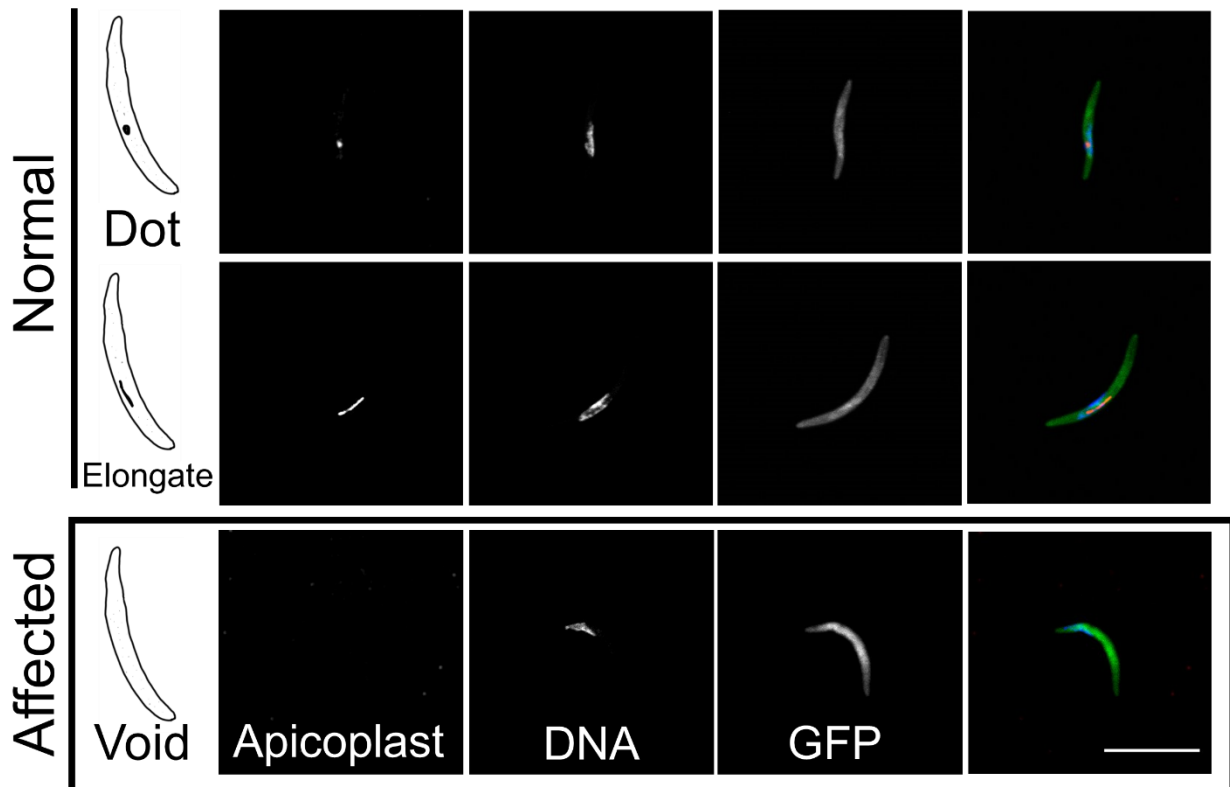
of the sporozoite organelles, apicoplast as a potential *Plasmodium* drug target. *P. berghei* sporozoites were isolated by manual dissection of salivary glands from infected *Anopheles* mosquitoes. An equal number of sporozoites were distributed tubes, containing varying concentrations of *Artemisia* infusions and incubated for 1 hour. These samples were then processed to visualize the apicoplast morphology.

The intracellular localization and morphology of apicoplast was observed carefully under confocal microscopy by immunolabeling against an apicoplast resident protein, the acyl carrier protein (ACP). ACP is extensively used as a positive marker of apicoplast luminal protein which acts as a hub for the type II Fatty acid biosynthesis pathway in the apicoplast by shuttling the nascent fatty acid between enzymes²⁰. It can be noted that FASII pathway has been demonstrated integral for the latter half of the liver stages in *P. falciparum* and not essential during the mosquito and asexual blood stages²¹⁻²³. We observed two distinguishable morphologies of apicoplast in both infusion treated samples and controls: dot and elongated shapes. In infusion treated samples only, an additional sporozoites group was detected totally lacking the apicoplast. This loss of apicoplast was termed as 'void'. Then we quantified apicoplast morphologies to assess whether infusions led to a direct or indirect effect on the apicoplast development in sporozoites. We assumed that the dot form represent the status of a less developed or an early stage of sporozoite growth and then it stretches to become elongated. It can be noted that sporozoites in the salivary glands of mosquitoes are not the same state of their development, this could explain why all sporozoites did not exhibit same apicoplast morphologies

In control group, a major population of sporozites were in elongated shape (81%) whereas the remaining sporozoites were found dot shaped (19%). In *Artemisia* infusion group, a third category of sporozoites, totally lacking the apicoplast and the percentage of void category had slightly increased with the increasing dose of infusions. In *A. annua* 5 g/L and 10 g/L infusion groups, large population of parasites shifted to the less developed dot forms (78% and 80% respectively). 11% and 19% parasites were identified without having apicoplast in infusion treated groups: such devoid forms of apicoplast was absent in controls. These could be explained in two ways 1) infusion blocked the dot form apicoplast to become elongated and 2) the less developed dot forms of apicoplast were diminished by infusion treatments causing complete disappearance of apicoplast. We observed quite similar effects in *A. afra* treated sporozoites. In comparison with control, elongated forms were decreased and dot forms were increased. In azithromycin (our positive control as a known apicoplast targeting molecule)

treatment, a considerable amount of sporozoites were identified with disappearance of apicoplast (33%) while the percentage of dot shape was 65%. Only few sporozoites displayed elongated forms of apicoplast.

1A



1 B

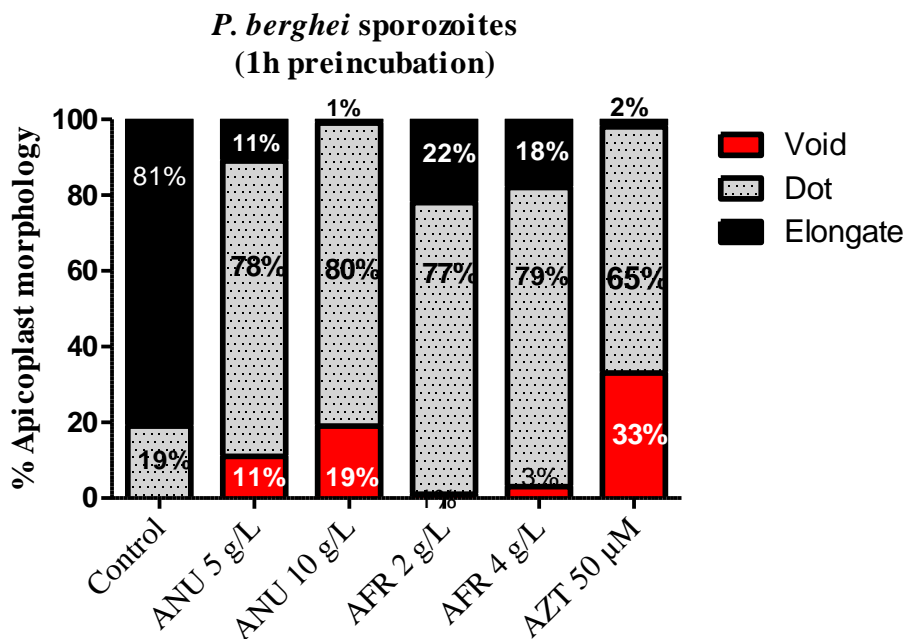


Figure 1: Schematic and confocal images of *P.berghei* sporozoites apicoplast following 1h pre-incubation with *Artemisia* infusions and quantification of morphologies

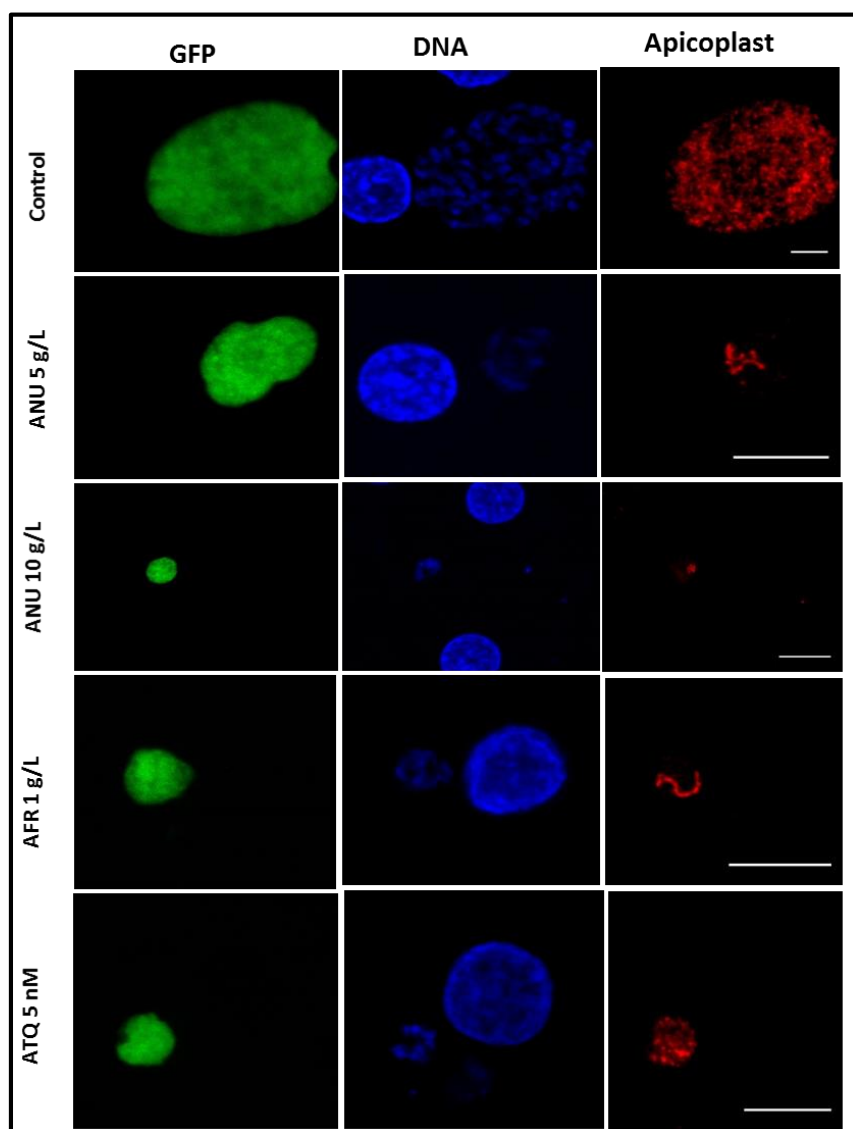
A) Three distinct morphological phenotypes of apicoplast were observed in control and infusion exposed *Pb*-GFP sporozoites. Apicoplast of sporozoites were immunolabelled with anti-*Py*ACP. Different morphologies of apicoplast were sketched and demonstrated in left panel where the right panel shows microscopic images. (Scale bar= 10 µm). Additional Images contained in the black box represent apicoplast morphology identified in only control group. **B)** The stacked chart plotted in percentages. Bar chart depicts the percentage of apicoplast morphology of sporozoites following 1h incubation with *Artemisia* infusions. ACP immunolabelled parasites were quantified and categorized based on morphological appearance in infusions treated versus control. For each conditions, 100 parasites were enumerated at 63X magnification of Leica SP8 white laser microscope.

2. *Artemisia* infusions disrupt the apicoplast integrity in *P. berghei* hepatic forms

To know whether *Artemisia* infusions could have any apicoplast damaging effect on the hepatic stage of *Plasmodium*, we took rodent infecting malaria species *P. berghei* as an experimental tool for this purpose. *Plasmodium berghei* infected simian hepatocytes cultures were treated with *Artemisia* infusions for 48 hours. We then analyzed the apicoplast forms at 48 h of post infection using confocal imaging microscopy. In control group, most of the hepatic *Plasmodium* forms displayed an orientated branching of apicoplast throughout the cytoplasm of the parasite. Such morphological exhibition of apicoplast distribution clearly implicated the status of schizont maturation. Next we compared those apicoplast morphology with infusion treated parasite populations. A close visual inspection in infusion treated groups revealed a defective apicoplast structure. A severely curtailed apicoplast structure was identified in infusion treated conditions. The affected apicoplast had limited association with its own machineries (ANU 5 g/L). The effect was more robust and evident with the highest concentration of *A. annua* treatment (10 g/L) where only a punctate shape of apicoplast was detected (**Fig 2A**). We hypothesize that this could be a clear visualization of apicoplast disruption due to infusion treatment. Morphological quantification revealed that 70 percent of apicoplast in ANU 5 g/L treatment group was affected. In addition the other parasite population in that group had more or less similar morphology like control (**Fig 2B**). We could quantify apicoplast numbers in other treatment groups, particular ANU (10 g/L) and *A. afra* 2 g/L but very low number of parasites were detected in these two groups following infusion treatments.

In the case of *A. afra*, treatment for 48 h rendered an effect on apicoplast morphology where infusion exposed schizonts had an elongated apicoplast shape compared to control. Atovaquone a schizonticidal drug, used in this study and showed size reduction effect without affecting the apicoplast (**Fig 2A**).

2 A



2 B

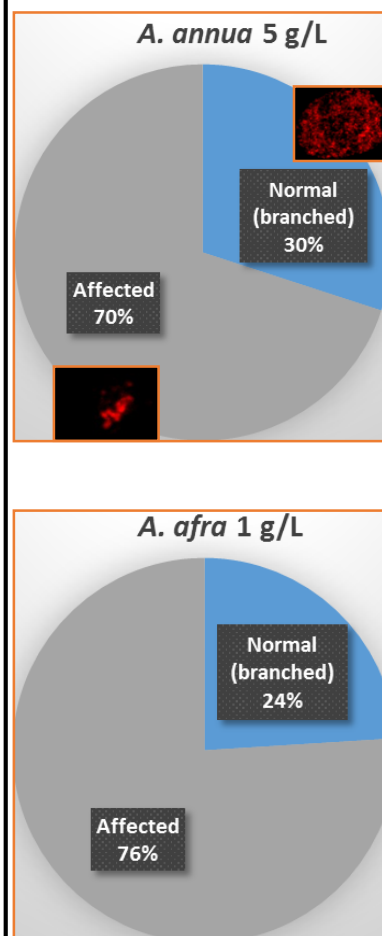


Figure 2: Confocal images showing disruption of apicoplast network in a rodent malaria model. A) Morphological alterations of *P. berghei* schizonts after infusion & atovaquone treatment. Apicoplast were immunolabelled with anti-Py ACP (scale bar 10 μ M). Host and parasite DNA were visualized with DNA marker DAPI B) % of morphological phenotypes observed in *A. annua* 5 g/L and *A. afra* 1 g/L treatment. Quantifications were not possible in *A. annua* 10g/L, *A. afra* 2 g/L and atovaquone treated conditions because of the presence of very minimal numbers of schizonts with high dose infusion treatments.

3. Quantitative PCR reveals an effect of infusion on *P. berghei* apicoplast

In previous experiments, we found *Artemisia* infusions affected apicoplast of *P. berghei* parasites. From the observation, we speculated that total apicoplast DNA could be affected by infusion treatments as apicoplast contains its own small circular genome (DNA)⁴. Next, quantified the apicoplast DNA in *Pb*-GFP ANKA infected hepatic culture. To do so, infected cultures were treated with *Artemisia infusions* for 48 h. After 48h we quantified the ratio of apicoplast DNA (Elongation factor Tu gene, TufA) or that of the parasite's chitinase gene in the nucleus to that of the GFP gene throughout hepatic development. Doxycycline, a classical apicoplast-targeting drug was used as a positive control. In cultures exposed to doxycycline or *Artemisia* infusions, qPCR quantification showed a constant nuclear/GFP DNA ratio over maturation (18, 27 and 48 hpi), indicating that the infusions had no effect on parasite nuclear DNA replication. On the other hand, both *Artemisia* infusions (5%) led to a strong inhibition of apicoplast DNA replication (reduction of apicoplast DNA when normalized to GFP), that was similar to that observed with 10 μ M doxycycline.

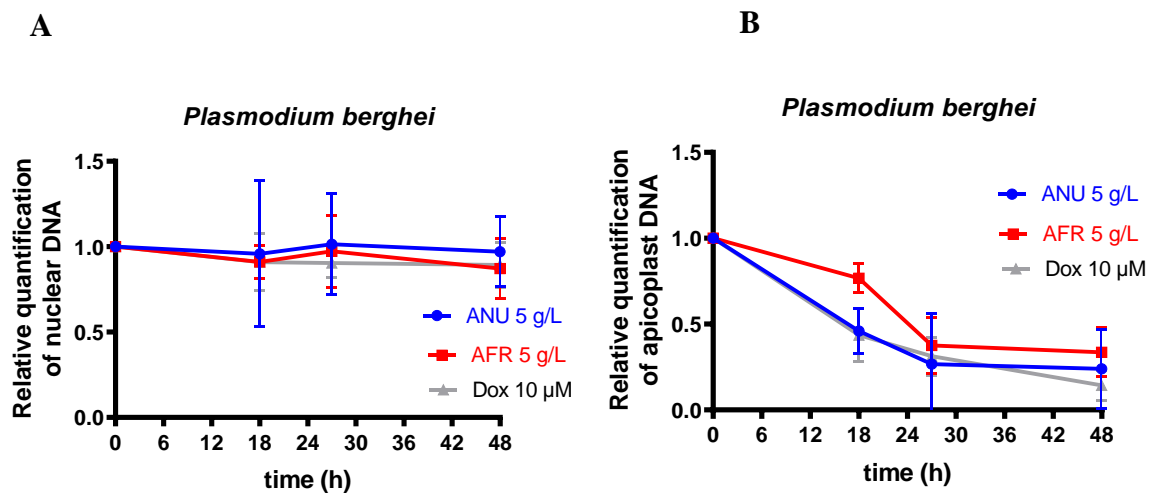


Figure 3: Quantification of nuclear and apicoplast genomes relative to GFP by qPCR during the hepatic cycle of *P. berghei* following infusion treatment. A) Nuclear genomic DNA B) Apicoplast genomic DNA was extracted from treated or control groups at different timings and qPCR was performed using oligos amplifying parasite genomic, apicoplast representing genes plus GFP. Each target gene was normalized to GFP, expressed as transgene in *P. berghei*.

4. Prophylactic infusions treatment disrupt the apicoplast of *P. falciparum* and *P. cynomolgi* in hepatic schizonts

Next we sought to decipher whether infusions treatment could render any damage on the apicoplast of malaria parasites with longer hepatic schizogony. Prophylactic infusion treatments (D0-D4) were applied on *P. falciparum* and *P. cynomolgi* infected hepatic cultures. In *P. falciparum* infected hepatocytes culture, schizonts of control wells showed a uniform appearance where ACP signals appeared like small dots densely decorating the periphery of the parasite organelles (Figure A, top panel). Apicoplast, at day 5 of *P. falciparum* hepatic stage, seemed to reach nearly its terminal stage of development (considering the 5-6 days of *P. falciparum* schizogony). We hypothesized that this could be the reminiscent of the preparation of apicoplast sequestration as matured parasites become ready to egress merozoites from day 6 of post infection¹⁶. However, in cultures treated with *Artemisia* infusions, two other distinct apicoplast morphologies were observed. We categorized them into aggregate and void type. Apicoplast was appeared with an accumulated ACP signal. Most surprisingly, we identified parasites with complete disappearance of ACP signal (void category) with the highest dose of infusion treatment (**Fig 4A**).

We enumerated apicoplast morphologies and represented the data in percentage derived from infusion treated and non-treated infected culture. The control group showed a complete uniform appearance of normal apicoplast (schematic figure A; top panel). However, we observed 3 distinct types of apicoplast morphologies in *A. annua* (2.5 g/L) treated group where normal apicoplast (31%), aggregated (65%) and void (1%) were identified. Treatment of *A. annua* 5g/L resulted in complete disappearance of apicoplast signal (void category). This disappearance was observed in 40 schizonts presented in the culture after the inhibitory effect of *A. annua* infusion. In addition, with lower dose of *A. afra* infusion, disruption of apicoplast seemed less affected with only 8% aggregated forms where a major population has unaffected apicoplast. Treatment of *A. afra* (2 g/L) completely cleared all parasites from culture wells and thereby quantification of apicoplast morphologies in that treatment group was not possible (**Figure 4B**).

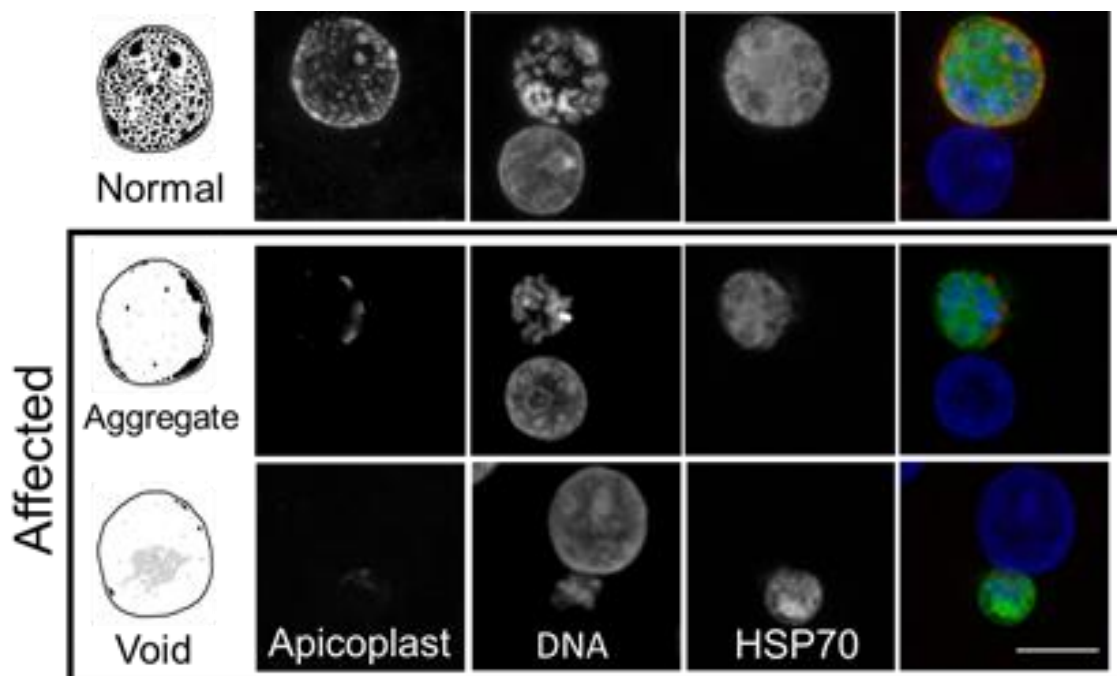
In *P. cynomolgi* infected model, prophylactic infusion treatment was applied at the time of infection. Cultures fixed at day 6 post infection, displayed two different forms of apicoplast in *Artemisia* infusion treated versus control. The elongated forms of apicoplast could represent the less developed schizonts whereas a highly coordinated and networking/branching of ACP signal might indicate the status of schizont maturation. Another category of apicoplast morphology appeared in all *Artemisia* infusion treatment groups where ACP labelling

demonstrated a diffused signal characteristics of a disrupted apicoplast (**Fig 4C**, black box). Considering the morphological alterations of apicoplast in connection with their irrespective parasite size, we hypothesize that growth of parasites in hepatic culture were not synchronous in the course of liver stage life cycle. Thereby it could be assumed that it had reflected on the morphology.

Next we approached for the quantification of apicoplast morphologies. In control wells, the majority of schizont population had network shape (84%) and the remainder parasites were elongated (16%). *A. annua* infusion treatment of 2.5 g/L and 5g/L led to an increasing number of parasites that were shifted to diffused forms with a percentage of 35% and 69% respectively by reducing the number of apicoplasts with network shaped morphologies. We observed similar effect by low dose of *A. afra* treatment. Such morphological alterations of apicoplast e.g shifting from well-developed apicoplast branching to diffused forms clearly indicated a disruption event occurred by infusion treatment in the apicoplast of hepatic parasites.

