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Various antimalarial strategies in Indonesia to fight *Plasmodium falciparum*

Arba Pramundita Ramadani

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THESIS

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Presented by:
Arba Pramundita Ramadani

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Various Antimalarial Strategies in Indonesia to Fight *Plasmodium falciparum*

JURY

Françoise BENOIT-VICAL Mustofa	Research Director INSERM, Toulouse University Professor, Indonesia	Thesis Promotor Thesis Co- Promotor Examiner
Alexis VALENTIN	University Professor – Hospital Practitioner, Toulouse	Examiner
Mahardika Agus Wijayanti	Research Director Faculty of Medicine, UGM, Indonesia	Reviewer
Supargiyono	University Professor, Indonesia	Reviewer
Iwan Dwiprahasto	University Professor, Indonesia	Reviewer
Sofia Mubarika Harjana	University Professor, Indonesia	Reviewer

Doctoral Program Biologie – Santé – Biotechnologie
Research Unit LCC-CNRS, UPR8241 Toulouse, France
Thesis Promotors Françoise BENOIT-VICAL and Mustofa

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Yogyakarta, July 2017

Arba Pramundita Ramadani

*For indeed, with hardship (will be) ease. Indeed, with
hardship (will be) ease.*

[Q.S.94:5-6]

*A chaque problème il y une solution. S'il n'y a pas de
solution, c'est qu'il n'y avant pas de problème.*

[FBV]

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Various antimalarial strategies in Indonesia to fight *Plasmodium falciparum*

ABSTRACT

Malaria remains a global public health problem and worsening with the resistance of *Plasmodium falciparum* to Artemisinin-based Combination Therapies (ACTs), the latest and most effective antimalarial drugs. My project aimed to provide insight into malaria elimination in Indonesia. The first part was to look for new antimalarial drugs based on Indonesian ethnobotanical data. Among 25 crude extracts realized on Indonesian traditional medicinal plants, seven showed a good antimalarial activity ($IC_{50} < 5\mu\text{g/mL}$) and some of them were also active against *Babesia divergens* and *Leishmania infantum*. The second part of the study focused on chemosynthetic organometallic compounds. The structure-activity relationships study on organometallic gold(I)-NHC complexes led to a very active compound on *P. falciparum* with an IC_{50} of 320nM. The third part of this work was dedicated to the study of *P. falciparum* resistance to artemisinin and its derivatives. The correlation between *PfK13* polymorphism and artemisinin resistance has been clearly established thanks to reverse genetic with resistant and sensitive laboratory strains and clinical isolates from Cambodia. This resistance was evidenced *in vitro* throughout a parasite survival assay called $RSA_{(0-3h)}$. By the same genotypic and phenotypic methods, mapping of *PfK13* polymorphism distribution in Indonesia was performed in Kupang on *P. falciparum* malaria patients. However, at the time of *P. falciparum* blood samples collection, prevalence showed a dramatic decrease hindering the continuation of the clinical study. Facing to the very small number of eligible patients with a *P. falciparum* malaria, no conclusive results has been obtained. In conclusion, medicinal plants and synthetic compounds are potentially interesting as chemical starting point for new antimalarial drugs. Concerning artemisinin resistance, any treatment failure or delayed cure with ACTs has yet to be reported in Indonesia. However, because Indonesia is relatively close to the Southeast Asian areas of resistance, the possible occurrence of such cases in Indonesia must be anticipated by determining the variations of *P. falciparum* malaria chemo-sensitivity and by following *PfK13* polymorphism, responsible for artemisinin resistance.

Keywords : malaria, *Plasmodium falciparum*, traditional medicine, *PfK13*, artemisinin, resistance

Différentes stratégies en Indonésie pour combattre *Plasmodium falciparum*

RESUME

Le paludisme demeure un problème de santé publique mondial qui risque de s'aggraver avec la résistance de *Plasmodium falciparum* aux thérapies combinées à base d'artémisinine (ACT), médicaments antipaludiques les plus récents et les plus efficaces. Mon travail avait pour but de proposer différents axes d'élimination du paludisme en Indonésie.

Une première partie a consisté à rechercher de nouveaux médicaments antipaludiques à partir de données ethnobotaniques indonésiennes. Parmi les 25 extraits bruts réalisés à partir de plantes médicinales indonésiennes utilisées traditionnellement dans le traitement du paludisme, sept ont montré une activité antipaludique intéressante ($CI_{50} < 5 \mu\text{g/mL}$) et certains d'entre eux se sont révélés également actifs sur 2 autres pathogènes *Babesia divergens* et *Leishmania infantum*. La deuxième partie de ce travail était axée sur les composés organométalliques synthétiques. Les études de relations structure-activité de ces complexes organométalliques d'or (I) -NHC ont permis de sélectionner un composé actif sur *P. falciparum* avec une CI_{50} de 320nM. La troisième partie du travail a été consacrée à l'étude de la résistance de *P. falciparum* à l'artémisinine et à ses dérivés. La corrélation entre le polymorphisme de *PfK13* et la résistance à l'artémisinine a été clairement établie grâce à des études de génétique inverse avec des souches de laboratoire résistantes et sensibles et des isolats cliniques Cambodgiens. Cette résistance a été mise en évidence *in vitro* par un test de survie parasitaire appelé RSA_(0-3h). Par les mêmes méthodes génétique et phénotypique, la cartographie de la distribution du polymorphisme de *PfK13* en Indonésie a été réalisée dans la zone de Kupang sur des patients infectés par *P. falciparum*. Cependant au moment de la collecte des échantillons de sang, la prévalence de *P. falciparum* a montré une diminution spectaculaire empêchant la poursuite de l'étude clinique. Face au faible nombre de patients admissibles avec un paludisme à *P. falciparum*, aucun résultat concluant n'a pu être obtenu.

En conclusion, les plantes médicinales indonésiennes et les composés synthétiques sont potentiellement intéressants comme point de départ chimique pour de nouveaux médicaments antipaludiques. En ce qui concerne la résistance à l'artémisinine, aucun échec thérapeutique ou parasitologique après traitement par ACT n'a été signalé, pour le moment, en Indonésie. Cependant, les zones de résistance de *P. falciparum* aux ACT dans le Sud-Est asiatique sont relativement proches et nécessitent, en Indonésie, un suivi des variations de la chimiosensibilité du paludisme à *P. falciparum* et du polymorphisme de *PfK13*, responsable de la résistance à l'artémisinine.

Mot de clé : paludisme, *Plasmodium falciparum*, traditionnel médecine, *PfK13*, artemisinin, résistance

INTRODUCTION

Malaria is a tropical infectious disease, which continues to be one of the largest public health problems in developing countries. According to the World Health Organization (WHO), there were approximately 212 million cases worldwide in 2015 [1]. Most of them occurred in Africa (90%) followed by South East Asia (7%), including Indonesia that shared 9% malaria cases of SEA region in 2015 [1]. In Indonesia, based on the latest data collection, there were 252,027 positive cases reported in 2014 [2]. Although morbidity and mortality statistics are routinely under-reported [3], annual parasite incidence (API) is used to determine the morbidity trend and endemicity area in Indonesia [2]. The latest report from Indonesian Ministry of Health (MoH) stated that the national API in 2015 was 0.85 per 1,000 population. However, several provinces showed higher API level than national level, such as Papua (31.93), West Papua (31.29), East Nusa Tenggara (7.04), Maluku (5.81), North Maluku (2.77), Bengkulu (2.03), and Bangka-Belitung Island (1.08) [4]. Almost 60% of malaria cases in Indonesia are caused by *Plasmodium falciparum* followed by *Plasmodium vivax* (40% approximately) with different proportion in each endemic region [5].

Even though some reports stated minor prevalence in several endemic regions, the malaria remains a constant strike each year [6]. Hence, the continuous effort to eliminate malaria in Indonesia is imperative and many Indonesian scientists explore ethno-botanical data to find efficient medicinal plants allowing the discovery of new antimalarials. However, many studies were promptly stopped often due to un-standardized method of assays or sample preparations. Moreover, several promising medicinal plants are endangered due to lacking of knowledge on its cultivation process and uncontrolled harvesting. All of them make the process of discovering new antimalarials from plants come too far from clinical implementation.

Indeed, exploring medicinal plants remains a common way to discover new drugs. In endemic areas, medicinal plants are the most affordable treatments [7] and compounds such as alkaloids, terpenoids, flavonoids, and others possess antiplasmodial activity [8]. As Indonesia has abundant sources of medicinal plants for traditional disease treatments, people excessively use them until today. For example, malaria is commonly treated using herbal medicinal products, particularly to reduce fever simply by using them as patch, drinking brew preparation or decoction. Several plants naturally growing in Indonesia and widely used to treat malaria include *T. diversifolia*, *C. barbata*, *T. crispa*, *A. flava* and *P. cauliflora*. These plants offer an opportunity to discover new antimalarial drugs through assessment their antiplasmodial activities in conjunction with cytotoxic assays to determine their selectivity. Their activity profile can be extended to some other parasites like *Babesia* close to *Plasmodium* or *Leshmania* for which an overlapping in geographic distribution exists with *Plasmodium* and can be at the origin of co-infections in human [9].

The identification of new chemical entities (NCEs) can be sourced by chemical synthesis [10]. Among synthetic drugs, organometallic compounds, such as gold complexes, are particularly interesting due to the intrinsic activity of the metallic moieties able to increase the efficacy of organic drugs. As a result, it has been published that cationic lipophilic gold (I)-NHC complexes were active against chloroquine-resistant *P. falciparum* strain possibly due to the respective positive influence between cationic gold and the presence of nitrogen or sulfur atoms in the ligands [11].

Apart from discovering new drugs, the Indonesian Ministry of Health (MoH) actively launched the malaria elimination program. Comprehensively, the program covers surveillance and vector control, early diagnosis procedure and fast precise treatments in order to stop malaria spreading [12]. Concerning therapies, the Indonesian government follows and adjusts its treatment policy to the WHO recommendations. Accordingly, Artemisinin-based

Combination Therapies (ACTs) have been implemented as the first line therapy for uncomplicated malaria since 2006 especially as many studies revealed the resistance of *Plasmodium* to non-artemisinin therapies in several regions [13] [14].

ACTs, a combination of artemisinin or one of its derivatives (artesunate, artemether, or dihydroartemisinin) with an antimalarial drug partner belonging to a different chemical class [15], are recommended by the WHO [16, 17]. However, although artemisinin is the most potent antimalarial drug, several studies reported a decreasing parasite susceptibility to artemisinins characterized by delayed parasite clearance times, a sign that artemisinin resistance occur [18-20]. In addition, recent findings figured out that the resistance can be also extended to the companion drug of some ACTs, such dihydroartemisinin-piperaquine (DHP) [21], recently implemented in Indonesia.

The mechanism of artemisinin resistance is different from those involved in other antimalarial drug resistances. This mechanism is based on an entrance in a quiescence state, allowing parasites to survive against high doses of artemisinin at the ring stage. Parasites can resume a regular cycle after the removal of drug [22]. This phenomenon is not observable by a standard chemo-sensitivity assay, but by ring-stage survival assay (RSA), which evaluates the susceptibility of *P. falciparum* to artemisinin [23] and reflects the ability of parasites to survive ART treatment [22]. Further investigations found that mutations of K13 propeller, involved in protein ubiquitination, were correlated to artemisinin resistance [24]. In order to discover new targets to overcome this artemisinin resistance, it is necessary to clarify the role of K13 mutations. This is achieved by both insertion and removal of mutated or wild type *Pfk13* genes on clinical isolates and several *P. falciparum* laboratory lines [25].

Interestingly, for the moment, K13 mutations are found to play a role in artemisinin resistance only in Asia and have been studied and documented in Cambodia, Thailand,

Myanmar, Laos, Vietnam [26, 27], and China [28-32] but are not correlated with any case of resistance in Africa [26, 28]. Since Indonesia lies close to regions where artemisinin resistance emerges and spreads with a strong correlation with K13 mutations, it is highly likely that the phenomenon also occurs in Indonesia. The evaluation of clinical, *in vitro* and genotypic chemosensitivity to DHP in Kupang, a region of high malaria prevalence, could provide a new outlook on antimalarial resistance in Indonesia.

CHAPTER I

LITERATURE REVIEW

1. Malaria

Physicians have diagnosed and treated fevers for thousands of years. Until Robert Koch, Louis Pasteur, and their contemporaries uncovered that “germs” were the cause of most febrile illness, fevers were considered as a disease and not the result of the disease. Malaria-like febrile illnesses (with names like “the ague” or “paludisme”) have been described since Hippocrates as periodic fevers were and associated with marshes and swamps [33]. In-depth malaria studies were feasible after the discovery of the parasite responsible for the illness by Charles Louis Alphonse Laveran in 1880 and the parasite transmission to humans by mosquitoes by Ronald Ross in 1897 [34]. Globally, malaria is a disease caused by a parasite of the genus *Plasmodium*, which is transmitted to human and widespread through female *Anopheles* mosquito bites.

There are five species of human malaria parasite: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. knowlesi* and *P. malariae* which have different world distributions though overlapping exists. The first symptoms of malaria are nonspecific and similar to influenza (chills, headache, nausea, fatigue, muscle pain, and rigors). The onset of the symptoms is between 1 to 3 weeks after the infection consecutively to a mosquito bite. Fever may appear 2-3 days after initial symptoms and may follow a pattern of every 2 or 3 days for *P. vivax*, *P. ovale*, and *P. malariae* infections. With *P. falciparum* fever can be erratic and may not follow specific patterns [35].

Plasmodium vivax is mostly found in Middle America, North Africa, and Middle East. The other parasites, *P. ovale* is highly present in Africa and *P. malariae* is widespread around

the world. *P. knowlesi*, a *Plasmodium* species naturally living in macaques, has been positively found in human in Borneo [36], particularly in people living in the natural habitat of simian hosts [37]. *Plasmodium knowlesi* widely exists in west of the Wallace Line in Indonesia (which runs between Borneo and Sulawesi; as well as between Bali and Lombok) [3]. *Plasmodium falciparum*, the deadliest one, is common in West and East Africa, Haiti, the Dominican Republic, part of Amazon (South America), and South East Asia [35] including Indonesia.

Based on the WHO report, malaria caused 429,000 casualties with 303,000 deaths of children under 5 years old in 2015 worldwide [1]. In Indonesia, 80 % malaria cases were found in Papua, West Papua, East Nusa Tenggara (NTT), Maluku, North Maluku and Bengkulu [2, 4]. These provinces are the top-6 provinces with highest malaria rate and have higher level of annual parasite incidence than the national level (0.85/10,000 population) [4]. The Indonesian Ministry of Health stated that in 2014 there were 58 districts /cities considered as high risk areas with 3% population (6,331,381) living there [2]. Furthermore, malaria cases in Indonesia are mainly caused by *Plasmodium falciparum* (60% approximately) and by *P. vivax* (almost 40%) [5].

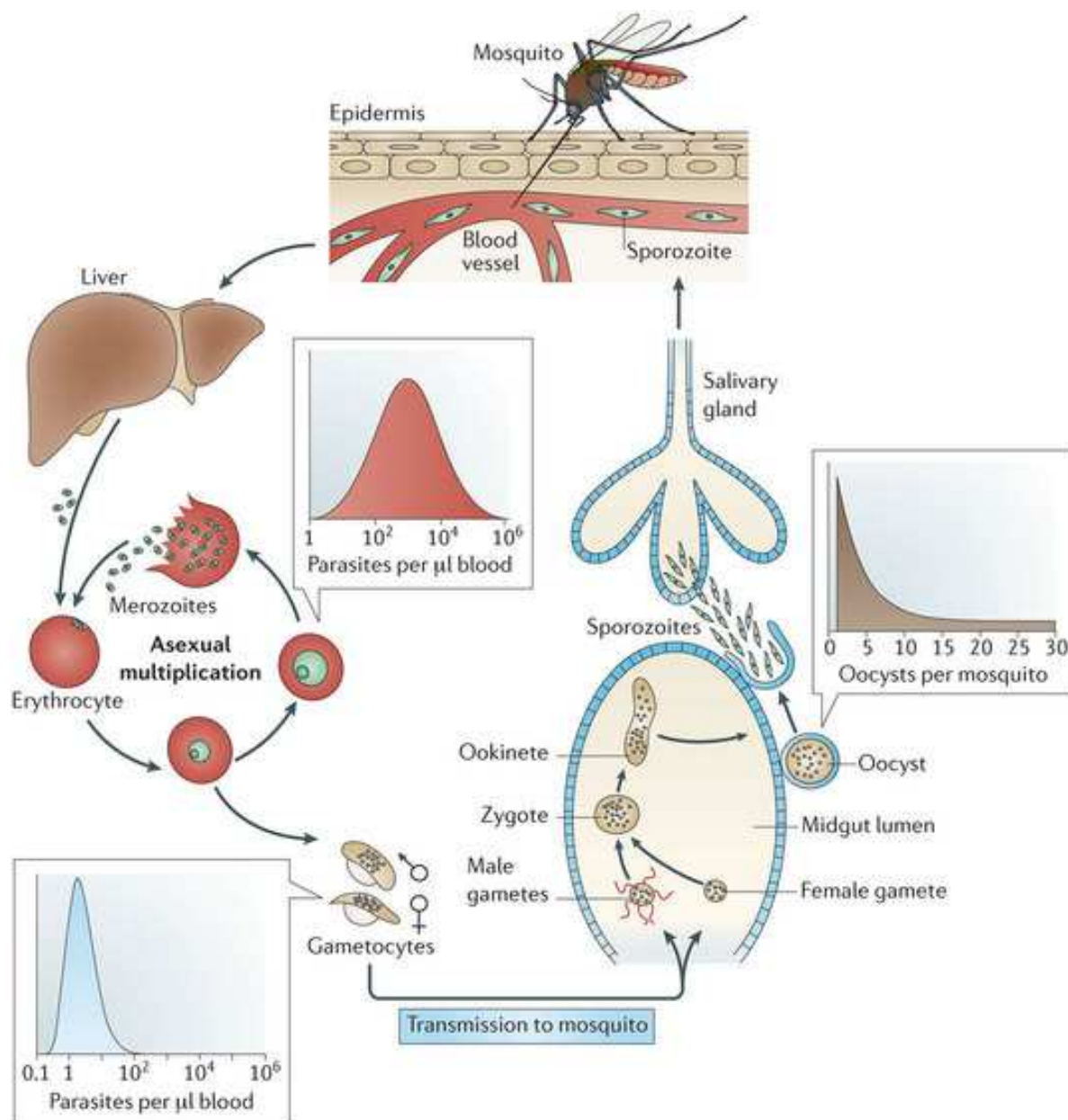
2. The Life Cycle of *Plasmodium*

The life cycle of malaria parasite is complex (Fig. 1). Motile sporozoites are transmitted directly into the human bloodstream by the bite of infected female mosquitoes of the genus *Anopheles* during its blood meal. Within minutes after the bite, the sporozoites circulate briefly in human blood before invading hepatocytes, where for the parasite an asexual life cycle and an important proliferation ensue. Between 5 and 15 days after (depending on the species) thousands of daughter merozoites are released into the blood stream after the rupture of infected

hepatocytes. The merozoites then invade circulating erythrocytes for the clinically important intra-erythrocytic cycle of 48-hour asexual replication [38, 39].

Plasmodium falciparum differs from other human malarial parasites species in that infected erythrocytes do not remain in the bloodstream. After a maturation of 24-32 hours parasites passing as ring stage at the trophozoite stage, parasitized erythrocytes adhere to micro-vessel endothelial cells of various organs (called sequestration). Trophozoites then mature into schizonts with an egress of 48 hours after the beginning of the erythrocytic infection, of 16-31 daughter merozoites that invade non-infected erythrocytes to perpetuate the asexual life cycle. Some parasites inside red blood cells differentiate (in response to stress or other cues) into male and female gametocytes. Consecutively to their ingestion by a female mosquito during its blood meal, they undergo a sexual development [38, 39] .

The formation of male and female gametes results in a rapid maturation of the gametocytes in the mosquito gut (10-20 min). One female gamete emerges from one female gametocyte, and three mitotic division cycles give rise to eight male gametes from a single male gametocyte. Fertilization of a female gamete by a male gamete produces a zygote (the only diploid stage), which undergoes rapid meiotic division and mitosis leading to numerous new haploid sporozoites in an oocyst. Then sporozoites migrate to the salivary gland and await introduction into a host during the next blood feeding, thereby ensuring the continuation of the parasite life cycle [40, 41].



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Figure 1. The life cycle of *P. falciparum* [42]

3. Antimalarial Drug

Malaria was among the first diseases to be treated by a pure chemical compound – quinine - isolated from the cinchona bark in 1820. Historically, two herbal treatments for malaria fevers, cinchona bark and qinghao, were used to effectively treat malaria for hundreds

of years. Today, both derivatives of quinine and artemisinin (from qinghao) remain major in the control of malaria [33].