***P. falciparum* schizonts (D0-D4 treatment)**

A



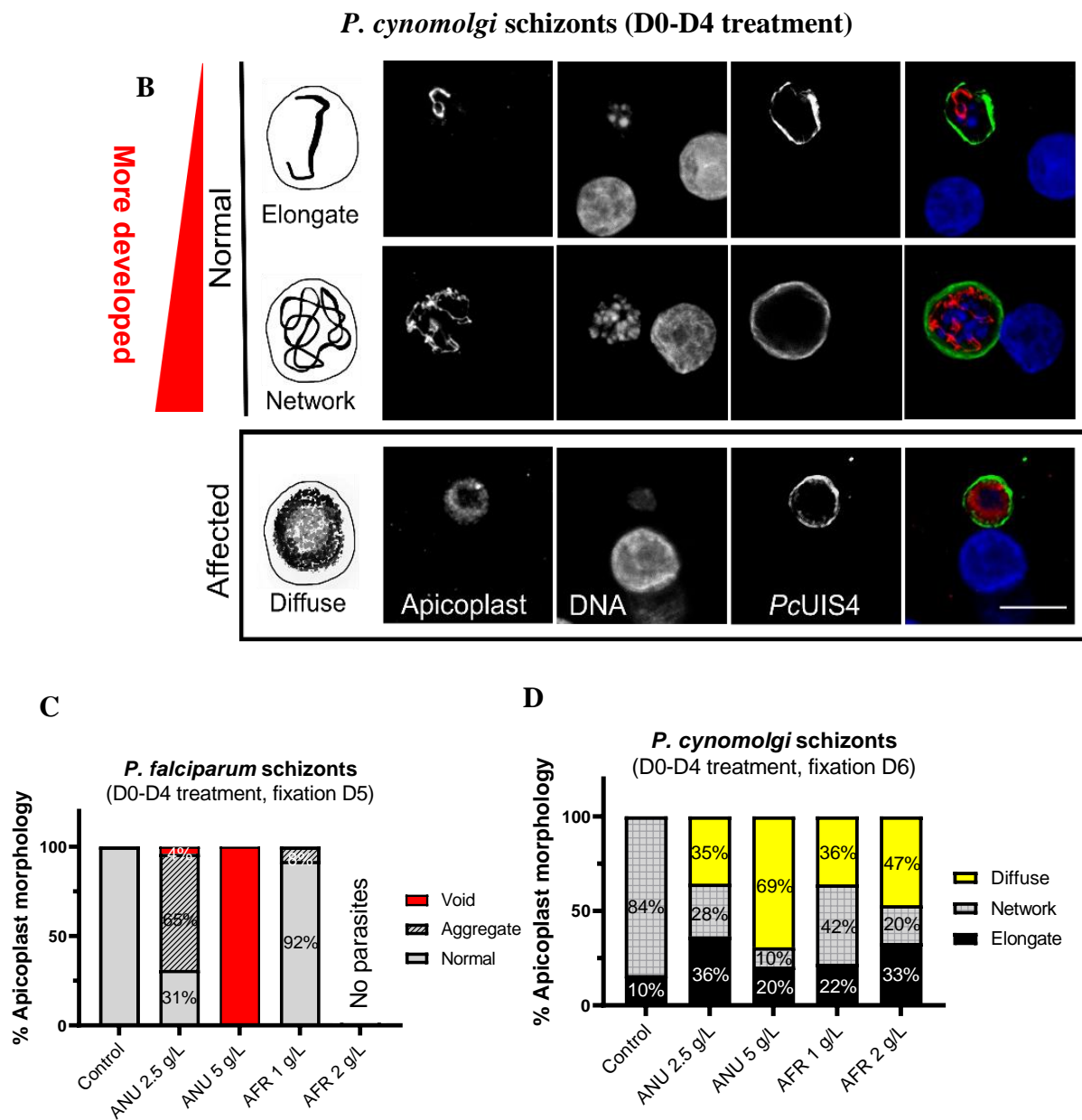


Figure 4 : Apicoplast network disruption in *P. falciparum* and *P. cynomolgi* schizonts mediated by infusion treatments. A) Top panel showing images (schematic left and confocal images at right) of normal apicoplast of *P. falciparum*, cultures fixed at day 5 of post infection. Fixed parasites were stained with anti-*PbHSP70* and apicoplast was labelled with anti-*PyACP* (scale bar =10 μ m). The below panel (highlighted in black box) represents the affected apicoplast by infusion treatments. Two distinct types of ACP signaling were appeared ; aggregate and void (had no ACP signal). B) Quantification of apicoplast morphologies (void , aggregate and normal) in with or without infusion treatments. Different apicoplast

morphologies were quantified in 100 parasites and presented. **C)** In *P. cynomolgi* model, two distinct patterns of apicoplast morphologies (elongated and network) were considered as normal phenotype. In addition, the lower panel highlighted with black box represent ‘affected’ apicoaplast. Schizonts were visualized with an anti-*PcUIS4*, host and parasite DNA were marked with DAPI (scale bar =10 μ M). **D)** Stacked chart depicted the morphological alterations of apicoplast observed in 100 schizonts in each infusion treatment group. Quantified data plotted in percentage.

5. Prophylactic *Artemisia* infusion treatment has an effect on hypnozoites apicoplast

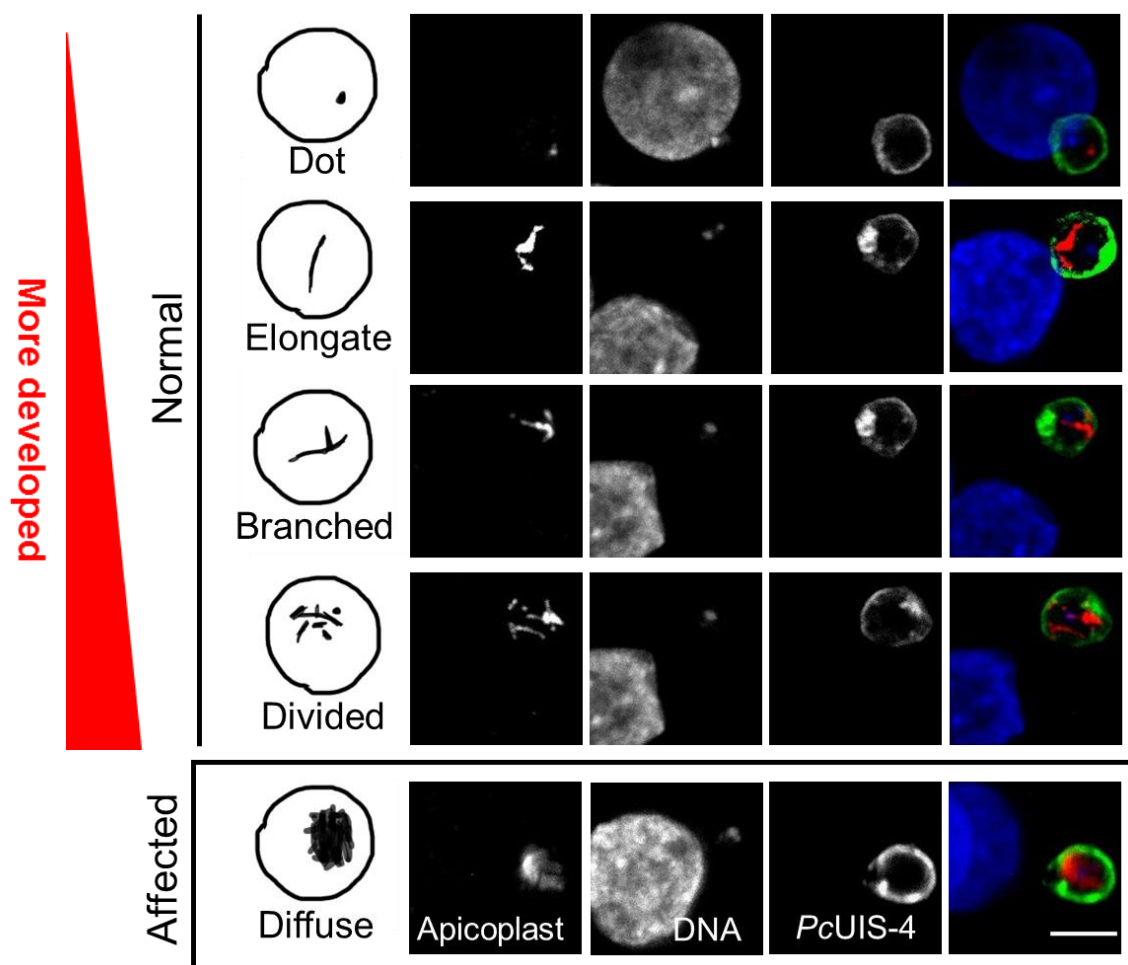
We tried to understand the infusions effect on hypnozoite apicoplast. To do so, *P. cynomolgi* infected hepatic culture was treated with *Artemisia* infusions (D0-D4) and immunolabelled with an anti-ACP marker. We extensively investigated the ACP marked hypnozoites under confocal microscope. In control group at day 6 of post infection, four categories of apicoplast morphologies could be defined. We hypothesize that hypnozoites don’t develop synchronously in the hepatocyte culture thereby the apicoplast morphologies did not appear to be uniform, consistent with our previous observation of different apicoplast morphologies and variations in parasite schizonts. We could assume that younger hypnozoites display a punctate (single dot) shape of apicoplast. The single dot apicoplast then evolves into an elongate organelle. Next the elongate apicoplast undergoes branching and finally a divided morphology could be observed in oldest hypnozoites (which could be a reactivated hypnozoites or matured one). Such morphological alterations probably indicate an early stages of apicoplast biogenesis occurring in hypnozoites. We addressed them as normal developmental state of apicoplast in hypnozoites. Interestingly, an additional ‘diffused apicoplast’ form was observed in hypnozoites following infusion treatment which was not identified in control group. Apparently that form was affected one resulting in aggregated apicoplast contents (**Figure 5A**).

Next we quantified apicoplast morphologies of 100 hypnozoites from each experimental group. In control group, 54% of hypnozoites had elongated or branched apicoplast whereas almost similar percentage of other forms e.g. divided (26%) and dot forms (18%) were observed in the same group. Interestingly the percentage of hypnozoites with less developed, punctate apicoplast was increased following *Artemisia* infusion treatments. In opposite manner, the

percentage of divided and elongated forms had been decreased in treatment groups compared to controls. Most strikingly, considerable percentages (36% in *A. annua* 5 g/L group and 29% in *A. afra* 2 g/L) of affected apicoplast with diffused ACP signal were appeared in infusion treatment groups which was completely absent in control. This clearly indicates that *Artemisia* infusions treatment affect apicoplast of hypnozoites.

A

P. cynomolgi hypnozoites (D0-D4 treatment)



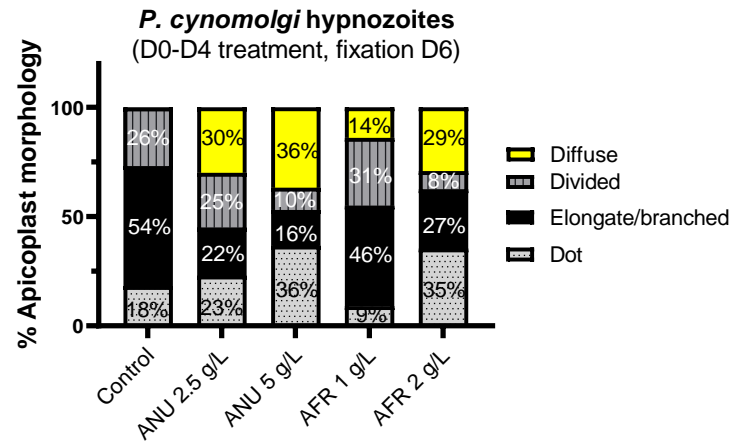
B

Figure 5: *Artemisia* infusions interfere with apicoplast integrity in hypnozoites. **A)** Illustrative (left) panel depicts different apicoplast morphologies observed in hypnozoites culture with or without infusion treatments. Three distinct forms of apicoplast (dot, elongated and divided) were identified in control and infusion treatment group. An additional ‘diffused’ morphology, highlighted in affected panel, was observed following infusion treatment which was not identified in control group. **B)** Quantification of the various apicoplast morphologies in 100 parasites/ treatment condition. Hypnozoites were visualized with an anti-*Pc*UIS4 and apicoplast were labelled with anti-*Py* ACP (scale bar= 10 μ m)

6. Curative infusion treatment lead to a morphological alteration of apicoplast in hypnozoites

To expand our critical observation regarding the infusion mediated effect on apicoplast, we investigated the apicoplast morphologies of late stage hypnozoites of *P. vivax*. Prior to the morphological assessment, *P. vivax* infected human hepatocytes culture was treated with *Artemisia* infusion from day 4 post infection until day 10. Next we approached for quantification by evaluating apicoplast morphologies under a confocal microscope. Day 10 old hypnozoites displayed a slight difference in apicoplast morphology (**Fig 6 A**) compared

to day 6 hypnozoites (**Fig 5 A**). Almost all of the hypnozoite population had a divided pattern of apicoplast which we defined them as normal apicoplast. In the *Artemisia* treated group, most of the apicoplast forms appeared to be affected indicating an aggregation of apicoplast machinery towards the periphery of parasitophorous vacuole membrane (PVM). The percentage of affected apicoplast were in 76% *A. annua* and for 86% *A. afra*. To note, we did not observe any anti apicoplast activity of DHA treatment on hypnozoite.

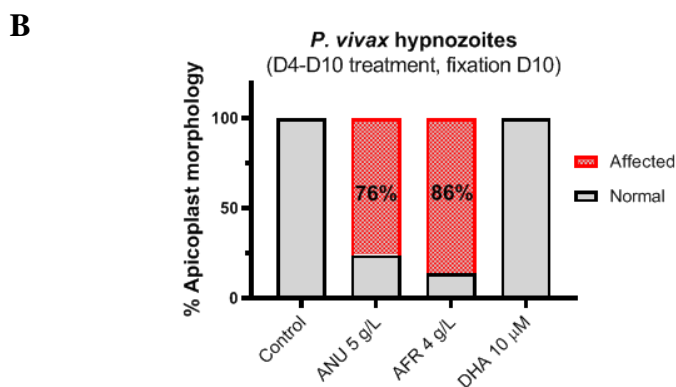
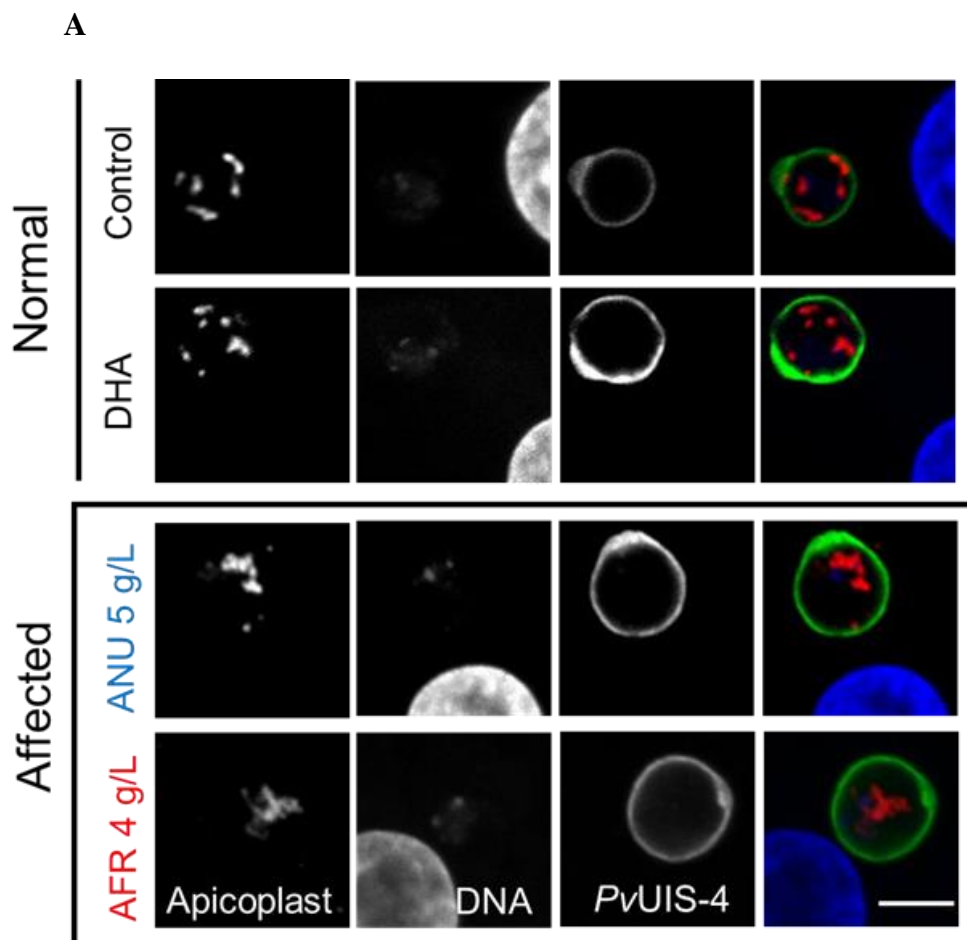


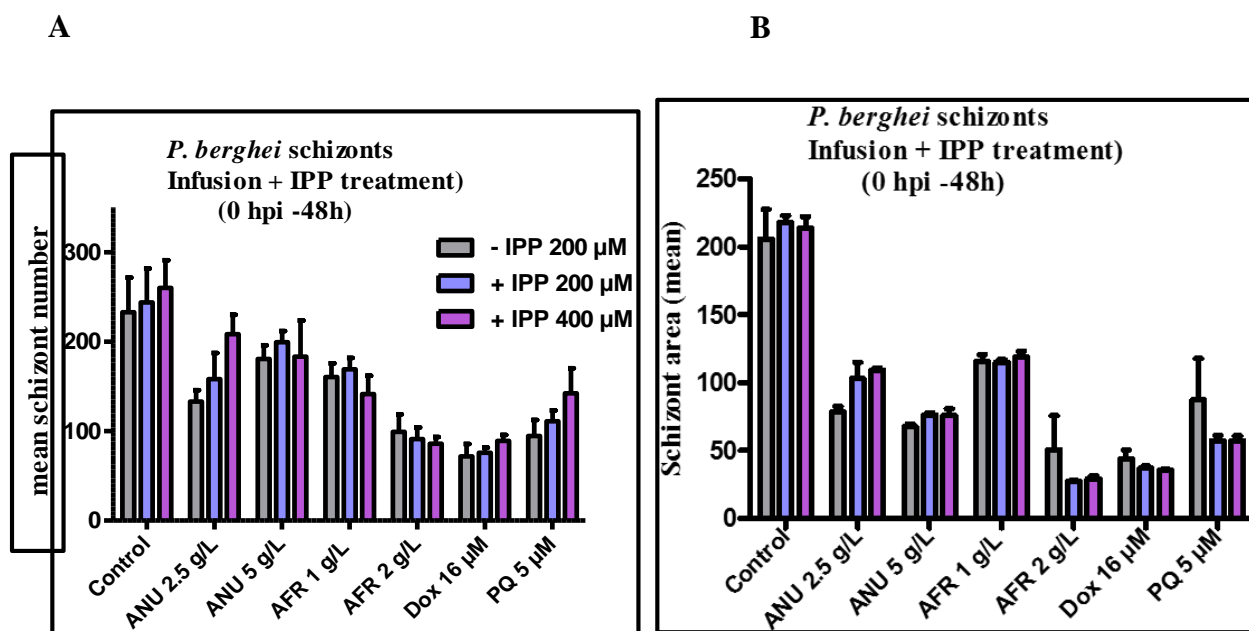
Figure 6: Delayed treatment of infusions affects the apicoplast of *P. vivax* hypnozoites.

A) Confocal images demonstrating the apicoplast morphologies of *P. vivax* hypnozoites following D4-D10 infusions treatment. Apicoplast were immunolabelled with anti-PyACP. Upper panel apicoplast forms are representative of normal apicoaplast observed in control and DHA treatment groups. The highlighted box panel depicts the affected apicoplast forms following infusion treatments (Scale bar=10µM). **B)** Quantifications of apicoplast morphologies control versus infusion treatment. Results expressed in percentages after counting 100 hypnozoites per condition.

7. Exogenous IPP treatment fails to rescue the infusion mediated damaged apicoplast in *Plasmodium* liver stage

Detailed morphological investigations of apicoplast in hepatic stages of all *Plasmodium* species and DNA quantifications of *P. berghei* apicoplast, altogether revealed an effect of infusion on parasite apicoplast. Isopentenyl pyrophosphate (IPP) production is the sole required function of the apicoplast in the blood stage parasites. Supplementation of IPP was shown to rescue the parasites from apicoplast perturbing drugs for instances antibiotics e.g azithromycin, doxycycline etc^{24, 25}. To decipher the role of isoprenoid precursor in hepatic stage of *Plasmodium*, we infected simian hepatocytes with rodent malaria species *P. berghei* and started the infusion and IPP treatment simultaneously in infected cultures. Both IPP and infusion treatments lasted for 48 hours during the full hepatic cycle of rodent species. IPP treatment had no anti- plasmodial effect neither it could improve the rate of hepatocytes infection. In addition, IPP could not rescue the parasites from infusion induced damage to apicoplast. However, 2.5 g/L *A. annua* infusion treatment with 400µM of IPP showed a slight tendency to increase the parasite number (**Fig 7A**). Doxycycline was used during this liver stage assay as it is known as apicoplast targeting drug in blood stage of *Plasmodium* parasite. *Artemisia* infusions and doxycycline affected the surface area of schizonts. Addition of IPP in each treatment groups couldn't prevent the size reduction effect of parasites (**Fig. 7B**). It can be mentioned that the concentration of IPP (200µM) was chosen on the basis of previous blood stage findings where 200µM of IPP rescued the blood stage parasites.

Hepatic stages of rodent parasites have only two days of schizogony and we anticipated that IPP treatment was not sufficient enough to show a rescue effect within this short period treatment. Next, we applied IPP treatment in *P. falciparum* model as it has longer schizogony. Infected hepatic culture was treated with *Artemisia* infusions for first three days. The treatment window was based on our previous experiments where 3 days infusion treatment led to a significant reduction of parasite numbers. However, in this assay we extended IPP treatment for 7 days in accordance with the duration of *P. falciparum* hepatic life stage. Consequently we observed a tendency of IPP to rescue parasites from *A. annua* infusion mediated death but such tendency was not observed in 1 g/L *A. afra* treated culture (**Fig 7C**).



C

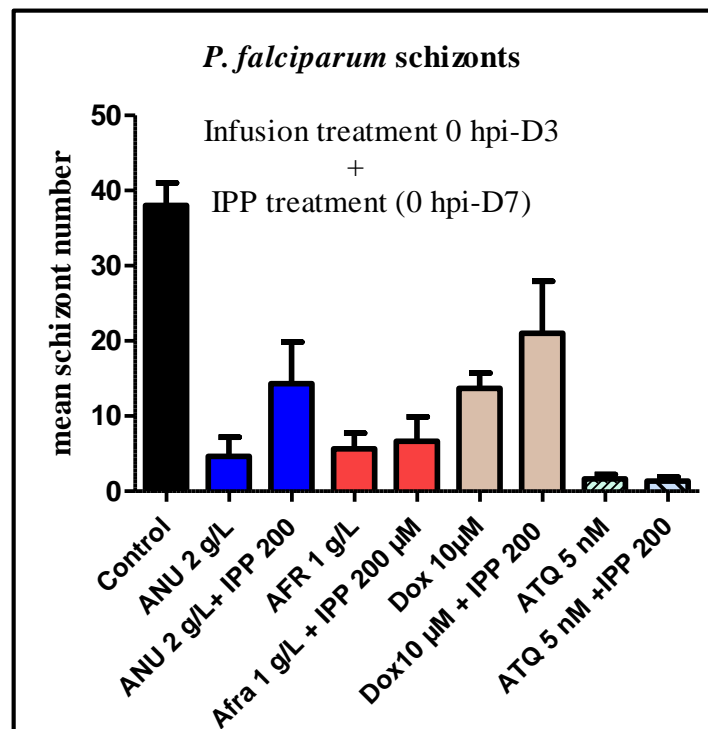


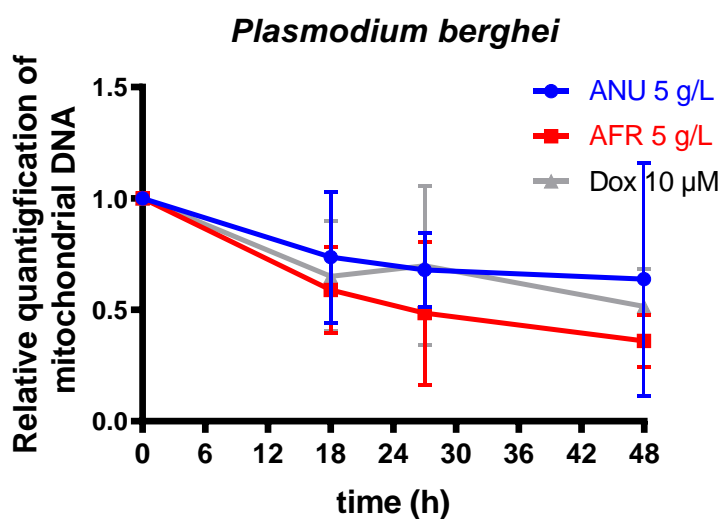
Figure 7: IPP treatment does not improve parasite developmental defects caused by infusions. **A)** Bar charts represents the mean number of schizonts following *Aretmisia* infusions and IPP treatment. The results of IPP supplementation effects on infusion exposed *P. berghei* hepatic culture. During the time of hepatic infection and infusion treatment, the cultures were also treated with IPP or without IPP. Doxycycline is a positive control drug known to affect the apicoplast of blood stage parasites. Primaquine (PQ), a control schizonticidal drug, used in this experiment. **B)** Parasite surface area following infusion treatment and IPP supplementation. The surface area was calculated computationally in high content screening platforms. **C)** *P. falciparum* hepatic culture where infusion treatment was applied from D0-D3 pi. 200 μM IPP supplementation was also started from D0-D7. Atovaquone (ATQ) as control schizonticidal drug used for this experiment.

8. Infusion treatment leads to a partial effect on parasite mitochondria in hepatic stage

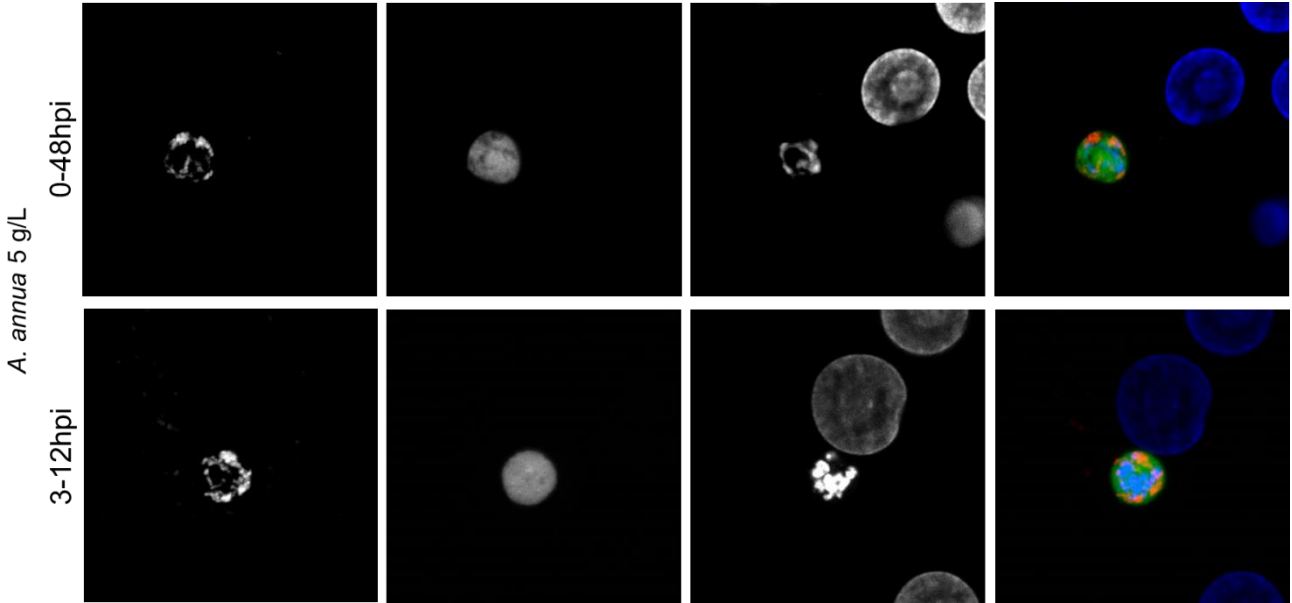
In addition to the apicoplast, apicomplexans also harbour another endosymbiotic organelle, the mitochondrion. Various electron microscopy images of apicoplast showed an association with

mitochondria which was more prominent during schizogony. However, there is no direct evidence of functionality shared by two's has been demonstrated²⁶⁻²⁹. We further assessed infusion effect on parasite mitochondria in rodent malaria species *P. berghei*. Quantitative PCR revealed a reduction of mitochondrial DNA in infusion treated samples compared to control (**Fig 8 A**). This prompted us to investigate the morphology of *P. berghei* mitochondria following *Artemisia* infusion treatment. At 48h post infection, parasitic growth in the control group maintained uniform distribution - bigger and multinucleated syncytium. Almost all parasites obtained extensive mitochondria branching with a special attribute finger-like projections. However in treated conditions including ATQ and Doxycycline, parasite surface area was largely reduced. In *A. annua* 5 g/L treatment, we observed an obvious stunted morphological change of mitochondria where branching was tightly shrunk and mitochondrial machinery seemed aggregated and located peripherally close to the plasma membrane. This abnormal deformity complex of mitochondria was also observed with a shorter treatment window (3h-12h) by *A. annua* 5g/L (**Fig 8C**). In the case of *A. afra* 2 g/L infusion, the mitochondrial dysfunction was more obvious where branching was totally arrested yielding mitochondrial rudiments. ATQ which is a Cyt-b inhibitor of mitochondria clearly diminished mitochondrial labeling at 25 nM concentration (**Fig 8B**).

A



B



C

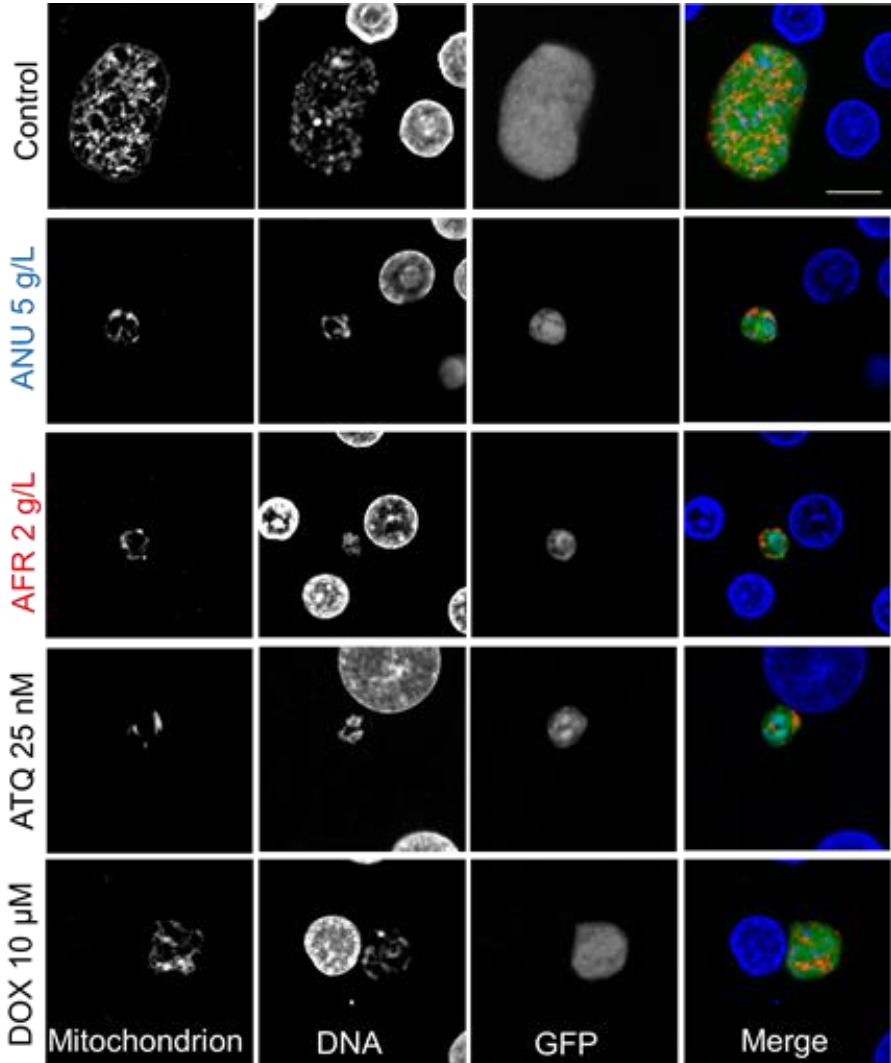


Figure 8: Quantification of mitochondrial DNA and confocal imaging evoke an effect on parasite mitochondria by infusion treatment. **A)** Relative quantification of mitochondrial DNA was done by qPCR from infusion exposed *P. berghei* hepatic culture. Cytb3 was used here as mitochondria marker **B)** Confocal images illustrating morphologies of mitochondria of *P. berghei* liver parasites following *Artemisia* infusions, atovaquone and doxycycline treatment. Parasite mitochondria was labelled with anti-*Pf*hsp60, host and parasite DNA were marked with DAPI (Scale bar=10 μ m) **C)** A short treatment window (3-12 hpi) of *Artemisia* infusion compared to 0-48 hpi treatment window.

Discussion

Plasmodium parasites rely on apicoplast for their growth and survival but the mechanism is still fully not understood. Parasite specific processes that are essential for their survival but absent in the host are instinctively attractive drug targets. To date, understanding the apicoplast biology is largely confined to erythrocytic stages of malaria and the essential role of the apicoplast in *Plasmodium* was understood by the effect of antibiotics on blood stage parasites³⁰⁻³¹. On the other hand, apicoplast function and identification of drugs against this organelle in *Plasmodium* liver stage is truly scarce. However, transcriptome and proteome analyses in the hepatic stage of rodent parasites revealed that apicoplast association in the development of early liver parasite forms³²⁻³³. So it could be interesting to study this organelle in these stages. One of crucial functions of apicoplast is fatty acid biosynthesis. This pathway received considerable attention since many antibiotics already exist to target this pathway. Gene knockout studies showed that this apicoplast resident FAS II pathway was essential for the development of liver stage *Plasmodium* parasites. Central to the type II FAS pathway, a protein called acyl carrier protein (ACP) which acts as a hub for the type II FAS on order to shuttle the nascent fatty acid between enzymes²¹⁻²². Using immunofluorescence microscopy, we investigated apicoplast morphology by examining the intracellular localization of the acyl carrier protein (ACP) in *Artemisia* infusion treated *Plasmodium* liver parasites. In the normal parasite, ACP is localized to a stroma and considered a cellular marker of apicoplast. Hence infusion treated and control cultures of all *Plasmodium* hepatic forms were immunolabeled with anti-ACP antibody to visualize this plastid organelle. First we examined the apicoplast of *Artemisia* infusion exposed sporozoites following a co-incubation assay.

In rodent and human malaria causing *Plasmodium* species, we found that *Artemisia* infusions curtailed sporozoites' ability to infect hepatocytes and thus the development of liver parasite forms were arrested in the culture. Consequently we examined the reasoning behind this deleterious effect of *Artemisia* infusions on freshly dissected sporozoites. It was reported that ablation of different secretory pathways in sporozoite stage could limit the hepatocyte infection. Recently an apicoplast resident folate transporter (FT2) identified in sporozoite and gene knockout of FT2 resulted in reduced hepatocytes infection. Clearly sporozoite apicoplast contribute to the infectivity of hepatocytes³⁴. Previously, AZT effects on mosquito stages were conducted where administration of AZT resulted in 50% reduction of sporozoite production in salivary glands suggesting an effect of AZT on mosquito stage sporogony and apicoplast replication. The speculative AZT effect on apicoplast from that study could be based on AZT binding ability to the apicoplast 50 s ribosomal subunit which could inhibit protein synthesis of this organelle³⁵⁻³⁶. We could not rule out the possibility that *Artemisia* infusion component (s) might interact with apicoplast machineries and could impede normal phenotypes.

We found a rapid activity of *Artemisia* infusions against the liver parasite forms as treatment was not required to the full hepatic cycle *Plasmodium*. This rapidity was more evident against human malaria parasites by low concentration of *A. afra* where three days treatment cleared all liver forms. A detailed morphological investigations of these affected parasites revealed an altered apicoplast structure. We hypothesized that this could be partially mediated by the disruption of apicoplast. The hallmark of apicoplast perturbing drugs mostly antibiotics are associated with a phenotype of delayed death. Such slow antimalarial activity is thought to be fundamental limitation for effective drug strategy of antibiotics. However in our *Plasmodium* liver stage experiments, the quick action of *Artemisia* infusions could be different than the so called 'delayed death' phenomena. Moreover doxycycline, which anti-malarial activity was associated with delayed death phenotype, now has been reported to kill the parasites quickly at the first cycle. This clearly indicates an off target mechanism could exist outside the apicoplast. In addition, parasites treated with high concentration of doxycycline, were not able to rescue with the supplementation of exogenous IPP whereas such rescue could be attained with low concentration (1-3 μM) of doxycycline. In addition authors showed that parasite killing activity at first cycle relied on a metal dependent mechanism as supplementation of FeCl_3 rescued the parasites¹³. It is not known to what extent antibiotics affect the apicoplast of *Plasmodium* liver parasites. In a rodent model, it was showed that treatment of 10 μM fosmidomycin affected

apicoplast of *P. berghei* hepatic parasites and arrested merosome formation³⁷. This effect was also mirrored by the other drugs that target apicoplast in *Plasmodium* liver stages. In addition, gene knockout studies revealed that deletion of apicoplast protein PALM (*Plasmodium*-specific apicoplast protein for liver merozoite formation) and single pyruvate dehydrogenase (PDH) enzyme reduced the merosome formation and arrested blood stage infection^{38,39}.

In *Artemisia* infusion treated parasites, we observed apicoplast deformity where branching of ACP signaling had profusely shrunk or in aggregated forms compared to control. We were wondering if *Artemisia* infusion could damage the apicoplast integrity of hepatic *Plasmodium* forms albeit apicoplast DNA could be intact. Thus qPCR experiment was conducted and results showed that parasite nuclear DNA to GFP ratio was remained constant in the control group and infusion did not exert any effect on parasite nuclear DNA replication. On the other hand, *Artemisia* infusions and doxycycline strongly inhibited DNA replication in the apicoplast (reduction ratio of apicoplast DNA: GFP). In all respects, the effect of infusions over the *P. berghei* hepatic stage phenocopied doxycycline effect on apicoplast.

In our assays, *Artemisia* infusions killed the parasites and parasites that were not susceptible to treatments had reduced surface area. We quantified apicoplast morphologies with that remaining parasites in the culture and compared to controls to estimate the effect on apicoplast. Quantitative analysis confirmed that a strong effect on apicoplast could be exerted by the *Artemisia* infusions treatment. The observed effect on apicoplast morphology could also reflect by a block in parasite development. Consequently we could question the *Artemisia* infusions effect on parasite growth in respect to the apicoplast growth in two ways -

- i) *Artemisia* infusions somehow directly arrested the parasites development which led to limit the normal growth of apicoplast during hepatic schizogony.
- ii) *Artemisia* infusions could directly impair the apicoplast biogenesis and perturb the apicoplast function related to the rapid nuclear division of schizonts. This could affect the growth of schizonts yielding a reduced surface area. Indeed it was described that following extensive nuclear division of schizogony, apicoplast displayed highly intertwined branched structures although no close association of apicoplast with nuclei was identified⁴⁰.

Atovaquone (ATQ) and *Artemisia* infusion both demonstrated an effect on parasite area. When we compared the apicoplast morphology from these two drug groups, we found ATQ treated

parasites had smaller surface area with unaffected ACP signaling of branching network whereas infusion exposed parasites were resulted in defective apicoplast morphology with diffused ACP signaling.

To date hypnozoites resident apicoplast is partially described in literature but most of the description is related to the ACP immunolabeling to localize the apicoplast in fixed liver tissue samples. Recent transcriptomics data of hypnozoite predicated apicoplast mediated functions involved in maintaining the hypnozoite quiescence⁴². We examined apicoplast behavior in hypnozoites utilizing two relapsing malaria species *P. cynomolgi* and *P. vivax* following *Artemisia* infusion treatment. ACP labelling demonstrated a diffused or aggregated signaling display in *Artemisia* infusion treated hypnozoites. Moreover we observed a clear shifting paradigm of apicoplast morphologies following *Artemisia* infusion treatment. The first information we could get from the confocal images that hypnozoites do not develop synchronously in *in vitro* hepatic culture. At day 6-12 post infection, hypnozoite population could display different apicoplast morphologies relative to its growth. Young hypnozoites could have a punctate or dot shaped globular apicoplast structure which could be also observed in other early liver parasite forms including sporozoites. The single dot apicoplast could evolve into a single line elongated shape and subsequently they could attain a division form which could be defined as the matured or persistent hypnozoite. These hypnozoites could be found slightly bigger than the usual hypnozoites. Moreover it was also shown that hypnozoites could steadily grow both *in vitro* and *in vivo*. We speculated that this divided forms of apicoplast could be the terminal stage of apicoplast evolution in hypnozoites which presumably could be defined as a reactivated hypnozoite. However we observed a pronounced apicoplast disruption in hypnozoites due to *Artemisia* infusion treatment even at day 10 hypnozoites that resisted the infusions treatment albeit found having defective apicoplast.

Apicoplast function is essential to *Plasmodium* parasites thus mutant parasites lacking apicoplast are not viable¹. Exactly what causes parasites to die during apicoplast disruption is intriguing. Interestingly it was demonstrated that apicoplast minus strain or parasites with drug induced defective apicoplast could also grow and replicate in culture with the supplementation of IPP, the product of isoprenoid precursor biosynthesis^{24,43,44}. Taken into consideration, we applied IPP treatment in our hepatic experiments with equivalent concentration (200 μ M) of IPP used in blood stage experiments²⁴. We observed that IPP treatment could not rescue the liver parasites from being killed by *Artemisia* infusions treatment. In addition, it can be mentioned

that drugs that particularly inhibit the biosynthesis of IPP for instance fosmidomycin don't alter structural integrity of the apicoplast rather affect its function⁴². In several studies it has been showed that IPP is not only the essential pathway in the parasite apicoplast as supplementation of FeCl₃ rescued the doxycycline exposed parasites¹³. Thus we hypothesized that the faster killing activity of *Artemisia* infusions could be involved in targeting multiple organelles of *Plasmodium* parasites. Similar observation was also found in few antibiotics where the rapid killing of *Plasmodium* parasites could have off targets beyond apicoplast. In the case of *Artemisia* infusions, the metabolites and other natural bioactive compounds they possess could have other sites of action for instance the mitochondrion as apicoplast was found close proximity to the mitochondrion⁴⁵. In addition, if a given compound or drug is active on metabolic pathways, it is also imperative to define to what extent other related redundant pathways could be compensated for it⁶. The speculation is not surprising considering the diversity of phytochemicals and chemical identity produced by these two *Artemisia* species. Metabolic and phytochemical investigation of *A. annua* revealed that these plants contain significant amounts of terpenoids and flavonoids⁴⁶⁻⁴⁷. Terpenoids have gained particular interest due to it's ability to block the intraerythrocytic proliferation of *P. falciparum* parasites by interfering with the isoprenoid biosynthetic pathway in the apicoplast⁴⁸. Immunolabeling with a mitochondrial marker HSP60, we observed mitochondria following *Artemisia* infusion treatment. A distorted morphology of mitochondria was observed in *Artemisia* infusion exposed parasites.

Overall, our data highlights *Artemisia* infusion rapidly kills *Plasmodium* hepatic parasites by the partial interruption in the apicoplast biogenesis. The apicoplast inhibitory activity of *Artemisia* infusions evidenced in our study presumably comes from yet unidentified antiplasmodial compounds. Hence it warrants further investigation to find out the anti apicoplast component(s) from this medicinal plant.

Future research perspectives

1. Once hypnozoites are settled in *in vitro* culture or inside the host, killing them is quite impossible by the licensed antimalarials, except primaquine. However, it has not been

investigated whether these licensed drugs could have any effect on the hypnozoites apicoplast? Next question would come whether apicoplast deformity could lead to hypnozoite death? Does hypnozoite initiate the reactivation process with a defective apicoplast? Is hypnozoite apicoplast involved in maintaining the hepatic stage quiescence or any role playing activity in the reactivation process? What about the housekeeping apicoplast function maintained in hypnozoite during its reactivation?

2. IPP synthesis is one of the important apicoplast functions in blood stage parasites. To what extent IPP synthesis is essential in the liver stage as it is still unsettled.

3. Apicoplast plays an important role in haem synthesis. Previously haem synthesis was used to consider an essential pathway in *Plasmodium* blood stage parasites. However gene knock out and biochemical studies revealed that this pathway is not necessary in the blood stage; rather it's important for the development of liver stage parasites and sporozoite stage^{49, 50}. It could be also interesting to decipher the role of haem synthesis in hypnozoite apicoplast.

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Chapter 5

***in vitro* activity of *Artemisia* infusions against antimalarial drug resistant blood stage parasites**

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Abstract

Artemisinin, extracted from *Artemisia annua* (sweet wormwood), or its derivatives are considered to be solely responsible for the potent activity against the parasite's blood-stages. Recently decoctions of *Artemisia* dried leaves have been controversially advocated as cheaper traditional plant-based treatments for malaria. Such self-administered, unregulated and unsupervised use of treatments with variable and potentially sub-optimal artemisinin content is strongly discouraged, not least as this would promote the emergence of drug resistance. It has been suggested that the efficacy of such remedies also rests with additional plant compounds that can synergise to enhance parasite killing. This claim is supported by the purported anti-malarial traditional herbal teas based on the African wormwood *A. afra*; a species having minimal contents of artemisinin. We have exploited this difference to explore this issue through comparative *in vitro* assessment of the inhibitory activity of infusions from both plants on *Plasmodium*.

Introduction

Antimalarial drug resistance in the *Plasmodium falciparum* parasite poses a constant challenge as it has led to the declining efficacy of several first line treatments. Endorsed by the World Health Organization (WHO), ACTs (Artemisinin combination therapy) is now the first-line antimalarial treatment in many malaria endemic countries because of drug pressure from the conventional antimalarials^{1,2}. This ACT treatment consists of artemisinin derivatives, which kill blood stage parasites rapidly but have short biological half-lives in the blood. Thus combinations with a partner compound such as mefloquine, lumefantrine, amodiaquine or piperaquine are prescribed, which have longer half-lives and could clear the remaining parasites. The rationale behind the use of drug combinations is both to maximize treatment potency and to minimize the potential for resistance selection³⁻⁵. Unfortunately, over the last decade evidence has been made that artemisinin resistance has emerged on Thai-Cambodia border and then spread in the whole Greater Mekong Regions (GMS). In parallel, the emergence of multiresistant parasites leading to a dramatic decrease of ACT therapeutic efficacy have been demonstrated. ART-R is now emerging in Africa. This feature was recently described in Rwanda, with an increased circulation of K13 R561H mutant associated with artemisinin resistance. This clearly constitutes an early warning sign and could undermine malaria eradication effort⁶. In addition, the dominant strain, *pfk13* C580Y mutant lineage, originally

emerged in Cambodia with resistance to ART, but it acquired resistance subsequently to the partner drug piperazine. This surpassed other strains of drug-resistant malaria to exhibit dominance in the affected regions⁷⁻⁸. Thus activity against kelch13 mutants is a prerequisite for the development of new anti-malaria drug candidates

Most of the antimalarial medicines, like quinolines and endoperoxides derived from natural products⁹. *Artemisia annua* from which the first line antimalarial artemisinin (ART) class is extracted had been traditionally used as infusion forms to treat intermittent fever and malaria-like symptoms in the ancient world as folk medicine. Such traditional practices are still included and embellished in Chinese pharmacopoeia and also widely popular in a part of Africa subcontinent. Now a days, there has been a growing interest in the possible benefits of *Artemisia* infusions in particular areas where conventional drug therapies are not readily accessible or are too expensive to purchase¹⁰. *Artemisia afra*, another species of *Artemisia* family and indigenous to Africa reported having a negligible amount of artemisinin contents (even no trace amount) theoretically but phytochemical investigations of these plants revealed a rich amount of essential phytochemical constituents (e.g. at least 28 classes of monoterpenes, 30 sesquiterpenes, 36 individual flavonoid components, 7 coumarins and phenols¹¹. Many of these have antimalarial properties though they are far less potent than artemisinin, for example caticin, chrysoplenetin, chry-sosplenol-D and circilineol demonstrated to potentiate *in vitro* activity of artemisinin against *falciparum* malaria. Extracts of *Artemisia afra* were also found effective against *P. falciparum in vitro*¹². The activity could be guided by the complex mixture of flavonoids present in *A. afra* suggesting that non-ART compounds in this plant are still able to kill *Plasmodium* parasites. Hence the phytochemical research of this plant is very important in terms of discovering bioactive profiles that may have therapeutic importance against malaria. Moreover it was reported that *Afra* samples collected from different geographical localizations and dissolved into various solvents demonstrated different antioxidant properties leading to higher antimalarial activities. The IC₅₀, derived from hexane extract of *A. afra* treatment against *P. falciparum* strains D6 (CQ sensitive) and W2 (CQ resistant) are 3.18 µg/ml and 0.71 µg/ml respectively¹³.

In two *in vivo* rodent malaria models, mice were infected with artemisinin-resistant strain of *P. yoelii* to assess whether *A. annua* could overcome drug resistance and with another *P. chabaudi* strain (relevant *P. falciparum* model) to track the development of resistance. The result revealed a reduced parasitemia observed in mice treated with dried whole plant *Artemisia annua*

compared to the mice receiving pure artemisinin. Furthermore it took at least three times longer for a second rodent malaria parasite to select a stable resistance in the case of *A. annua* treatment compared to artemisinin¹⁴. Hence it is suggesting that the whole plant treatment would have an advantage compared to ACTs. The hypothesis for this superior activity would lie in the presence of non-ART antiparasitic compounds. Other features such as synergy with ART and ART bioavailability enhancer are also possible.

Altogether, these findings suggest that therapeutics based on the whole plant would thus not be equivalent to a monotherapy and the activity based not only on ART but on increase of its potency, improvement of its PK/PD parameters or acting against additional parasites targets.

Individuals infected with *P. falciparum* exhibited slower parasite clearance rates following ACT treatment due to kelch 13 mutations^{15,16}. Generally in the case of sensitive strains, *in vivo* parasite clearance rates by ART are typically 1-3 h but a subset population of rings induce dormancy in the resistant strains which yields prolonged parasite clearance rate (termed as resistance)¹⁷. It has been demonstrated that Kelch13 mutations do not lead to trophozoites resistance but associated with survivals of rings following ART monotherapy¹⁸. Mathematical modeling also hinted artemisinin resistance was due to ring stages that refractory to drug function¹⁹. So, *in vitro* RSA assay is considered a reliable indicator of the clinical parasite clearance after ACT treatment which added a great value in measuring clinical efficacy of drugs. In *in vitro* studies, artemisinin and DHA were described to involve in reduced susceptibility and dormancy²⁵.

Decoctions of *Artemisia* dried leaves have been controversially advocated as cheaper traditional plant-based treatments for malaria. Such self-administered, and unregulated use of, treatments with variable and potentially sub-optimal artemisinin content is strongly discouraged, not least as this would promote the emergence of drug resistance. The presence of numerous phytochemicals in these plants with proven evidence on *Plasmodium* blood stage activity in comparison with commercially available ART drug, no study to the best of our knowledge has been explored so far, on their activity on the ACT resistant parasites. We did RSA assay to compare infusions prepared from artemisinin containing species (*A. annua*) and a non-ART containing species *A. afra* against resistant rings of *falciparum malaria*.

Methods

Preparation of *Artemisia* infusion

Artemisia afra (PAR, voucher ID: LG0019528 Université de Liège) and *Artemisia annua* (LUX, voucher ID: MNHNL17733 Herbarium Luxembourg) were collected as leaves and twigs and were preserved at room temperature. Both products were protected from sunlight until *Artemisia* infusion preparation.

However, the final decoction recipe was followed by weighing 2.5 g *Artemisia* dried leaves along with twigs, pouring in pre-boiled 25 ml commercial drinkable water (Crystalline Company) in an Erlenmeyer flask. The mixture was boiled at boiling point with a magnetic stirrer for 5 minutes and allowed to cool for 10 minutes. The decoction was passed through a 40 µm cell strainer (Falcon, Corning Brand) in order to remove bigger debris of plant materials. After that, the decoction samples were centrifuged at 3000 rpm for 10 minutes to pellet down the smaller debris. Finally, 0.20 µm membrane filter (CA-Membrane) was used which provided debris free *Artemisia* decoction. The decoction was stored at 4°C (short term storage), -20°C (long term storage). However, during drug (*Artemisia*) preparation, the decoction was diluted with fresh blood.

Ring stage Survival assay (RSA 0-3h)

Following the modified protocol developed by Witkowski et al ²⁰ culture-adapted parasites were synchronised twice with 5% sorbitol (Sigma-Aldrich, Singapore) at 40 h intervals. Synchronous 10–12 nuclei schizonts were incubated for 15 min at 37°C in RPMI-1640 supplemented with 15 U/mL of sodium heparin (Rotexmedica, Luitre, France) to disrupt agglutinated erythrocytes, purified on a 75% Percoll (Sigma-Aldrich) discontinuous gradient, washed in RPMI-1640, and cultured for 3 h with fresh erythrocytes. Cultures were treated with 5% sorbitol to eliminate remaining schizonts, adjusted to 2% haematocrit and 1% parasitaemia by adding uninfected erythrocyte. The RSA^{0-3h} was done immediately with 0–3 h post invasion rings. In each assay, parasites were exposed to 700 nM dihydroartemisinin, 0.1 % dimethyl sulfoxide for 6 h, washed with 12 mL RPMI-1640 to remove drug, re suspended in complete medium (RPMI-1640, 0.5% Albumax II, 2% heat-inactivated B+ plasma, 50 µg/mL gentamicin), and cultured at 37°C in a tri-gas atmosphere (5% CO₂, 5% O₂, 90% Ar). Thin blood smears were prepared and stained with 10% Giemsa (Merck, Darmstadt, Germany) for 20 min.