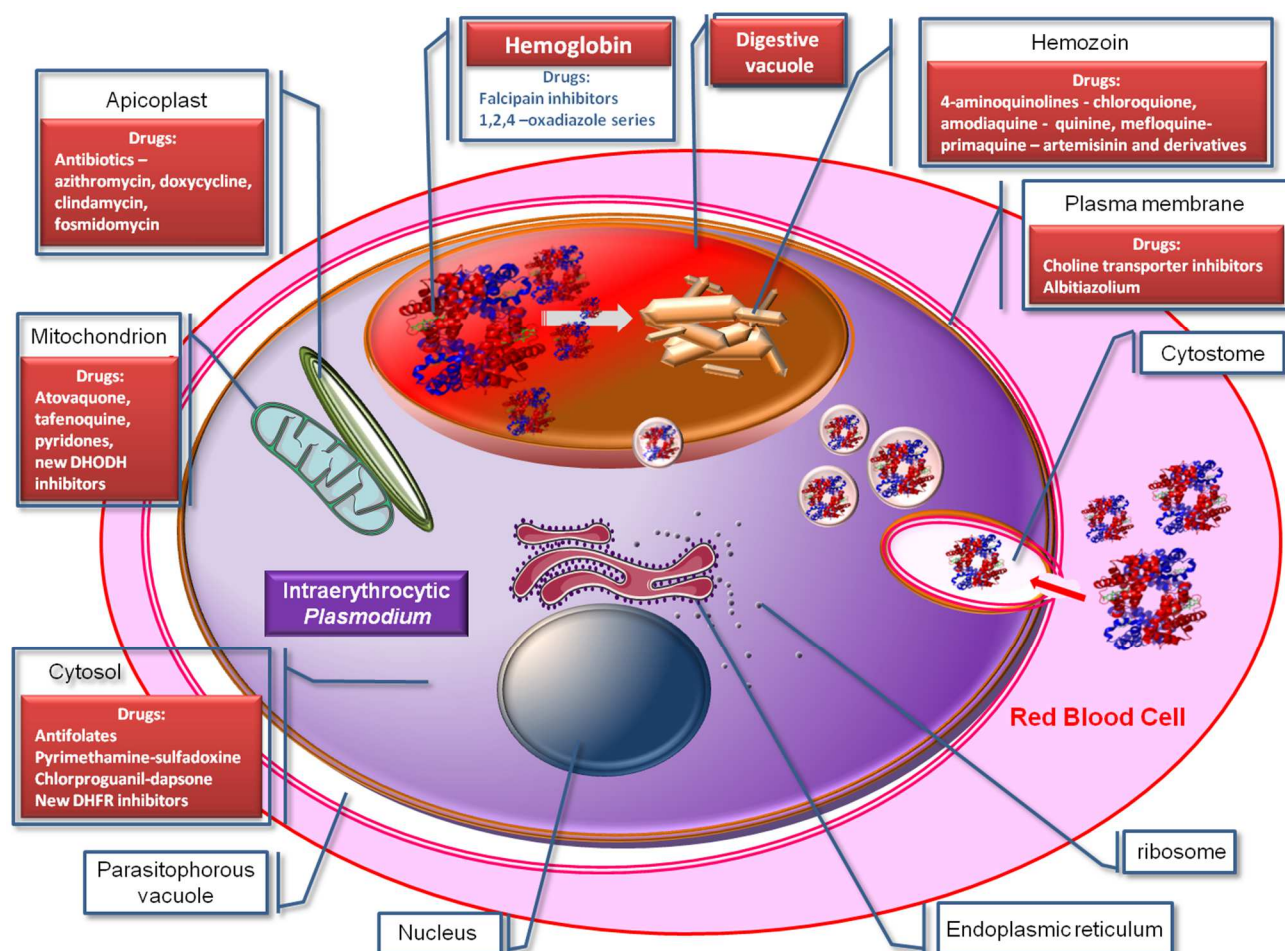


Figure 2. Mode of action of antimalarial drug at the erythrocytic [43-45]

Antimalarials belong to different chemical series with different modes of action (Fig. 2). They target the red blood stage and the merozoite production, some of the gametocytes, and for others also the hepatic stages. In the food vacuole, the polymerization of hemozoin corresponding to the detoxification process of wastes due to haemoglobin digestion is affected by quinolines [43] and artemisinins. Artemisinin and its derivatives are also responsible for alkylation of proteins and heme leading to oxidation damages [46]. At the mitochondrion level, atovaquone, targeting the cytochrome *bc₁* complex, inhibits the parasitic electron transport chain and respectively the dihydroorotate dehydrogenase (DHODH) linked to the respiratory

chain and implicated in the pyrimidine nucleotides biosynthesis. Antifolates like proguanil and pyrimethamine inhibit the folic acid action involved in the DNA and RNA synthesis by blocking the dihydrofolate reductase and dihydropteroate in the cytosol, (Fig. 2).

a. Quinine and related compounds (Fig. 3)

Quinine, along with its dextroisomer quinidine, continues to be used for the treatment of severe malaria. Chloroquine (CQ) is a 4-aminoquinoline derivative of quinine and the most widely used antimalarial drug. It has been the drug of choice for uncomplicated malaria therapy and for chemoprophylaxis. Yet, its use has dramatically reduced due to widely spread resistance. Chloroquine works by binding with ferriprotoporphyrin IX in the parasite, thereby antagonizing the polymerisation of this toxic metabolic product into inert crystal of hemozoin [47]. Another quinine derivative, amodiaquine, is an available compound closely related to CQ. Other quinine-related compounds in common use include primaquine (specially used to eliminate the exoerythrocytic forms of *P. vivax* and *P. ovale* that cause relapses) and mefloquine, a quinoline-methanol derivative of quinine [48]. The primaquine action mechanism is mediated by reactive metabolites which destroy mitochondrial structure of the parasite [49], while mefloquine is believed to work in the same way as CQ [50].

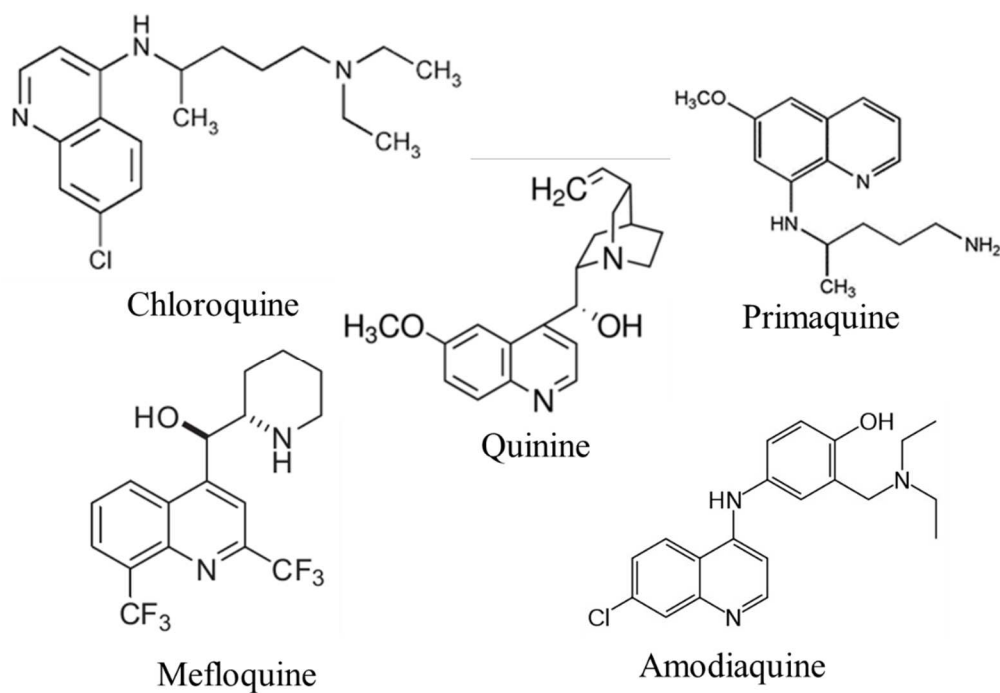


Figure 3. Aminoquinolines: quinine and related compounds

b. Antifolate drugs

These drugs are a variety of dihydrofolate-reductase (DHFR) inhibitors [50] that arrest DNA replication (proguanil, chlorproguanil, pyrimethamine, and trimethoprim) and dihydropteroate synthase (DHPS) inhibitors (dapson, sulfalene, sulfamethoxazole, sulfadoxine, and others) (fig. 4). Despite their use in combinations, parasite resistance to these drugs can develop rapidly.

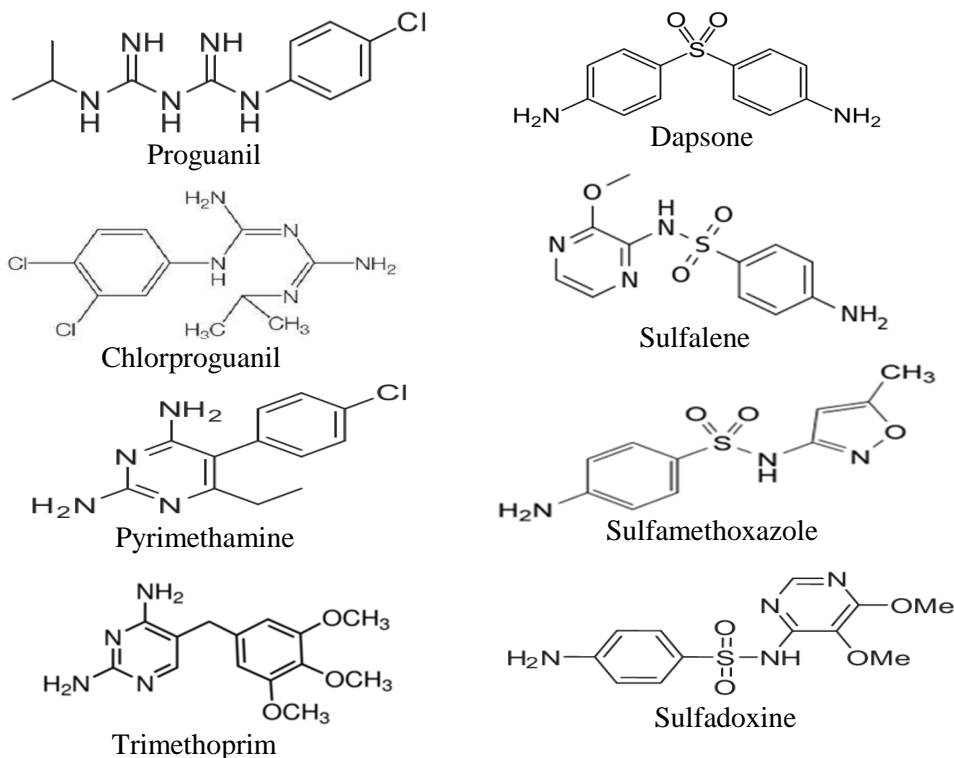


Figure 4. Antifolate drugs

c. Antibiotics

Tetracycline and its derivatives such as doxycycline are very potent antimalarials and used for both treatment and prophylaxis. In areas where response to quinine has deteriorated, tetracyclines are often used in combination with quinine to improve cure rates [51, 52] and effectively reduce the duration of treatment [53]. Another antibiotic, clindamycin, has been used as an effective treatment of malaria when given twice daily for five days but it is a slow-acting drug. With a mean parasite clearance time of four to six days its administration in monotherapy is likely to pose a danger [53]. Therefore, clindamycin treatment in combination with another antimalarial drug is highly recommended. The combination of clindamycin (5 mg/kg Body Weight) with quinine (10 mg/kg Body Weight) is an efficient and attainable choice for the treatment of malaria. Moreover, it shortens the duration of treatment for 3 days twice

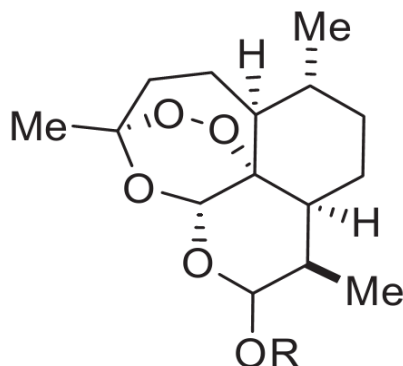
daily and is not contraindicated in children and pregnant women [53, 54]. The combination of clindamycin and CQ is also an effective and plausible treatment even in high rate of CQ resistance regions though requiring a higher dose of CQ [52, 53, 55, 56].

d. Artemisinin

In 1967, the Chinese government initiated the National Project against malaria under the direction project 523 office. Focus on exploration of more than 2000 Chinese herbs has resulted in 640 hits with probable antimalarial activity and 380 extracts studied against animal models of malaria. Promising results were found in *Artemisia annua* L. extracts with strong and reproducible parasite growth inhibitions. These activities were confirmed on animal models (mice and monkeys) and supported the studies which led to the isolation and identification of the active compound named artemisinin (qinghaosu). These remarkable results on *A. annua* activity against malaria parasite were published in 1982 [57].

Pure artemisinin (ART) (Fig. 5) has low solubility in both water and oil but it is possible to administrate it orally, rectally, and intramuscularly [58]. However, for severe malaria patients, oral route is often not possible considering the effect of intense vomiting. In order to overcome this obstacle and to limit the necessity of vegetal supply, several semi-synthetic ART derivatives have been developed [48, 59] like the water-soluble artesunate and the oil-soluble artemether and arteether [60]. The studies of drug metabolism found that ART derivatives are converted into the same active metabolite as artemisinin: dihydroartemisin (DHA) [61, 62]. These compounds are used in monotherapy for treatment of severe malaria and have shown very rapid parasite clearance time and fast fever resolution [48]. Artemisinins probably work by generation

of free radicals when Fe (II) cleaves the peroxide bond of the drug in parasitized red blood cells, followed by alkylation of parasite proteins [49, 63].



Dehydroartemisinin: R = H

Artemether: R = Me

Artesunate: R = -CO-CH₂-CH₂-CO-

Figure 5. Artemisinin, a sesquiterpene lactone with a peroxide bridge

e. Miscellaneous compounds

Atovaquone, a hydroxynaphthoquinone (Fig. 6), is an effective compound against CQ-resistant *P. falciparum*, but when used alone parasite resistance can develop quickly. That is why atovaquone is usually given in combination with proguanil [64, 65]. Atovaquone targets the electron transport chain (ECT) of the mitochondrion, and specifically the cytochrome bc₁ complex [49], while proguanil acts not only as DHFR inhibitor but also in a synergistic effect lowering the effective concentration at which atovaquone breaks down the parasite mitochondrial membrane potential [66].

Pyronaridine was reported 100% effective in Cameroon [67] but only 63%-88% in Thailand [68]. Pyronaridine is an acridine-based drug with unknown mode of action, but could act on haemoglobin degradation, haem polymerisation, and topoisomerase-2 activity [50].

Lumefantrine is another antimalarial compound administered in combination with artemether [69, 70]. Lumefantrine is an aryl-amino alcohol [71] that prevents polymerization of haem and free radicals to induce parasite death [72]. In order to prevent recrudescence, it is absorbed and cleared slowly ($t_{1/2}$ =3-6 days) [73] to eliminate residual parasite that may remain after artemether has been cleared from the body [72, 74, 75].

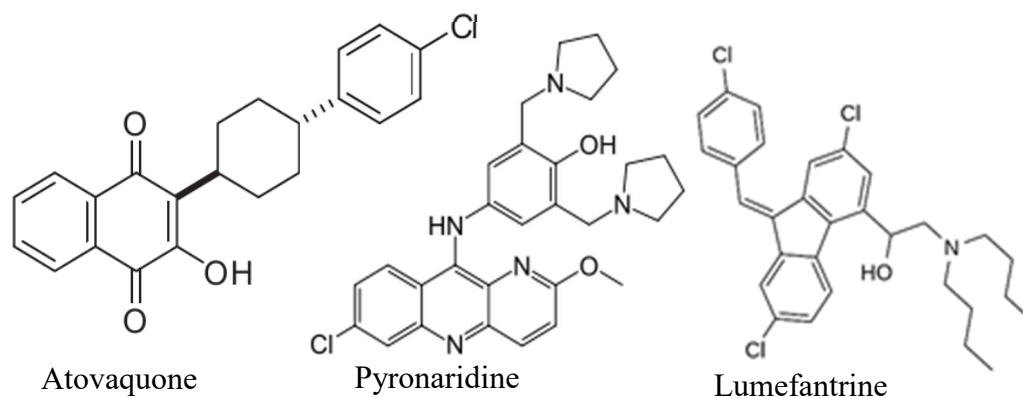


Figure 6. Miscellaneous compounds

f. Non-Artemisinin Combination Therapy

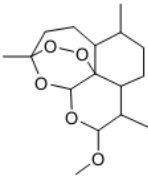
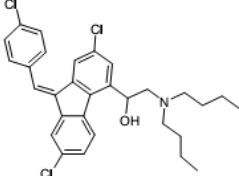
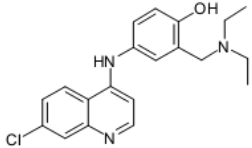
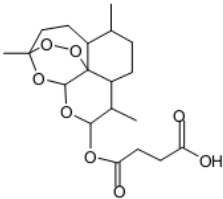
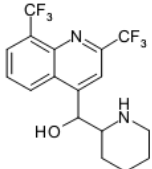
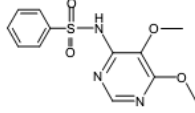
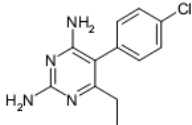
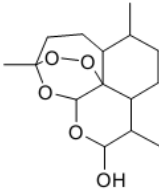
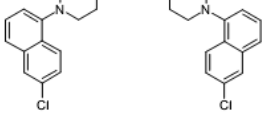
A two-antimalarial combination, especially when they have different mechanisms of action, has the potential to inhibit the development of resistance to each component [48]. The combination of a 4-aminoquinoline drug (CQ or amodiaquine) with sulfadoxine/pyrimethamine (SP) improves the parasitological clearance (compared with SP alone) [76]. However, high levels of resistance against these two chemical series indicate that these combinations are not recommended and no longer used [15].

g. Artemisinin based-Combination Therapies (ACTs)

Another combination therapy approach is artemisinin derivatives associated with longer half-life antimalarials [48]. These combinations have been recommended by the WHO as malaria front-line treatment to counter the resistance of *P. falciparum* to monotherapies and to improve treatment outcome [15].

Artemisinin-based combination therapies (ACTs) are the current standard treatment for uncomplicated malaria. Artemisinin and its derivatives (artesunate, artemether, dihydroartemisinin) are fast acting compounds but are rapidly cleared [15] (human $t_{1/2}$ ~ 1 hour), and are therefore combined with slow-clearing drugs to kill residual parasites. The typical partner drugs include lumefantrine (human $t_{1/2}$ = 3-4 days) and piperaquine (human $t_{1/2}$ = 8-16 days) [49]. When given in combination with other compounds, a 3-day course of treatment with an artemisinin compound is deemed effective. Five ACTs are currently recommended by the WHO: artemether + lumefantrine, artesunate + amodiaquine, artesunate + mefloquine, artesunate + sulfadoxine - pyrimethamine, and dihydroartemisinin - piperaquine [15]. ACTs have supplanted the previously recommended sulfadoxine – pyrimethamine, which in turn replaced CQ treatments [49].

Table 1. ACTs recommended by WHO
[77-80]

ACTs	Brand name (suppliers)	Artemisinin derivative	Companion drug
Artemether – lumefantrine	Coartem® Riamet® (Novartis)	Artemether 	Lumefantrine 
Artesunate – amodiaquine	ASAQ Winthrop® (DNDI/Sanofi)		Amodiaquine 
Artesunate – mefloquine	ASM FDC (DNDI/Cipla Ltd)	Artesunate 	Mefloquine 
Artesunate – sulfadoxine – pyrimethamine	Artesunate + Sulfadoxine + Pyrimethamine Tablets (Advacare PHARMA) Malosunate® (Anhui)		Sulfadoxine  Pyrimethamine 
Dihydroartemisinin - piperaquine	Eurartesim® (Sigma- Tau/MMV) Atrketin® (Holleykin) Diphos® (Genix Pharma)	Dihydroartemisinin 	Piperaquine 

4. Artemisinin-based Combination Therapies (ACTs) in Indonesia

The year 2004 was the beginning of ACTs use in the national malaria control program in Indonesia. This was due to the overwhelming burden of resistance of malaria parasites to previous conventional malaria drugs. This step was also in conjunction with WHO's recommendation of using ACTs to fight malaria [81, 82].

The first ACT adopted in Indonesia was artesunate-amodiaquine as first line therapy. This fixed dose regimen is active against *P. falciparum* as well as *P. vivax*, safe for all ages, and affordable though large number of pills to be ingested is a disadvantage. Moreover, in places such as Papua, Lampung, and North Sulawesi or in areas with high chloroquine resistance, treatment failures with artesunate-amodiaquine were reported [82].

Artemether-lumefantrine has been recently, implemented in Indonesia. In a study conducted by Sutanto *et al.* (2013), artemether-lumefantrine proved to be safe and highly efficacious in 59 residents of eastern Sumba Island presenting uncomplicated *P. falciparum* malaria [83]. Another study in southern Papua of Indonesia, an area with multidrug resistant *P. falciparum* also showed high cure rate (95.3%) with artemether-lumefantrine, but it was less responsive to *P. vivax* compared to other combination (43%) [84]. The disadvantages of this ACT are: i) it should be administered twice daily for three days, given with fatty foods, and ii) it is expensive. This limits the utilization of artemether-lumefantrine as second line therapy for *P. falciparum* malaria.