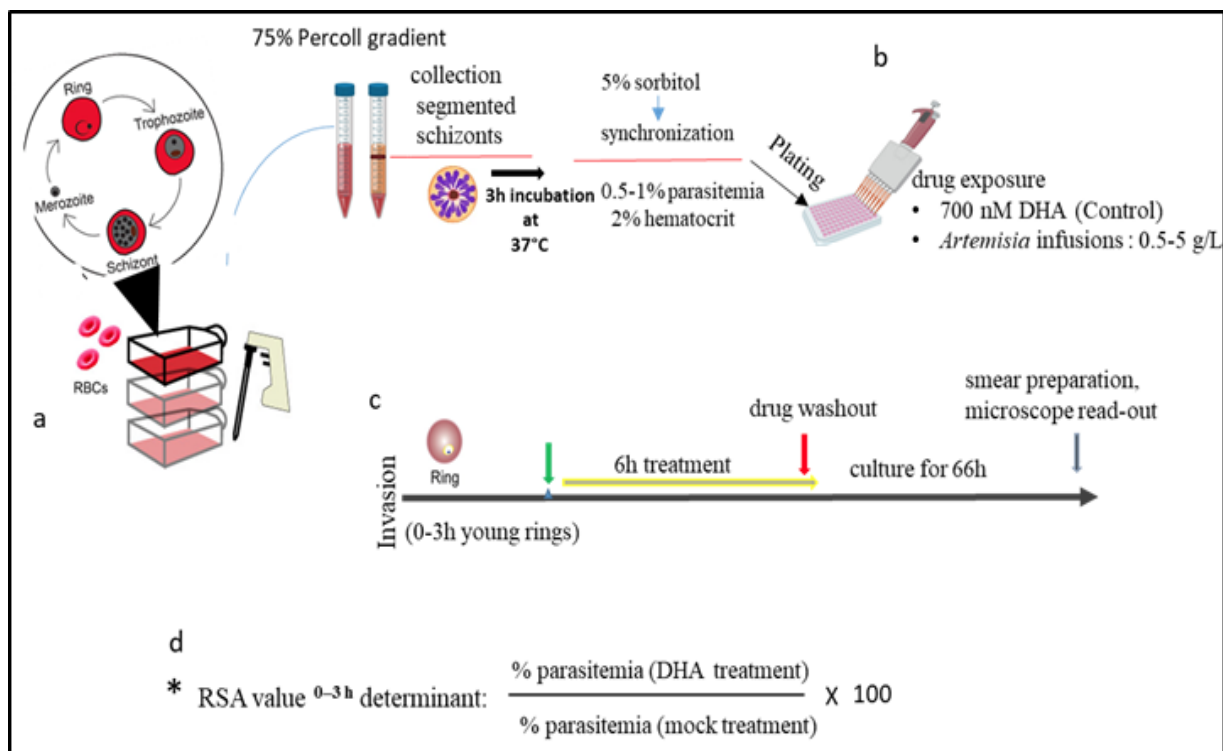


Figure 1: Schematic outline of parasite synchronization to 0-3h invasion rings and ring survival assay¹⁴ (RSA^{0.3h}) was performed with *P. falciparum* culture adapted parasites derived from patient blood. a) Cryopreserved *P. falciparum* parasites were thawed and allowed to increase the parasitemia by changing the culture medium. When ring stage parasitemia reached upto 2%, cultures were processed for D-sorbitol treatment after 30-35 hours of culture. By this time, the proportion of mature schizonts (10-12 nuclei) to be > 0.5%. A percoll gradient step allowed to isolate segmented schizonts that were kept in a 37°C incubator. After incubation, some schizonts might yet to invade; thus, parasites were treated with 5 % sorbitol again to remove remaining schizonts and isolate 0–3 h ring-stage parasites. Finally the parasite mixture was adjusted to 1 % parasitaemia b) drugging DHA and *Artemisia* infusions ranging from 0.5 g/L to 5 g/L c) young rings (0-3h) were exposed to DHA and *Artemisia* for 6h and drugs were washed out after twice wash. Cultures were maintained in a 37°C incubator in a drug free medium for another 66h. Blood smears were prepared and counted the parasitemia under an inverted microscope d) RSA value was calculated in percentage using this equation.

Quantification of ART in infusion: MS/MS Detection

MS/MS experiments were carried out using the Shimadzu UHPLC system described above coupled to a Shimadzu LCMS-8050 triple quadrupole mass spectrometer using the multiple reaction monitoring (MRM) technique operating in positive ion mode. The following parameters were used for all experiments: electrospray interface voltage was 3 kV; heat block temperature of 350 °C; desolvation line temperature of 300 °C; interface temperature of 300 °C; drying gas flow gas was set at 5 L/min; and nebulizing gas flow at 3 L/min, heating gas flow rates were set at 15 L/min. Q1 and Q3 were set to unit resolution. The dwell time was set at 1 ms for each MRM transition; optimal CE values were chosen to obtain the most characteristic fragments.

Initial <i>Artemisia</i> Infusion	ART concentration in infusion samples	ART concentration in the infusion used for hepatic cultures
100 g <i>A. annua</i> leaves and twigs/L	$6.07 \pm 0.01 \mu\text{g/mL}$ = $21.5 \pm 0.03 \mu\text{M}$	0.607 $\mu\text{g/mL}$ (from a 10 g/L infusion) = 2.15 μM
50 g <i>A. afra</i> leaves and twigs /L	ca. $0.071 \pm 0.007 \mu\text{g/mL}$ = $251 \pm 25 \text{ nM}$ ($<$ limit of quantification)	ca. 0.0028 $\mu\text{g/mL}$ (from a 4 g/L infusion) = 10 nM ($<$ limit of quantification)

Table 1. Quantification of ART in *A. annua* and *A. afra* infusion by UHPLC-MS.

Result and Discussion

In this context, we examine whether infusions prepared from *Artemisia* plants exert any *in vitro* antimalarial potency against drug resistant parasites. Very few studies reported previously that *A. annua* infusions showed potent activity against chloroquine resistant lines ²¹. Most of their results are contradictory and difficult to interpret. Moreover, to our best of knowledge, no study has been performed with *Artemisia* infusions against the ACT resistant strains of Greater Mekong Regions which is a great concern and center of malaria elimination. To overcome this, there is an urgent need to develop a novel drug that is safe, cost-effective and well tolerated among all for mass administration.

In our experimental RSA assay, blood stage parasite cultures were enriched with 0-3h fresh rings after tight synchronization. *A. annua* and *A. afra* infusions ranging from 0.5-5 g/L were tested against 0-3 hours post invasion young rings in a 6h treatment window. We chose 5 drug resistant strains and one drug sensitive strain (3D7). All of the resistant strains were freshly isolated from patient blood. Genomic analyses of these resistant strains revealed multiple copies of copy number variations of plasmepsin 2 gene family and multi drug resistance 1 gene family. In fact differences in *pfmdr* copy number are connected to changes in clinical efficacy of several drugs. Increases in copy number are associated with increased risk of treatment failure^{22,23}. A pronounced inhibitory activity of *Artemisia* infusions which was severe with *A. afra* was observed against experimental *P. falciparum* strains. Drug sensitive strain (3D7) was found highly susceptible to *A. annua* infusion. In 3D7 strain, nearly 25% of ring stage parasites could survive at 1.25 g/L exposure of *A. annua* infusion while a complete annihilation of rings was observed by 2.5 g/L of *A. annua* infusion treatment. However in drug resistant isolates, *A. annua* infusions showed a dose dependent inhibitory activity. All resistant isolates responded to similar antimalarial effect to *A. annua* infusion treatment. Treatment with 5 g/L *A. annua* against resistant isolates resulted in (<13%) survival rate of rings. DHA exposed culture showed reduced susceptibility to rings with 10-16% survival of rings. In addition, water as solvent used here in this study did not show any interference in the survival of ring parasites. It could be noted that both DHA and 5 g/L *A. annua* infusion exerted almost similar activity against young rings of the *kelch* 13 mutant strains (**Fig. 2A**).

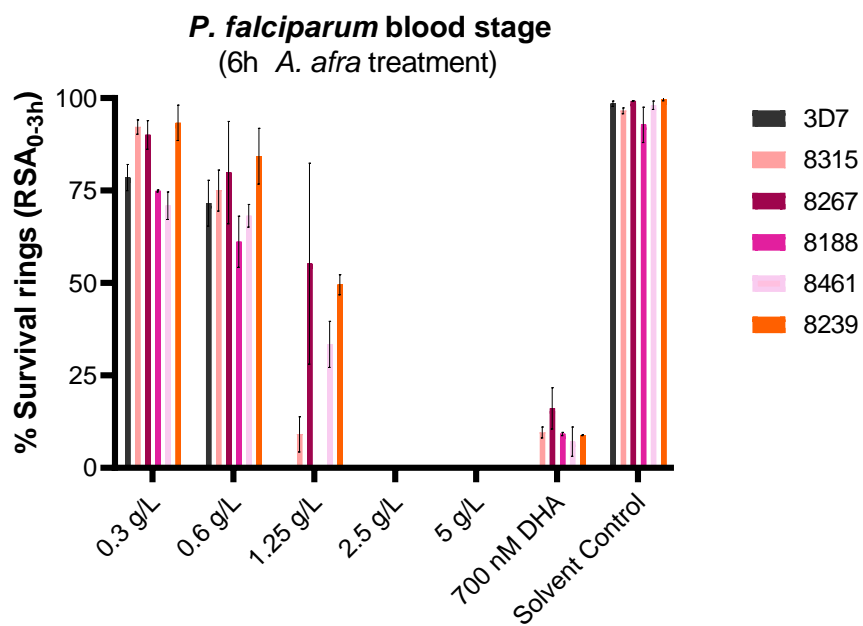
Furthermore, we also assessed *A. afra* activity on the resistant rings with the similar isolates used for *A. annua* experiments. In 3D7 strain, *A. afra* completely killed the rings at 1.25 g/L concentration and strain 8315 and 8188 also remarkably reduced the survival of rings. A complete clearance of rings was observed in all resistant strains at the 2.5 g/L concentration of *A. afra* infusion. In all resistant isolates, DHA retained its reduced susceptibility to rings ranging from 10-12 % survival of rings (**Fig. 2B**).

Altogether, our preliminary RSA assays revealed that *A. afra* involved in significant killing rate of rings than *A. annua* at the equal concentration of infusion against drug sensitive and resistant isolates of *P. falciparum*. At 2.5 g/L, *A. afra* killed all resistant rings from culture whereas *A. annua* 5 g/L was not sufficient enough to eliminate these forms which can be attributed as reduced killing activity of *A. annua*, similar to DHA. From the phenotypic point of view, DHA and *A. annua* exhibited several distinct morphologies of rings²⁵. Giemsa staining revealed most of the parasites were as pyknotic forms with damaged nuclei without any distinguishable cytoplasm. Ring parasites treated with 2.5 g/L *A. afra*, completely cleared the rings in the culture (Fig. 2C, D).

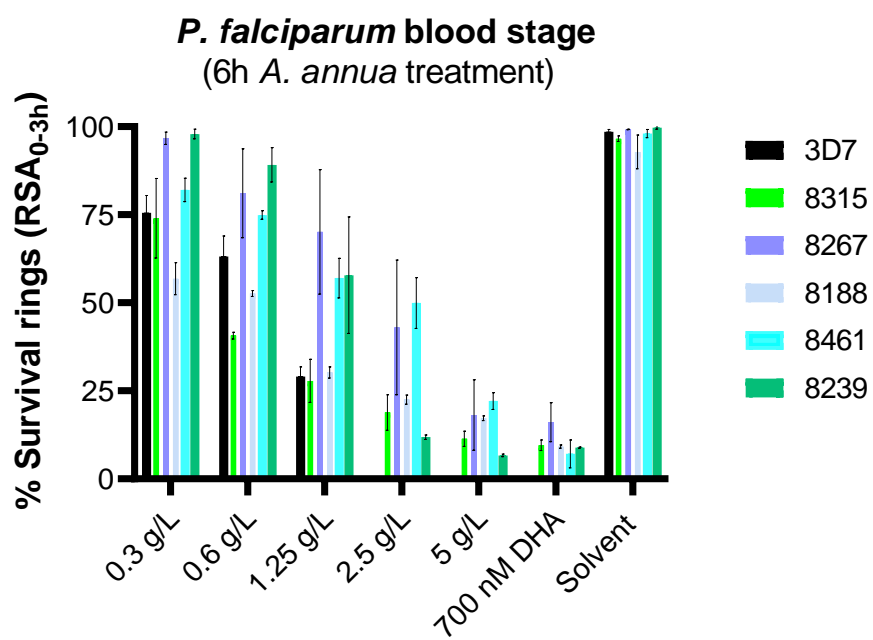
A

<i>P. falciparum</i> strains		K13	MDR1 CNV	PM2 CNV
3D7		Drug Sensitive		
8315	fresh	C580Y	1	2
8267	fresh	C580Y	1	1
8188	fresh	C580Y	1	4
8461	fresh	C580Y	3	1
8239	fresh	C580Y	1	4

B



c



D

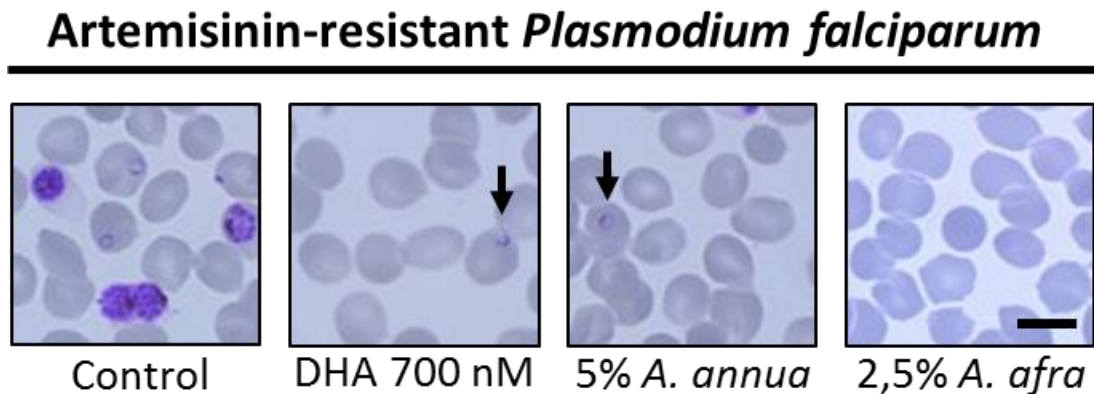


Figure 2: *Artemisia* infusion efficacy against the resistant rings of *P. falciparum*

A) Genomic information of resistant isolates identifying copy number variations (CNV) in different resistant isolates of *P. falciparum* (B-C) Bar chart depict the result of ring survival assay $0-3h$ performed with a view to understanding *Artemisia* infusion efficacy against drug resistant rings. The *Artemisia* treatment window was for 6h against $0-3h$ post invasion rings. Survival of rings expressed in percentage. Each isolate repeated twice for RSA experiments and results expressed as mean of two independent experiments of each isolate. Water as solvent for the infusion preparation of *Artemisia* D) microscopy images showing differential effect of *annua* and *afra* against ART-R rings.

The potent antimalarial activity of *Artemisia* infusions against drug sensitive blood stages was consistent with our liver stage findings (unpublished). Interestingly, we evidenced a marked contrast in terms of infusion activity against drug resistant parasites. At equal concentration of treatment, *A. annua* loss its potency while *A. afra* cleared the resistant rings. An exposure to 1.25 g/L *A. afra* clearly shown a pronounced anti-malarial activity against the resistant lines. However, faster parasite killing antimalarials are considered to have lower proneness to *de novo* resistance²⁴. In contrast, *A. annua* at maximal concentration of 5 g/L was not effective enough to completely eliminate the ring parasites. However, we could not rule out the probability of the fact that the limited activity of *A. annua* infusion might partially due to the presence of artemisinin in our infusion samples (approximately 1.075 μ M of artemisinin was quantified in

5 g/L *A. annua* infusion). In contrast *A. afra* was found effective at lower infusion percentage against resistant rings and more strikingly it reduced parasitemia below threshold detection in *Plasmodium* erythrocytic culture. Such antimalarial activity of *A. afra* against resistant parasites may greatly unfold for the identification of novel compound(s) that might active against artemisinin resistant parasites as the plant species reported to be negligible amount of artemisinin.

Conclusion & perspective

In line with discussion above, the artemisinin-independent anti-plasmodial effects exerted by *A. afra* infusions suggest the presence of uncharacterized and potent antiparasitic molecules. This hypothesis is worth being investigated through rigorous drug discovery investigations. Most antimalarial drugs that are now in use were not developed on the basis of rationally identified targets, but following the serendipitous identification of the antimalarial activity of natural products. Thus a systematic extraction-fractionation to identify other valuable non-pharmaceutical forms of artemisinin (ART) from these *Artemisia* plants that can overcome the resistance problem.

Artemisia infusions disrupted apicoplast biogenesis in *Plasmodium* liver and blood stages (unpublished data). Clearly *Artemisia* plant species contain compounds that could target the *Plasmodium* apicoplast. Nowadays apicoplast is considered as a unique drug target of malaria due to its absence inside the host. Genome wide association studies (GWAS) identified *P. falciparum* apicoplast ribosomal protein S10 in artemisinin resistant isolates²⁶. In contrast, no mutations were detected in the *P. falciparum* apicoplast ribosomal protein S10 precursor in newly emerged *Pfkelch13*-mediated artemisinin resistance in Rwanda⁶. Moreover, a study has demonstrated apicoplast link in artemisinin resistance²⁷. It has been described that DHA induced quiescent parasites also retain the apicoplast functions such as FAS II fatty acid synthesis type II pathway^{28, 29}. Thus, this metabolic activity of apicoplast could be targeted by *Artemisia* infusion component flavonoids³⁰ as *Artemisia* plant is reported to contain abundance of flavonoids¹². Overall, it could be interesting to explore further whether *Artemisia* infusion could disrupt the apicoplast of resistant parasites in a similar fashion in wild type that could additionally reveal underlying resistance biology linked to apicoplast.

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General Discussion

Hypnozoite culture of relapsing malaria species and radical cure drug screening sometimes encountered with technical difficulties associated with microbial contamination. That's why cultures crash phenotypically too quickly and no results can be extrapolated and restored particularly when fungus emerge in the culture. This causes a financial impact in frequent hypnozoite research as commercial cryopreserved hepatocytes and logistics tools are expensive. The mischievous contamination could arise from two sources: 1) contaminated culture reagents or hepatocyte processing during harvest 2) contaminated sporozoites. The first contamination source could be tackled by incorporating different antimicrobial cocktails in cell culture medium but some of them already demonstrated anti-parasitic activity especially with widely used antifungal agent amphotericin B¹. In addition, study showed that preventing fungal contamination in hypnozoite culture requires a great deal of antibiotics in combination with antifungal agents which is not cost effective². Contaminated sporozoite is another confounding factor that can cause hepatic contamination. The source is more evident as sporozoites are developed in the salivary gland of mosquitoes and *Anopheles* mosquitoes are always exposed to huge communities of microbiota in their breeding sites, in their aqueous feedings and inside midguts³. Previous studies reported that salivary glands of mosquitoes take part in food digestion⁴ and so, if their feeding sources are contaminated with microbes by any means that could contaminate sporozoites and thereby curtail parasite development and infection. Moreover, sporozoites are released from salivary glands with hand dissection which could also contribute to the contamination. To alleviate the contamination problem, we improvised mosquito feeding protocol with supplementation of antimicrobial agent methylparaben and penicillin-streptomycin in sucrose solution. We showed that modified feeding diet was effective in aborting unwanted pathogens in feeding solutions ensuring sterilized mosquito diet whereas regular feeding diet was filled with yeast, bacteria and other contaminating microbes. Moreover, the feeding strategy did not affect the normal oocyst development, maturation, and sporozoite production in the salivary glands of mosquitoes. In addition, we retrieved a significant number of sporozoite yield from the MP-PS feeding mosquitoes compared to the non-treated group. Besides, microbial analyses confirmed yeast free sporozoite production in the salivary glands due to MP-PS feeding. It can be noted that our employed MP-PS strategy could not completely annihilate bacterial load particularly *Elizabethkingia miricola* in sporozoite suspension. Practically, compared to yeast, this is not a serious concern as routine hepatocyte culture always includes penicillin-streptomycin or gentamycin and these can efficiently mitigate the risk of bacterial contamination. Recently, an antibiotic vancomycin was reported to improve *P. yoelii* hepatic cultures by controlling *Elizabethkingia* sp⁵. We also

showed that hepatocyte cultures infected with sporozoites, isolated from MP-PS mosquitoes retained hepatic physiology for weeks without contamination. Thus MP-PS protocol favored the yeast free sporozoite production which facilitated maintenance of a long term culture, essential for hypnozoite study and drug screening against this parasite stage.

Currently, there are very few novel antimalarial in clinical trials that could solve the relapse and resistance problems of current drugs. Undoubtedly relapse and resistance (2R) compromise malaria eradication efforts⁶. Clinical data suggest that the problems arise from the treatment failure, drug resistance and lack of a safe drug for the community especially *vivax* infected G6PD individual patient. Those patients cannot be treated with licensed anti-hypnozoite drugs; primaquine and tafenoquine⁷. Thus hypnozoite mediated relapses evoke recurrent *P. vivax* infections. So the demand for well tolerated and globally deployable anti-hypnozoite malarial drugs are in demand. Due to the high cost of developing novel drugs, drug repurposing or natural sources could be promising alternative methods of treatment.

There is a consensus among the malaria community that natural products have a dominant presence in discovering new leads for the development of drug treatment. Drugs derived from natural sources, especially herbs and bacterium represent a significant proportion of the pharmaceutical market, particularly in malaria therapy where 11 drugs out of the 15 antimalarials included in WHO therapeutic schemes for malaria treatment are either natural products or their analogues or were design-based on the pharmacophores from natural products⁸. The great importance of natural sourced based drugs for malaria treatment is highlighted by the discovery of quinine (derived from Cinchona tree), artemisinin (derived from *Artemisia annua*) and synthetic compound atovaquone (Malarone[®]), analogue of lapachol from the *Tabebuia* species and antibiotics like Azithromycin, doxycycline are also derived from bacterium⁹.

The frontline antimalarial artemisinin, purified from the plant *Artemisia annua*, and its synthetic derivatives are the most potent and rapidly acting anti-malarials known to date. Unfortunately the compound is not adequate for the treatment of *vivax* malaria particularly ineffective against relapse causing hypnozoites¹⁰. On the other hand, plant derived compounds such as morphanin,¹¹ and metaformin¹² showed potent antimalarial activity against the *Plasmodium* liver stage. The plant *Artemisia sp.* is enriched with phytochemicals mainly flavonoids, mono-terpenes and secondary metabolites including yet unknown compounds

which have been reported with anti-malarial properties. Thus the plant offers a great avenue to screen its' parts for future need in the context of next generation anti-malarial drug discovery.

To date, the investigation of *Artemisia* infusions mainly were conducted on the erythrocytic stages. The clinical results are not consistent and difficult to interpret. In addition there is no relevant information related to hepatic stage efficacy of *Artemisia* infusions. Although artemisinin was tested against the hepatic stage of rodent malaria and found a limited activity¹³¹⁴. Consequently utilizing diverse *Plasmodium* species including hypnozoite forming species, we have exploited this difference to explore this issue through comparative and extensive *in vitro* assessment of the inhibitory activity of infusions from both plants on *Plasmodium*. Unexpectedly we observed a pronounced inhibitory activity against both malaria stages and we showed that this is partially mediated by the disruption of apicoplast in the liver and blood stage parasites most strikingly in the hypnozoites which compromise the global malaria eradication efforts. Thus the antiplasmodial effects were essentially artemisinin independent. Furthermore, the infusions significantly debilitated human malaria causing *P. falciparum* sporozoites *in vitro* and also reduced liver load of relapsing parasites. This provides the first indication that compounds other than 8-aminoquinolines could be effective against relapsing malaria and thus *Artemisia* sp. merit to further investigations

We also tested *Artemisia* infusions in *P. falciparum* erythrocytic stage against the young rings of drug resistant Cambodian isolates. A 6h treatment window of *A. afra* cleared rings in the culture whereas RSA_{0-3h} value of *A. annua* was similar to DHA. We could hypothesize that the reduced activity of *A. annua* could be the presence of artemisinin in this sample. In addition. *A. afra* activity suggest that non-ART extracts or compounds could be effective against the resistant parasites.

Taken together all insightful observations, therefore we believe that it is imperative to scrutinize *Artemisia* plants and identify another artemisinin like lead component(s) that was discovered by Tu Youyou few decades ago. Moreover, commercial artemisinin extraction from *Artemisia annua* plant is considerably low (0.5-1%)¹⁵ and considering the plant materials minimization and realizing the decreased sensitivity of artemisinin may be *Artemisia afra* can be an alternative source to develop next generation antimalarial. For this, a systematic bio-guided fractionation from the *Artemisia* infusion may lead to develop synthetic and semi synthetic compounds that may confer multi stage protection of malaria and thereby enrich our

antimalarial arsenal. Noted that in our preliminary study with *Artemisia* extracts demonstrated significant inhibitory activity of rodent parasites (See ANNEX)

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CONCLUSION & PERSPECTIVE

The core objective of this study is not to promote or encourage the herbal infusions as pre-clinical validation of antimalarial therapy or to discourage the current efficient ART-based therapies. We rather tried to discern whether the infusion component(s) could exert any anti-hypnozoite activity thus it can facilitate the discovery of next generation antimalarial. Hence, the idea to screen this treasured plant for a specific effect (most strikingly for an anti hypnozoite effect) without any clue is not rational. In fact the pros and cons of ACT over the holistic infusion-based medicines against *Plasmodium* infection is beyond the discussion of this paper and has been done before by WHO and other health experts.