Dihydroartemisinin – piperaquine (DHP) can be another option to overcome the failure of other combinations such as artesunate-amodiaquine [82]. Based on the data of previous ACTs trials in Indonesia, DHP was found to be the best combination to treat uncomplicated malaria in areas with multi-drug resistance. In comparison with all the existing forms of ACTs, a 3-day DHP is the best substitute for the Indonesian ACT program. Moreover, DHP is safe

and effective for both *P. falciparum* and *P. vivax* malaria. The cure rates of DHP reported for the treatment of *P. falciparum* and *P. vivax* were 95.2% and 92.7%, respectively. DHP has been used for more than 2 years in Papua, a high multi-resistance area, as first line therapy [85]. In 2006, the national drug policy was changed and DHP became the first line treatment for any uncomplicated cases of all four parasite species and for treatment of malaria in the second and third trimesters of pregnancy [14].

The dosage of dihydroartemisinin is 2-4 mg/kg of body weight (BW), and 16-32 mg/kg BW for piperazine. The dose of DHP must be based on body weight rather than on age classification, except if the weight scale is not available.

Table 2. The dosage of DHP based on body weight classification for *falciparum* malaria [86]

Days	Treatment	Number of tablets per days based on body weight classification						
		≤ 5 kg	6 -10 kg	11-17 kg	18-30 kg	31-40 kg	41-59kg	≥ 60 kg
		0-1 month	2-11 month	1-4 year	5-9 year	10-14 year	≥ 15 year	≥ 15 year
1-3	DHP	¼	½	1	1 ½	2	3	4
1	Primaquine	-	-	¾	1 ½	2	2	3

5. Antimalarial Drug Resistance

a. Non-Artemisinin Resistance

The obstacle to malaria eradication is parasite resistances to antimalarial drugs. The WHO defines resistance to antimalarials as the ability of parasite strain to survive and/or multiply despite the administration and absorption of a medicine given in doses equal to -or higher than- those usually recommended but within the tolerance of the subject, with the caveat that the form of the drug active against the parasite must be able to gain access to the parasite or the infected red blood cells for the duration of the

time necessary for its normal action [15]. Malaria resistances was reported with chloroquine, antifolates, and atovaquone [87, 88].

Chloroquine resistant *P. falciparum* began to emerge in 1957 in South America, South East Asia to Africa [89]. In the end of 1980, resistances to sulfadoxine-pyrimethamine and mefloquine settled well in Thai-Cambodia and in 2006, resistance to artemisinin appeared [19, 88]. Commonly, the resistance mechanism of antimalarial agents is mediated by multidrug resistance gene polymorphism, for example *Pfmdr1* (chloroquine, mefloquine, amodiaquine, halofantrine, lumefantrine), *Pfcr1* (chloroquine and amodiaquine), *Pfdhps* (sulfadoxine), and *Pfdhfr* (pyrimethamine and proguanil) [90-99].

Two main mechanisms of *Plasmodium* resistance to almost all anti-malarial drugs are: i) modifications in transporter genes reducing drug availability at its site of action or ii) modifications of the drug target by mutations in the corresponding genes (Table 3). For antimalarials targeting the haemoglobin digestion in the food vacuole two transporters are mainly involved in the resistance: the chloroquine resistance transporter (*Pfcr1*) and the multi-drug resistance transporter 1 (*Pfmdr1*). Another possibly related transporter is the multi-drug resistance associated protein (*Pfmrp*) [100]. The mechanism of resistance for these antimalarial agents is mediated by single nucleotide polymorphisms (SNPs) of the genes that encode these transporters or by an increased number of copies of some of them [101, 102]. Modifications of the drug target concern more with antimalarial drugs targeting metabolic pathways, such inhibition of the cellular respiration and the dihydroorotate dehydrogenase (DHODH) involved in the pyrimidine nucleotide biosynthesis at mitochondrion level [103, 104] or the dihydrofolate reductase (DHFR) involved in the production of purines, thymidine and therefore in the DNA biosynthesis in the cytosol [105, 106].

Table 3. Resistance genes in antimalarial drug

Anti-malarial		Resistance	Main resistance mechanisms	Refs
Class	Drug	gene		
Quinolines	Quinine	<i>Pfmdr1/other</i>	Disruption of drug accumulation inside food vacuole by reducing propensity of the drug transporter PfMDR1 to bind to and transfer anti-malarial	[100, 101]
	Chloroquine	<i>Pfcrt/pfmdr1</i>	Drug extrusion from digestive vacuole by mutating drug transporter PfCRT due to higher lipophilicity and negativity of the transporter allowing ionized chloroquine efflux	[107-109]
	Mefloquine	<i>Pfmdr1/other</i>	Reduction of parasite susceptibility to mefloquine by amplification of <i>Pfmdr1</i> copies	[96]
Natural endoperoxide compounds and semi-synthetic derivatives	Artemisinin Artesunate Artemether	<i>Pfk13</i>	Quiescence	[22, 24]
Antimetabolites	Sulfadoxine - pyrimethamine Proguanil	<i>Pfdhps</i> - <i>Pfdhfr</i> <i>Pfdhfr</i>	Modification of the drug target by reducing inhibition of enzymatic activity by the drug	[110]
Other	Atovaquone	<i>Pfcytb</i>	Modification of the drug target by disruption of cytochrome <i>bcl</i> complex	[103]

b. Artemisinin Resistance

The working definition of artemisinin resistance is based on observations from routine therapeutic efficacy studies of ACTs and clinical trials of artesunate monotherapy. An artemisinin resistance area is suspected by, consecutively to an increased parasite clearance time, $\geq 10\%$ of patient cases with parasites detected 3 days after treatment with ACT and more than 10% of treatment failures on day 28 or 42 [111].

In 2006, declining parasite clearance rates with ART were found, marking an initial detection of ART resistance. This resistance emerged in multiple locations in the Greater Mekong subregion (GMS) and has later triggered another resistance to antimalarial drugs, including partner drugs of ART such as piperazine [112]. Realizing that ART resistance would be an obstacle for the malaria elimination program, our laboratory team (Group V, LCC-CNRS, Toulouse) reviewed the underlying molecular mechanism of this particular resistance to provide a conceptual framework of cellular network involved in parasite survival. Several cases of ART resistance have been reported. In 2009 *in vitro* studies performed on blood samples of patients with uncomplicated *P. falciparum* malaria suggested an increasing artemisinin failures in western and eastern Thailand, also in Cambodia, apparently correlated with increased artemisinin IC₅₀ values (mean of IC₅₀ :1.87nM, 3.18nM, and 3.34nM respectively, compare to 1.51nM of artemisinin-sensitive clone W2 [20]. Dondorp's study [19] figured out reduced *in vivo* susceptibility to artesunate in Pailin (western Cambodia) as opposed to Wang Pa (northwestern Thailand). As many as 30% patients followed in Pailin showed 10% recrudescence of artesunate monotherapy, and 5% of them indicated mefloquine combination Wang Pha. This resistance is characterized by slow parasite clearance *in vivo* without corresponding reductions on conventional *in vitro* susceptibility testing, in contrast to the Noedl's observation about increased IC₅₀ values the artemisinin in regions with a high failure rate of artesunate-mefloquine.

A number of recent research revealed that the resistance happened to not only artemisinin alone but also with its combinations, such as dihydroartemisinin-piperazine. In Cambodia parasite recrudescence after dihydroartemisinin-piperazine treatment was associated with a higher prevalence of *kelch13* mutations

and a higher piperazine IC₅₀. The treatment failure was caused by both artemisinin and piperazine resistance [21].

It is well established that ART resistance is mediated by mutations of the *Pfk13* propeller which trigger a sequence of events leading parasites to enter in a quiescent state during the presence of ART or its derivatives; the parasite restarts its cell cycle after drug elimination. The *Pfk13* belongs to the kelch super-family of proteins and is likely to have a similar function to Keap 1 protein which plays an essential role in human in protecting against oxidative stress through an unfolded protein response (UPR).

A putative transcription factor (uTF) associated with K13 is probably involved in the parasite, such as like the human transcription factor Nrf2 normally linked to Keap1. In unstressed conditions Nrf2, linked to Keap1, is degraded in the proteasome by a polyubiquitination process [113]. Under stress conditions or in the case of K13 mutations, similar to Nrf2 in human, uTF could be constitutively activated by inhibition of its proteasomal degradation. It was recently found that PfPI3K is a ligand of *Pfk13*. In the case of *Pfk13* mutations, the PI3K ubiquitination is inhibited leading to an increased level of basal PI3P. The PI3P role in ART resistance is confirmed by PI3P transgenic expression that endows artemisinin resistance in non-mutated K13 parasites [114].

The underlying molecular mechanisms linked to K13 mutations and artemisinin resistance have yet to be completely understood. The *in vivo* transcriptome analysis of *Plasmodium falciparum* revealed that artemisinin resistance, caused by single K13 propeller mutations, is associated with increased expression of a network of molecular chaperones and major protein complexes belonging to the Unfolded Protein Response

(UPR) pathways. Some of the upregulated genes of these complexes are involved in protein folding and repair in the endoplasmic reticulum (ER) especially BiP (immunoglobulin-binding protein). The misfolded proteins bind to BiP and trigger a cascade of events probably similar to the BIP-PERK-eIF2 α pathway observed in mammals and leading to a limitation of global protein synthesis and a cell cycle arrest via cyclin dependent kinases.

The parasite ART-induced quiescence state is characterized by a suspended glycolysis and associated ATP and PEP productions. However, a minimum ATP and PEP production is ensured by apicoplast and mitochondrion that remains active during the quiescence stage [115].

Although the mitochondrial tricarboxylic acid cycle is partly down regulated, the transcription of proteins of the electron transport chain remains active and a FASII pathway is implemented in the apicoplast consecutively after a decrease in host fatty acid incorporations. For those reasons, quiescent parasites can be killed by drugs targeting the respiratory chain of the mitochondrion such as atovaquone, or its FASII metabolism such as haloxyfob, and triclosan.

In conclusion, *Plasmodium falciparum* resistance to artemisinin and its derivatives is not due to a common mechanism of antimalarial resistance but based on the increasing ability of *Pfk13*-mutant parasite to restrain oxidative damages. In a quiescence state, even though main metabolism pathways slow down, the apicoplast and mitochondria keep supplying minimum energy for metabolism to resume growth as soon as the drug is eliminated.

Indeed, the well-defined cellular connections between the different elements involved in artemisinin resistance are a prerequisite to the definition of the best

strategy to fight this resistance and the search for efficient drugs targeting the quiescence phenomenon.

c. A review was published in order to present the whole cellular and molecular network of artemisinin resistance.

L. Paloque, **A. P. Ramadani**, O. Mercereau-Puijalon, J.-M. Augereau, F. Benoit-Vical.

Plasmodium falciparum: multifaceted resistance to artemisinins.

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REVIEW

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Plasmodium falciparum: multifaceted resistance to artemisinins

Lucie Paloque^{1,2}, Arba P. Ramadani^{1,2,3}, Odile Mercereau-Puijalon⁴, Jean-Michel Augereau^{1,2†} and Françoise Benoit-Vical^{1,2*†}**Abstract**

Plasmodium falciparum resistance to artemisinins, the most potent and fastest acting anti-malarials, threatens malaria elimination strategies. Artemisinin resistance is due to mutation of the Pfk13 propeller domain and involves an unconventional mechanism based on a quiescence state leading to parasite recrudescence as soon as drug pressure is removed. The enhanced *P. falciparum* quiescence capacity of artemisinin-resistant parasites results from an increased ability to manage oxidative damage and an altered cell cycle gene regulation within a complex network involving the unfolded protein response, the PI3K/P13P/AKT pathway, the PPK4/eIF2 α cascade and yet unidentified transcription factor(s), with minimal energetic requirements and fatty acid metabolism maintained in the mitochondrion and apicoplast. The detailed study of these mechanisms offers a way forward for identifying future intervention targets to fend off established artemisinin resistance.

Keywords: Malaria, Artemisinin-based combination therapy, Resistance, Pfk13, Quiescence

Background

Artemisinin (ART) and its derivatives, introduced in 1980s, are the most potent and fastest acting anti-malarials, producing rapid clearance of parasitaemia and rapid resolution of symptoms. The discovery of artemisinin by Professor Youyou Tu has been rewarded the Nobel Prize for Physiology or Medicine 2015, recognizing that it has radically improved treatment against malaria [1]. *Plasmodium falciparum* artemisinin resistance was first detected in 2006 [2], after decreased clearance rates were observed in patients receiving an artemisinin therapy in clinical studies performed in 2006. It is nowadays widespread across southeast Asia [2, 3]. The delayed clearance of artemisinin-resistant infections exposes larger numbers of parasites to anti-malarial drugs, potentially driving selection to higher-grade artemisinin or to the partner drug. In Asia, treatment failures of artemisinin-based combination therapy (ACT) have recently

emerged, including resistance to piperazine [4]. This represents a serious threat for malaria eradication, widespread artemisinin resistance being predicted to cause in excess of 116,000 deaths annually, with medical costs and productivity losses evaluated as 146 million US\$ and 385 million US\$ per year, respectively [5]. The resistance to artemisinins is due to mutation of the Pfk13 propeller domain endowing the parasites with an increased ability to enter a quiescent state. Piecemeal evidence associates artemisinin resistance with increased unfolded protein response (UPR), dysregulation of the pre-replication phase and the PI3K/P13P/AKT pathway. Yet, an overall picture about how these modifications result in quiescence-associated artemisinin resistance is lacking. A conceptual framework for the cellular networks involved from oxidative stress to quiescence and parasite survival is proposed here, opening novel avenues for future research.

Rapid overview of antiplasmodial drugs

Currently used anti-malarials belong to different chemical series and all *Plasmodium falciparum* stages can be targeted (asexual red blood cell stages, gametocytes, hepatic stages) by one or another anti-malarial. Each

*Correspondence: francoise.vical@inserm.fr

†Jean-Michel Augereau and Françoise Benoit-Vical contributed equally to this work

¹CNRS, LCC (Laboratoire de Chimie de Coordination) UPR8241, 205 route de Narbonne, BP 44099, 31077 Toulouse Cedex 4, France

Full list of author information is available at the end of the article



antiplasmodial drug series has its own specific mode(s) of action. In the food vacuole, haemozoin synthesis, corresponding to detoxification of the waste from haemoglobin by the parasite, is specially affected by quinolines [6] and artemisinins. Artemisinin and its derivatives are also responsible for alkylation of proteins and haem leading to oxidative damages [7]. At the mitochondrial level, atovaquone, targeting the cytochrome *bc₁* complex, inhibits the parasite electron transport chain and thus the dihydro-orotate dehydrogenase (DHODH) activity linked to the respiratory chain and involved in pyrimidine nucleotide biosynthesis [8]. In the cytosol, inhibition of dihydrofolate reductase (DHFR) by proguanil or pyrimethamine or of dihydropteroate synthase (DHPS) by sulfadoxine [9], blocks the biosynthesis of folate involved in DNA and RNA synthesis. Unfortunately, most anti-malarial drugs have lost their efficacy as resistance has emerged and spread.

The World Health Organization (WHO) defines resistance to anti-malarials as “the ability of *Plasmodium* to survive and/or multiply despite the administration and absorption of a medicine given in doses equal to -or higher than- those usually recommended but within the tolerance of the subject”, with the subsequent statement that “the form of the drug active against the parasite must be able to gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action” [10]. For some anti-malarial drugs, resistance occurred very quickly after their introduction (Table 1).

Among all mechanisms of resistance described in various organisms, from bacteria, parasites, fungi to human cancer cells, two main mechanisms drive *Plasmodium* resistance to almost all anti-malarial drugs: (i) reduced

drug availability at its site of action, essentially due to mutations in transporter genes; and, (ii) modification of the drug target by mutations in corresponding genes (Table 1). Artemisinin resistance results from a different cellular process, quiescence, which is detailed below. The quiescence-based cellular mechanisms comply with the WHO definition of *P. falciparum* drug resistance [10] (see also WHO global report on drug resistance 2010), as artemisinin-resistant parasites survival exposure to therapeutic, lethal concentrations of artemisinin derivatives.

Recommendations for the use of dual and triple therapy

Parasite resistance to drug monotherapy prompted the WHO to recommend dual or triple therapy, which combines molecules with independent modes of action or distinct target enzymes. Drug combination is usually more effective and in the event of resistance to one component, the second one kills residual resistant parasites. However, some combination therapy, such as sulfadoxine-pyrimethamine plus chloroquine or amodiaquine, must be avoided due to the high levels of resistance established to these drugs already extensively used in monotherapy. For the same reason and because of the rapid acquisition of atovaquone resistance, atovaquone-proguanil is not recommended [2, 11, 12]. Clinical resistance to artemisinin was first detected in Cambodia in 2006 and reported in 2008 [13]. In 2009, Noedl et al. reported artemisinin resistance of uncomplicated falciparum malaria cases in Thailand and Cambodia and correlated delayed clearance with increased in vitro artemisinin IC₅₀ values compared to the artemisinin-sensitive clone W2 [14]. Dondorp et al. [15] documented reduced in vivo susceptibility to artesunate in Pailin (western Cambodia) compared to Wang Pha (northwestern Thailand),

Table 1 Date of introduction and first reports of anti-malarial drug resistance, resistance genes involved [33, 86, 87] and main mechanisms of resistance

Anti-malarial drug	Introduced	First reported resistance	Resistance genes	Main resistance mechanisms	Refs
Quinine	1632	1910	<i>Pfmdr1/other</i>	Disruption of drug accumulation inside the food vacuole [88, 89] by reduced propensity of the drug transporter PfMDR1 to bind to and transfer the anti-malarial	
Chloroquine	1945	1957	<i>PfCRT/Pfmdr1</i>	Drug extrusion on from digestive vacuole by mutated drug transporter PfCRT due to higher lipophilicity and negativity of the transporter allowing ionized chloroquine efflux	[90–92]
Proguanil	1948	1949	<i>Pf dhfr</i>	Modification of the drug target: reduced inhibition of enzymatic activity by the drug	[9]
Sulfadoxine-pyrimethamine	1967	1967	<i>Pf dhps-Pf dhfr</i>		
Mefloquine	1977	1982	<i>Pfmdr1/other</i>	Reduction of parasite susceptibility to mefloquine by amplification of <i>Pfmdr1</i> copy number	[93]
Atovaquone	1996	1996	<i>Pf cyt b</i>	Modification of the drug target by disruption of Cytochrome <i>bcl</i> complex	[8]
Artemisinins	1980s	2006	<i>Pf k 13</i>	Quiescence	[31, 33]

characterized by a slow parasite clearance time (72–84 h in Pailin and 48–54 h in Wang Pha) and higher failure rates (recrudescence) after artesunate monotherapy (in Pailin 30 % of patients showed recrudescence compared to 10 % in Wang Pha). These altered clinical characteristics were not associated with reduced in vitro susceptibility, contrasting with observations by Noedl et al. [15]. Resistance to artesunate monotherapy first reported in western Cambodia and along the Thailand-Myanmar border, resulted in lengthening of the parasite clearance half-lives from 2.6 h in 2001 to 3.7 h in 2010 [16]. Artemisinin resistance is now also established in Myanmar, Vietnam, Lao PDR, and China [3].

In order to avoid the spread of *P. falciparum* resistance, monotherapy with artemisinin or its derivatives should be banned [17]. ACT has been recommended by the WHO as first-line treatment for uncomplicated malaria since 2001 and is nowadays widely used (Additional file 1). However, in December 2014 eight countries, mainly in Africa (Angola, Cabo Verde, Colombia, Equatorial Guinea, Gambia, Sao Tome and Principe, Somalia, Swaziland) still allowed artemisinin-based monotherapy [18].

Treatment failures with ACT have only been observed in Asia where, in addition to artemisinin resistance, parasites were also resistant to companion drug, e.g. mefloquine along the Thai-Myanmar border [19] or piperaquine in Cambodia [20–22]. In such cases, the ACT used is replaced by another one and the therapeutic efficacy of the replacement ACT must be monitored every 2 years as recommended by the WHO [2] for timely adaptation of the treatment policy. The threshold of 10 % cases with detectable parasites on day 3 after ACT treatment used by the WHO to define areas of artemisinin resistance [2] was established in Asia, a region of low/moderate transmission. In Africa, where transmission is usually much higher, artemisinin resistance has not yet been reported. However, a recent study suggested that a threshold of 5 % cases on day 3 parasite positivity is more suited to artemisinin resistance monitoring in Africa due to higher levels of acquired immunity against *Plasmodium* in African populations contributing to faster parasite clearance [23].

Phenotypic and genotypic basis of artemisinin resistance

It is striking to note that from chloroquine resistance in 1957 to artemisinin resistance in 2006 [13], all anti-malarial drug resistance plaguing Asia was first reported at the Thai-Cambodian border and more especially in Pailin Province. Poor access to medicines, generating traffic of counterfeit drugs, with sub-clinical quantities, use of monotherapy, combined with an intense and extensive migrant labour system, are potential contributors

[24–26]. No correlation was found between an increased mutation rate of the parasite genome and drug resistance in these regions, invalidating the hypothesis of the existence of a 'hypermutator' parasite [27]. Resistance in this region has a typical multi-resistance profile, with resistance mutations in *Pfprt*, *Pfdhps*, *Pfahfr*, *Pfmdr1* fixed in the parasite populations [28, 29].