We present the first comparative assays on the activity of infusions from the two plants (one with and without artemisinin) on the pre-erythrocytic stages of four different species of malaria parasites including those that form hypnozoites, the dormant liver stages that cause relapses. The data clearly demonstrates potent inhibitory activity that is independent of artemisinin, most likely mediated by disruption of the biogenesis of the parasite's apicoplast and mitochondrion, an activity consistent with both prophylactic and therapeutic action. Of particular interest, this activity extended to the formation and development of hypnozoites that hitherto could only be achieved by 8-aminoquinolines (primaquine and the recently licensed tafenoquine) whose widespread use is restricted by potential severe toxicity. Our *in vitro* observations are clearly insufficient to support claims of *in vivo* prophylactic or curative efficacy for any traditional remedy from the two *Artemisia* plants. They do, however, confirm the presence of hitherto unknown plant components with potent inhibitory activity. This warrants systematic bio-guided screening to identify novel lead antimalarials, and in particular those that can safely prevent *P. vivax* relapses. Drugs that could supplant 8-aminoquinolines, the only drugs currently capable of exerting hypnozoitocidal activity. In fact *Artemisia* plants contains vast amount of flavinoids. Flavonoids such as fisetin, kaempferol, luteolin, myricetin and quercetin were reported to inhibit Fab1, FabZ. These enzymes are required for fatty acid biosynthesis. Fatty acid biosynthesis II was reported to be involved in liver apicoplast functions. Considering this, we anticipate that the data will be of significant interest to the malaria community as they open the prospect of uncovering novel antimalarial compounds, not least one that would be effective at preventing the relapses that impede the control of *P. vivax*.

ANNEX 1

ARTICLE 2:

A thienopyrimidinone derivative shows multi-stage activity against *Plasmodium falciparum*, including artemisinin-resistant parasites

(Manuscript under revision in Microbiology Spectrum)

Gamhepathiopine”, based on an original pharmacophore belonging to the thienopyrimidinone series and highlighting a remarkable anti-malarial activity profile toward *Plasmodium falciparum*. In previous studies it was showed that the molecule showed profound inhibitory activity against erythrocytic, hepatic and asexual stages of *P. falciparum*.

Till date a vast number of new potential antimalarial compounds have been developed to overcome the eventual failure of the frontline treatments. But many of the antiplasmodium drugs are stage specific where they target target pathogenic blood stages of the parasite in humans and the efficacies of these drugs are compromised by the inevitable resistance. Hence drugs active other life stages of malaria including asymptomatic liver stage is highly desirable. It can be mentioned that drugs effective to inhibit the growth of sexual stages and liver parasites show the potential to reduce the transmission and chemoprotection. Several antimalarial thienopyrimidine derivatives, and their chemical moieties showed antipalmodial activity. Based on previous activity of Gamhepathiopine, it could be the good starting tool for the development of next generation antimalarials. Although its rapid metabolitic activity by cytochrome liver enzymes suggesting to develop chemotypes and refinement of this compound may be that effective as more potent multi-stage antimalarials.

A thienopyrimidinone derivative shows multi-stage activity against *Plasmodium falciparum*, including artemisinin-resistant parasites

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ABSTRACT

Human malaria infection begins with a one-time asymptomatic liver stage followed by a cyclic symptomatic blood stage. For decades, the research for novel antimalarials focused on the high throughput screening of molecules that only targeted the asexual blood stages. In a search for new effective compounds presenting a triple action against erythrocytic and liver stages in addition to the ability to block the transmission of the disease *via* the mosquito vector, 2-amino-thienopyrimidinone derivatives were synthesized and tested for their antimalarial activity. One molecule named Gamhepathiopine (M1) showed potent *in vitro* good activity against both erythrocytic and liver forms of the malaria parasites. Furthermore, Gamhepathiopine efficiently blocked the development of the sporogonic cycle in the mosquito vector by inhibiting the exflagellation step. Moreover, M1 is active against artemisinin-resistant forms and especially at the quiescent stage. Nevertheless, in mice, M1 showed modest activity due to its rapid metabolization by P450 cytochromes into inactive derivatives, calling for the development of new parent compounds with improved metabolic stability and longer half-lives. These results indicate that Gamhepathiopine, a new chemical entity belonging to the thienopyrimidinone series, is an excellent starting point to search for novel drug candidates displaying multi-stage activity and original mechanism of action with the potential to be used in combination therapies for malaria elimination in a context of artemisinin-resistance.

Keywords: Antimalarials, 2-amino-6-phenyl-thienopyrimidin-4(3*H*)-one, multi-stage activity, artemisinin resistance.

INTRODUCTION

Malaria remains the most prevalent tropical disease worldwide with a high mortality and morbidity: 229 million clinical cases and 409,000 deaths in 2019 according to the WHO (1). Beyond such human burden, malaria also has a dramatic social and economic impact on the development of 87 malaria-endemic countries (1). Resistance to antimalarials, especially in *P. falciparum*, is a first serious problem that threatens to undermine the efficacy of all commercial antimalarial drugs, including the artemisinin components of the first-line artemisinin-based combination therapies (ACTs) that are recommended by the WHO (1-3). A second main issue is that very few antimalarial drugs are effective against the liver stage of the parasite, mainly 8-aminoquinolines (primaquine and tafenoquine) and atovaquone (naphthoquinone derivative). However atovaquone tends to select resistant clones among blood stages of *P. falciparum* while primaquine and tafenoquine carry a high risk of acute life threatening hemolysis in G6PD-deficient patients, limiting the prophylactic use of these drugs (4-6). Moreover, recent studies showed that primaquine is ineffective in people with low metabolizing cytochrome P450 2D6 genotypes (7). A third main limitation to the chemotherapeutic fight against malaria is the fact that, among commercial antimalarial drugs that are available today, 8-aminoquinolines are the only ones able to block transmission by mosquito vectors, thanks to their activity on gametocytes, but are not clinically used in this indication (6). Thus, in May 2015, the WHO developed the global technical strategy for malaria 2016-2030. The pillar 2 of this strategy is to accelerate efforts toward the elimination of malaria and attainment of malaria free status. Evidence is the fact that malaria elimination will require new strategies to target the hepatic stage, the erythrocytic stage and the gametocyte development stages to prevent transmission to vectors, but also to target the sporogonic stages inside the vector to prevent transmission to a new human host (2, 8, 9). The hepatic and sporogonic stages of the malaria parasite have remained largely under exploited as antimalarial targets, due to the poorly understood biology

of these life cycle stages and the inherent technical difficulties in studying them (9, 10). Thus, the scientific community is nowadays actively developing and running drug screening campaigns that target liver and/or sporogonic stages in addition to the erythrocytic stage. In 2021, considering the drug portfolio of Medicines for Malaria Venture (MMV) (11), these research efforts allowed five novel chemical entities (**Fig. 1**) displaying new mechanisms of action and multi-stage activities to enter clinical studies. Artefenomel (OZ439) is a 1,2,4-trioxolane derivative (12) that has reached phase II in combination with ferroquine (13). Ganaplacide (KAF156) is an imidazolopiperazine derivative (14) that has reached phase II in combination with lumefantrine (15). Cipargamin (NITD609) is a spiro-indolone derivative inhibiting the P-type ATPase *Pf*ATP4 (16) and that is studied in phase II (17). MMV048 (MMV390048) is a bipyridine derivative inhibiting the phosphatidylinositol-4-kinase *Pf*PI4K (18) and that is clinically studied in phase II (19). Finally, M5717 (DDD107498) is a quinoline derivative showing multi-stage activity (20) through the inhibition of the translocation elongation factor 2 eEF2 and has reached phase I. Regarding the promising molecules that are studied at a pre-clinical stage, it appears that plasmodial kinases are quite promising targets (21, 22), as recently demonstrated by Birkholtz and co-workers in the search for novel gametocytocidal compounds (23) or by Tobin *et al.* who identified the cyclin-dependent like protein kinase *Pf*CLK3 as a main drug target for developing novel multi-stage antimalarial drugs (24). In 2015, our group reported that a new compound from a chemical library containing kinase inhibitor candidates and belonging to the thienopyrimidinone series was active *in vitro* toward the blood stage of *P. falciparum* ($EC_{50} = 35\text{-}200$ nM) and the liver stage of *P. yoelii* ($EC_{50} = 35$ nM) infecting HepG2-CD81 cells (25). It was also shown that the mechanism of action (still under evaluation) of this new compound was not common with the main ones described for commercial antimalarials (inhibition of heme crystallization, inhibition of *Pf*DHFR, action on mitochondrial membrane, free radical production). This compound was

named “Gamhepathiopine” (noted “M1” in Tables and Figures) because of its multi-stage antiplasmodial activity that will be described in the present work: “Gam” for its gametocytocidal activity, “Hepa” for its activity on the hepatic stage and “Thiopine” as a contraction of thienopyrimidine. It is to note that several antimalarial thienopyrimidine derivatives, especially bearing a phenyl substituent at position 6 of the scaffold, were reported in the literature (25-28), one of them being presented as a potential *Plasmodium* kinase inhibitor (26); their structures are presented in **Fig. 2**. Then, we decided to extend the study of the antimalarial activity of M1 toward i) hepatic stages of *P. yoelii*, *P. cynomolgi* and *P. falciparum* in murine, simian and human primary hepatocytes *in vitro* along with *P. yoelii in vivo*, ii) gametocytes and sporogonic stages along with the transmission-blocking effect and iii) artemisinin-resistant *P. falciparum* parasites in proliferation or quiescence state, to evaluate the global potential of M1 as an antimalarial candidate.

RESULTS

1) *In vitro* experiments

1.1) Inhibition of the liver forms of *Plasmodium*

In vitro liver stage assays were conducted to determine if M1 possessed activity against *Plasmodium* parasites. M1 was found to be quite active against the liver stage of *P. yoelii*, *P. falciparum* and *P. cynomolgi in vitro*. As presented in **Table 1**, the activities of M1 ($EC_{50} = 0.45 - 1.5 \mu\text{M}$) were very close to the ones of primaquine ($EC_{50} = 0.6 - 2.7 \mu\text{M}$). Regarding *P. falciparum* in human hepatocytes and *P. cynomolgi* in simian hepatocytes, the liver stage development was fully inhibited from $14 \mu\text{M}$ ($EC_{50} = 450 \text{ nM}$ and 1500 nM respectively). Nevertheless, at all tested concentrations ($0.1 - 50 \mu\text{M}$), M1 was not active on *P. cynomolgi*

hypnozoites. This inhibition property unlikely results from a cytotoxic effect of M1 on hepatic cells as the drug concentration affording 50% of cell viability (also called CC_{50} , Table 1) was found higher than 60 μM towards all primary hepatocytes (murine, simian and human), concentration higher than most of the ones used in the experiments.

In another approach, we evaluated the effect of M1 on parasite size (μm^2). Comparison of the size of schizonts developing in primary hepatocyte cultures after infection showed that M1 significantly reduced the size of the parasites (**Table 1**). The effect of primaquine and M1 on parasite size was significant only from 10 μM . At this concentration, M1 led to a size reduction of 100% for *P. yoelii* and 80% both for *P. falciparum* and *P. cynomolgi* while primaquine led to a 100% reduction for *P. falciparum* and 70 % for *P. cynomolgi*. In order to determine the effect of M1 on the invasion of hepatocytes by sporozoites, we exposed *P. falciparum* sporozoites to 1, 10 and 100 μM of the drug for 1 h at room temperature before incubating them with primary human hepatocytes. Three hours later, after sporozoites penetration into hepatocytes, cultures were washed and further incubated with or without the presence of the drug for 6 days (**Fig. 3**). Our results indicated that M1 inhibited the invasion of sporozoites in hepatocytes in a dose-dependent manner: 30% at 1 μM , 55% at 10 μM and close to 100% at 100 μM . When cultures were additionally treated, the inhibition of invasion was quite good, varying from 80% at 1 μM to 100% at both 10 μM and 100 μM .

1.2) Transmission blocking effect: gametocytocidal activity assays

Sexual stage gametocytes are responsible for the transmission of malaria parasites from the infected host to the mosquito vector. Only 8-aminoquinolines are proven effective at eliminating mature gametocytes (5). To examine if M1 possessed activity against sexual stage of *P. falciparum*, we conducted several assays (See supporting info **Fig. S1**). In the first assay, M1 (10 μM) was added at D0 with *N*-Acetylglucosamine (NAG) for gametocyte induction, to

evaluate the conversion rate of the asexual parasites into gametocytes. In the second assay, M1 (10 μ M) was added at D3 after gametocyte induction, to observe the effect on the gametocyte development. In these two assays, the treatment was renewed every day until D10. In the last assay, at D10, before induction of gamete formation, mature gametocytes were pre-incubated with M1 (10 μ M) for 2 or 24 h. Thus, our results showed that M1 had the ability to reduce the conversion rate of asexual parasites into gametocytes by 87.8% (**Fig. 4**). M1 also influenced the development of gametocytes when treating after the induction. At day 6 and 10 post induction, corresponding to gametocyte stages III and stage V respectively, we noticed a difference between the control and the culture treated with M1: Stage III mean control = 1.29 , M1 = 0.48 (p -value = 0.0465) and Stage V mean Control = 0.70, M1= 0.26 (p -value = 0.0122) (**Fig. 5A** and **5B**).

We also investigated the effect on mature stage V gametocyte development into gametes. The exflagellation blocking assays were performed in triplicate. The mean percentage values for male exflagellation were 57% at 2 h and 7.5 % at 24 h (**Fig. 6A** and **6B**). These results suggested that M1 inhibited exflagellation, either interfering directly during the process of gamete formation or “sterilizing” the mature gametocyte in such a way that it was metabolically viable but unable to form gametes by somehow.

1.3) Activity toward artemisinin-resistant *P. falciparum* strains

As shown in **Table 2**, M1 showed high antiplasmodial activity on both *P. falciparum* F32-ART5 and F32-TEM lineages with EC₅₀ values ranging from 227 to 283 nM. M1 EC₅₀ values did not significantly differ between these two laboratory strains. However despite artemisinin-resistant genotype of F32-ART5 (29), EC₅₀ values of artemisinin were similar for both strains, due to the specific *P. falciparum* quiescence-based mechanism of resistance to artemisinins leading to parasite cell cycle arrest during drug exposure (30, 31, 32). These data confirmed

that the standard proliferation chemo-sensitivity assay is not appropriated for the detection neither of artemisinin-resistance nor cross-resistance with artemisinin, as already reported (30, 31, 32). In contrast artemisinin-resistance can be evidenced *via* the recrudescence assay with a faster recrudescence for F32-ART5 than for F32-TEM parasites after 48 h artemisinin exposure (p -value = 0.0003) (**Fig. 7A**). Interestingly M1 presented no cross-resistance with artemisinin since there was no significant difference in recrudescence times between F32-TEM and F32-ART5 lines after 48 h exposure to M1 (p -value = 0.346 at 2.5 μ M; p -value = 0.096 at 10 μ M) (**Fig. 7B-C**). Moreover, M1 showed activity against quiescent parasites. Indeed when quiescence is induced by a 6 h pre-treatment with 3 μ M artemisinin before the addition of the drug to be tested, recrudescence time of F32-ART5 quiescent parasites treated by M1 was significantly different (p -value = 0.002) from recrudescence time of F32-ART5 quiescent parasites treated by artemisinin alone with a median delay in recrudescence time of 6 days (**Fig. 8A**). By contrast, when the tested molecule is inactive against parasites, like chloroquine (CQ) (33), there was no difference in these recrudescence times of parasites treated with the combination artemisinin / artemisinin + CQ and of the parasites treated with ART alone, although the drug was active against proliferating parasite (**Fig. 8B**).

2) *In vivo* experiments

2.1) Inhibition of the liver forms of *P. yoelii* in a mouse model

In an *in vivo* liver stage assays, M1 was administered at 50 mg/kg i.p. and liver stage development was assessed at 44 h post-infection on day 2. Results showed that, contrary to the data obtained *in vitro*, M1 did not prevent the development of liver stage parasites *in vivo* at day 2 (**Fig. 9A**). Thus at D3 and D6, blood stage parasites were observed in the bloodstream. However the time of appearance of the parasites is delayed in the group treated with M1 (to D4

instead of D3 for the control). In M1 treated mice, parasites are retained at the level of the spleen at days 3 and 4 (**Fig. 9A**). All mice were followed for the development of the blood stage infection. Parasitemia on days 3 and 6 post sporozoite infection were determined on blood smears. The results indicated that M1 reduced the burden of blood stage parasites (**Fig. 9C**). Mice treated with control compound primaquine (50 mg/kg) remained clear of parasitemia throughout the assay. To understand the discrepancy between *in vitro* and *in vivo* efficacy of M1 on the hepatic stage, the same experiment was done on mice which P450 cytochromes were inhibited by the i.p. administration of 1-aminobenzotriazole (ABT) at 50 mg/Kg (34, 35) from D-1 to D3. The results presented in **Fig. 9B** and **9D** showed that, with ABT co-treatment, a significant statistical difference is observed ($p < 0.0065$) between the luminescence level of the control group and the treated group: *in vivo* activity of M1 on the hepatic stages was quite improved when inhibiting P450 cytochromes with ABT co-treatment but did not allow full eradication of hepatic schizonts in the tested conditions.

2.2) Transmission blocking evaluation: mosquito feeding assay

M1 was tested for its ability to inhibit oocyst formation in a rodent malaria model. On day 4 after injection of 10^7 infected red blood cells, mice treated with M1 at 50 mg/kg during D0 to D3 showed a 50 % reduction in parasitemia before mosquitoes feeding (**Table 3**). The mouse group treated with M1 at 50 mg/kg at day 3, 2 h before mosquito feeding had significantly the same parasitemia and gametocytemia as in the untreated group (**Table 3**). Two hours after this treatment, the mosquitoes were allowed to feed on mice. The results showed that M1 reduced the infection rate (the percentage of mosquitoes with oocysts) from 82.8% (untreated) to 37.1%. Among the infected mosquitoes, M1 affected the sporogonic development of *P. yoelii*. The mean oocyst density (oocysts burden) was 106 oocysts per mosquito which ranged from 0 to 300 oocysts per mosquito. M1 demonstrated potent activity, up to a 95 % reduction of the total

oocyst count relative to the control group ($p < 0.0001$), as presented in **Table 3** and **Fig. 10A**. The effect of M1 appeared stronger when treating mice just before (2 h) mosquito feeding. The reduction of mean oocysts density logically led to a major decrease of the number of sporozoites in the M1 groups compared to the untreated group (**Fig. 10B**).

3) **Microsomal stability, metabolism and metabolites of Gamhepathiopine (M1)**

When incubated with female mouse microsomes, M1 showed a poor stability: $T_{1/2} = 11$ min. Searching for the microsomal metabolites of M1, by incubating M1 with female mouse microsomes and analyzing the resulting medium by LC/MS/MS approach, it appeared that 2 sites of M1 were targeted by the P450 cytochromes: the tolyl (methylphenyl) moiety and the *tertio*-butyl group were oxidized, successively leading to alcohol, aldehyde and carboxylic acid derivatives. The metabolites formed on the tolyl moiety that were proposed by the LC/MS/MS analysis were synthesized, using a new optimized organic synthesis pathway (See supporting info **Fig. S2**). The preparation of these 3 metabolites (**7**, **8** and **10**) allowed to confirm the structures proposed by the LC-MS-MS analysis (confirmation of retention times and fragmentations) and to evaluate their *in vitro* activity on the blood stage of *P. falciparum* (K1) along with the hepatic stage of *P. yoelii* in HepG2-CD81 for **7** and **10**. As presented in **Table 4**, all metabolites (especially carboxylic acid **10**) were quite less active (1 log to 2 log increase of EC_{50}) than M1, both on the blood and hepatic stages.

DISCUSSION

To realize the goal of malaria eradication, identification of new potent compounds active against multiple life stages of the parasite is necessary. Because most of the currently licensed antimalarials only target the asexual intra-erythrocytic stage, we urgently need to expand our antimalarial arsenal. Beyond efficacy against blood stages, drugs which can effectively target

hepatic stage and gametocyte stages, responsible for the transmission to the mosquito vector, will be required for malaria elimination (2). Furthermore, in a context of artemisinin-resistance, these new drugs must be active on artemisinin-resistant parasites, including quiescent ones.

In this demanding context, the data presented herein demonstrated that Gamhepathiopine (M1), a potent (*Pf*K1 $EC_{50} = 45$ nM) antiplasmodial molecule toward the erythrocytic stage (25), also displayed *in vitro* activity against artemisinin-resistant quiescent parasites, was active against the liver stage of *P. falciparum* in human primary hepatocytes, inhibited the formation and development of gametocytes and showed transmission blocking activity.

In vitro*, Gamhepathiopine showed activity toward the liver stages of *Plasmodium spp

We first conducted *in vitro* assays against the liver stages of *P. yoelii*, *P. falciparum* and *P. cynomolgi* in corresponding primary hepatocytes. As presented in **Table 1**, the activities of M1 were similar to the ones of primaquine (EC_{50} M1 = 450-1500 nM versus EC_{50} primaquine = 600-2720 nM). M1 acted both on the number of parasites but also on their development by reducing their size. As the number of parasites developing in hepatocytes depends on the number of sporozoites that can invade them, we studied the effect of M1 on the penetration and development of sporozoites into hepatocytes. Incubation of sporozoites with M1 reduced the number of parasites by 30% at 1 μ M and 60% at 10 μ M (**Fig. 3A**) whereas complete inhibition of sporozoite invasion was achieved by treating both sporozoites and infested hepatocyte cultures with M1 at 10 μ M (**Fig. 3B**). This action on sporozoites coupled with that on the hepatic stages make M1 an interesting model for developing novel antimalarials with chemoprophylaxis potential.

***In vitro*, Gamhepathiopine inhibited the induction and development of gametocytes along with the exflagellation of gametes**

Antimalarials that prevent the transmission of malaria parasites (from human host to mosquito vector or from mosquito to human) have an important role to play in malaria control programs and may help to contain the spread of drug resistance (36). In fact, at this day, a single low dose of 0.25 mg/kg of primaquine remains the only gametocytocidal treatment recommended by the WHO. Unfortunately, its deployment was limited because of concerns over haemolytic effects in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency. To explore the potential transmission blocking activity of M1, we investigated its ability to i) prevent the induction of gametocytes, ii) to affect their development and iii) to disturb the exflagellation, following a protocol using *P. falciparum* gametocyte cultures that is summarized in **Fig. S1** (supporting info). As presented in **Fig. 4**, M1 used at 10 μ M was a significant inhibitor of gametocyte induction. Then, early stage gametocytes (stages I-II) were exposed to M1 at 10 μ M. As shown in **Fig. 5A** and **5B**, M1 significantly inhibited the development of gametocytes, both at days 6 and 10. Moreover, when exposing stage V gametocytes to M1 for 2 or 24 h, an inhibition of the rate of exflagellation was observed (**Fig. 6**). These data highlighted M1 as a transmission-blocking drug with inhibitory activity on more than one critical step in the sexual stages of the parasite life cycle.

***In vitro*, Gamhepathiopine was active on artemisinin-resistant and quiescent parasites**

In a context of worldwide threat of artemisinin resistance spreading, it is essential to assess efficacy of new antimalarial compounds toward this very special drug resistance. M1 exerted a similar activity against *P. falciparum* artemisinin-resistant and artemisinin-sensitive parasites (F32-ART5 and F32-TEM respectively) evaluated by a standard chemo-sensitivity assay (**Table 2**). Very interestingly, M1 presented no cross-resistance with artemisinin, whatever the

dose tested (**Fig. 7**) and showed activity against artemisinin-resistant parasites at the quiescent state (**Fig. 8**), contrary to several antimalarial drugs (31, 33). A drug bearing such properties could be a gold standard partner in ACTs allowing to counteract artemisinin resistance and avoid risk of selection of partner drug resistance.

***In vivo*, Gamhepathiopine showed transmission blocking activity but was rapidly metabolized by P450 cytochromes into inactive metabolites.**

It was previously reported (25) that M1, administered twice a day at 2.5 mg/kg for 3 days to mice infected by *P. berghei*, was moderately active, in comparison with chloroquine used at the same dose and untreated group (parasitemia at day 4 = 9% in untreated group, 4.9% in M1 group and 1.5% in chloroquine group). This *in vivo* lack of efficacy was confirmed when studying the activity of M1 on the liver stage of *P. yoelii*. Effectively, when infecting mice with sporozoites on day 0, there was a decrease at day 3 in the number of hepatic parasites in the group treated with M1 (versus untreated group), but M1 was not efficient enough to clear hepatic parasites so that erythrocytic schizogonia could take place, leading to a high parasitemia at day 6, contrary to what was observed in the primaquine group (**Fig. 9 A and C**). By repeating the same experiment after treating mice with 1-aminobenzotriazole (ABT), a multi-P450 CYP inhibitor (34, 35), the efficacy of M1 was quite significantly improved at day 2, 3 and 6 (**Fig. 9 B and D**), confirming that the loss of *in vivo* efficacy of M1 was probably due to its metabolization by hepatic cytochromes into inactive derivatives.

Effectively, in an *in vitro* microsomal stability assay, M1 presented a short half-life of 11 min. To validate this hypothesis, the metabolites of M1 formed after incubation with mouse microsomes were first searched by LC/MS/MS, revealing 2 metabolic hot spots in the structure of M1: the tolyl (methylphenyl) and *tertio*-butyl moieties that could afford oxidized metabolites (alcohols, aldehydes and carboxylic acids). Then, 3 of the suspected metabolites were

synthesized (see supporting info **Fig. S2**), allowing confirmation of the proposed structures, and were evaluated for their *in vitro* antiplasmodial activity toward the erythrocytic K1 *P. falciparum* strain and hepatic stage of *P. yoelii* in HepG2CD81 (**Table 4**). In comparison with M1, alcohol **7**, aldehyde **8** and carboxylic acid **10**, microsomal metabolites of M1 on the tolyl moiety, showed reduced to abolished antiplasmodial activities ($EC_{50} = 0.4$ to $>10 \mu\text{M}$), proving that the partial *in vivo* activity of M1 was due to its rapid metabolization into less active and inactive derivatives, arguing for the conception of new M1 derivatives bearing metabolic blocker groups on the 2 metabolized sites of the pharmacophore.

Finally, we evaluated the effect of M1 on the formation of *P. yoelii* oocyst in *Anopheles stephensi* mosquitoes (37) via a feeding assay on infected mice. The presence of M1 in the infectious blood feed had a significant impact on the mean oocyst numbers per mosquito (**Table 3** and **Fig. 10**). In view of the results obtained *in vivo* showing the metabolization of M1, the tests were carried out by treating the mice either from D0 to D3, or 2 h before feeding the mosquitoes in order to cancel the effect of the metabolization. The prevalence of oocyst infections in the control group was 82.8%, yielding a geometric mean of 106 oocysts/mosquito (**Table 3**). With 95 % reduction of the total oocyst count relative to controls, M1 reduced oocyst burden in mosquito midguts. The reduction in the number of oocysts inevitably leading to those of sporozoites (**Fig. 10**). This reduction was more marked when the treatment with M1 was done only 2 h before feeding the mosquitoes compared to the group treated from D0 to D3, further highlighting the metabolism of M1 into inactive derivatives.

In conclusion, these results indicate that Gamhepathiopine is an excellent starting point to develop novel thienopyrimidinone derivatives with the potential to be used in malaria eradication campaigns since this pharmacophore displays all the desired antiplasmodial multistage activity characteristics. Gamhepathiopine showed high antiplasmodial activity on

both erythrocytic and liver stages of *P. falciparum* and remained active toward artemisinin-resistant strains, including quiescent parasites. In addition, Gamhepathiopine showed efficient activity against the formation and development of gametocytes and exflagellation of gametes which are responsible for a quite interesting transmission blocking activity. Additional medicinal chemistry works are now awaited to transform this very promising antiplasmodial hit-compound, presenting an original pharmacophore, into a potential antimalarial candidate with improved hepatic metabolic stability.

MATERIAL AND METHODS

1) Ethics Statement

All procedures involving murine models complied with European regulations. The protocol was ethically approved by the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche (Authorization Number 01737.03).

The use of nonhuman primates at CEA is in accordance with recommendations of the Weatherall report. Experimental procedures were conducted in strict accordance with the recommendations of the European guidelines for the care and use of laboratory animals (European directive 63/210). The protocols and the use of hepatocytes for the purpose of the work described here were approved by the Ethical Animal Committee of the CEA (permit number A 92-032-02).

2) Study on the liver stage

2.1) Parasite strains, sporozoites isolation and animals

P. falciparum (NF54 strain) sporozoites were obtained from infected salivary glands of *Anopheles stephensi* 14-21 days after an infective blood meal (Department of medical microbiology, University Medical Centre, St Radboud, Nijmegen Netherland).

P. cynomolgi (Strain M) sporozoites were obtained from salivary glands of in-house infected *Anopheles stephensi* 14 -16 days after an infective blood meal. *P. cynomolgi* infected blood was obtained from *Macaca fascicularis* monkeys (the natural host of *P. cynomolgi*) 10 to 15 days after their infection with cryopreserved blood stages of this parasite (CEA, Fontenay aux Roses, France).

P. yoelii 230-GFP-Luc sporozoites are mutant parasites expressing a GFP-luciferase cassette generated by the GOMO strategy “Gene Out Marker Out” (38). Sporozoites were obtained from salivary glands of in-house infected *Anopheles stephensi* 14 -16 days after an infective blood meal on a mouse previously infected with cryopreserved blood stage parasites.

All infected salivary glands were removed by hand dissection and crushed in a potter for sporozoites isolation and filtrated through a 40 µm filter to remove mosquito debris (Cell Strainer, BD BioSciences, USA). The sporozoites were counted using a disposable Glasstic microscope slide (KOVA, USA). All mice used in these experiments were female BALB/c mice (the average weight was approximately 18 g) purchased from René Janvier (Le Genest-Saint-Isle, France).

2.2) Hepatocytes

Primary human hepatocytes were isolated from liver segments obtained from adults' patients undergoing partial hepatectomy. (Service de Chirurgie Digestive, hépato-bilio pancréatique, Hôpital Pitié Salpêtrière, Paris France). Primary simian hepatocytes were isolated from liver

segments collected from healthy *Macaca fascicularis* from CEA, Fontenay aux Roses, France. All hepatocytes were obtained using collagenase perfusion as previously described (39). Simian hepatocytes were immediately cryopreserved with a Nicool- Freezal (Air liquide Santé, Marne la Vallée) and then used when needed after fast thawing at 37 °C. Primary murine hepatocytes were isolated from liver segments collected from BALB/c mouse. Human and murine hepatocytes were used directly after their isolation. Cells were seeded in 96-well plates (Falcon by Becton Dickinson Labware Europe, France) coated with collagen I (BD Bioscience, USA), at a density of 80,000 cells per well. Simian hepatocytes were maintained at 37°C in 5% CO₂ in a complete William medium supplemented with 10% of Foetal Bovine Serum FCIII, 1% penicillin-streptomycin (Sigma Aldrich, USA), 5.10⁻³ g/L insulin (Sigma Aldrich, USA) and 5.10⁻⁵ M hydrocortisone (Upjohn laboratories SERB, France) until infection with sporozoites. For human hepatocytes, the complete medium was supplemented with 2% dimethyl sulfoxide (DMSO, Sigma- Aldrich, USA) before infection to preserve their differentiation.

HepG2-CD81 cells (ATCC HB-8065 with CD81 induced expression) were cultured in 96-well plates coated with collagen I, at the density 250.000 cells/cm² in DMEM supplemented with 10% foetal calf serum and antibiotics (40).

2.3) *In vitro* cell viability assay toward primary hepatocytes, HepG2-CD81 and HepG2-HB-8065 cell lines

The toxicity of M1 toward primary hepatocytes and HepG2CD81 was evaluated through the MTT test (41). Cells were plated (80.000 cells/well) and incubated with different concentrations of each drug in complete medium. The protocol for changing the medium was the same as drug assay: at 3 h post-initiation and then daily for 2, 5 and 8 days respectively for HepG2CD81, murine, human and simian hepatocytes. Cultures were washed twice with PBS to eliminate complete medium. 10% MTT in Williams's medium was added and on the cells for 4 h at 37

°C. Then, MTT was replaced by a mix of DMSO-ethanol (1:1) to dissolve formazan crystals and the absorbance was read at 540 nm on a flexstation plate reader.

HepG2 cell line (ATCC, ref HB-8065) was maintained at 37 °C, 5% CO₂ with 90% humidity in MEM supplemented with 10% fetal bovine serum, 1% L-glutamine (200 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL) (complete MEM medium). The evaluation of the tested molecules cytotoxicity on the HepG2 cell line was performed according to the method of Mosman (41) with slight modifications. Briefly, 5.10³ cells in 100 µL of complete medium were inoculated into each well of 96-well plates and incubated at 37 °C in a humidified 5% CO₂. After 24 h incubation, to obtain adherent cells, 100 µL of medium with various product concentrations dissolved in DMSO (final concentration less than 0.5% v/v) were added and the plates were incubated for 72 h at 37 °C. Triplicate assays were performed for each sample. Each plate-well was then microscope-examined for detecting possible precipitate formation before the medium was aspirated from the wells. 100 µL of MTT solution (0.5 mg/mL in medium without FBS) were then added to each well. Cells were incubated for 2 h at 37 °C to allow MTT oxidation by mitochondrial dehydrogenase in the viable cells. After this time, the MTT solution was removed and DMSO (100 µL) was added to dissolve the resulting blue formazan crystals. Plates were shaken vigorously (700 rpm) for 10 min. The absorbance was measured at 570 nm with 630 nm as reference wavelength using a TECAN Infinite F-200 Microplate Reader. DMSO was used as blank and doxorubicin as a positive control. Cell viability was calculated as percentage of control (cells incubated without compound). The 50% cytotoxic concentration (CC₅₀) was determined by non-linear regression analysis processed on the dose–response curve by using the TableCurve software 2D v.5.0. CC₅₀ values represent the mean value calculated from three independent experiments.

2.4) Assessment of liver stage development *in vitro*

Hepatocytes were seeded into collagen-coated black 96-wells plates and maintained at 37 °C in 5% CO₂ in complete medium. The day of infection, 3.10⁵ sporozoites of *P. falciparum*, *P. yoelii* and *P. cynomolgi* were added to their respective host cell cultures (human, murine and simian). The infected culture plates were centrifuged 10 min at 900 g allowing fast parasites sedimentation and further incubated with serial dilutions of M1. Culture medium containing the appropriate drug concentration was refreshed daily and cells were fixed at 48 h post-infection (p.i.) for infections with *P. yoelii*, 6 days p.i. for *P. falciparum* or 8 days p.i. for *P. cynomolgi*. For the *in vitro* sporozoites invasion experiments, *P. falciparum* sporozoites were preincubated with 1, 10 and 100 µM of M1 for 1 h at RT, before hepatocytes infections. Three hours later, after sporozoites penetration into hepatocytes, cultures were washed and further incubated with or without the presence of the drug during 6 days. DMSO was used as a negative control.

2.5) Parasite counting and schizont size determination using High-Content Imaging

The number, size and shape of the parasites were determined using a CellInsight High Content Screening platform equipped with the Studio HCS software (ThermoFisher Scientific). The parasite immunostaining was done using a α PfHSP70 antibody raised in mice and a secondary anti-mouse antibody coupled to AlexaFluor 488 (42). Host cell and parasite nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI). Thirty-seven images, representing more than 95% of the total bottom surface area of a culture well in 96-well plates, were captured for analysis. The custom script used for counting and measuring parasites first required identification of the objects based on a fluorescence intensity threshold. The object identity was then further validated based on size and morphological (shape) criteria. The presence of DAPI-associated fluorescence in the selected objects allowed their final selection and the rejection of false positives. EC₅₀ values are the drug concentration at which a 50% reduction of the

exoerythrocytic form (EEF) number was observed, as compared to the control cultures. We then estimated the EC₅₀ with ICEstimator software version 1.2 (43). The parasite size reduction is calculated on the average object area using the total surface area of each selected object (μm^2) and is related to the threshold value used for object identification.

2.6) *In vivo* assessment of the effect toward the liver stage

Five mice per treatment group were used. Experimental groups were treated with 50 and 100 mg/kg intra-peritoneal (i.p.) of M1. Untreated infected mice, non-infected mice (infection controls) as well as primaquine-treated (50 mg/kg) mice (control drug) were also included. The drugs were administrated on days -1, 0, +1, and mice were challenged on day 0 by retro-orbital injection of *P. yoelii* (GFP-luc strain) sporozoites (10,000 per mouse). *In vivo* bioluminescence imaging was performed 44 h post-infection on day 2, to assess liver stage development. When parasites were detected in the liver, treatment was continued until day 5 and *in vivo* imaging was performed on days 3 and 6, to evaluate a prepatent period for the blood stage parasites in mice after the sporozoite inoculation. *In vivo* imaging and blood smears were performed to monitor blood stage development using methods described previously (44). This experiment was performed twice.

IVIS Spectrum (Caliper Life Science, Hanover, MD) was used to capture bioluminescent signals. Prior to analysis the mice were injected i.p. with D-luciferin (100 mg/kg), anesthetized with isoflurane and imaged 10 min post injection. Images were analyzed using the living Image 3.0 software (Capiler Life Science, Hanover, MD).

In order to study the *in vivo* efficacy of M1 without any hepatic CYP metabolism, we conducted an experiment using 1-aminobenzotriazole (ABT), a non-selective inhibitor of cytochrome P450 enzymes (34, 35). ABT was administered i.p. on mice 1 h before each treatment with Gamhepathiopine 50 mg/kg for 3 days starting from the day before sporozoite inoculation.

3) *In vivo* transmission blocking activity assays

Experimental mice were infected by i.p. inoculations of 10^7 erythrocytes parasitized with *P. yoelii* GOMO 15 to test the effect of M1 following two different protocols of drug treatment. Infected mice were randomly allocated in 6 groups (5 mice per group) corresponding to the 3 conditions (Control: Solvent, M1 50 mg/kg and PQ 50 mg/kg) in both drug administration protocols. For the first protocol, mice were treated daily from day 0 to day 3 while for the second protocol, treatment was administered only once, 4 days after the infection, 2 h before mosquitoes feeding (37). At day 3 p.i., the presence and maturity of gametocytes was checked by microscopic examination of Giemsa-stained thin blood films and live blood observation to assess microgamete exflagellation density. Mice were then anesthetized and placed according to the treatment on the top of individual cages containing 100 glucose-starved *Anopheles stephensi* female mosquitoes, which were allowed to feed for 1 h. Unfed mosquitoes were removed from the cages. Seven days after the infectious blood meal, 30 mosquitoes were dissected and their midguts examined using a phase contrast microscope (400x) to assess the presence of oocysts. Two weeks after the blood meal, salivary glands of 30 mosquitos were dissected to estimate the average number of sporozoites per mosquito in each group.

4) *P. falciparum* gametocytes induction and *in vitro* drug susceptibility assays

To produce gametocytes, *P. falciparum* NF54 was cultured *in vitro* as described by Trager and Jensen (45) with minor modifications. Briefly, parasites were maintained in human type O-positive red blood cells (RBC) at 5% hematocrit (HCT) in RPMI 1640 medium supplemented with 10% heat-inactivated human serum, hypoxanthin 10 mM, gentamicin (20 µg/mL), The cultures were maintained at 37°C in a standard gas mixture: 5% O₂, 5% CO₂, 90% N₂. Synchronous production of gametocytes was achieved as previously described (46). Briefly, asexual parasite cultures were synchronized by purifying schizonts with MACS separation

columns CS (Miltenyi Biotec) and using them to reinvade erythrocytes. When parasitemia reached 10-15% rings, culture medium was supplemented with 50 mM *N*-acetyl-glucosamine (NAG, Sigma Aldrich) for five days in order to clear residual asexual parasites and obtain a virtually pure gametocyte culture.

To assess the effect of gamhepathiopine on the induction of gametocytes, M1 (10 μ M) was added at the same moment with NAG. For gametocyte development, gamhepathiopine was added two days after the addition of NAG. Medium replacement was performed every day. All cultures were prepared in parallel with controls corresponding to cultures that were treated with 0.1% DMSO. The percentages of each stage of gametocytes in cultures were assessed by microscopy of Giemsa-stained thin blood smears.

5) Exflagellation-blocking assays

Exflagellation-blocking assays were performed according to published protocols (47, 48). Assays were performed using gametocytes cultures providing high enough exflagellation centers for meaningful measures (> 30 centers of exflagellation in 50 fields of the microscope x 40).

To show the suitability of field isolates for exflagellation-blocking assays, the activity of 10 μ M of M1 was evaluated. Assays were carried out in 6-well plates containing 400 μ L of gametocyte culture medium with 10 μ M of M1 dissolved in DMSO. Then, 100 μ L of stage V mature gametocyte at 2% HCT were dispensed into each assay well. The 6-well plate was incubated at 37 °C. After treatment with M1 during 2 h or 24 h, exflagellation was induced by decreasing temperature to 25°C and replacing culture medium with AB serum. The number of exflagellation centers were recorded between 10 and 30 min after induction and compared to the control (DMSO). Activity was expressed as percentage of exflagellation inhibition compared to control.

6) Statistical analysis for the studies regarding the hepatic stage, gametocytes and sporozoites

Excel 2007 spreadsheet (Microsoft Office) and GraphPad Prism 6 statistical Software (GraphPad. Software, San Diego, CA, USA) were used for data analysis. All values were expressed as means and standard deviations (SD). A *p*-value of 0.05 or less was considered as statistically significant.

7) *In vitro* chemo-sensitivity assay regarding the blood-stage of *P. falciparum*

In this study, a K1 culture-adapted *P. falciparum* strain resistant to chloroquine, pyrimethamine and proguanil was used in an *in vitro* culture. Maintenance in continuous culture was done as described previously by Trager and Jensen (45). Cultures were maintained in fresh A+ human erythrocytes (EFS, French Blood Bank) at 2.5% hematocrit in complete medium (RPMI 1640 with 25 mM HEPES, 25 mM NaHCO₃, 10% of A+ human serum) at 37 °C under reduced O₂ atmosphere (gas mixture 10% O₂, 5% CO₂, and 85% N₂). Parasitaemia was maintained daily between 1% and 3%. The *P. falciparum* drug susceptibility test was carried out by comparing quantities of DNA in treated and control cultures of parasites in human erythrocytes according to a SYBR Green I fluorescence-based method (49) using a 96-well fluorescence plate reader. Compounds, previously dissolved in DMSO (final concentration less than 0.5% v/v) were incubated in a total assay volume of 200 µL of synchronized culture suspension (2% hematocrit and 0.4% parasitaemia) for 72 h in a humidified atmosphere (85% N₂, 10% O₂ and 5% CO₂) at 37 °C, in 96-well flat bottom plates. Duplicate assays were performed for each sample. After incubation, plates were frozen at -20 °C for 24 h. Then, the frozen plates were thawed for 1 h at 37°C. 15 µL of each sample were transferred to 96-well flat bottom black plates (Greiner Bio-one) already containing 15 µL of the SYBR Green I lysis buffer (2X SYBR Green I, 20 mM Tris base pH 7.5, 20 mM EDTA, 0.008% w/v saponin, 0.08% w/v Triton X-100). Negative

control, treated by solvents (DMSO or H₂O) and positive controls (atovaquone, chloroquine and doxycycline) were added to each set of experiments. Plates were incubated for 15 min at 37 °C and then read on a TECAN Infinite F-200 spectrophotometer with excitation and emission wavelengths at 485 and 535 nm, respectively. The concentrations of compounds required to induce a 50% decrease of parasite growth (EC₅₀K1) were calculated by non-linear regression analysis processed on the dose–response curve by using the TableCurve software 2D v.5.0. EC₅₀K1 values represent the mean value calculated from three independent experiments.

8) Evaluation of M1 activity in the framework of resistance to artemisinin

8.1) Parasite culture

The *Plasmodium falciparum* artemisinin-resistant strain F32-ART5 and its twin sensitive line F32-TEM (30, 31) were cultivated at 37 °C with 5% CO₂ in human red blood cells (EFS, French Blood Bank) diluted to 2% hematocrit in RPMI-1640 medium supplemented with 5% human serum (EFS, French Blood Bank) as previously reported (46).

8.2) Standard *in vitro* chemo-sensitivity assay

In vitro susceptibility of *P. falciparum* parasites was determined by using the standard hypoxanthine uptake inhibition assay as described previously (50) with some modifications (51). After D-sorbitol treatment, parasites synchronized at the ring-stage were exposed for 48 h, with [³H]-hypoxanthine (Perkin Elmer, France), to a range of concentrations of the different tested compounds. [³H]-hypoxanthine incorporation was then measured with β -counter and EC₅₀ values (concentrations of drugs that inhibited 50% of the parasite growth) were determined in concentration versus percent inhibition curves. Each result represents 3 independent experiments.

8.3) Recrudescence assay

Recrudescence assay was performed to evaluate the ability of the F32-ART5 parasite line to survive drug exposure comparatively to the F32-TEM parasite line (30, 31) in order to evaluate the risk of cross-resistance between artemisinin and M1. D-sorbitol synchronized ring-stages parasites at 3% parasitemia (2% hematocrit) were exposed to the drug for 48 h. After drug exposure, parasite cultures were then washed with RPMI-1640 medium and replaced in normal drug-free culture conditions. The time to parasite recrudescence was determined as the time required to reach initial parasitemia. If no parasites were observed in the following 30 days, the culture was considered “not recrudescence” (30, 31). Each experiment, performed in parallel for F32-ART5 and F32-TEM cultivated in the same conditions to generate paired results, was carried out at least three times independently and data statistically analyzed. The tested doses corresponded to 10-50-fold the EC₅₀ values (31).

8.4) Quiescent-stage survival assay (QSA)

Chemosensitivity evaluation of artemisinin-resistant parasites at the quiescent stage was performed on the F32-ART5 strain thanks to the QSA (33). D-sorbitol synchronized ring-stages parasites at 3% parasitemia (2% hematocrit) were first exposed to 3 μ M of artemisinin for 6 h to induce quiescence of artemisinin-resistant parasites. Then quiescent parasites were exposed or not (as control condition) to 2.5 μ M of M1 for 24 h. After drug exposure, parasite cultures were washed with RPMI-1640 medium and replaced in normal drug-free culture conditions. The time to parasite recrudescence was determined as the time required to reach initial parasitemia. If no parasites were observed in the following 30 days, the culture was considered “not recrudescence”. In each experiment, results obtained with the condition “artemisinin 3 μ M 6 h / artemisinin 3 μ M 24 h” were compared to those obtained with the condition “artemisinin 3 μ M 6 h / (artemisinin 3 μ M + M1 2.5 μ M) 24 h” in order to determine the delay in recrudescence time. This delay is representative of the capacity of the tested compound to be

active on quiescent parasites and it is assumed that a 6-day threshold is necessary to classify a compound as active on quiescent parasites (33). Each experiment was carried out three times independently and data statistically analyzed. The same experiment was done once with chloroquine (CQ) at 200 nM as negative control because despite its activity on proliferating *Plasmodium* parasites, CQ failed to kill quiescent parasites due to its therapeutic target which is not implemented in quiescent parasites (33).

8.5) Data Analysis

A statistical analysis (log-rank test) was carried out on data from the recrudescence assay and the QSA thanks to a Kaplan-Meier survival analysis considering censored data. The final event was defined as the time necessary for cultures to reach initial parasitemia. Observations were considered as censored if no recrudescence was observed within the experiment timeframe (30 days).

9) Microsomal stability and metabolite identification

The tested product and propranolol, used as reference, were incubated in duplicate (reaction volume of 0.5 mL) with female mouse microsomes (CD-1, 20 mg / ml, BD Gentest™) at 37 °C in a 50 mM phosphate buffer, pH 7.4, in the presence of MgCl₂ (5 mM), NADP (1 mM), glucose-6-phosphate dehydrogenase (0.4 U/mL) and glucose-6-phosphate (5 mM). For the estimation of the intrinsic clearance: 50 µL aliquot at 0, 5, 10, 20, 30 and 40 min were collected and the reaction was stopped with 4 volumes of acetonitrile (ACN) containing the internal standard. After centrifugation at 10000 g, 10 min, 4 °C, the supernatants were conserved at 4 °C for immediate analysis or placed at -80 °C in case of postponement of the analysis. Controls (t₀ and t_{final}) in triplicate are prepared by incubation of the internal standard 4 with microsomes denatured by acetonitrile. The LC-MS used for this study was a Waters® Acquity I-Class / Xevo TQD, equipped with a Waters® Acquity BEH C₁₈ column, 50 x 2.1 mm, 1.7 µm. The

mobile phases were (A) ammonium acetate 10 mM and (B) acetonitrile with 0.1% formic acid. The injection volume was 1 μ L and the flow rate was 600 μ L/min. The chromatographic analysis, total duration of 4 min, was made with the following gradient: $0 < t < 0.2$ min, 2% (B); $0.2 < t < 2$ min, linear increase to 98% (B); $2 < t < 2.5$ min, 98% (B); $2.5 < t < 2.6$ min, linear decrease to 2% (B); $2.6 < t < 4$ min, 2% (B). The quantification of each compound was obtained by converting the average of the ratios of the analyte/internal standard surfaces to the percentage of consumed product. The ratio of the control at t_0 corresponded to 0% of product consumed. The calculation of the half-life ($T_{1/2}$) of each compound in the presence of microsomes was done according to the equation $T_{1/2} = (\ln 2)/k$ where k was the first-order degradation constant (the slope of the logarithm of compound concentration versus incubation time). The intrinsic clearance *in vitro* (Cl_{int} expressed in μ L/min/mg) was calculated according to the equation: $Cl_{int} = (\text{dose}/AUC_{\infty}) / [\text{microsomes}]$ where dose was the initial concentration of product in the sample, AUC_{∞} was the area under the concentration-time curve extrapolated to infinity and [microsomes] was the microsome concentration expressed in mg/ μ L.

Gamhepathiopine (0.5 mL of a 37.5 μ M solution in water/methanol 99:1) was incubated with female mouse microsomes (CD-1, 20 mg / ml, BD GentestTM) at 37 °C in a 50 mM phosphate buffer, pH 7.4, in the presence of MgCl₂ (5 mM), NADP (1 mM), glucose-6-phosphate dehydrogenase (0.4 U/mL) and glucose-6-phosphate (5 mM). Reaction is stopped by adding 2 mL acetonitrile at $t = 0$, $t = 2$ min, $t = 5$ min and $t = 15$ min. After centrifugation (12 000 rpm, 10 min, 4 °C), supernatants were evaporated *in vacuo* and reconstituted in a methanol/acetonitrile mixture (1:1) before analysis. Controls ($t = 0$, $t = 2$ min, $t = 5$ min and $t = 15$ min) were prepared by incubation of gamhepathiopine with microsomes denatured by acetonitrile. Analytical methods used for metabolite identification are LC/MS TOF (Waters® LCT Premier XE) and UHPLC-MS Triple quadrupole (Waters® Acquity I-Class/Xevo TQD). Among identified metabolites of M1, alcohol **7**, aldehyde **8** and carboxylic acid **10** were

synthesized and studied in the same chromatography and mass spectrometry analysis conditions, for validation purposes.

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FIGURES AND TABLES

Figure 1. New chemical entities which have reached clinical trials and display multi-stage antimalarial activity in 2021.