Resistance to artemisinins was selected from such multi-resistant parasites circulating in western Cambodian provinces and was shown to be an inheritable genetic trait of the parasites [16, 30]. The mechanism driving artemisinin resistance was discovered using an experimental model, the F32-ART line -a highly artemisinin-resistant line- established in vitro after 5 years of exposure to escalating concentrations of artemisinin [31]. Artemisinin resistance of F32-ART is mediated by a resistance mechanism quite distinct from those previously described [15, 31–33] (Table 1), since these parasites score susceptible using the standard in vitro susceptibility assays [31, 33–35]. *Plasmodium* resistance to artemisinins is due to the enhanced number of young ring forms to enter into a quiescence state upon exposure to artemisinins, and quickly resume growth once the artemisinins are removed. This capacity is conferred by mutations of a gene called *Pfki3* [15, 31, 33]. This gene is now currently monitored to follow artemisinin resistance spread according to WHO recommendations [2, 18, 36].

Plasmodium falciparum resistance to artemisinin is quiescence-based

The first hypothesis of artemisinin resistance based on a partial cytostatic effect of the drug was evoked thanks to a mathematical model in 2000 [37]. The classical definition of quiescence is 'a reversible absence of proliferation', i.e., a non-dividing cell which eventually restarts its cell cycle when conditions become appropriate. Various quiescent states may depend on the cell history; alternatively, quiescence can be the convergence of an adaptive process to cope with an adverse environment and an active preparation to efficiently resume proliferation [38]. The development cycle of *P. falciparum* presents normal arrest at the sporozoite stage in the mosquito salivary glands or at the gametocyte stage in red blood cells. In vitro, *P. falciparum* cell cycle progression can be strongly delayed at the trophozoite stage in response to isoleucine starvation [39]. A fraction of *P. vivax* and *Plasmodium ovale* sporozoites is able to stop its cell cycle during the hepatic phase to enter into a particular state, named hypnozoites, another form of dormant or quiescent parasites, and responsible for relapses, all under an epigenetic control of gene expression by histone modification enzymes [40].

Quiescence and artemisinin-resistant parasite stages

Data obtained with the highly artemisinin-resistant F32-ART line and *P. falciparum* isolates showed that artemisinin pressure induces developmental arrest of a sub-population of very young (0–3 h) ring stages which enter a quiescent state, while killing all other stages [31, 41]. Quiescence has also been correlated with K13-mediated artemisinin resistance in field isolates [33].

Quiescent parasites are difficult to identify under the microscope but staining mitochondrial activity with fluorescent dyes, unambiguously labels live quiescent cells in artemisinin-treated cultures [31, 42, 43]. Cell cycle arrest leads to overestimations of the parasite clearance time in patients [44], and a defect of H³-hypoxanthine incorporation in standard chemosensitivity assay leads to an overestimation of the effectiveness (IC₅₀) of anti-malarials [31, 42]. As a result, classical susceptibility assays based on parasite proliferation are not suitable to differentiate parasites that are artemisinin-resistant from those that are artemisinin-sensitive. Recrudescence assays and ring stage survival assay (RSA_{0–3h}) using highly synchronized *P. falciparum* parasites in culture, based on a 6-h dihydroartemisinin exposure in vitro, followed by culture in drug-free conditions until microscopic read-out at 72 h, are able to detect and quantify artemisinin resistance [31, 35, 41]. Quiescence takes place at a moment of the parasite cycle (young ring stage) where artemisinin is less efficient [43]. Indeed, artemisinin toxicity is potentiated by products of haemoglobin digestion inside the food vacuole, which begins in the mid-ring stage. Haemoglobin degradation products, such as haem and ferrous iron, react with artemisinin to cause oxidative stress and irreversible damages [45]. The low levels of haemoglobin endocytosis and therefore its absence or low digestion level in very young rings, could explain their reduced sensitivity. At this stage, which is approximately 14 h longer in resistant parasites than in wild-type parasites [46], a lower (moderate) oxidative stress is likely more manageable by the parasite, and particularly so by Pfk13 mutants, while at the trophozoite stage, artemisinin-dependent damage exceeds the parasite's response abilities [43]. The ring stage arrested development allows parasites to survive during the relatively short period of elevated artemisinin concentration (the elimination half-life in humans varies from one to 11 h depending on the derivative) [31, 41, 47]. Why only a small sub-set population (<1 %) of resistant parasites [42] is able to enter a quiescence stage despite their common genetic pattern remains to be elucidated.

Response of non-mutated Pfk13 parasites to artemisinin exposure

It appears that all parasite lines are able to enter quiescence or dormancy-mediated processes [48]. In vitro,

this is monitored by the capacity to proliferate after drug removal, i.e., to recrudescence. Artemisinin-resistance is characterized by a short time to recrudescence as demonstrated with the F32-ART (artemisinin-resistant) and F32-TEM (sensitive) sibling laboratory lines. After exposure to up to 18 μM of artemisinin, F32-TEM was able to resume growth and reach 5 % of parasitaemia 17 days after artemisinin removal compared to 10 days for F32-ART [31]. Other studies reported that several artemisinin-sensitive *P. falciparum* strains restarted their cell cycle from 4 to 25 days after the drug removal [48, 49]. Thus, some wild-type parasites are able to survive artemisinin exposure but artemisinin-resistant strains restart faster with more quiescent parasites and/or quicker awakening [31, 35].

Involvement of the Pfk13 locus in Plasmodium falciparum resistance to artemisinins

Pfk13 polymorphism

Whole genome-sequencing of F32-ART and its sibling-sensitive line F32-TEM as well as of artemisinin-resistant *P. falciparum* isolates from Cambodia, allowed artemisinin resistance to be associated with mutations of the K13 protein (accession number PF3D7_1343700), belonging to the kelch super-family of proteins. All mutations found in the *Pfk13* gene and correlated with artemisinin resistance are non-synonymous, and are present after position 440 in the propeller domain (K13-propeller) [33, 36, 50]. Just one of these mutations is sufficient to confer artemisinin-resistance [50]. There is a strong genetic correlation in Asia between K13-propeller mutation and a slow parasite clearance time (half-life longer than 5 h) [3, 28, 33, 36, 51–53]. In Myanmar, among the K13 mutations observed, C580Y and M476I (the mutation acquired by F32-ART parasites) were associated with delayed clearance-time [54]. In Southern China, at the Myanmar border, the single F446I mutation predominates and is associated with delayed clearance [52]. The current picture is that artemisinin-resistant parasites circulating in different geographic areas of Southeast Asia stem from several independent emergences of unique mutations in the K13-propeller domain [28, 36, 53] where the parasite genetic background could play a role in the propensity of K13 mutants to emerge. Three prevalent K13-propeller mutations -C580Y, R539T and Y493H- were shown to correlate strongly with increased parasite clearance in vivo and with increased survival rates in the in vitro RSA_{0–3h} [33, 55, 56]. Gene editing showed that introduction of the wild-type allele into resistant parasites procured a sensitive, very low RSA_{0–3h} survival rate (0.3–0.7 %) whereas the parent isolates harbouring a K13 mutation (R539T, I543T or C580Y) displayed 40–49 % RSA_{0–3h} survival. Conversely C580Y introduced into wild-type

artemisinin-sensitive Cambodian clinical isolates and reference lines conferred varying degrees of in vitro resistance, suggesting additional contribution from the genetic background [50]. Importantly, Asian K13 mutations are generally not found in Africa where numerous additional but rare *Pfk13* alleles were identified [36, 57–59]. An exception is the SNP A578S, observed in many African countries [58–60] as well as Bangladesh [51] and Thailand [36], however this mutation is not associated with artemisinin resistance [36].

Apart from one study in a limited number of African children with severe malaria [61], none of the K13 mutations has been associated with clinical artemisinin resistance for the time being, despite evidence that introducing the C580Y mutation generates artemisinin resistance in vitro in the NF54 parasite strain considered to be of African origin [62]. Genetic determinants of artemisinin resistance have been reviewed recently by Fairhurst [63]. A search of mutations in the *k13* *P. vivax* orthologue showed reduced polymorphism compared to *Pfk13* and the V552I mutation identified cannot be currently associated with any *P. vivax* drug resistance [64].

PfK13 activity

The exact function of PfK13 in *Plasmodium* is not known yet, but analogies are possible with the human Keap1 protein function, in particular, in the cell response to oxidative stress (Fig. 1). PfK13 and Keap1 share homologies in the C-terminal BTB/POZ and the 6-kelch propeller domains [33, 65]. The presence of a BTB/POZ domain suggests that PfK13 could dimerize like Keap1. Formation of a dimer has been experimentally confirmed in the recently solved crystal structure of the PfK13 BTB/POZ propeller domain [66]. Based on Keap1 function, a hypothetical model could be that in steady state conditions, the wild-type K13-propeller domain binds to uTF (a putative, unidentified transcription factor functionally equivalent to human Nrf2) allowing its ubiquitination and proteosomal degradation. This ubiquitination could be mediated by one of the *Plasmodium* ubiquitin ligases but this has not yet been investigated. Importantly, the putative uTF transcription factor remains to be identified in the *Plasmodium* genome [67]. Recently, *P. falciparum* phosphatidylinositol 3 kinase (PfPI3K) was shown to undergo a PfK13-dependent ubiquitination. It could be immunoprecipitated in a complex with PfK13 [68] and has been included as a complex with PfK13 illustrated in Fig. 1, but direct binding of PfPI3K to PfK13 remains to be demonstrated [68].

Biochemical consequences of a mutant K13 propeller

Based on Keap1 involvement in human lung cancer [69] and hypertension [70], it is possible to predict that

K13-propeller mutations altering the propeller structure impair its biological function and interaction with partner proteins [33]. In particular, K13-propeller mutations would prevent fixation of uTF to the propeller domain, and as a consequence reduce the ubiquitination-dependent turnover, promoting translocation of uTF to the nucleus (Fig. 2).

In C580Y mutant parasites, PfPI3K was no longer ubiquitinated, resulting in 1.5–twofold increased basal PI3P levels. This may reflect disrupted interactions between PfPI3K and PfK13 or disrupted interactions within the PfK13 associated protein complex (Fig. 2). Elevated basal phosphatidylinositol 3 phosphate (PI3P) levels appear central to artemisinin resistance, as K13 wild-type parasites became resistant upon elevation of PI3P levels induced by transgenic expression of human VPS34. Similarly transgenic elevation of PFAKT (also known as protein kinase B) in a wild-type parasite confers in vitro artemisinin resistance in connection with increased levels of PI3P by a feedback mechanism supposed [68]. As PfPI3K enzymatic activity is inhibited by artemisinins, the levels of PI3P likely drop rapidly upon exposure to artemisinins in sensitive parasites, whereas the PI3P level is already elevated in resistant parasites [68]. Additional downstream effectors likely come into play to orchestrate the quiescence response to artemisinin and withstand its toxicity.

PfK13 polymorphisms are also associated with modifications of endoplasmic reticulum homeostasis (Fig. 2). Indeed, transcriptome analysis of *P. falciparum* isolates revealed that artemisinin resistance is associated with increased expression of a network of molecular chaperones and major protein complexes belonging to the UPR pathway. In particular, immunoglobulin-binding protein (BiP) and cyclophilin B (CYPB), belonging to the *Plasmodium* reactive oxidative stress complex involved in protein folding and repair in the endoplasmic reticulum, are upregulated. Along the same line, the endoplasmic reticulum-resident calcium-binding protein (ERC) involved in endoplasmic reticulum Ca²⁺ homeostasis is also overexpressed [71, 72]. This upregulation of UPR in PfK13 mutants likely endows the parasite with increased ability to repair or degrade proteins damaged by alkylation and oxidation generated by artemisinin [73]. Based on homology with the human BiP-PERK-eIF2 α pathway involved in the cell cycle arrest under stress conditions (Fig. 3) [74, 75], BiP bound to misfolded/altere proteins could dissociate from the PERK homologue, PfPK4, leading to its activation, and phosphorylation of eIF2 α , which could trigger parasite cell cycle arrest via cyclin-dependent kinases, inhibition of protein synthesis, and the translocation of an unidentified transcription factor uTF into the nucleus. In mammalian cells, the PI3K/AKT

a The human Keap1 complex. The human Keap1 (Kelch-like ECH associated protein 1) is a repressor protein of Nrf2 (Nuclear factor erythroid 2-related factor 2) activity. The BTB/POZ domain (Broad-Complex, Tramtrack and Bric a Brac or Poxvirus or Zinc-finger domain) and the N-terminal portion of the IVR (Intervening Region) mediate Keap1 homodimerization allowing Cul3 (Cullin 3) binding with BTB domains, and Nrf2 binding, through its ETGE and DLG motifs, with kelch-propeller domains. In basal conditions the Keap1-Nrf2 complex leads to polyubiquitination (Ub) of Nrf2 mediated by the E2-ubiquitin ligase activity of Cul3 promoting Nrf2 degradation by the proteasome. Under stress conditions, modifications of cysteine residues of Keap1 induce a conformational change disrupting the interaction between the ETGE/DLG motifs and Kelch domains, and thus a release of Nrf2, which is no longer degraded and can translocate to the nucleus [96]. Inside the nucleus, Nrf2 controls, through the Antioxidant Response Elements (ARE), the expression of approximately 200 genes involved in the preservation of a healthy intracellular redox balance including the regulation of the expression of molecular chaperones as well as of proteasome subunits. The regulation of Nrf2 activity can also be mediated by the PI3K (phosphatidylinositol 3-kinase) pathway promoting Nrf2 translocation [97]. Thus the Keap1-Nrf2 complex is the major regulator of the cytoprotective response to any endogenous and exogenous stress caused by reactive oxygen species (ROS) and electrophiles. Some mutations of the Keap1-propeller cause a constitutive Nrf2 nuclear translocation increasing chemo-resistance and enhancing tumor cell growth [96]

b The *P. falciparum* K13 complex. A putative wild type PfK13 protein complex based on analogy with human Keap1 in steady state conditions. Known features are the capacity of PfK13 to form dimers, presence of PfPI3K in the PfK13 protein complex, and polyubiquitination of PfPI3K in steady state conditions [68]

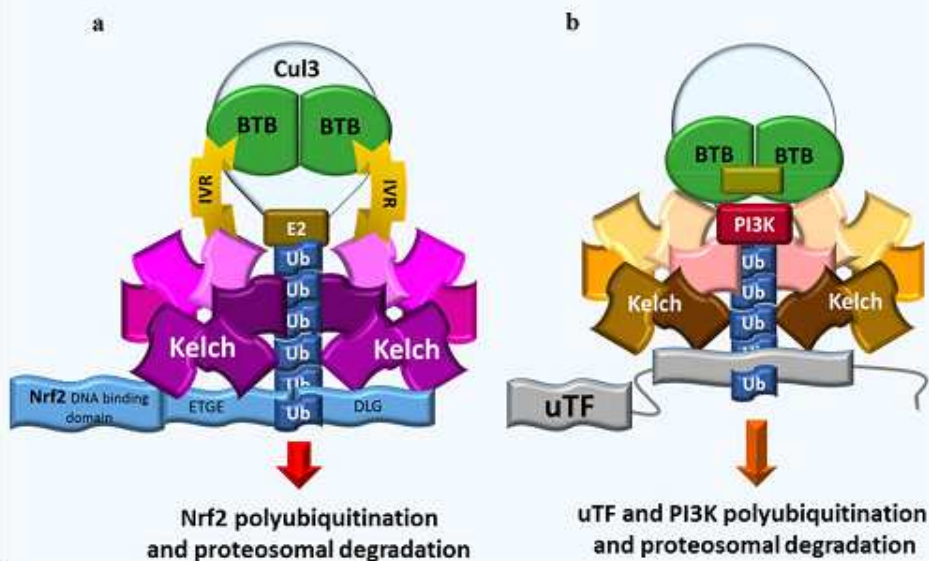
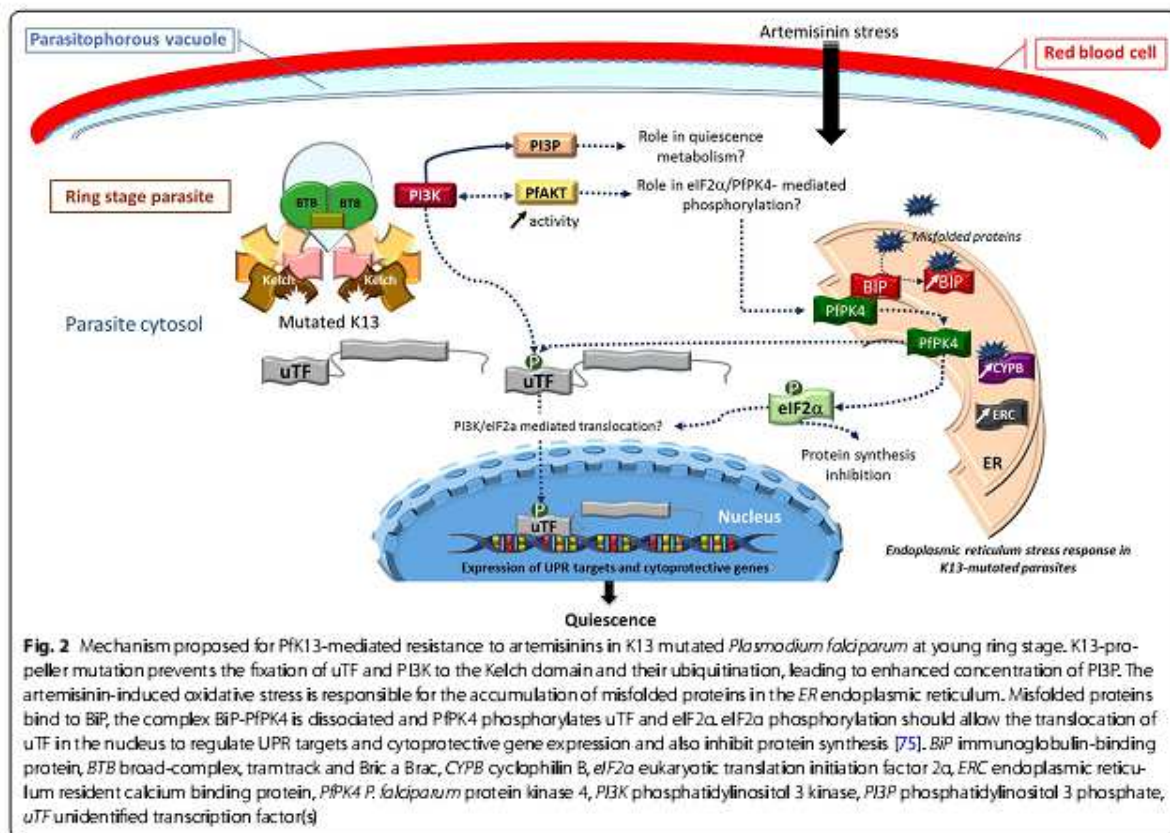


Fig. 1 The Keap1 complex in human cells and a hypothetical PfK13 complex

pathway participates in UPR regulation and the activated AKT protein (also known as protein kinase B) seems to be required for Protein kinase RNA-like endoplasmic reticulum kinase (PERK)-mediated eIF2 α phosphorylation [76]. This is reminiscent of the *Plasmodium* cell cycle

slow down and activation of eIF2 α kinases (PfeIK1 and PfeIK2) induced by amino acid starvation and observed in sporozoite latency inside mosquito salivary glands [39, 77]. Translocation of uTF could, in addition, be favoured by its phosphorylation by PfPK4 and also, like in humans,



by PfPI3K, and could activate the expression of *Plasmodium* UPR and cytoprotective genes (Fig. 2).

This model does not exclude the existence of more than one uTF. Moreover, the PfPK4/PfPI3K downstream regulation cascades remain to be elucidated and experimentally demonstrated (Fig. 2). The involvement of the PfAKT pathway in artemisinin resistance, like PI3P involvement, still remains to be detailed. Moreover, the exact kinetics of events once the parasites are exposed to artemisinin oxidative and alkylating damage needs to be clarified, in particular as PI3K is inhibited by artemisinins [68] and should no longer contribute to refuelling PI3P levels.

Artemisinin resistance possibly includes a combination of the increased degradation of misfolded proteins/repair of damaged proteins especially those involved in the cell cycle progression and the 'PfPK4/PfPI3K' mediated response to the artemisinins' oxidative stress. This cellular response of K13 mutant parasites to artemisinin stress could lead to a sub-set of ring stage parasites to enter into a quiescence state (Fig. 2). Why only a sub-set and a variable fraction of ring stages is involved needs to be understood. It may reflect a finely tuned balance between

different cellular effectors that is unequally distributed between individual infected cells.

Artemisinin-induced quiescence and *Plasmodium* metabolism

Dihydroartemisinin (DHA)-induced quiescence in non-mutated PfK13 parasites is associated with suspended RNA, DNA and protein synthesis [49]. Folate metabolism, isoprenoid metabolism, lactate dehydrogenase activity and glycolysis, the main metabolic pathway producing adenosine triphosphate (ATP) and phosphoenolpyruvate (PEP) in *Plasmodium*, are severely down-regulated [49]. But, even though the metabolism of quiescent parasites is largely down-phased, some pathways remain active and require another source of ATP and PEP, depending on pyruvate production by the apicoplast and on mitochondrial activity (Fig. 4) [49, 78]. In DHA-induced quiescent rings, among the genes that remain actively transcribed, are genes coding for enzymes of the pyruvate metabolism (pyruvate kinase 2, pyruvate dehydrogenase E1 beta subunit), the fatty acid metabolism by FASII -fatty acid synthesis type II- pathway (biotin acetyl-CoA carboxylase, enoyl-acyl carrier

Bip-PERK-eIF2 α pathway

In mammals the oxidative stress generated in the endoplasmic reticulum (ER) with an accumulation of misfolded proteins causes activation of the Protein kinase RNA-like endoplasmic reticulum kinase (PERK) upon its dissociation from the BiP-PERK complex. PERK directly phosphorylates eIF2 α (eukaryotic translation initiation factor 2 α) leading to:

- i) limitation of the global protein synthesis and degradation of specific proteins like cyclin D1, the regulatory subunit of the cyclin-dependent kinases CDK4 and CDK6 contributing to the induction of G1 cell cycle arrest [98];
- ii) translocation of ATF4 (activating transcription factor 4) to the nucleus driving the expression of UPR target and cytoprotective genes [75].

Three eIF2 α kinases have been identified in *Plasmodium*: PflK1, PflK2 and PflK4 [99]. PflK4, the PERK homologue, is known to phosphorylate eIF2 α during the different parasite blood stages and plays an important function in the arrest of global protein synthesis in schizont and gametocyte stages [100]. Phosphorylation of eIF2 α under ER stress was confirmed in *P. falciparum* after exposure to DTT (dithiothreitol), a disruptor of ER homeostasis leading to the accumulation of unfolded proteins. Although several genes involved in the UPR pathway of other eukaryotes are missing from the parasite, a non-canonical *Plasmodium* stress response exists, depending on eIF2 α phosphorylation by PflK4 [101]. The *Plasmodium* CDK-like kinase PflK5 is activated in the presence of the cyclin Pfcyc-1 and likely participates in the regulation of the nuclear division cycle i.e. at the schizont stage [102].

Fig. 3 Bip-PERK-eIF2 α pathway

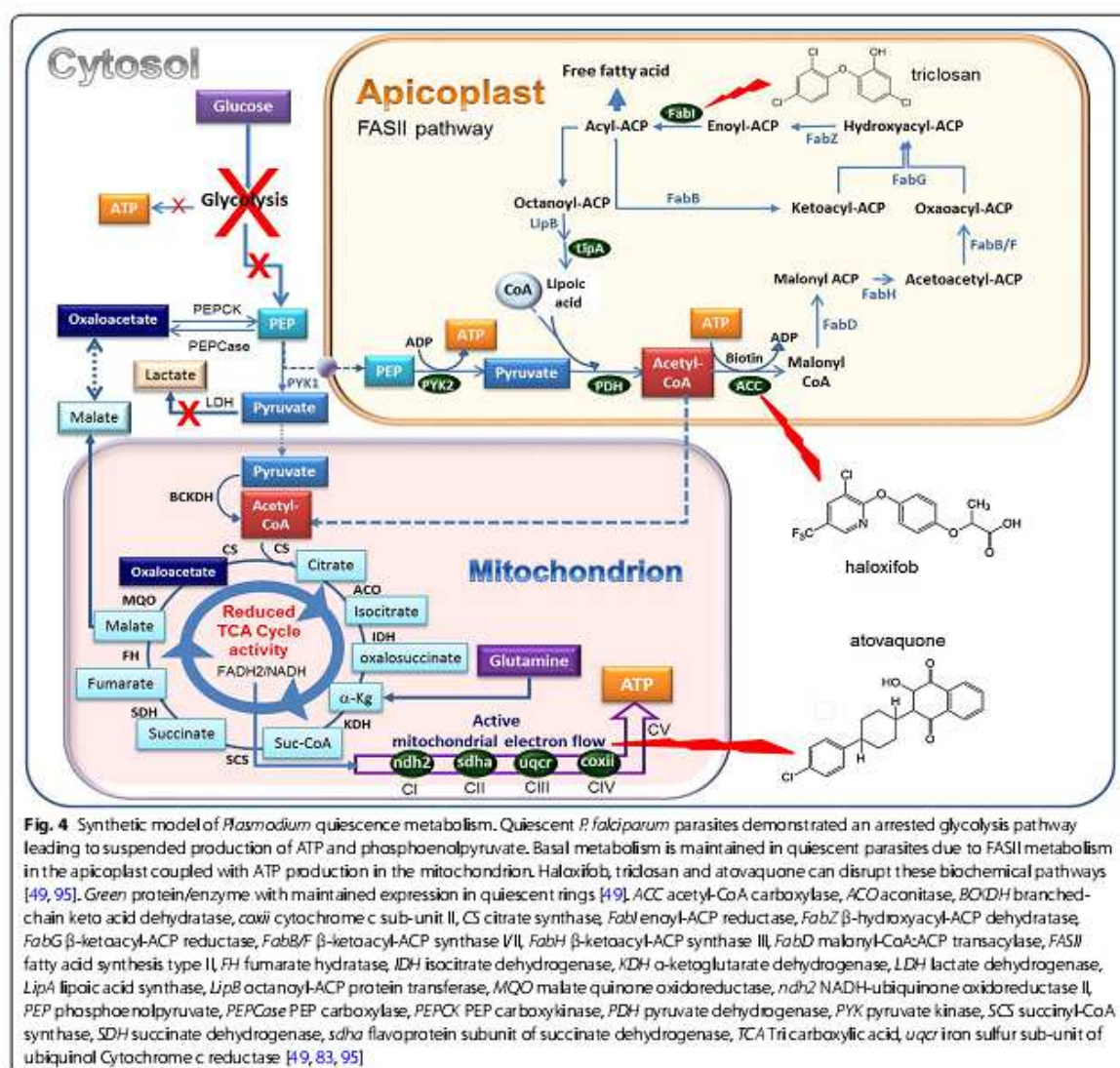
reductase/FabI) and for the lipoic acid metabolism (lipoic acid synthase) [49]. The mitochondrial tricarboxylic acid cycle is down-regulated but remains active and genes encoding the proteins of the electron transport chain also remain transcribed at a normal level (cytochrome c subunit II, NADH-ubiquinone oxidoreductase II, flavo-protein sub-unit of succinate dehydrogenase and ubiquinol cytochrome c reductase iron sulfur sub-unit) [49]. This was confirmed by rhodamine staining of parasite after DHA treatment: only rhodamine-positive parasites resumed growth [42]. In stress conditions, depletion of host-derived fatty acids induces an upregulation of the *Plasmodium* FASII pathway [79]. PI3P is present in the apicoplast membrane and can play a role in exchanges of protein and likely lipids [80–82] with the adjacent mitochondrion. The elevated production of PI3P in resistant parasites [68] could play a role in the maintenance of this minimal energetic metabolism based on mitochondrial and apicoplast activity and also found in quiescent resistant parasites [49]. Indeed these parasites cannot survive and resume growth in the presence of atovaquone, an inhibitor of the mitochondrial activity, and the use of haloxyfob, an acetyl-CoA carboxylase inhibitor, as well as triclosan, an inhibitor of FabI delays the recovery of DHA-induced quiescent parasites [35, 42, 49, 83, 84], demonstrating that this minimum active metabolism during the induced quiescence state by artemisinins is essential for the survival and the recovery of the parasites from dormancy.

Thus, the quiescent state induced at the young ring stage by artemisinin in sensitive and resistant parasites

likely seems to involve the same cellular mechanisms, but the PflK13 mutation shifts the intra-population distribution so as to allow more parasites to become quiescent in the case of resistant parasites, and to quickly resume growth after the drug removal in vitro or drug elimination in patients.

Conclusions

It is increasingly clear that the *P. falciparum* resistance to artemisinin and its derivatives is not due to efflux modulation or target modifications as described for other anti-malarials, but is based on increased capacity of PflK13-mutant parasites to manage oxidative damage thanks to greater UPR mobilization. The over-expression of UPR target genes should impact three key points: (an) unidentified transcription factor(s) (uTF) regulating transcription of UPR/oxidative response genes, the PI3 K/PI3P/AKT pathway activity and the PflK4/eIF2 α cascade. This would allow parasite entrance into a quiescence state with minimal energy metabolism provided by the apicoplast and the mitochondrion, maintained by alternative tricarboxylic acid cycle and FASII metabolism, until drug removal/excretion when parasites can resume growth. Recently, the artemisinin-resistant F32-ART line, selected by long-term drug pressure with solely artemisinin, was shown to display an extended age range of stages surviving artemisinin treatment extending to older ring stages (13–16 h) and even trophozoite stages. These parasites were also able to survive lethal doses of diverse classes of anti-malarial drugs, including molecules used as partners in currently recommended ACT, in the



absence of the 'classical' mutations of the target genes for these drugs. Thus, long-term in vitro artemisinin exposure selects a novel multidrug tolerant phenotype, which could represent a major threat to anti-malarial drug policy in the field [35]. This threat is exacerbated by the fact that artemisinin-resistant parasites seem able to infect and be transmitted by a large panel of *Anopheles* species, including the major African species *Anopheles gambiae* [85]. This is yet another reason to urgently clarify the cellular network of ART resistance in order to identify new therapeutic modalities (to protect the molecules currently used), and/or novel drug development, in order

to avoid entrance into a quiescent state, target quiescent parasites and promote restart of the cell cycle, rescuing drug susceptibility of parasites.

Additional file

Additional file 1. ACT recommended by WHO and the newly approved combination DHA-piperaquine.

Authors' contributions

JMA and FBV designed the review; all authors contributed to acquisition, analysis and interpretation of data. LP, APR, OMP, JMA, and FBV have been

involved in drafting and revising the manuscript critically. All authors read and approved the final manuscript.

Authors' information

OM-P and FB-V are co-inventors on the pending patents #U561/904651 and U562/062439, filed by Institut Pasteur. These patents cover the use of K13 mutations as a molecular marker of *P. falciparum* ART resistance.

Author details

¹CNRS, ICCC (Laboratoire de Chimie de Coordination) UPR8241, 205 route de Narbonne, BP 44099, 31077 Toulouse Cedex 4, France. ²Université de Toulouse, UPS, INPT, 31077 Toulouse Cedex 4, France. ³Present Address: Department of Pharmacology and Therapy, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. ⁴Unité d'Immunologie Moléculaire des Parasites, Institut Pasteur, 75015 Paris, France.

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Competing interests

The authors declare that they have no competing interests.

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6. Medicinal Plants for New Antimalarial Drug Discovery

Malaria control is based on different strategies, among others are developing an effective vaccine, eradicating mosquito-vectors and discovering new drugs [116, 117]. In fact, the development of vaccine proved to be very problematic. Moreover, the attempt to control *Anopheles* mosquito populations has had a limited success, although the use of insecticide bed nets is highly correlated to malaria death rates reduction [118]. The limitation of vaccine and vector control as well as the increasing resistance of malaria parasites to antimalarial drugs pointed the importance of the discovery of new antimalarial agents [119]. The main concern over antimalarial drug development, beyond the antimalarial activity, is that the drugs must be affordable for the population in endemic regions of developing countries [7, 120].

Regarding these problems, plant-derived compounds play an important role in new antimalarial drug discovery. The knowledge of medicinal plants by native people of malarious regions can initiate medicinal chemistry exploration based on specific antimalarial clinical data. Many plants were screened or are under evaluation for their antiplasmodial activities [121-123], in the image of the discovery of quinine from *Chincona* bark [124] and artemisinin from *Artemisia annua*.

When the Spanish and Portuguese began to colonize South America, they discovered the use of the bark of *Chinchona* tree by native Indians to treat fevers such as those caused by malaria. Tea made from the bark cured the people suffering from malaria. The main antimalarial compound was isolated from the bark of *Cinchona succiruba* (Rubiaceae) in 1820 and identified as quinine, one of the 31 alkaloids contained in the plant [125].

The search for new antimalarial agents continued and found qinghaosu or artemisinin, a sesquiterpene isolated from *Artemisia annua* (Asteraceae), a plant used in Chinese traditional

medicine to treat fevers including those provoked by malaria [126, 127]. Artemisinin has proven to be effective in chloroquine-resistant strains of human malaria [128].

Another species used as an antimalarial drug in Chinese traditional medicine is *Dichroea febrifuga* (Saxifragaceae) [129] with the active principle, febrifugine. It has been evaluated clinically against *P. vivax* and *P. ovale* but its liver toxicity has limited its use for malaria treatment [130]. The NAPRALERT natural product database has listed plant species from 152 genera from popular usages for antimalarial properties in Africa, Americas, and Asia [131].

Antiplasmodial activities of plant crude extracts can be classified based on the IC_{50} criteria proposed by Basco and collaborators. Good activity refers to an $IC_{50} < 10 \mu\text{g/mL}$, $10 < IC_{50} < 50 \mu\text{g/mL}$ is classified as moderate, $50 < IC_{50} < 100 \mu\text{g/mL}$ indicates low activity, and $IC_{50} > 100 \mu\text{g/mL}$ is considered inactive [132, 133]. For others, inactive criteria are reached if the $IC_{50} > 100 \mu\text{M}$, $20 < IC_{50} < 60 \mu\text{M}$ is classified as low activity, and $1 < IC_{50} < 20 \mu\text{M}$ refers to moderate or limited activity [134]. A recent study gave another classification but with closed criteria. Very good activity refers to an $IC_{50} < 2 \mu\text{g/mL}$, $2.0 < IC_{50} < 5.0 \mu\text{g/mL}$ is classified as good, $5.1 < IC_{50} < 10 \mu\text{g/mL}$ indicates good to moderate, $11 < IC_{50} < 25 \mu\text{g/mL}$ is determined as weak, $26 < IC_{50} < 50 \mu\text{g/mL}$ is specified as very weak, and $IC_{50} > 100 \mu\text{g/mL}$ is considered inactive [135].

7. Indonesian Medicinal Plants for Malaria Therapy

Indonesia is rich in medicinal plants widely used by the population for any diseases including malaria. Basically, the use of medicinal plants for malaria treatment is based on fever therapy like in South America with *Cinchona* or *Artemisia* in China. Some studies hypothesized that antimalarial plants may act on unique biochemical targets for protozoa [131].

The ethno-botanical approach is the most common strategy for screening plant activities. From plants, belonging to various families, which are traditionally used for malaria fever therapy and naturally grow in Indonesia, five species were selected as potential sources of antimalarial agents due to their widely well-known use and for some of them are not well chemically characterized: kembang bulan (*Tithonia diversifolia* (Hemsl.) A. Gray), cincau rambat (*Cyclea barbata* Miers), brotowali (*Tinospora crispa* (L.) Hook. F. & Thomson), kayu kuning (*Arcangelisia flava* Merr), and sengkubak (*Pycnarrhena cauliflora* (Miers.) Diels).

a. *Kembang bulan* (*Tithonia diversifolia* (Hemsl.) A. Gray)

Tithonia diversifolia is a woody herb or succulent shrub locally called *kembang bulan* and also known as Mexican sunflower. It is characterized by opposite leaves, attenuate base, acute apex, and crenate margin. It belongs to the kingdom Plantae (plants), subkingdom Tracheobionta (vascular plants), superdivision Spermatophyta (seed plants), division Magnoliophyta (flowering plants), class Magnoliopsida (dicotyledons), subclass Asteridae, order Asterales, family Asteraceae, genus *Tithonia*, and species *Tithonia diversifolia* (Hemsl) A. Gray [136]. It is commonly used for several traditional medication purposes including malaria fever [137]. Its infusion is suitable for constipation, stomachache, indigestion, sore throat, and liver pain [138]. Some research showed that its ethanolic leaf extract exhibited anti-malarial activity [139]. In addition, the ether extract from its aerial parts demonstrated an *in vitro* antiplasmodial activity (IC₅₀: 0.75µg/mL on chloroquine-sensitive strain (FCA)). This finding led to extraction of active compound, the sesquiterpene lactone tagitinin C with 0.33µg/mL IC₅₀ against *Plasmodium* FCA strain [140]. Moreover, *in vivo* study showed that the ethanol extract of *T. diversifolia* is active *in vivo* at 200 mg/kg BW in a malaria Swiss albino mice model [141].

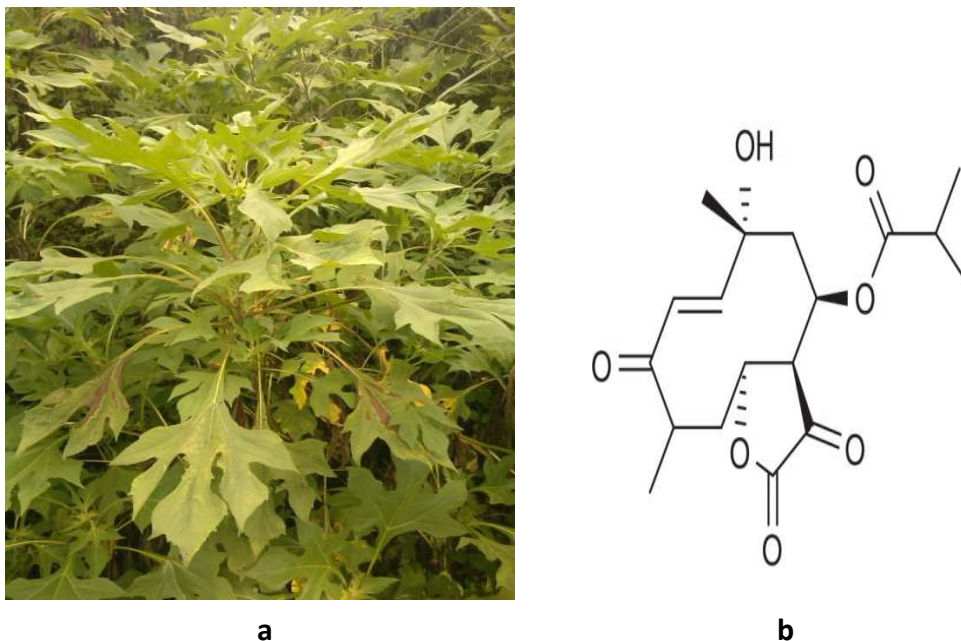


Figure 7. The leaves of *T. diversifolia* (a) and tagitinin C structure (b)

b. *Cincau rambat* (*Cyclea barbata* Miers)

Cyclea barbata (Menispermaceae) can be easily distinguished by its deltoidovate, hispid leaves with acuminate apex, finely mucronate acumen, and long male and female inflorescences with dense capitate flowers. It is in the kingdom Plantae, division Magnoliophyta, class Magnoliopsida, order Ranunculales, family Menispermaceae, genus *Cyclea*, and species *Cyclea barbata* Miers [136]. In Java, the leaves of *C. barbata* are typically prepared in the form of jelly consumed as stomach medicine and a brew prepared from the dried roots is used as a prophylactic against fever [142]. It has been shown that *C. barbata* leaves had a protective effect on aspirin-induced gastric ulcer in Balb/c mice [143]. The alkaloid extract of *C. barbata* roots demonstrated both antimalarial and cytotoxic activities, and among the five active bisbenzylisoquinolines alkaloids that were isolated, the most active was cycleapeltine with ED₅₀ of 29 and 41 ng/mL on *Plasmodium* strains D-6 and W-2 respectively [144]. Continuous studies of the alkaloidal fraction of *C. barbata* roots led to identification of two new bisbenzylisoquinoline alkaloids: (-)-2'-

norlimacine and (+)-cycleabarbazine [145] but their antimalarial properties were not evaluated.