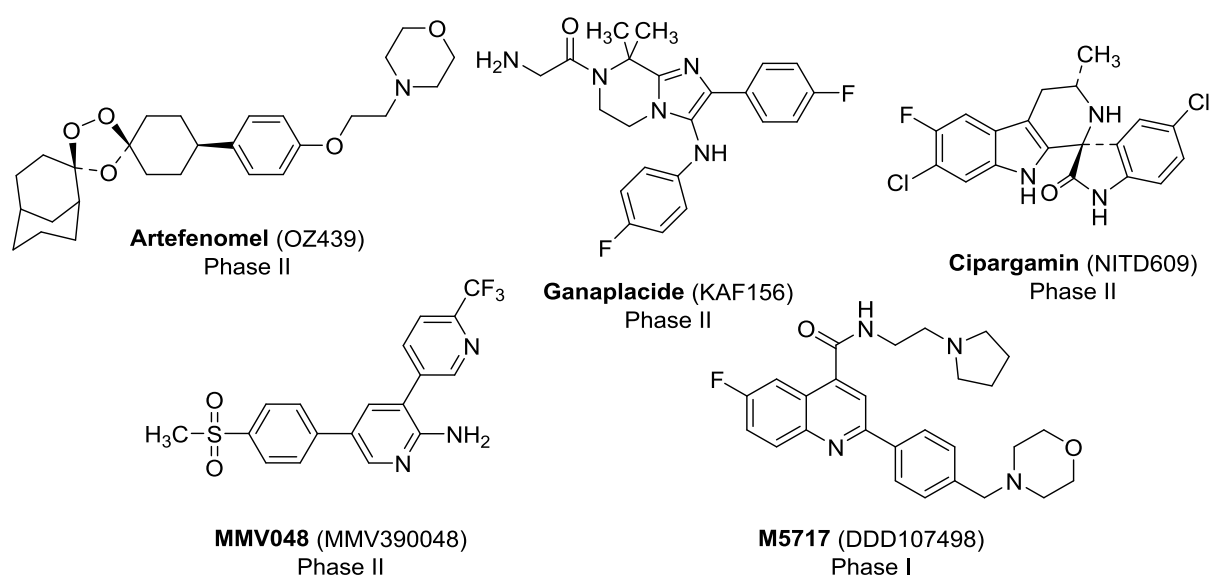


Figure 2. 6-Phenylthienopyrimidine derivatives reported as antiplasmodial compounds: A & B = 2,4-diamino-6-phenylthieno[3,2-*d*]pyrimidine derivatives (27, 28); C = 6-phenylthieno[3,2-*d*]pyrimidinone derivative (26) and D = 2-amino-6-phenylthieno[3,2-*d*]pyrimidinone derivative = Gamhepathiopine = M1 (25).

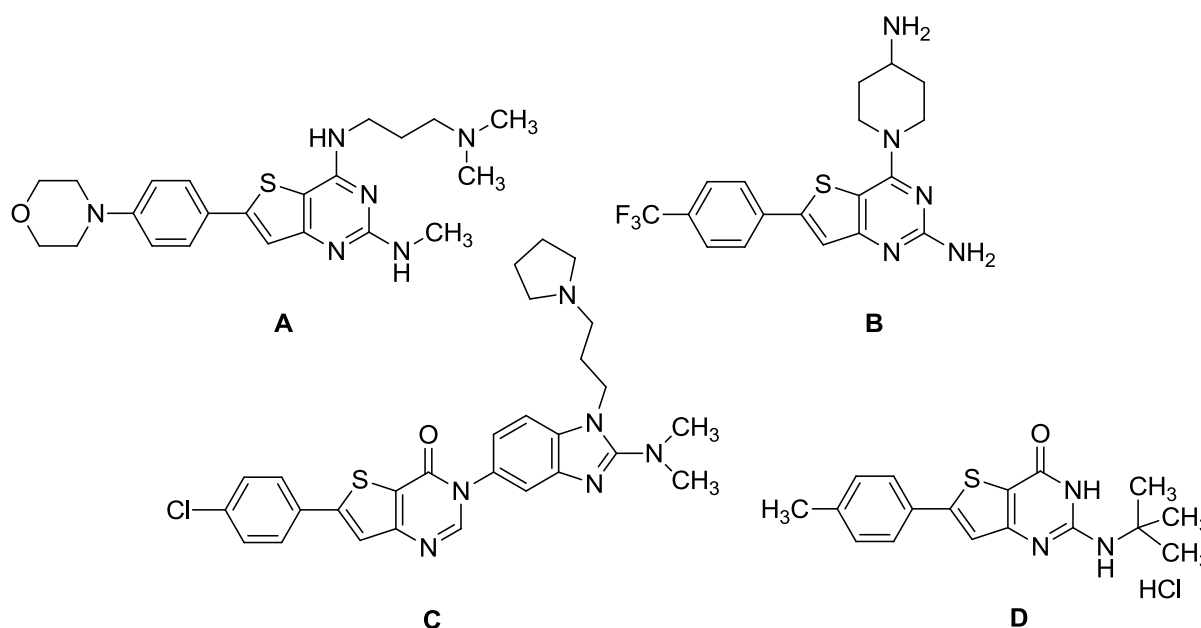


Table 1. Estimated half-maximal effective Concentration (EC_{50}) values of Gamhepathiopine (M1) and primaquine on *Plasmodium* liver stage.

<i>Plasmodium</i> species	Gamhepathiopine (M1)			Primaquine (control)		
	EC_{50} (μ M)	SI*	Size reduction at 10 μ M (%)	EC_{50} (μ M)	SI*	Size reduction at 10 μ M (%)
<i>yoelii</i> (MH)	0.45 +/-0.1	>133	100	0.64 +/-0.1	110	ND
<i>falciparum</i> (HH)	0.45 +/-0.15	>133	80	0.6 +/-0.06	66	100
<i>cynomolgi</i> (SH)	1.5 +/-0.3	>40	80	2.72 +/-0.34	12	70

MH = murine primary hepatocytes, HH = human primary hepatocytes, SM = simian primary hepatocytes

*SI = selectivity index = hepatocyte CC_{50} / EC_{50} (CC_{50} values of M1 were > 60 μ M for all primary hepatocytes)

Figure 3. Effect on hepatocyte invasion by *P. falciparum* sporozoites. Sporozoites were pre-incubated for 1 h at rt with the drug at 1, 10 or 100 μM , prior to be added to the hepatocytes. Three hours later, after sporozoites penetration into the hepatocytes, cultures were washed and incubated in the presence of medium without M1 (A) and with M1 (B) at 1, 10 or 100 μM every day until day 6. EEFs = exoerythrocytic forms.

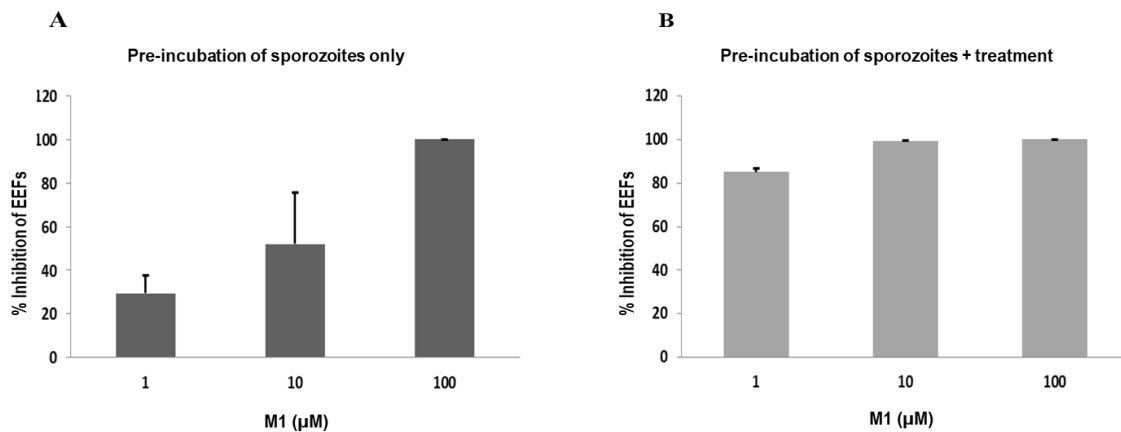


Figure 4. Effect of Gamhepathiopine (M1) *in vitro* on the induction of *P. falciparum* gametocytes. The drug and NAG were put at the same time on the culture at day 0 (Control = untreated). We estimated conversion rate (% gametocytemia day 6 / % parasitemia day 0) in gametocytes (* p -value = 0.0209).

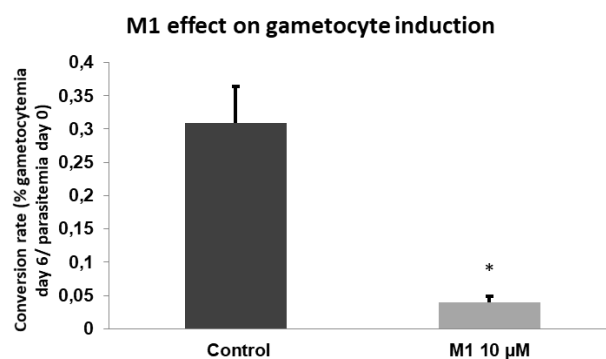


Figure 5. Effect of Gamhepathiopine *in vitro* on *P. falciparum* gametocytes development. (A) Stage III gametocytes at day 6 (* p -value = 0.0465). (B) Stage V Gametocytes at day 10 (* p -value = 0.0122). Control= untreated.

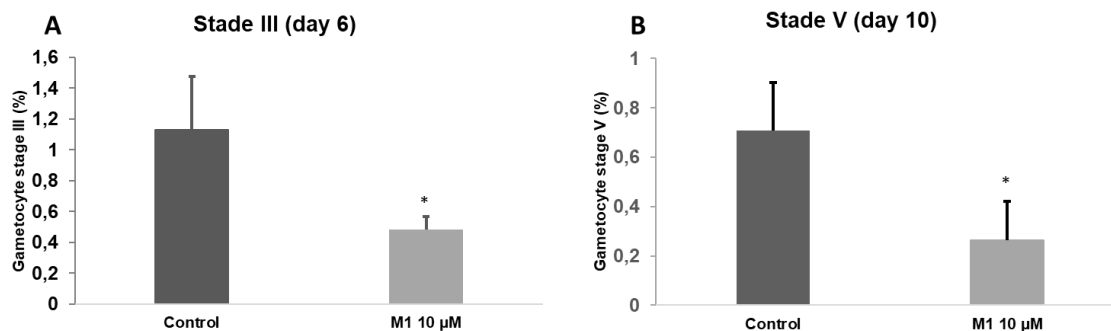


Figure 6. Effect of M1 *in vitro* on *P. falciparum* gametocytes. (A) Effect on the exflagellation of mature gametocytes (stage V) after 2 h of treatment with M1 (10 µM), mean of 3 independent experiments. (B) Effect on the exflagellation of mature gametocytes (stage V) after 24 h of the treatment M1 (10 µM). Control = untreated. (** p -value= 0.065)

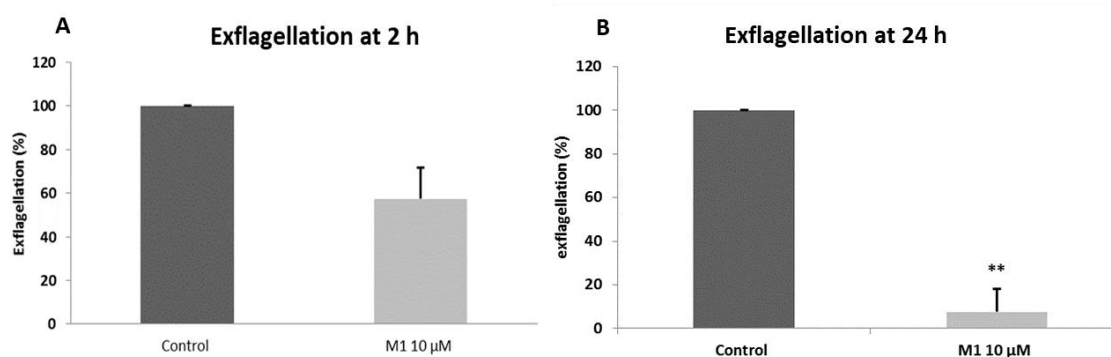


Table 2. Chemo-sensitivity by standard assay of *P. falciparum* F32-ART5 and F32-TEM lineage to M1

	EC ₅₀ values, nM		<i>p</i> -value [§]
	Mean ± SD		
	F32-ART5	F32-TEM	
M1	227 ± 40	283 ± 125	0.547
Artemisinin	11 ± 0.5	11.1 ± 1.6	0.894

[§] Paired t test. A *p*-value < 0.05 was considered as statistically significant. Results represent the mean of three independent experiments. Artemisinin was used as drug control.

Figure 7. Recrudescence curves of synchronous ring-stage parasites of F32-ART5 (solid lines) and F32-TEM (broken lines) after a 48-h drug exposure using Kaplan-Meier survival analysis. The final event was defined as the time necessary for *P. falciparum* cultures to reach initial parasitemia. Observations were considered censored if no recrudescence was observed at day 30. Log-rank test was performed (p -value < 0.05 is considered as significant). A) Treatment with 18 μM artemisinin ($n = 7$); B) Treatment with 2.5 μM M1 ($n = 4$); C) Treatment with 10 μM M1 ($n = 3$).

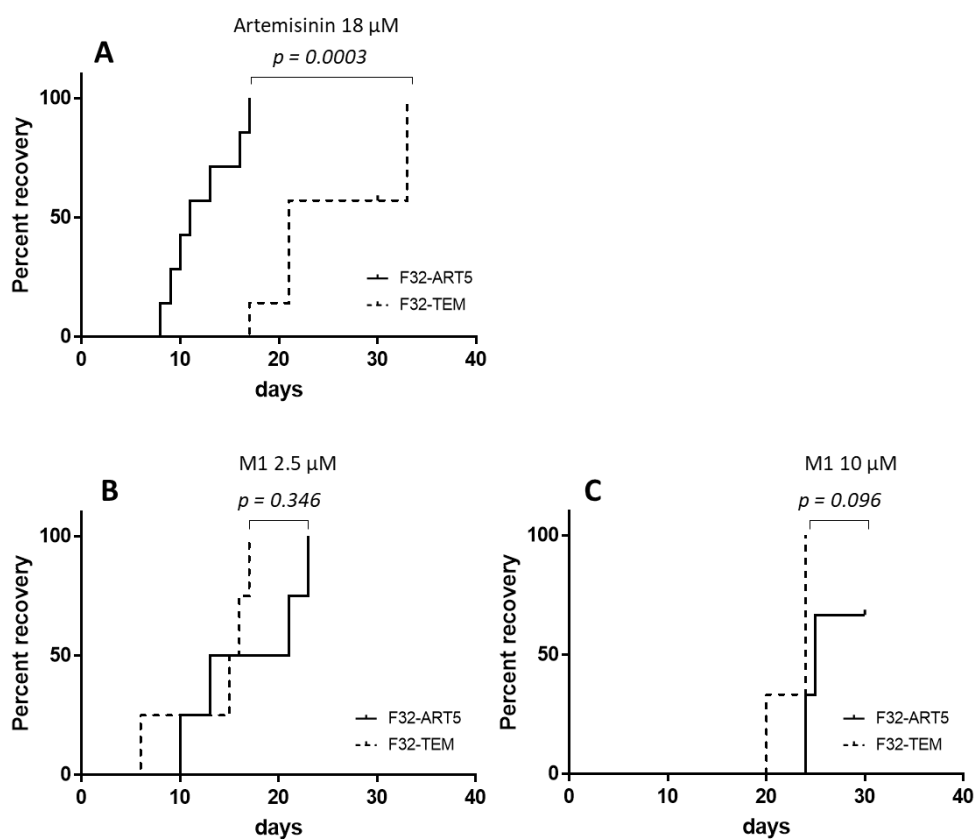


Figure 8. Recrudescence curves of synchronous ring-stage parasites from the F32-ART5 line tested by QSA, after treatment with “artemisinin 3 μ M 6 h / artemisinin 3 μ M 24 h”, “artemisinin 3 μ M 6 h / (artemisinin 3 μ M + drug) 24 h” and “mock 6 h/ drug 24 h” using Kaplan-Meier survival analysis. The final event was defined as the time necessary for parasite cultures to reach initial parasitemia. Observations were considered censored if no recrudescence was observed at day 30. Log-rank test was performed (p -value < 0.05 is considered as statistically significant). A) Treatment with 2.5 μ M M1 (n=5); B) Treatment with 200 nM CQ (n=1), recrudescence curve of parasites treated with the combination artemisinin / artemisinin + CQ overlap with that of the parasites treated with artemisinin alone.

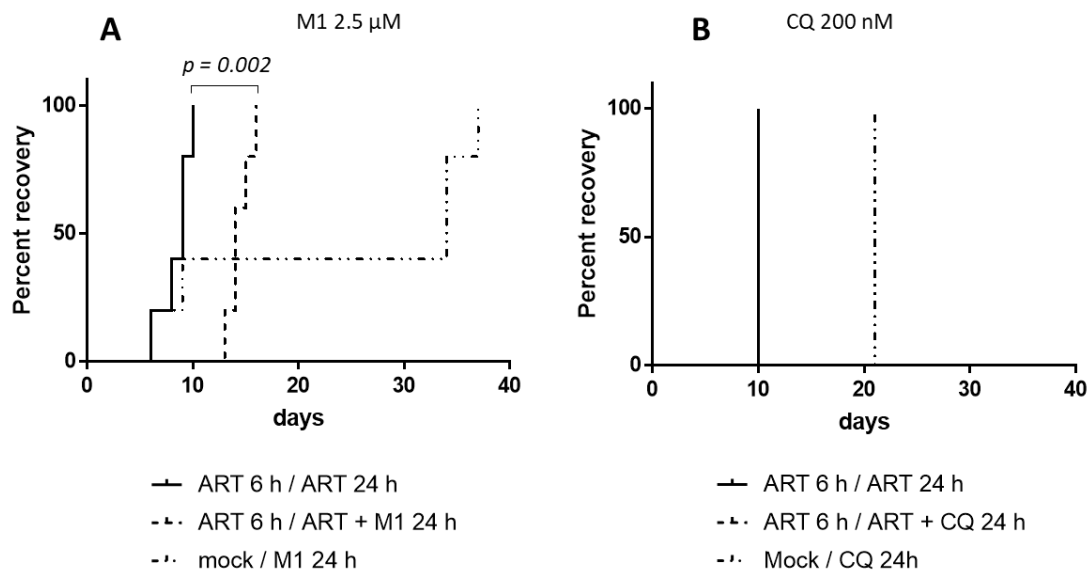


Figure 9. *In vivo* antimalarial activity of M1 against liver and blood stage. Mice were infected with 10,000 sporozoites. M1 (50 mg/kg) and Primaquine = PQ (50 mg/kg) were administered on day -1, 0 and 1 to monitor activity against liver stage and on day 3 and 4 to monitor blood stage activity. (A) Representative *in vivo* images (IVIS) of luminescence show in the liver of live Balb/C mice at different time points. Rainbow images show the relative levels of luminescence ranging from low (blue), to medium (green), to high (yellow/red). (B) Activity of M1 with 1-aminobenzotriazole (ABT). (C) Activity of M1 and PQ: mean luminescence levels (photos/sec) of each groups at D2, D3 and D6. (D) Activity of M1 and ABT: mean luminescence levels (photos/sec) of each groups at D2, D3 and D6 (***) p -value < 0.0056). Control = treated with DMSO.

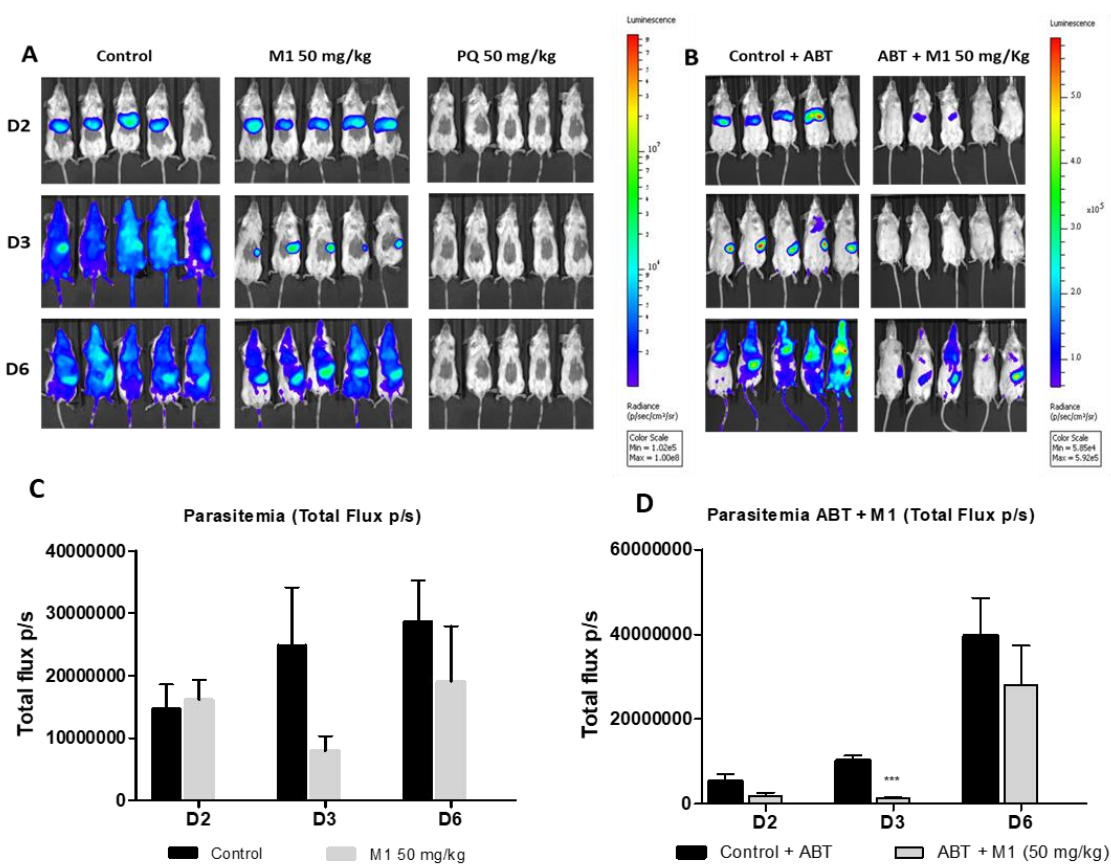


Table 3. Parasitemia and gametocytemia before feeding and prevalence of infected mosquitoes in each group. Mice from the group “M1 D3 2 h” received the treatment on D3, 2 h before mosquito feeding. Mice from the group “M1 D0-D3” received the treatment every day from D0 to D3.

Parasitemia at day 4	(M1, 50 mg/Kg i.p.)		
	Untreated	M1 D3 2 h	M1 D0-D3
Parasitemia (%)	0.97	0.99	0.47
Gametocytemia (%)	0.05	0.04	0.04
Sporogonic stage			
Infected mosquitoes (%)	29/35 (82.8)	13/35 (37.1)	25/35 (71.4)
Oocysts per mosquito	106	1	32

Figure 10. Impact of M1 50 mg/kg on sporogonic development. Mice from the group “M1 D3 2 h” received the treatment on D3, 2 h before mosquito feeding. Mice from the group “M1 D0-D3” received the treatment every day from D0 to D3. (A) Number of oocysts per midgut. Each point represents the oocyst number from an individual mosquito. Oocysts density in M1 is significantly different compared with the untreated group (***) p -value < 0.0001). (B) Average of sporozoites extracted from salivary glandes of mosquitoes (Total of sporozoites/number of mosquitoes, ** p -value < 0.11). The results showed are representative of one assay among the three independent assays performed.

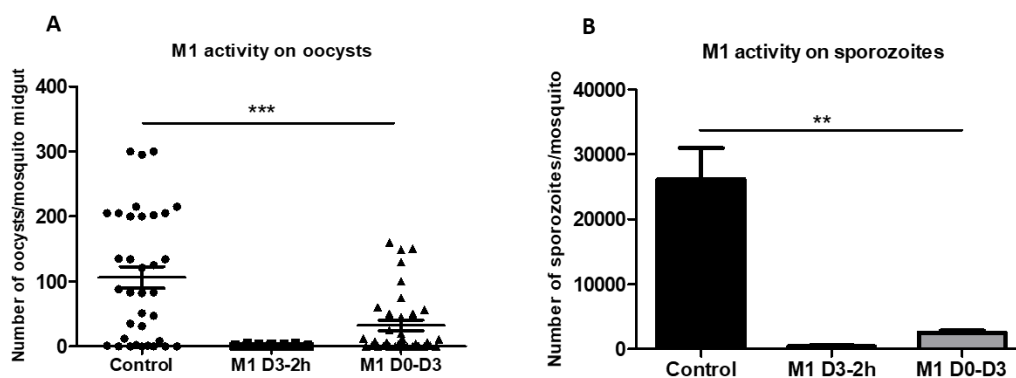
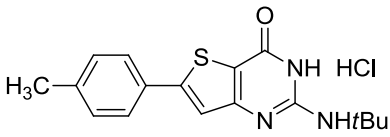
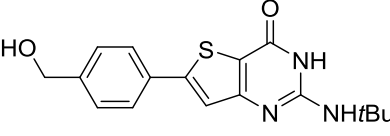
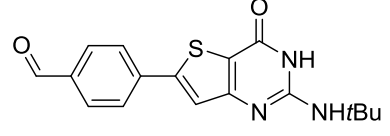
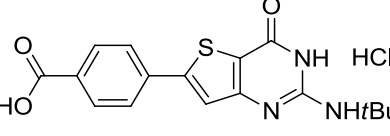


Table 4. *In vitro* activities of gamhepathiopine (M1) and some of its metabolites (**7**, **8**, **10**) on the erythrocytic stage of *P. falciparum* (K1), hepatic schizontes of *P. yoelii* in HepG2-CD81 cells and corresponding cytotoxicities on the human HepG2 cell line.