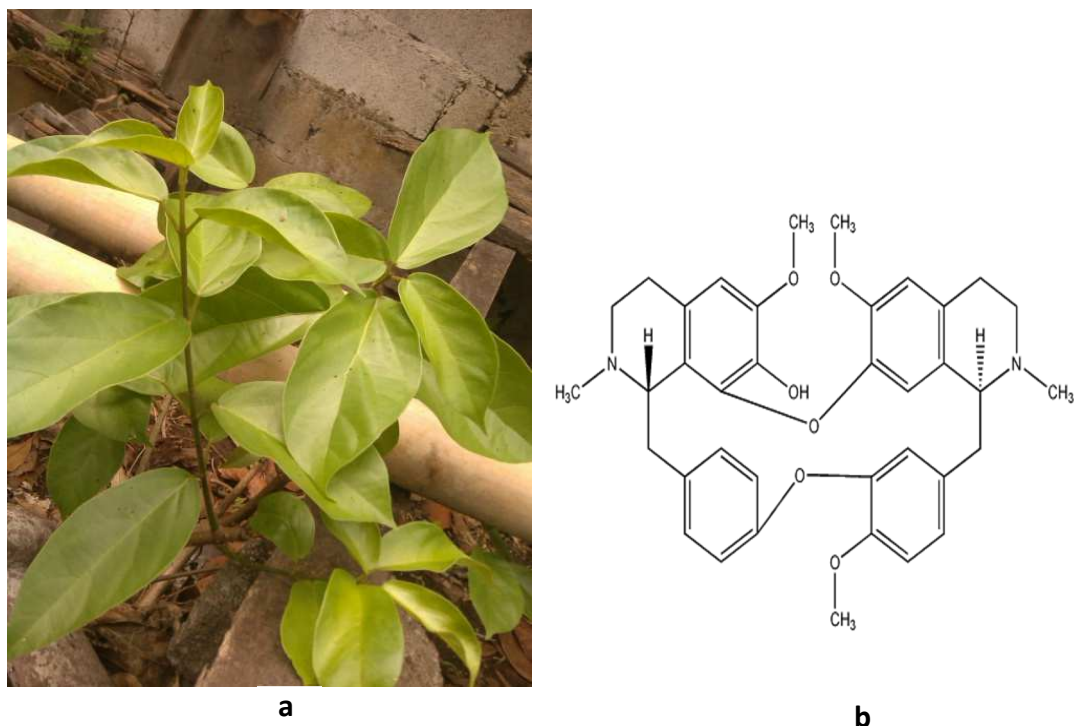


Figure 8. The leaves of *C. barbata* (a) and cycleapeltine structure (b).

c. *Brotowali* (*Tinospora crispa* (L.) Hook. F. & Thomson)

Tinospora crispa (L.) (Menispermaceae) is a tropical liana (woody) with heart shaped large shiny green leaves and yellow coloured flowers which are fascicled [146]. This plant is predominant in primary rainforests or mixed deciduous forests of South East Asia and Africa including Thailand, Malaysia, and Indonesia [147]. It is endemic in several areas of Indonesia particularly in Java. This plant is part of the kingdom Plantae, phylum Tracheophyta, class Magnoliopsida, order Ranunculales, family Menispermaceae, genus *Tinospora*, and species *Tinospora crispa* (L.) Hook. F. & Thomson [148]. *T. crispa*, known as brotowali in traditional folklore, is used for various therapeutic purposes such as treatment for diabetes, hypertension, stimulation of appetite and protection from mosquito bites, fever and malaria, stomachache and jaundice. An infusion of the stems is

prescribed as a worming, and a decoction of the whole plant is used as a general tonic [147, 149-151].

Phytochemical investigations of the stems of brotowali led to the isolation of aporphine alkaloid, including N-formylasimilobine, N-formylanonaine, N-formyldehydroanonaine, N-formylnomuciferine, magnoflorine, paprazine, N-trans-feruloyltyramine, and cytidine [152, 153]. Further investigation revealed that methanol extract of *T. crispa* displays an antioxidant activity [154-156], methanol crude extract of *T. crispa* shows antiproliferative activity [156, 157], aqueous extract possesses hypoglycemic effect [149], and ethanol extract and its isolated fraction stimulate murine macrophage cell (RAW264.7) viability and intracellular expressions of cytokines [158], and stem extract of *T. crispa* shows both *in vitro* antiplasmodial activity [159] and *in vivo* against *P. berghei* in dose-dependent manner [160].

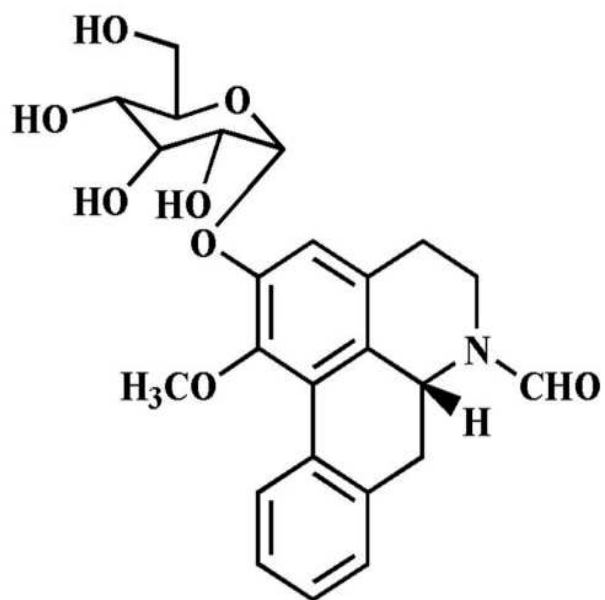
**a****b**

Figure 9. The stem of *T. crispa* (a) and one of the aporphine structures, N-formylasimilobine 2-O-β-D-glucopyranoside (b)

d. *Akar kuning* (*Arcangelisia flava* Merr)

Akar kuning (*A. flava*) found in Sumatra, Java, Borneo, and Sulawesi in Indonesia is a large, woody, glabrous, dioecious liana up to 20m long. The stem is up to 5 cm in diameter, yellow wood, and exuding yellow sap when cut. It is a member of the kingdom Plantae, division Magnoliophyta, class Magnoliopsida, order Ranunculales, family Menispermaceae, genus *Arcangelisia*, and species *Arcangelisia flava* Merr [161]. Traditionally, the sap of cut stems of *A. flava* is drunk to treat typhoid fever and sprue [162], hepatitis, stomach disturbance, and malaria [163]. *A. flava*, also known as katola by the communities of Muna district in Southeast Sulawesi, is traditionally used as antidiarrheal and yellow fever treatment by Wawoni tribes.

Some alkaloids yielded from *A. flava* are berberine, 8-hydroxyberberine, columbamine, jatrorrhizine, palmatine, thalifendine, dehydrocorydalmine, shobakunine, (-)R,R-limacine, (+)R,S-homoaromaline, and pycnarrhine [162]. A previous study mentioned that berberine of *A. flava* has antimicrobial activity [164], antioxidant and cytotoxic activity [165]. Its antiplasmodial activity is also figured out against *P. falciparum* with IC₅₀ ranging from 0.4 to 8.6 µg/mL [166]. In addition, the berberine extracted from *A. flava* inhibits telomerase activity in *P. falciparum* as a potential target for future malaria chemotherapy [167].

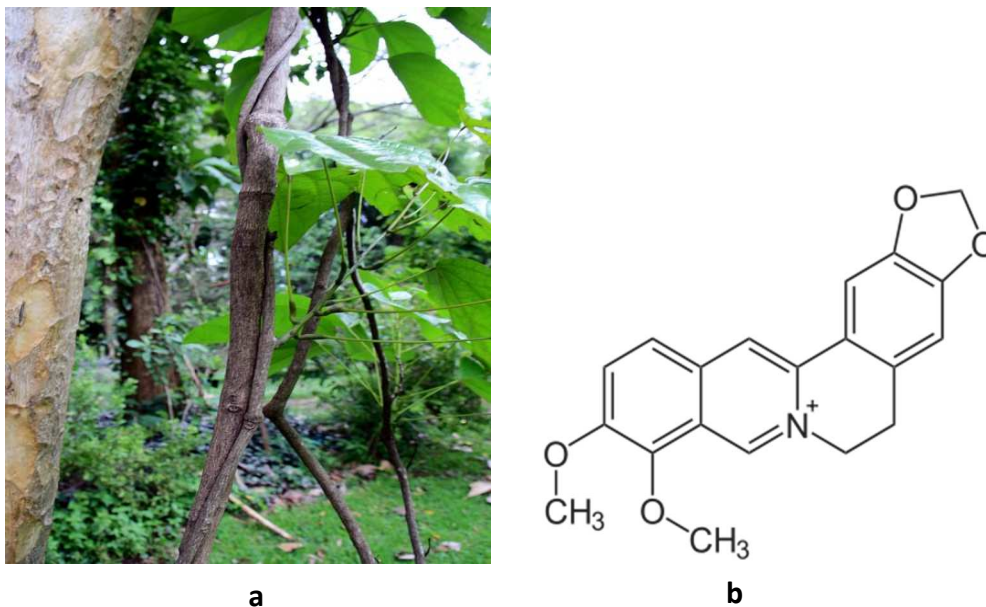


Figure 10. Stem of *A. flava* (a) and berberine structure (b)

e. *Sengkubak* (*Pycnarrhena cauliflora* (Miers.) Diels)

Sengkubak (*Pycnarrhena cauliflora* (Miers.) Diels) from West Borneo has been used widely by Dayak tribes as a cooking flavor and treatment for flatulence (by placing soaked leaves on the stomach), fever and malaria (by drinking leaf infusion). This plant can be found in primary or secondary forests with spatial distribution and tend to grow in clusters. It is a slender twining shrub and frequently climbing up on a tall tree [168]. *Sengkubak* belongs to the kingdom Plantae, phylum Magnoliophyta, class Magnoliopsida, order Ranunculales, family Menispermaceae, genus *Pycnarrhena*, and species *Pycnarrhena cauliflora* (Miers.) [148]. The bisbenzylisoquinoline alkaloid compounds of *P. cauliflora*, like obaberine, picnarine [169], are responsible for many activities including antioxidant and cytotoxic properties [170, 171] but the antimalarial activity has yet to be

experimentally proved. From the eight species in the genus *Pycnarrhena*, only four (*P. australiana*, *P. ozantha*, *P. manilensis*, *P. longifolia*) have been deeply investigated.

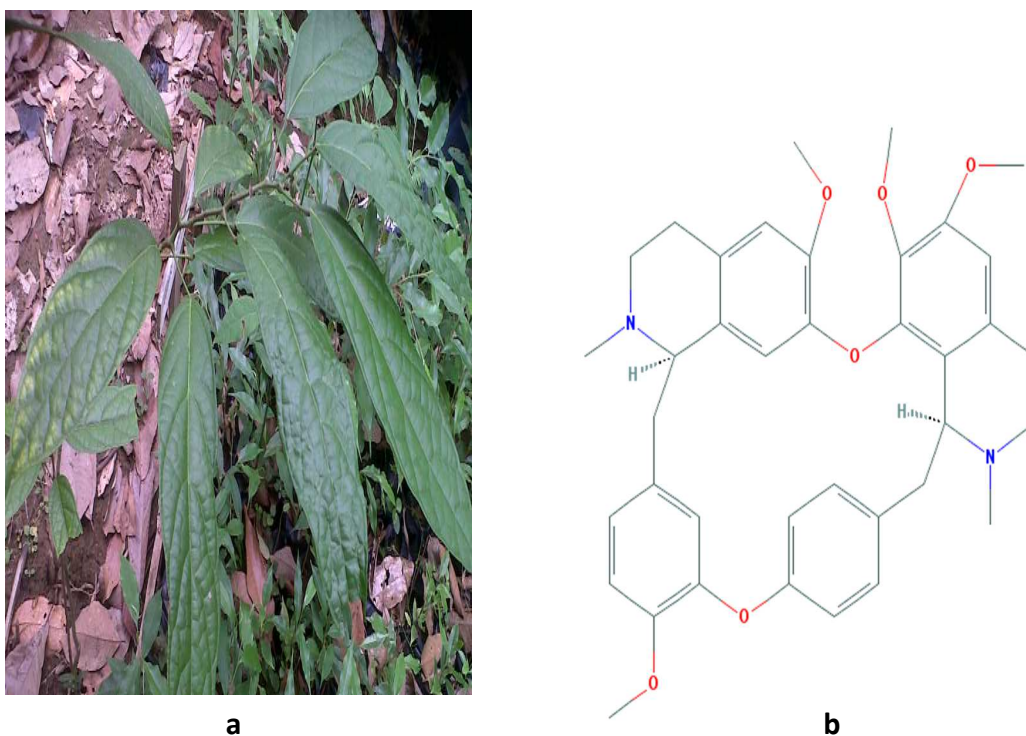


Figure 11. The plant of *P. cauliflora* (a) and obaberine structure (b)

All these five selected plants are naturally growing in Indonesia. *T. diversifolia*, *C. barbata*, and *T. crispa* are easily found in gardens or in cultivation centers. *A. flava* and *P. cauliflora* are found in forests. These species are now endangered despite the clinical interest of some of them, such as *A. flava* which has been studied for its numerous activities and *P. cauliflora* that is considered as a promising source of medicinal plants. Deforestation for various reasons, and limited direct economic benefits for the people are the main factors of their extinction. In order to confirm the antiplasmodial activity and to increase their economic benefit, those selected plants were collected and extracted, and their activities were investigated using standard *in vitro* assays with various parasites and human cell lines for cytotoxic studies. Further study on isolation and characterization of active compounds will be required before developing a new antimalarial drug.

8. Gold Complexes as Antimalarial Agents

Metal-based therapeutic agents have become an increasingly important research effort toward the development of new compounds with less toxic side effects and with an understanding of their mechanism of action [172-176]. In particular, the use of metal containing drugs as antiparasitic agents has not been very much explored. The emergence of resistance to antiparasitic drugs induces an urgent need for new, effective, non-toxic anti-parasitic drugs. Moreover, an immense number of rational combinations of appropriate organic molecules (used in traditional medicine) with different transitional metal-drug synergism can enhance the activity of parental organic drugs due to bonding with the metal ion. The stabilization of the drug by coordination with the metal ion may be related to this activity enhancement that leads to a prolong contact of the drug with the organism and more efficient biological targeting. Furthermore, the toxicity diminution of the metal ion may occur since its complexation with the organic drugs makes it less available for toxic reactions [177].

The same approach, metal-drug synergism, has also been applied for antimalarial metal agent discovery. It was reported that in 1987, using the drugs primaquine and amodiaquine, 32 metal complexes were synthesized with two of those being based on gold (III). The activity study proved that all compounds were active against *P. falciparum* as well as the parental drugs [178].

Gold is among the most ancient of all metals used in medicine and its current use has allowed information regarding toxicological and clinical administration to become available along with valuable studies concerning its metabolism and molecular targets. An attempt of attaching gold-containing fragment to CQ showed a strong variation of the electronic properties. Moreover, the diversity of possible gold-ligand fragments that can be attached to base CQ or chloroquine diphosphate (CQDP) yields a widespread exploration of their anti-malarial properties [178].

One of the CQ-metal complexes, $[\text{Au}(\text{PPh}_3)(\text{CQ})]\text{PF}_6$ potentially inhibited the growth of *P. berghei* and was also very effective against two CQ-resistant FcB1 and FcB2 strains of *P. falciparum*. It has been documented that the activity was 5-10-fold greater than the corresponding CQDP, displaying that gold coordination leads to a significant enhancement of the activity of the parental drug against resistant parasites without any adverse reaction or apparent acute toxic responses observed [179]. The principal mechanism proposed for the antimalarial action of $[\text{Au}(\text{CQ})(\text{PPh}_3)]\text{PF}_6$ against resistant strains of *P. falciparum* is the interaction with heme and the inhibition of β -hematin formation. Both of the enhanced activity and the ability of this compound to lower CQ-resistance are related to the high lipophilicity of the metal complex and the important structural modification of the CQ structure imposed by the presence of the metal-containing fragment [180].

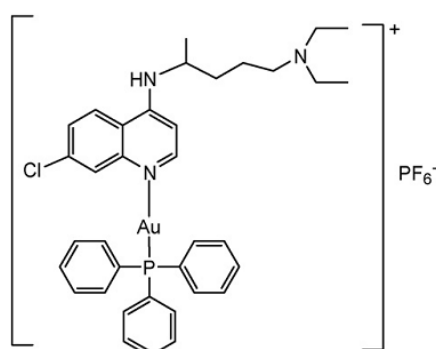


Figure 12. Structure of $[\text{Au}(\text{CQ})(\text{PPh}_3)]\text{PF}_6$

This result then stimulates a derivatization process of CQ-Au complex by structure modification of $[\text{Au}(\text{CQ})(\text{PPh}_3)]\text{PF}_6$ complex. The modification included variation of the phosphine ligand with the purpose of inducing changes in the electronic and steric properties, variation of counter anion, variation of the gold oxidation state (both Au(I) and Au(III)), and use of other biologically important ligands. The result demonstrates that Gold (III) complexes are more active than CQDP against CQ-resistant strain K1. The highest activity for this series

is found for complex $[\text{Au}(\text{Pet}3)(\text{CQ})]\text{PF}_6$, which is 5-fold more active than CQDP against CQ-resistant strain FcB1 [178].

Recently, another ligand widely studied for its coordination with gold complex is NHC (NHC = *N*-heterocyclic carbene). NHCs represent one of the most important classes of ligands in organometallic chemistry. The easy preparation of NHC-precursors has allowed an almost infinite access to new organometallic complexes, in which the nitrogen atoms of the azoliums rings can be functionalized by organic groups, organometallic moieties and biological entities [11]. Moreover, because of their easy preparation via Ag carbene transfer route, the interest in Au^{I} - and Au^{III} -NHC complexes has surged in the past decade [181] and interesting biological activities of numerous NHC-metal complexes have been documented in several articles [182].

Au^{I} - and Au^{III} -NHC compounds are generally readily prepared, stable to air and moisture, and some of them, especially dinuclear gold(I) and polynuclear gold(I)-heterometal species display long-lived intense photoluminescence at room temperature [181]. In particular, given that these ligands are extremely good σ -donors, they form strong Au-carbene bonds, giving stable Au^{I} -NHC complexes that are insensitive to biologically important thiol groups. Au^{I} -NHC have shown potential medical applications [183], especially as anticancer [182, 184-187], antiarthritis [188], and antimicrobial agents [189]. It has to be mentioned that gold-based compounds, including some gold(I)-NHCs, show anti-mitochondrial activity with great specificity [183]. In addition, pharmaco-modulations of *N*-functionalized bis(imidazolium) proligands and their corresponding silver(I), gold(I) and gold(III) complexes leading to the second series of complexes with IC_{50} values up to 330nM against *P. falciparum* strain, without any hemolysis [190].

The study by Micale and colleagues explored the possible mechanism of gold complexes as antimalarial agents. It was noted that the parasite enzyme Falcipain 2 (Fp2) may

involve in this regard. This protein is correlate to the degradation of host's hemoglobin that produce significant amounts of peptide and amino acids for parasite growth. This process is strongly occurring at the early throphozoite stage. Although the direct correlation of gold compounds to Fp2 inhibition has been investigated *in vitro*, further findings displayed that the inhibition of Fp2 only represent one of the diverse mode of action of gold compounds inhibit *P. falciparum* replication [11, 191].

CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

1. Antiplasmodial Activity of Indonesian Medicinal Plants

a. Plants & reagents

The parts of the medicinal plants studied here were leaves of *T. diversifolia*, leaves and stems of *C. barbata*, stems of *T. crispa*, stems of *A. flava*, as well as leaves and radices of *P. cauliflora*.

Other materials used were ethanol 70%, n-hexane, ethyl acetate, and methanol.

b. *P. falciparum* strains and cell lines

FcM-29 Cameroon chloroquine-resistant strain was employed for assessing the antiplasmodial activity, while VERO cells were used for testing the cytotoxic.

c. Antimalarial drug controls & reagents

We used Chloroquine (Sigma), DMSO (Sigma), RPMI-1640 (Biowest, France), HEPES, L-glutamine, AB⁺ human serum (French blood bank, EFS, Toulouse, France), O-type human RBC (French blood bank, EFS, Toulouse, France), ³[H] hypoxanthine (Perkin Elmer, France), and complete medium (MEM, FCS, L-glutamine, penicillin, streptomycin, NEAA 1x).

d. Tools

The tools employed were maceration chamber, water bath, rotary evaporator, glass instruments, conical tubes, centrifuge, vortex, freezer, refrigerator, CO₂ incubator, 96-well plates, cell counter (Perkin Elmer) and Class II Biosafety cabinet.

2. Antiplasmodial Activity of Gold (I) Complexes

a. Tested compounds

Twenty-five synthesized compounds were tested in this study consist of the imidazolium salts (proligands: compounds **1 – 9**, and **11**; zwitterionic proligand: compound **10**) and the mononuclear gold (I) bis(NHC) complexes (compound **12 – 25**).

All these compounds were obtained from group V UPR 8241 LCC-CNRS, Toulouse, France.

b. *Plasmodium falciparum* strains and cell lines

Chloroquine-resistant *P. falciparum* FcM-29 Cameroon strain was used for assessing the antiplasmodial activity while VERO cells were used for testing the cytotoxic.

c. Antimalarial drugs and reagents

The materials used in this study were Chloroquine (Sigma), artemisinin (TCI), DMSO (Sigma), RPMI-1640 (Biowest, France), HEPES, L-glutamine, AB⁺ human serum (French blood bank, EFS, Toulouse, France), O-type human RBC (French blood bank, EFS, Toulouse, France), ³[H] hypoxanthine (Perkin Elmer, France), and complete medium (MEM, FCS, L-glutamine, penicillin, streptomycin, NEAA 1x).

d. Tools

The tools utilized were water bath, rotary evaporator, glass instruments, conical tubes, centrifuge, vortex, freezer, refrigerator, CO₂ incubator, 96-well plates, cell counter (Perkin Elmer) and Class II Biosafety cabinet.

3. K13 Gene and Artemisinin Resistance

a. Strains & reagents

The *P. falciparum* strains used in this research were *P. falciparum* clinical isolates from Cambodia (Cam3.I, Cam3.II, Cam5, Cam2, CamWT) and reference laboratory lines (F32-TEM, F32-ART, V1/S, FCB, Dd2) provided by four different laboratories. In our laboratory, we conducted the research using FCB, Dd2, and F32 strains.

Other materials used were RPMI-1640 (Biowest, France), HEPES, L-glutamine, AB⁺ human serum (French blood bank, EFS, Toulouse, France), O type human RBC (French blood bank, EFS, Toulouse, France), Dihydroartemisinin (TCI), DMSO (Sigma), Percoll[®] (Sigma-Aldrich), PBS, Heparin, D-sorbitol (Sigma-Aldrich), immersion oil, and Giemsa stain (Diff quick[®]).

b. Materials

The tools utilized were Class II biosafety cabinet, CO₂ incubator, water-bath, scale, pipette and tips, flask culture, centrifuge, conical tube, filter 0.22µM pore size, 48-well plates, glass slides, microscope, and counter.

4. Dihydroartemisinin-Piperaquine Resistance in Kupang

a. Materials

The materials used were dihydroartemisinin-piperaquine tablets (Darplex), dihydroartemisinin (TCI), RPMI-1640 (Sigma), ACD anticoagulant, O type RBC, culture medium (RPMI-1640, HEPES, Gentamycin, Hypoxanthine, Albumax II, heat-inactivated human serum), DMSO (Sigma), immersion oil, Giemsa stain (Merck), syringe (Terumo), filter paper (Whatmann), K13-propeller primers,

dNTP (Biodynes), MgCl₂, *Taq* DNA polymerase (Solis Biodyne), and ethidium bromide stain.

b. Strains

The *P. falciparum* strain employed in this study was *P. falciparum* isolates from malaria patients in Kupang, East Nusa Tenggara.

c. Tools

The tools involved were conical tubes, glass instruments, glass slides, microscope, counter, 48-well plates, CO₂ incubator, candle jar, Class II biosafety cabinet, water bath, balance, pipette and tips, Duran® bottles, flask cultures, centrifuge, and filters 0.22µM pore size.

B. METHODS

1. Antiplasmodial Activity of Indonesian Medicinal Plants

a. Plant collections and identification

Tithonia diversifolia (Asteraceae; voucher specimen 007-02/L-PB/UII/2013) was collected in Sleman District of Yogyakarta, and *Cyclea barbata* (Menispermaceae; voucher specimen 008-03/L.S-PB/UII/2013) was gathered in Bantul District of Yogyakarta, while *Tinospora crispa* (Menispermaceae; voucher specimen 009-03/S-PB/UII/2013) was obtained in Yogyakarta City. Meanwhile *Arcangelisia flava* (Menispermaceae; voucher specimen 010-04/S-PB/UII/2013) was gathered in South Borneo, and *Pycnarrhena cauliflora* (Menispermaceae; voucher specimen 011-01/S.R-PB/UII/2013) was collected in Sintang District, West Borneo.

The plants were identified in the Laboratory of Biology, Department of Pharmacy, Universitas Islam Indonesia, Yogyakarta, Indonesia (Herbarium of Laboratory of Biology, Department of Pharmacy, Faculty of Science and Mathematics, UII, Yogyakarta, Indonesia), and their samples were air-dried (50-60°C) and powdered.

b. Plant extraction

Similar to the traditional ways, the parts of the plant taken for the extraction included leaves of *T. diversifolia*, stem and leaves of *C. barbata*, stem of *T. crispa*, stem of *A. flava*, as well as stem and radix of *P. cauliflora*.

Powdered samples of *T. diversifolia* (leaves), *C. barbata* (stem and leaves), *T. crispa* (stem), and *A. flava* (stem) were extracted by maceration in 70 % ethanol for 24 hours, 300 g of plant powder with 1.5 L of solvent. This process was repeated for two days consecutively using fresh 70% ethanol. Following 3 days re-maceration in ethanol, the extracts were filtered and the filtrates evaporated using a rotary vacuum evaporator, to attain ethanol extract. Some of the ethanol extract were then fractionated by n-hexane by liquid-liquid separation method which create two separate layers (n-hexane and first ethanol layer). The n-hexane fraction was acquired after the n-hexane layer was evaporated. The fractionation process was continued by fractionated the ethanol layer using ethyl acetate (liquid-liquid separation) which produce ethyl acetate and second ethanol layer. Both layer was then evaporated separately yielded ethyl acetate fraction from ethyl acetate layer and fraction residue from second ethanol layer, in case there were active compounds left behind.

Contrary to previous plants, radix part of *P. cauliflora* was extracted by n-hexane to attract the lipophilic compound from the plant. The filtrate then evaporated and become n-hexane extract. Meanwhile, the precipitate of n-hexane extraction was further extracted using dichloromethane to obtain dichloromethane extract. Then, the precipitate from previous dichloromethane extraction was employed for methanol extraction. The methanol extract was gained from the evaporation of the filtrates.

At the end of extraction process, from each part of leaves of *T. diversifolia*, stem and leaves *C. barbata*, and stem of *T. crispa* were gained ethanol extract, n-hexane fraction, ethyl acetate fraction, and fraction residue. Meanwhile, from stem of *A. flava* there were ethanol extract, n-hexane fraction and ethyl acetate fraction. Yet, for each part of stem and radix of *P. cauliflora* produce n-hexane, dichloromethane and methanol extracts. Finally, it yielded 25 extracts and fraction that ready for investigation its antiplasmodial activities.

c. Reagent preparation

To evaluate the antiplasmodial activities, the stock solutions of the extracts (1 mg/mL) were prepared in dimethylsulfoxide (DMSO, Sigma, France) and then diluted in RPMI 1640 (Biowest, France). The dilutions were checked to confirm that the extracts did not re-precipitate under these conditions. The extracts were then tested immediately after their dissolution in DMSO and RPMI, kept at 4°C for 3-8 days, and re-tested. The residual DMSO in the assay was 2% and this was evaluated individually to check that this solvent percentage had no activity on parasite cultures. The extract concentrations for both chemo-sensitivity and cytotoxic assays were 0.1µg/mL, 1µg/mL, 10µg/mL, and 50µg/mL.

d. Chemo-sensitivity assay

The antiplasmodial activity was evaluated with the FcM29-Cameroon chloroquine-resistant *Plasmodium* strain and cultured continuously according to the Trager and Jensen method [192], in a 5% CO₂ atmosphere at 37°C by considering the modifications previously reported [193]. In brief, the parasites were kept *in vitro* in human red blood cells and diluted in RPMI 1640 medium, added by 25 mM HEPES, L-Glutamine and completed with 5% human serum (French blood bank, EFS, Toulouse, France). The antiplasmodial activity was assessed similarly to what was previously reported by Desjardins et al. [194] and modified as follows. Extract dilutions and reference compounds were tested in triplicate for at least 3 times independently, in 96-well plates with cultures at 1% parasitaemia and 1% haematocrit. For each test, the plates of parasite culture were incubated with extracts or reference compounds for 48 h and ³[H]-hypoxanthine (Perkin Elmer, France) was added to the medium 24 h after the beginning of incubation [193]. The parasite growth was estimated from [³H]-hypoxanthine incorporation. The control parasite culture (without drug and with 2% DMSO) was referred to as 100% growth. The IC₅₀ values (50 % inhibitory concentration) were graphically determined as concentrations versus percentage of parasite growth.

e. Cytotoxic evaluation on macrophage and VERO cell lines

The cytotoxicity tests were evaluated on macrophages and VERO cell lines for compounds showing the best anti-leishmanial and antiplasmodial activities, respectively. The evaluation of cytotoxicity by MTT assay on the J774A.1 cell line (mouse macrophage cell line, Sigma-Aldrich) was performed according to Mosmann [195] with slight modifications, and cytotoxicity on VERO cells

(monkey epithelial cell line, Sigma-Aldrich) was assessed using [³H]-hypoxanthine incorporation. In short, cells ($5 \cdot 10^4$ cells/mL) in 100 μ L of complete medium were seeded into each well of 96-well plates and incubated at 37°C in a humidified 5 % CO₂ with 95 % air atmosphere. The complete medium consisting of RPMI 1640 was supplemented with 10 % foetal calf serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin) for J774A.1 cell line and MEM with 10 % foetal calf serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin), NEAA 1X for VERO cell line. After 24 h incubation, 100 μ L medium with various product concentrations and appropriate controls (DMSO and doxorubicin) were added and the plates were incubated for 72 h at 37°C, 5 % CO₂. For cytotoxic evaluation on macrophages, each plate well was then microscope-examined to detect any precipitate formation before the medium was aspirated from the wells. One hundred μ L of MTT solution (0.5 mg/mL in RPMI) was then added to each well and the cells were incubated for 2 h at 37°C. Then, the MTT solution was removed and DMSO (100 μ L) was added to dissolve the resulting formazan crystals. The plates were shaken vigorously for 5 min and absorbance was measured at 570 nm with a microplate spectrophotometer (EON).

For cytotoxic evaluation on VERO cells, the plates were incubated with the test compounds for 48 h and [³H]-hypoxanthine (Perkin Elmer, France) added to the medium 24 h after the beginning of the incubation [193] to estimate VERO cell growth. The control VERO cell culture (drug-free but with 2 % DMSO) was referred to as 100 % of growth. The IC₅₀ values (50 % inhibitory concentration) were graphically determined as concentrations *versus* percentage of parasite growth.

f. Anti-*Babesia* activity

Babesia divergens (Rouen 1986 strain) was cultured continuously according to Précigout *et al* [196]. The parasites were maintained *in vitro* in human red blood cells (O⁺; EFS; Toulouse, France), diluted to 6 % haematocrit in RPMI 1640 medium (Biowest, France) supplemented with 30 nM NaHCO₃ and complemented with 10 % human AB⁺ serum (EFS).

The antibabesial activity of extracts was evaluated by the same radioactive micromethod as that for *Plasmodium* in 96-well culture plates except for the incubation time, which was reduced to 24 h. The drug-free control parasite cultures were considered as 100 % growth, and an imidocarb sensitivity control was included in every plate. IC₅₀ was determined graphically from concentration *versus* percentage of inhibition curves.

g. Anti-leishmanial activity on promastigote state

The effect of the compounds tested on the growth of *Leishmania infantum* (line MHOM/MA/67/ITMAP-263) promastigotes was assessed by Luciferase Assay. Briefly, promastigotes in log-phase in RPMI 1640 medium (Biowest, France) were supplemented with 10 % fetal calf serum (Hyclone, ThermoFischer, France), 2 mM L-glutamine and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL geneticin (Sigma, France)) were incubated at an average density of 10⁶ parasites/mL in sterile 96-well plates with various concentrations of compounds dissolved in DMSO or MeOH (final concentration less than 0.5 % v/v), in duplicate. Appropriate controls treated by DMSO, MeOH and amphotericin B (reference drug purchased from Sigma Aldrich) were added to each set of experiments. After a 72h incubation period at 24°C, each plate well

was then microscope-examined for any possible precipitate formation. To estimate the luciferase activity of promastigotes, 80 μ l of each well was transferred to white 96-well plates, Steady Glow reagent (Promega) was added according to the manufacturer's instructions, and the plates were incubated for 2 min. Luminescence was measured in a Microbeta Luminescence Counter (PerkinElmer). Inhibitory concentration 50 % (IC_{50}) is defined as the concentration of drug required to inhibit 50 % of the metabolic activity of *Leishmania infantum* promastigotes compared to the control. IC_{50} was calculated by non-linear regression analysis processed on dose-response curves, using TableCurve 2D V5[®] software. IC_{50} values are the geometric mean values calculated from three independent experiments.

2. Antiplasmodial Activity of Gold (I) Complexes

a. Synthesized compounds

All the synthesized compounds (the imidazolium salts and the mononuclear gold (I)-bis(NHC) complexes) have been prepared in powders and ready to be tested.

b. Reagent preparation

To evaluate the antiplasmodial activities, the stock solutions of the synthesized compounds (1 mg/mL) were prepared in dimethylsulfoxide (DMSO, Sigma, France) and then diluted in RPMI 1640 (Biowest, France). The dilutions were checked to confirm that the synthesized compounds did not re-precipitate under these conditions. The synthesized compounds were then tested immediately after their dissolution in DMSO and RPMI, kept at 4°C for 3-8 days, and re-tested. The residual DMSO in the assay was 2% and this was evaluated individually to

check that this solvent percentage had no activity on parasite cultures. The synthesized compounds concentrations for both antiplasmodial and cytotoxic assay were 0.1 µg/mL, 1 µg/mL, 10 µg/mL, and 50 µg/mL.

c. Antiplasmodial assay

The antiplasmodial activity was examined for the chloroquine-resistant strain FcM29-Cameroon and cultured continuously using the Trager and Jensen method [192], in a 5% CO₂ atmosphere at 37°C, and considering the modifications previously reported [193]. In brief, the parasites were kept *in vitro* in human red blood cells and diluted in RPMI 1640 medium, added by 25 mM HEPES, L-Glutamine and completed with 5% human serum (French Blood Bank, EFS, Toulouse, France). The antiplasmodial activity was assessed similarly to what was previously reported by Desjardins *et al.* [194] and modified as follows. Synthesized and reference compounds were tested in triplicate at least 3 times independently, in 96-well plates with cultures at 1% parasitaemia and 1% haematocrit. For each test, the plates of parasite culture were incubated with synthesized or reference compounds for 48 h and ³[H]-hypoxanthine (Perkin Elmer, France) was added to the medium 24 h after the beginning of incubation [193]. The parasite growth was estimated from [³H]-hypoxanthine incorporation. The control parasite culture (without drug and with 2% DMSO) was referred to as 100% growth. The IC₅₀ values (50% inhibitory concentration) were graphically determined as concentrations versus percentage of parasite growth.

d. Cytotoxic evaluation on VERO cell lines

The cytotoxicity tests were performed on VERO cell lines for compounds showing the best antiplasmodial activities. The evaluation of cytotoxicity on VERO cells (monkey epithelial cell line, Sigma-Aldrich) was assessed using [³H]-

hypoxanthine incorporation. In short, cells (5.10^4 cells/mL) in 100 μ L of complete medium were seeded into each well of 96-well plates and incubated at 37°C in a humidified 5% CO₂ with 95% air atmosphere. The complete medium consisting MEM with 10 % foetal calf serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin), NEAA 1X.

The plates were incubated with the test compounds for 48 h and [³H]-hypoxanthine (Perkin Elmer, France) added to the medium 24 h after the beginning of the incubation [193] to estimate VERO cell growth. The control VERO cell culture (drug-free but with 2% DMSO) was referred to as 100% of growth. The IC₅₀ values (50% inhibitory concentration) were graphically determined as concentrations *versus* percentage of cell growth.

3. K13 Gene and Artemisinin Resistance

a. Continuous culture of *P. falciparum*

The artemisinin resistance was assessed against various clinical isolates (Cam3.I, Cam3.II, Cam5, Cam2, and CamWT) and laboratory lines (F32 TEM, V1/S, FCB, and Dd2). All strains were cultured continuously according to Trager and Jensen method [192], in a 5% CO₂ atmosphere at 37°C, taking into account the modifications previously described [193]. The parasites were briefly maintained *in vitro* in human red blood cells and diluted in RPMI-1640 medium, supplemented with 25 mM HEPES, L-Glutamine and completed with 5% human serum (French Blood Bank, EFS).

b. Reagent preparations

1) Stock and test solution of Dihydroartemisinin

Dihydroartemisinin (DHA) stock solutions were prepared in DMSO to obtain 1mg/mL of stock solution. To have a 200 μ g/mL (700nM) of stock solution, the initial stock solution 1mg/mL was diluted 5-fold in DMSO. A total of 50 μ L aliquots of 700nM stock solution was stored in sterile 1.5 mL micro centrifuge tubes at -20°C. For DHA test solution, 20 μ L of DHA 700nM stock solution was added to 2 mL of culture medium.

2) Control solution of Dimethylsulfoxide (DMSO)

Dimethylsulfoxide (DMSO) control solution was prepared by adding 20 μ L of DMSO to 2 mL of culture medium.

3) Percoll® solutions

Percoll® solutions were prepared at room temperature, in a Class II biosafety cabinet, using sterile reagents. Nine volumes of Percoll® (Sigma-Aldrich) were mixed with one volume of 10x PBS to make a Percoll® 90% solution (stored at 4°C, up to 2 months). For RSA assay, 7.5 volumes of Percoll® 90% solution were mixed with 1.5 volumes of heparinized RPMI-1640 (freshly prepared 15 μ L heparin mixed with 10 mL RPMI-1640) to obtain a Percoll® 75 % solution prepared directly prior to the assay.

4) Sorbitol solution

A total of 50 grams of D-sorbitol (Sigma-Aldrich) was dissolved in 1L of distilled water resulting in a 5% D-Sorbitol solution. Using a 0.22 μ M filter, the homogenized solution was sterilized and stored at 4°C (up to 1 year).

c. *In vitro* RSA assay

These assays were carried out as previously described [197], with minor modifications. In summary, 10-15 mL parasite cultures were synchronized 1-2 times using 5% sorbitol (Sigma-Aldrich). Synchronous multinucleated schizonts were incubated in RPMI-1640 containing 15 units/mL sodium heparin for 15 min at 37°C to disrupt agglutinated erythrocytes, concentrated over a gradient of 75% Percoll® (Sigma-Aldrich), washed once in RPMI-1640, and incubated for 3 hours with fresh erythrocytes to allow time for merozoite invasion. Cultures were then subjected again to sorbitol treatment to eliminate remaining schizonts. The 0-3 hours of post-invasion rings were adjusted to 1% parasitemia and 2 % hematocrit in 1 mL volume (in 48-well plates) and exposed to 700nM DHA or 0.1% DMSO (solvent control) for 6 hours. Duplicate wells were established for each parasite line ± drug. As much as 1 mL of the cultures was then transferred to 15 mL conical tubes and centrifuged at 1600 rpm for 5 min to pellet the cells, and the supernatants were carefully removed. As a washing step to remove drug, 9 mL of culture medium was added to each tube, the cells were re-suspended and centrifuged, and the medium was aspirated. Fresh lacking drug medium was then added to the cultures, and this mixture was returned to standard culture conditions for 66 hours.

Parasite viability was assessed through microscopic examination of Giemsa-stained thin blood smears by counting the parasites developed into second-generation rings or trophozoites with normal morphology. To obtain a homogenous smear for all slides, the culture was re-suspended, transferred into an Eppendorf tube, and briefly centrifuged (2000 rpm for 30 seconds). Exactly 2 µL of the pellet was then used for each smear. Parasitemias were calculated from a

total of at least 40,000 erythrocytes per assay. Slides were read from the two duplicate wells per assay by two separate microscopists, each of them was examined for at least 10,000 erythrocytes per slide. In the case of more than 20% discrepancy in parasite counts, the slides were further examined by a third microscopist. The percentage of survival was calculated as the parasitemia in the drug-treated sample divided by the parasitemia in the untreated sample multiplied by 100.

$$\% \text{ survival} = \frac{\text{drug treated sample parasitemia (DHA wells)}}{\text{untreated sample parasitemia (DMSO wells)}} \times 100$$

The artemisinin resistance was described as $\geq 1.5\%$ of survival percentage.

4. Dihydroartemisinin-Piperaquine (DHP) Resistance in Kupang

a. Ethics approval submission

The study protocol and informed consent have been approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Gadjah Mada University, Yogyakarta (ref number KE/FK/222/EC). Therefore, the entire process of the study has been conducted under ethical considerations.

b. Study population

The population of this study covered several areas in Kupang East Nusa Tenggara which has a high case incidence (HCI) based on the Annual Parasite Incidence (API). The samples followed the inclusion criteria, such as patients diagnosed for non-complicated malaria caused by particularly *P. falciparum* (slide-confirmed malaria), those having fever or history of fever 48 h prior to presentation, patients seeking treatment in primary health center or district referral

hospital, and those agreeing to sign the informed consent and follow the study. The exclusion criteria were pregnant or lactating women, children under 5 kg, anyone already taking malaria treatment for 4 weeks beforehand, diagnosed for complicated malaria (co-infection), infected by other *Plasmodium* species than *P. falciparum*, having signs of severe malaria, severe malnutrition, serious underlying disease (renal, cardiac, or hepatic), or known to be allergic to the research drugs.

c. Drug administration and follow-up

The dihydroartemisinin-piperaquine (DHP) was administered 3 times daily for 3 days with a dosage of 2-4 mg/kg of body weight (BW) of dihydroartemisinin and 16-32 mg/kg BW of piperaquine. Subsequent follow up appointments were scheduled for days 2, 3, 7, 14, 21, 28, 35, and 42; in addition, the blood smear was examined during the follow-up periods and collected on filter paper for DNA extraction. During the follow-up visits, patients were asked about the general condition of well-being as well as open questions about possible adverse effects that might occur since the last visit and examined by the research physicians.

d. Parasite isolate preparation

Venous blood was collected aseptically into an ACD anticoagulant tube from the patients before a treatment administration (H_0). Using a drop of blood, parasitemia evaluation was performed by a Giemsa-stained thin blood smear. Following parasitemia determination, blood centrifugation (800g for 5 minutes) separated RBC pellet from the plasma and buffy coat. The RBC pellet was then

washed using RPMI (20 mL of RPMI, centrifugation at 800 g for 5 minutes and removal of supernatant, consecutively) for 6-8 times, preparing infected RBCs to be processed for *ex-vivo* RSA.

e. The *ex-vivo* Ring-stage Survival Assay (RSA)

The *ex-vivo* Ring-stage Survival Assay (*ex-vivo* RSA) was performed on the parasite isolates freshly collected from patients with uncomplicated *P. falciparum* malaria. The parasites were exposed to 700nM dihydroartemisinin (DHA) for 6 hours – approximating their drug exposure in patients treated with artemisinin – and their survival was assessed 72 hours later [198].