Molecule	Structure	<i>P. falciparum</i> (K1) in red blood cells EC ₅₀ (μM)	<i>P. yoelii</i> in HepG2- CD81 EC ₅₀ (μM)	HepG2 Cell viability CC ₅₀ (μM)
Gamhepa- thiopine = M1		0.045	0.067	24.0
Metabolite 7		0.4	1.0	25.0
Metabolite 8		3.1	ND	25.3
Metabolite 10		>10 ^a	>10 ^a	>10 ^a
Atovaquone ^b		0.001	0.003	>15.6 ^a
Chloroquine ^b		0.8	-	30
Doxycycline ^b		6	-	20
Primaquine ^b		-	3.6	-
Doxorubicin ^c		-	-	0.2

^aThe EC₅₀ or CC₅₀ value could not be reached because of a lack of solubility in the culture media

^bAtovaquone, chloroquine, doxycycline and primaquine were used as positive antimalarial controls

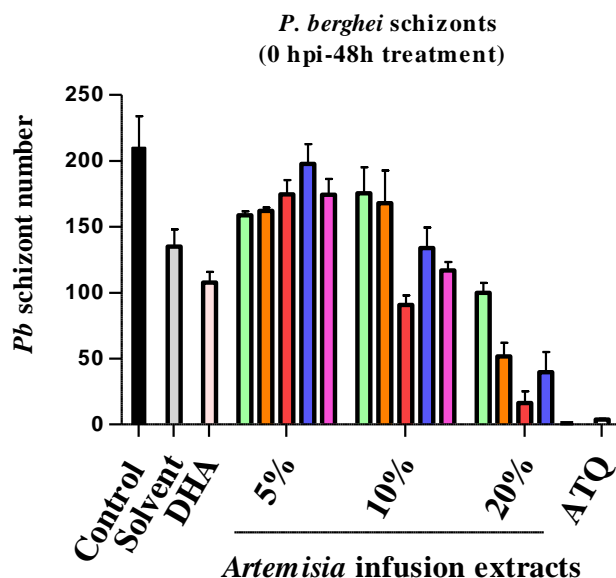
^cDoxorubicin was used a positive cytotoxic control

ANNEX 2

I. Extracts derived from *Artemisia* infusions show potent inhibitory activity against rodent malaria species

In a collaboration, we acquired *Artemisia* extracts that were systematically isolated from *Artemisia* infusions following subsequent and stepwise fractionation. We executed the experiment with *P. berghei* infected simian hepatocytes. The extracts treatment was started at the same time of sporozoite infection to cells. However, few extracts were identified active against the hepatic stage of rodent parasites. A complete elimination of hepatic parasites was observed with a high percentage of extracts 5a; the extract was isolated from *A. afra* infusions, whereas significant reduction of exo-erythrocytic forms (EEFs) were also observed with other extracts at high concentrations. Atovaquone (ATQ) used as positive drug control, had similar activity like extracts 5a at 10 nM ATQ concentration. In addition, 20% water also slightly inhibited the number of EEFs. The inhibition, shown by 20% water, was not surprising as that amount of water might dilute the culture media that indirectly affected the nutrient composition required for parasite survival. In addition, the parasite area was also reduced in a considerable way.

A



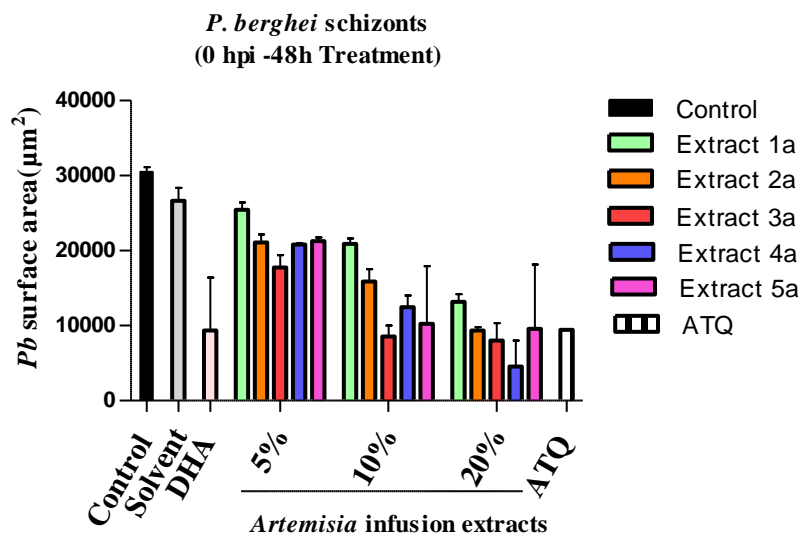


Figure: The effect of extracts of *Artemisia* infusion on the rodent *Plasmodium* parasites

A) Bar chart represents the result of *P. berghei* schizonts while the *P. berghei* infected simian hepatocytes culture was treated with infusion extracts. Atovaquone (ATQ) used here as positive drug control. B) Area reduction of *P. berghei* schizonts after extract treatments. The experiments were performed in triplicates and repeated two times, representative data are shown here.

***Artemisia* extract concentrations used in this assay don't render hepatotoxicity**

The profound killing efficacy of *Artemisia* extracts provoked us to monitor the health of infective primary cryopreserved simian hepatocytes treated with *Artemisia* extracts.

The experimental infusion concentrations tested in these liver stage assays demonstrated any hepatotoxicity neither on human hepatocytes nor on simian hepatocytes. The evaluation was observed by a 'High throughput screening' approach that facilitated to evaluate the candidate drug toxicity in addition to the activity profile.

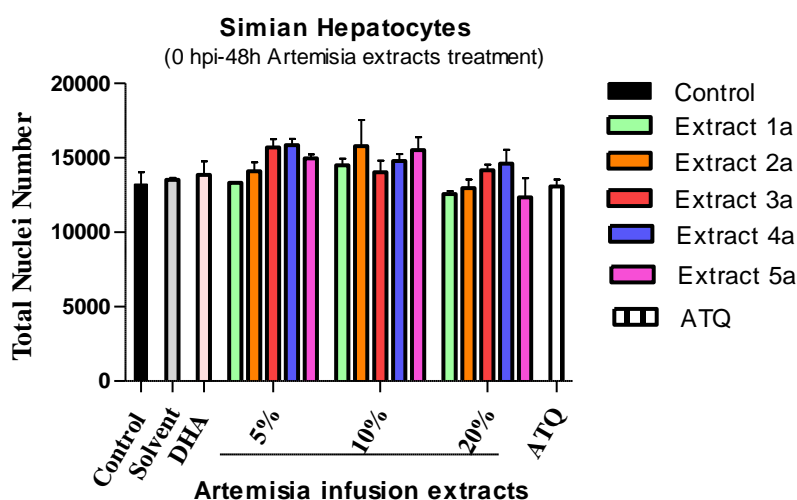


Figure 8: High throughput scanning assesses hepatotoxicity of *Artemisia* infusion treatments

A) Toxicity of different infusion concentrations on primary simian hepatocytes. The number of hepatocyte nuclei quantified from a certain number of microscopic scanning fields from control and infusion treated group's shows level of hepatotoxicity. Total nuclei counts of simian hepatocytes treated with *Artemisia* extracts derived from infusions.

Preliminary studies of *in vitro* rodent malaria assays have shown that *Artemisia* extracts retained the antimalarial activity like infusions. Antimalarial activity of the extracts particularly extract 5a was equivalent to the well-known prophylactic drug atovaquone (Fig. A & B). It can be mentioned that our close monitoring of toxicity evaluation based on high content screening platforms clearly indicate that the antimalarial effects observed with infusions were not due to the toxicity on human and monkey hepatocytes

II. Screening of epigenetic inhibitors against liver stage *Plasmodium*

Background

Recently, epigenetic event in late stage hypnozoites of *P.cynomolgi* has been reported (**Bertschi et. al, 2018**) and for the first time, a possibility for an epigenetic basis of hypnozoite dormancy has been investigated (**Dembele et. al., 2014**). Beyond this, there is no extensive epigenetic study in liver stage has been reported. So screening of potential HDACi with low activity against human HDAC is likely to be good starting point. It could be great interfering the hypnozoite mediated dormancy by selective epigenetic inhibitors to provoke reactivation and also quite challenging to break the dormancy of hypnozoites and which can be eliminated by potent schizonticides like “Wake & kill strategy”.

Result and Discussion

Epigenetic inhibitor as anti-malarial

Cpd84 was screened *in vitro* at hepatic stage of four *Plasmodium* species. For murine parasites likely *P.berghei* & *P. yoelii*, cpd84 showed potent activity during the standard treatment (0h-48h pi) whereas it also showed strong sporocidal activity during 1h pre-incubation with sporozoites (**Fig. 2A-C**). This effect also was in accordance with human parasite *P.falciparum* (IC₅₀ 1.8 µM) and *P.cynomolgi* (IC₅₀ 430.9 nM) which is a genetically close relative of *P. vivax*. Moreover, development of both replicating (schizonts) and non-replicating (hypnozoites) forms in *P.cynomolgi* culture were completely arrested during 1h pre-incubation at 4 µM concentration (**Fig. 2C**). One possible answer may be liable to this, as HDACi's are faster acting small molecules which may quickly interact with the sporozoites, affecting its' transcriptional machineries that are essential for gliding and invasion to host hepatocytes. However, mechanisms underlying of it, of course warrants further investigations. Apart this, standard treatment (D0-D7) of cpd84 against *P.cynomolgi* and *P. falciparum* infected hepatocytes showed marked inhibitory activity IC₅₀ 320.7nM and IC₅₀ 2.7 µM respectively. Dembélé, Franetich et.al, showed a *Plasmodium* specific” wake & kill “strategy by utilizing a methyltransferase inhibitor that was demonstrated to be involved in the quiescence or liver stage dormancy. Interestingly, 4 µM of cpd84 treatment initiated from day 4 to day 7, there is a slight

increase of schizonts number (62.7 %) compared to control (52%) (**Fig. 2D**) which was not statistically significant although but questions arise is it first provoking to “wake up” tendency of hypnozoites by HDACi. Indeed a single histone positive structure was identified in *P. vivax* infected humanized mice liver sections at day 5 of post infection describing it was not ready further for DNA replication and even at day 21 (**Mikolajczak et. al, 2015**).

Another novel histone deacetylase inhibitor (HDACi) called FR2352225 (a cyclic tetrapeptide fungal metabolite) was screened against *P. vivax* liver stage. FR2352225 (E3) showed potent activity by arresting the development of *P. vivax* schizont and hypnozoite particularly when treatment was initiated from D0-D5. Size of remainder schizonts were also curtailed by the compound E3. It could be note that the antimalarial effect was not associated with E3 mediated host cell toxicity which was validated by counting the total nuclei of human hepatocytes (**Fig. 3**). Recent transcriptomic study of *P.cynomolgi* hypnozoites revealed that acetylation-methylation event is profoundly active specially at late stage (**Bertschi et. al., 2018**). If it is, administration of epigenetic inhibitors likely DNMT, HDAC may render an effect inducing earlier DNA replication, modulating transcriptional machineries to regulate gene expression or repression that can break the dormancy or may facilitate re-activation of hypnozoites.

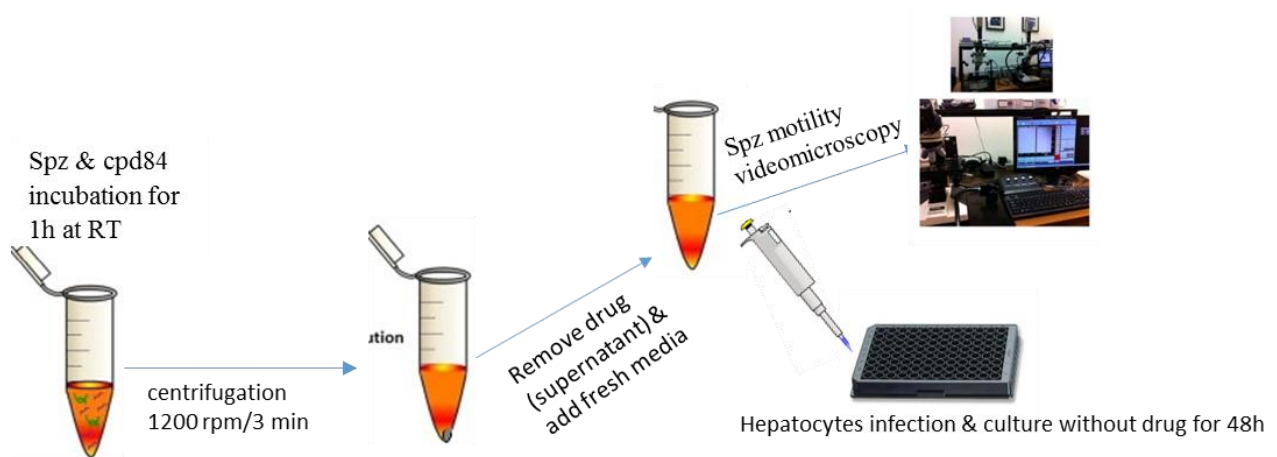
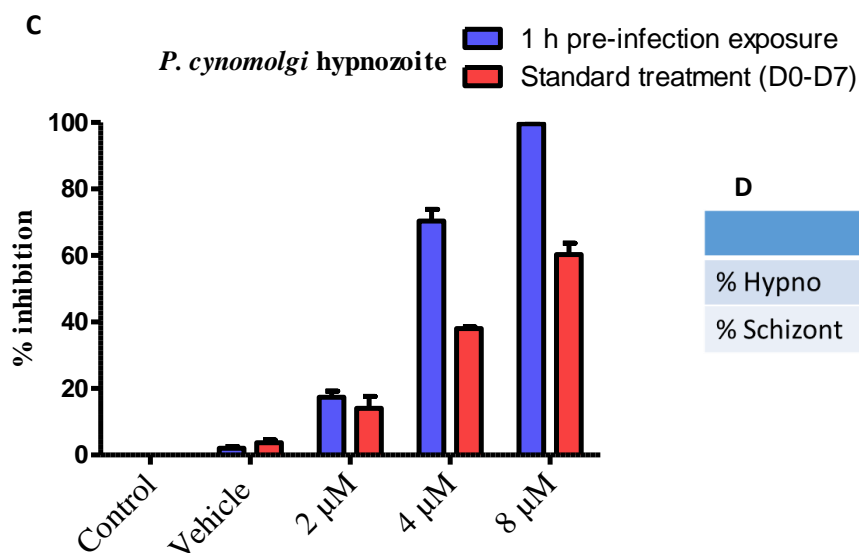
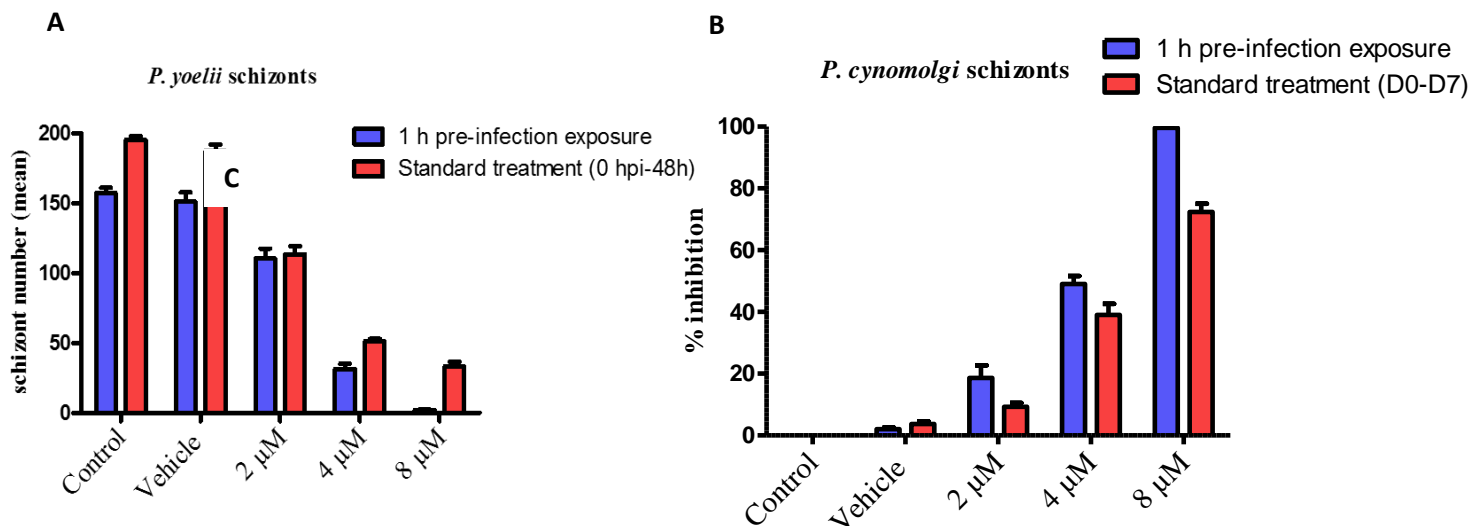


Figure 1: Schematic outline of pre-infection exposure of sporozoite with cpd84 and hepatocytes infection. Freshly isolated sporozoites were incubated with varying concentration of compound 84 at room temperature for 1h. Cpd84 exposed sporozoites were centrifuged at 1200 rpm for 3 minutes and washed three times to remove the drug contents. By replacing fresh medium, sporozoite suspensions were allowed to infect hepatocytes seeded in 96 well plate and continued the culture for 48h without cpd84 treatment. A part of sporozoite suspensions were processed for videomicroscopy to analyze sporozoite motility following 1 h exposure of Cpd84.



D

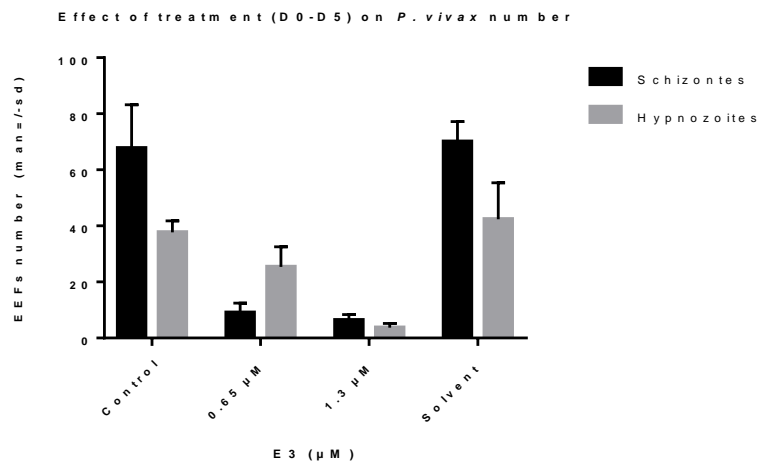
	Control	Cpd84 (4 μ M)
% Hypno	48%	37%
% Schizont	52%	63%

E

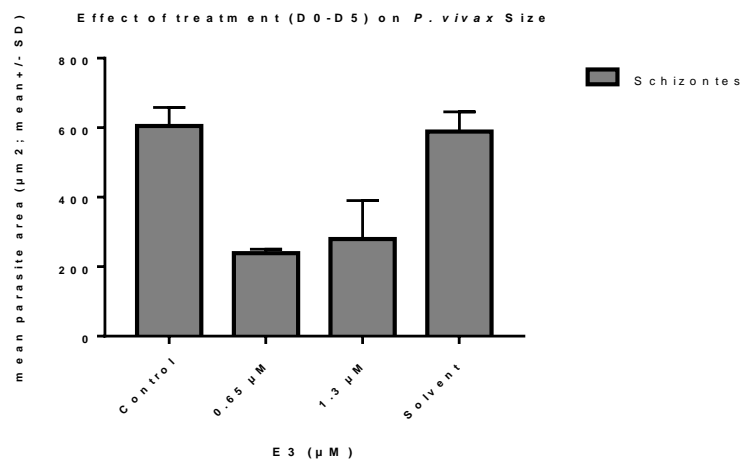
<i>Plasmodium</i> sp.	1h pre-infection exposure of sporozoites with Cpd84	Standard treatment
<i>P. berghei</i>	410.58 nM	600.13 nM
<i>P. yoelii</i>	388.32 nM	>800 nM
<i>P. cynomolgi</i>	430 nM	320.7 nM
<i>P. falciparum</i>	1800 nM	2700 nM

Figure 2: Pre-infection exposure of sporozoites with cpd84 interferes sporozoite to schizont development A &B) in two independent assays, *P. yoelii* and *P. cynomolgi* sporozoites were incubated with cpd84 for 1h before infection to hepatocytes. After removing the drug contents; cpd84 exposed sporozoites were allowed to infect simian hepatocytes. Apart from this, cpd84 and freshly isolated sporozoites were added simultaneously as prophylactic in the hepatocytes culture and continued the treatment till 48h for *P. yoelii* and until Day 7 for *P. cynomolgi*. Bar chart expressing mean schizont number of *P. yoelii* (Fig 2A) and % inhibition of schizonts of *P. cynomolgi* (**Fig. 2B**) following cpd84 treatment. DMSO was used as control vehicle to dissolve cpd84. **C**) % inhibition of hypnozoites of *P. cynomolgi* following 1h pre-infection exposure and standard treatment (D0-D7). **D**) % ratio of hypno and schizont following D4-D7 treatment of cpd84 against *P. cynomolgi* liver parasites. **E**) IC50 of different *Plasmodium* species following Cpd 84 treatment were calculated by malaria IC50 estimator.

A



B



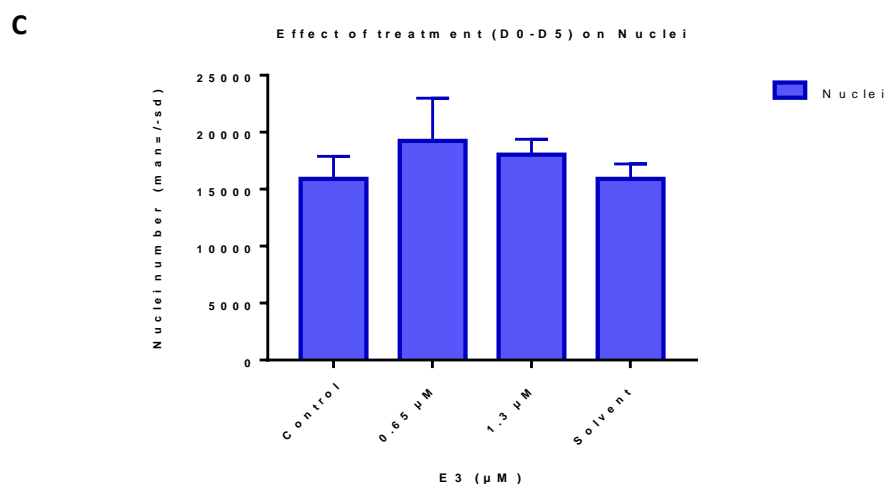


Figure 3: E3 treatment arrest *vivax* sporozoites to hypnozoite and schizont development in human hepatic culture. A) D0-D5 prophylactic treatment of E3 curtailed the liver stage parasite load of *P. vivax* compared to control. **B)** Size estimation of *P. vivax* schizonts following E3 treatment. Parasite area were measured in μm^2 **C)** Total nuclei number were counted by high throughput scanning machine utilizing the drug assay plates.

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<https://doi.org/10.7554/eLife.29605>

List of abréviations

ABS	Asexual blood stage
ACT	Artemisinin combination therapy
ACP	Acyl carrier protein
ATQ	Atovaquone
ART	Artemisinin
AZT	Azithromycin
CSP	circumsporozoite protein
DHA	Di hydro artemisinin
hpi	Hour post infection
LB	Lysogeny broth
EEF	Exo erythrocytic forms
MP	Methyl paraben
PS	Penicillin-streptomycin
HH	Human hepatocytes
GFP	Green fluorescent protein
nM	Nano molar
PQ	Primaquine
TVN	tubulovesicular network
UIS4	Upregulated infectious sporozoite 4
LSIP2	Liver specific protein 2
TQ	Tafenoquine
PI4K	phosphatidylinositol 4-kinase inhibitor
G6PD	Glucose 6 phosphate dehydrogenase
PVM	parasitophorous vacuole membrane
HTS	high throughput screening
HDAC	Histone deacetylase
MB	Methylene blue
RSA	Ring survival assay

RESUME

Ma thèse de doctorat s'intitule « Une étude complète des effets antiplasmodiaux d'infusions d'*Artemisia* spp contre plusieurs stades de développement du parasite, y compris l'hypnozoïte ». Le parasite dormant résidant dans le foie, appelé hypnozoïte, peut persister pendant des semaines, voire des mois, puis « s'éveiller » pour provoquer une infection récurrente au stade sanguin. Ce réservoir d'hypnozoïtes est l'un des obstacles critiques à l'éradication du paludisme, principalement en raison du manque de médicaments capables de l'éliminer. Le développement de nouveaux médicaments contre ces hypnozoïtes, est limité car les criblages phénotypiques sont entravés par le manque de plates-formes de culture *in vitro*. Dans des conditions de culture conventionnelles, les cultures d'hypnozoïtes sont souvent contaminées par des microbes qui affectent négativement la culture des hépatocytes. Ces contaminants sont introduits lors de l'inoculation des cultures par les sporozoïtes, récupérés par dissection manuelle et broyage des glandes salivaires de moustiques infectés. En abordant cette question, dans mon introduction générale (**chapitre 1**), je me suis d'abord concentré sur le développement d'un protocole facile à mettre en place à base d'un cocktail antimicrobien composé de méthylparaben, de pénicilline et de streptomycine. Ce cocktail, ajouté à la solution nutritive des moustiques à base de saccharose, permet de récupérer des sporozoïtes exempts de contaminants fongiques dans les glandes salivaires des moustiques infectés et est décrit en détails dans le **chapitre 2**. Après avoir développé une solide stratégie de culture, je suis passé au criblage de composés contre le stade hypnozoïte. Nous avons sélectionné deux *Artemisia* sp. La raison du choix de ces plantes a été décrite en détail dans ce manuscrit. J'ai testé l'effet d'infusions d'*A. annua* et d'*A. afra* sur les stades pré-érythrocytaires de différentes espèces de *Plasmodium*. J'ai trouvé une forte activité antipaludique des infusions d'*Artemisia*, en particulier sur l'infectivité des sporozoïtes, le blocage de la formation d'hypnozoïtes, et une clairance des parasites hépatiques *in vitro*. De toute évidence, cet effet n'est pas dû à l'artémisinine. Ces effets sont décrits en détail dans le **chapitre 3**. Nous pensons que cette profonde activité inhibitrice de l'infusion d'*Artemisia* sp est partiellement médiée par la perturbation de la biogenèse des apicoplastes et des mitochondries du parasite. Dans le **chapitre 4**, j'ai montré diverses images prises au confocal, montrant différents types d'altération observées sur ces organelles. Les données de PCR quantitatives de l'ADN des apicoplastes confirment ces observations. La résistance aux médicaments de *P. falciparum* est un problème croissant en Asie du Sud-Est et il est urgent de trouver de nouveaux médicaments pour faire face à ce problème. Dans le **chapitre 5**, j'ai décrit l'activité des infusions d'*Artemisia* contre des isolats de *P. falciparum* résistants aux dérivés d'artémisinine. Ma thèse a été effectuée en temps partagé entre l'Institut Pasteur du Cambodge, à Phnom Penh, où j'ai effectué des expériences sur les stades érythrocytaires de *P. falciparum*, et le stade hépatique de *P. vivax*, et le CIMI à Paris où j'ai travaillé sur les stades hépatiques des modèles rongeur et simiens, et du parasite humain *P. falciparum*.