To perform the *ex-vivo* RSA, $\geq 0.5\%$ parasitemia was required. The parasite solution was prepared by adding 50 μ L of infected RBCs to 2.5 mL of culture medium. If the parasitemia was $> 1\%$, pellet was diluted with uninfected RBCs to obtain 0.5-1% parasitemia and with culture medium to obtain 2% hematocrit. A thin smear was made for determining initial parasitemia. The culture medium consisted of RPMI-1640 medium, HEPES, gentamicin, hypoxanthine, Albumax II, and heat-inactivated human serum.

In a 48-well culture plate, 900 μ L of parasite solution was transferred to each well and added with 100 μ L of DMSO solution in the control well or 100 μ L of DHA solution (700nM) in the treatment well. The 48-well culture plate was maintained in an incubator under 37⁰C, humid atmosphere, 5% O₂, 5% CO₂, and 90% N₂ for exactly 6 hours. After 6 hours, the contents of each well were transferred into 15mL centrifuge tubes for a following centrifugation at 800g for 2 minutes. Then, 12 mL of pre-warmed RPMI was added for washing the RBCs pellet prior to a transfer into new wells in the 48-well culture plate with 1 mL of

culture medium to resuspend the pellet. The new 48-well culture plate was maintained in an incubator (37°C, humid atmosphere, 5% O₂, 5% CO₂, and 90% N₂) for 66 hours. Finally, the Giemsa-stained thin smear of each well was made at the end of incubation and determined for the percentage of survival under a microscope examination.

At 100x magnification in immersion oil, the number of infected RBCs containing viable parasites were counted in a total of 10,000 RBCs. Only viable parasites were scored; so, vacuolated and pyknotic forms were not considered. To determine the proportion of viable parasite (% survival), the parasitemia of DHA exposed was compared to the parasitemia of non-exposed (DMSO).

The *ex-vivo* RSA would be performed also at any days of treatment failure emerging during the follow-up.

f. Sequencing the K13-propeller domain

The molecular marker the *PfK13*-propeller domain was amplified using the following primers: for the primary PCR (K13-15'-cggagtgaccaaactctggga-3' and K13-4 5'-gggaatctggtgtaacagc-3') and the nested PCR (K13-2 5'-gccaaagctgccattcatttg-3' and K13-3 5'-gccttgtgaaagaagcaga -3'), one µL of DNA was amplified with 1µM of each primer, 0.2 mM dNTP (Solis Biodyne), 3 mM MgCl₂ and 2U *Taq* DNA polymerase (Solis Biodyne), using the following cycling program: 5min at 94°C, then 40 cycles of 30 s at 94°C, 90 s at 60°C, 90 s at 72°C and final extension of 10 min at 72°C. For the nested PCR, 2 µL of primary PCR products was amplified under the same conditions, except for the MgCl₂ concentration (2.5 mM). The PCR products were detected using 2% agarose gel electrophoresis and ethidium bromide staining. Meanwhile, the double-strand

sequencing of PCR products was performed using Macrogen, and the sequences were then analysed using MEGA5 software version 5.10 to identify specific SNP combinations.

CHAPTER III

RESULTS

This chapter points out various strategies investigated for malaria elimination, particularly in Indonesia. The first part is dedicated to discover new antimalarial drugs either from Indonesian medicinal plants or with synthesis of organometallic compounds. Moreover, as artemisinin resistance has emerged in South-East Asia, it is important to confirm the major role of molecular markers involved in this resistance. Defined by reverse genetic of the mutation involvement of *Pfk13* gene in this resistance, the study of its spread in South East Asia regions is used to monitor the possible appearance of artemisinin resistance in malaria endemic areas in Indonesia.

A. Antiplasmodial Activity of Indonesian Medicinal Plants

1. Results obtained

The emerging resistance of artemisinin, the basis of recent first-line combination therapy, has threatened the global effort to reduce malaria caused by *P. falciparum*. Moreover, there are no current antimalarial drug to replace artemisinin, hence discovering new antimalarial drug is inevitable. One of the strategies to find new antimalarial agents is the *in vitro* evaluation of crude extracts activities of medicinal plants against *P. falciparum* strains. Indonesia's rich biodiversity features medicinal plants used traditionally for a variety of disease treatments, including malaria. In cooperation with CNRS - LCC in Toulouse, France, the Faculty of Medicine of Universitas Gadjah Mada conducted the assessment of antiplasmodial activities of five medicinal plants: *T. diversifolia*, *C. barbata*, *T. crispa*, *A. flava* and *P. cauliflora*. These plants were selected due to their popularity as a fever therapy among Indonesians and because they naturally grow in Indonesia. In addition, some of these plants possess not only medicinal

advantages but also economic benefits, such as *C. barbata* prepared for drinks and sold in sidewalks. In contrast, some of them offer limited information on their medicinal and economic benefits, such as *P. cauliflora*, making people ignore their cultivation and the plants become endangered.

This France-Indonesia collaboration aimed to conduct a standardized study targeting antiplasmodial activities of these extracts. In addition, since *Plasmodium* has similar biochemical pathways with *Babesia*, another apicomplex parasite, we intended to discover shared active compounds on these two close pathogens. Moreover, as malaria patients can also be co-infected by leishmaniasis in areas where the two parasites coexist, especially in tropical and subtropical countries such as in South America as one of the endemic regions [9], it was also interesting to assess the antileishmaniasis activities of the selected extracts. This study was completed by an assessment of the cytotoxicity on mammalian cells to determine the specificity of their activities.

Our team gathered the plants from different areas in Indonesia and identified them in the Biology Laboratory of the Pharmacy Department of Universitas Islam Indonesia in Yogyakarta. A maceration process with various solvent polarities was utilized to extract the active compounds of the plants. The chloroquine-resistant strain of *P. falciparum* FcM29-Cameroon was selected to evaluate the antiplasmodial activity of these various extracts according to Desjardins's method with slight modifications. The same assay method was used for anti-babesial activity, except that *B. divergens* was cultured following the method by Précigout *et al* [196]. The anti-leishmanial activity was examined by a luminescent assay based on luciferase activity on *L. infantum* blood stage preceding the macrophage invasion (promastigotes). For the extracts with the best antiplasmodial and anti-leishmanial activities, cytotoxicity tests were conducted against macrophages and VERO cell lines using MTT assay and [³H]-hypoxanthine incorporation, respectively.

The results figured out that, among the 25 crude extracts and fractions, seven indicated promising antiplasmodial activities ($IC_{50} < 5\mu\text{g/mL}$) [135]: *T. diversifolia* ethanol extract and ethyl acetate fraction, *A. flava* ethanol extract, N-hexane and ethyl acetate fractions, *P. cauliflora* radix part dichloromethane, and methanol extracts. Additionally, these best antiplasmodial extracts were then tested on the VERO cell line to investigate the specificity of their activity which was then translated into selectivity index (SI) as a ratio between cytotoxicity and activity (antiplasmodial activity, in this regard). However, only *A. flava* and *P. cauliflora* radix dichloromethane extracts featured high selectivity against VERO cell lines ($> 50\mu\text{g/mL}$).

Tithonia diversifolia or locally known as kembang bulan was active against *P. falciparum* (ethanol extract and ethyl acetate fraction), but their cytotoxic values were similar, with less than 1 selectivity index. This low selectivity index (SI) indicated that this plant offers limited interest for further investigation although several previous studies demonstrated its activity. The ether extract of aerial parts showed *in vitro* antiplasmodial activity (IC_{50} : 0.75 $\mu\text{g/mL}$ against CQ-sensitive strain FCA) which led to extraction of active compound, tagitinin C, a sesquiterpene lactone possessing 0.33 $\mu\text{g/mL}$ IC_{50} against FCA [140]. Not only *in vitro* activity, *T. diversifolia* was also reported on its *in vivo* antiplasmodial activity in a mouse malaria model at concentration of 200 mg/kg [141].

Meanwhile, with the IC_{50} around 3 $\mu\text{g/mL}$, *A. flava* (also known as kayu kuning) ethanol extract, n-hexane and ethyl acetate fractions displayed a good antiplasmodial activity. In addition, their cytotoxicity against VERO cell showed high selectivity level (SI: >50). Based on literature, the antiplasmodial activity of *A. flava* extract has been published with the range of IC_{50} from 0.4 to 8.6 $\mu\text{g/mL}$ against *P. falciparum* [166] and one of its active compounds, berberine, has been proved to inhibit telomerase activity of *P. falciparum* [167]. The result of

this study supports those previous findings with additional information about selectivity of the extracts.

Sengkubak or *P. cauliflora*, a medicinal plant that has not been widely explored, demonstrated the best IC₅₀ values on its dichloromethane and methanol extracts, especially from radix part (3.3 µg/mL for both solvents) even though the high selectivity index only showed for the radix dichloromethane extract (SI:>15). This plant was reported having antioxidant [171] and cytotoxic activity [170] with bisbenzylisoquinoline alkaloids (obaberine and pycnarrhine) as active compounds [169]. Yet, no study has revealed its antiplasmodial activity to date. This plant then appears particularly promising and deserves further study since its active compound, bisbenzylisoquinoline alkaloids, has been reported to have antiplasmodial activity [199].

Unexpectedly, even though it is commonly used for traditional malaria treatment, extract and fraction from *C. barbata* (stem and leaf) or *T. crispa* (stem) did not show antiplasmodial activity. After 3 independent experiments, its IC₅₀ values were constantly beyond 5 µg/mL, a threshold to classify good activity of the extract. Previous studies found that bisbenzylisoquinoline alkaloids (tetrandrine, limacine, thalrugosine, homoaromaline, and cyclepeltine extracted from the root of *C. barbata* have both cytotoxic and antimalarial activities [200]. Other report also stated that those bisbenzylisoquinoline alkaloids demonstrated high antiplasmodial activity [201, 202]. On the other hand, a study on *T. crispa* reported its antiplasmodial activity of the stem against *P. berghei* [203] and *in vitro* activity from methanol stem extract with the IC₅₀ value of 0.8 mg/mL [159]. That IC₅₀ value correspond to the result of this study, which indicate low interest in pharmacological activity.

Eight extracts also displayed anti-babesial activities with IC₅₀ values ranging from 1.2 to 14.1 µg/mL, with 3 of them close to the control babesial inhibitor, imidocarb (0.5µg/mL).

With a good selectivity index *A. flava* extract and fractions showed that an antiplasmodial activity can also be coupled with an anti-babesial activity.

Concerning the extract assays on *L. infantum*, only 3 had IC₅₀ values less than 50µg/mL: the ethyl acetate fraction of *T. diversifolia*, and, dichloromethane and methanol fractions of *P. cauliflora* radix (IC₅₀ 21.9, 1.7 and 4.4 µg/mL, respectively). The results showed that *P. cauliflora* radix methanol extract was active against *P. falciparum*, *Babesia* and *L. infantum* at similar concentrations and relatively low indexes of selectivity, suggesting a global cytotoxic effect in contrast to the dichloromethane fraction at which the cytotoxicity was low on VERO cells.

In conclusion, extracts or fraction with a good antiplasmodial activity can be positively correlated to a good and specific antibabesial activity. Among the most active extracts against *P. falciparum*, only dichloromethane and methanol extract of *P. cauliflora* were active against *L. infantum*. The specific anti-parasite activities of some extracts were confirmed by their weak cytotoxicity on VERO cell line. These particular extracts need an in-depth phytochemical assessment in order to identify and characterize the active compounds either to standardize plant preparations or to chemically develop new anti-parasitic compounds as detailed in the article below submitted for publication.

2. Corresponding publication



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Research paper

Antiprotozoal properties of Indonesian medicinal plant extracts

Arba P. Ramadani^{a,b,c,d}, Lucie Paloque^{a,b}, Hugo Belda^{a,b,e}, Hady Anshory Tamhid^d, Masriani^f, Jumina^g, Jean-Michel Augereau^{a,b}, Alexis Valentin^{b,e}, Mahardika Agus Wijayanti^h, Mustafa^{c,*}, Françoise Benoit-Vical^{a,b,**}^a CNRS, LCC (Laboratoire de Chimie de Coordination) UPR8241, F-31077 Toulouse Cedex 4, France^b Université de Toulouse, UPS, INPT, F-31077 Toulouse Cedex 4, France^c Department of Pharmacology and Therapy, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia^d Department of Pharmacy, Faculty of Science & Mathematics, Islamic University of Indonesia, Yogyakarta, Indonesia^e UMR 152 Pharma-DEV IRD-UPS, Toulouse, France^f Study Program Chemistry of Education, Department of Mathematics and Natural Science Education, Tanjungpura University, Pontianak, Indonesia^g Department of Chemistry, Faculty of Mathematics and Natural Science, Gadjah Mada University, Sekip Utara, Yogyakarta, Indonesia^h Department of Parasitology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

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ABSTRACT

Tithonia diversifolia, *Cyclea barbata*, *Tinospora crispa*, *Arcangelisia flava*, *Pycnarrhena cauliflora* are plants used in Indonesia for the traditional treatment of malaria. In the search for new antiparasitic drugs, the parts traditionally used of these 5 plants were extracted with ethanol and then fractionated with various solvents and evaluated *in vitro* against *Plasmodium falciparum* and also against *Babesia divergens* and *Leishmania infantum*. Seven crude plant extracts out of 25 tested displayed high antimalarial activities with $IC_{50} < 5 \mu\text{g/ml}$ and in the cases of some of them an interesting selectivity regarding their cytotoxicity against mammalian cells. *A. flava* appeared to be the most promising antiplasmodial plant with the highest antiplasmodial activity (IC_{50} values less than $3 \mu\text{g/ml}$) and the weakest cytotoxicity. By contrast, only *P. cauliflora* radix, through its dichloromethane and methanol fractions also demonstrated a high activity against *L. infantum*, with IC_{50} values around $3 \mu\text{g/ml}$; their high selectivity index, especially on VERO cells, hypothesises a specific parasiticidal action. Moreover, for all the extracts showing antiplasmodial activity, a positive correlation was demonstrated with antibabesial activity, suggesting that these antiplasmodial extracts could be a potential source of antibabesial compounds. These preliminary results confirm the antiplasmodial interest of some of these plants used in traditional medicine but also their effects on leishmaniasis and babesiosis. Ongoing phytochemical investigations should allow identification of the chemical series responsible for these activities.

1. Introduction

Malaria is the most prevalent parasitic disease in the world and continues to be one of the largest public health problems, especially in the developing countries. There is an estimated 3.2 billion people at risk of being infected and leading to an estimated 584,000 malaria deaths 78 % of which concern children under 5 years (WHO, 2014c). *Plasmodium falciparum*, the most deadly species of malaria parasite already shows resistance to all antimalarial drugs, including artemisinin and its derivatives, which are the latest and the most effective treatments of malaria (Straimer et al., 2015; WHO, 2014c; Witkowski et al., 2009; Wongsrichanalai et al., 2002). Unfortunately the use of artemisinin-based combination therapies (ACTs) advocated by the World Health

Organization (WHO) did not allow for the emergence and the spread of multi-resistance in the Greater Mekong region (Cambodia, Vietnam, Thailand, Myanmar and Laos) threatening the strategies of malaria eradication in the other endemic areas (WHO, 2014b). Consequently there is an urgent need to discover new antimalarial agents, and crude extracts of natural products appear to be a promising route to follow as they are more affordable for people in developing countries (Benoit-Vical, 2005; Benoit-Vical et al., 2008, 2003).

Indonesia is rich in medicinal plants which the population use traditionally for curing diseases including malaria. This study, based on ethnobotanical data, was carried out on 5 medicinal plants selected as potential sources of antimalarial agents: *Tithonia diversifolia*, *Cyclea barbata*, *Tinospora crispa*, *Arcangelisia flava*, *Pycnarrhena cauliflora*.

* Corresponding author at: Department of Pharmacology and Therapy, Faculty of Medicine, Universitas Gadjah Mada, 55281 Yogyakarta, Indonesia.

** Corresponding author at: CNRS, LCC (Laboratoire de Chimie de Coordination) UPR8241, 205 route de Narbonne, BP 44099, F-31077 Toulouse Cedex 4, France.

E-mail addresses: mustofajogja@yahoo.com (Mustofa), Francoise.Vical@inserm.fr (F. Benoit-Vical).<http://dx.doi.org/10.1016/j.hermed.2017.06.004>Received 23 May 2016; Received in revised form 13 June 2017; Accepted 28 June 2017
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Except *T. diversifolia*, the 4 other plants are from the Menispermaceae family studied largely for their use in traditional medicine and principally for malaria treatment (Verpoorte et al., 1982b).

Some of these plants have already been investigated for their anti-plasmodial activities and pharmacological properties but no standardised studies have yet been performed with them. The infusion of *T. diversifolia* leaves is traditionally used for lowering blood glucose (Miura et al., 2005), flatulence, malaria fever (Njoroge and Bussmann, 2006), and also the healing of wounds. An ethanol extract of *T. diversifolia* exhibited anti-malarial activity by its leaf extracts (Oyewole et al., 2008). *C. barbata* leaves have been used by the Javanese for gastric problems and as a prophylactic against malaria fever (Manilal and Sabu, 1985; Saxena et al., 2003). *T. crispa* is traditionally used for the treatment of fever, rheumatic arthritis, hepatitis, anti-hyperglycaemia, and also malaria by using stem infusions (Pathak et al., 1995; Pushpangadan and Atal, 1984). *T. crispa*, which is abundant in the Philippines, is used freely by the natives under the name of *makabuhay* (meaning “You may live”), as a panacea, especially valuable in malarial fevers (The Southwest School of Botanical Medicine, 1918). Supporting the traditional uses, *T. crispa* scientifically proved as anti-hyperglycaemia (Noor and Ashcroft, 1998) and also both *in vitro* and *in vivo* anti-malarial activity (Najib Nik et al., 1999; Rungruang and Boonmars, 2009). *A. flava* is traditionally used as a stem decoction for typhoid fever (Mandia, 1999), hepatitis, gastric disturbance and malaria (Subeki et al., 2005). The anti-hepatotoxicity (Wongbutdee et al., 2003) and anti-malarial activity have been profiled by several studies (Lovin et al., 2012; Nguyen-Pouplin et al., 2007; Vennerstrom and Klayman, 1988; Verpoorte et al., 1982a). *P. cauliflora* is a Dayak tribe folk medicine used for flavouring, flatulence (by placing the soaked leaves on the stomach), fever and malaria (by drinking the leaf infusion). Only local accounts published in Indonesian can be cited for these uses. One study of *P. cauliflora* has just revealed its cytotoxic activities related to apoptosis and cell cycle arrest (Masriani and Adnyana, 2011), but its anti-parasitic properties have not yet been established. Indeed, despite widespread traditional use of *P. cauliflora* reported on malaria treatment in Indonesia, to the best of our knowledge, no studies have been conducted on this plant. Other *Pycnarrhena* species have been studied for their biologically active principles (Siwon et al., 1981b; Verpoorte et al., 1978).

Furthermore, because *Plasmodium* and *Babesia* belong to the Apicomplexa phylum they share many common biochemical pathways. We can therefore hypothesise the possibility of finding common active compounds for both pathogens. *Babesia divergens* babesiosis is a widespread illness transmitted by livestock ticks. This pathogen is the main agent of bovine babesiosis in Europe but can also affect splenectomized humans (Ceci et al., 2014; L’Hostis et al., 1995; Zintl et al., 2014). Infections can occur without producing symptoms, but babesiosis may also be severe and sometimes fatal due to the intraerythrocytic development of the parasite (Melhorn and Schein, 1984). There are effective babesiacides, but imidocarb dipropionate is practically the only drug available on the market and therefore the most widely used. More specific new fast-acting treatments for babesiosis should now be developed (Vial and Gorenflot, 2006).

Leishmaniasis, identified as a “Neglected Tropical Disease” by WHO, is another parasitic disease. The pathogen is endemic in 98 countries and territories, with the number of new cases estimated at 1.3 million per year (WHO, 2014a). Nowadays, malaria and leishmaniasis are responsible for over a million deaths a year and threaten more than 350 million people worldwide, mostly, but not only in tropical and subtropical countries.

Here we offer a standardised study that focuses on the search for new antiparasitic drugs targeting *Plasmodium*, *Leishmania* and *Babesia*. Indeed, even though the five selected plants are traditionally used for the treatment of malaria in Indonesia, the antiplasmodial activities of their different extracts were not totally investigated. The extracts showing the best antiplasmodial activity were also tested on *Babesia*

divergens cultures to investigate another Apicomplexa parasite. Moreover, in some tropical zones, malaria and leishmaniasis infections are largely co-endemic, the extracts were thus tested on both *P. falciparum* and *L. infantum* to evaluate their anti-parasite activities as potential sources of new active compounds. The cytotoxicity of these extracts was assessed in parallel in order to evaluate the specificity of their anti-parasitic activities.

2. Materials and methods

2.1. Plant material

The plants were identified in the biology laboratory of the Department of Pharmacy, Islamic University of Indonesia, Yogyakarta-Indonesia (Herbarium of Laboratory of Biology, Department of Pharmacy, Faculty of Science and Mathematics, UII, Yogyakarta, Indonesia). Samples were air-dried and powdered.

Tithonia diversifolia (Hemsl.) A. Gray. (Asteraceae; voucher specimen 007-02/L-PB/UII/2013) was collected in the Sleman district of Yogyakarta. *T. diversifolia* is a shrub, locally called “kembang bulan”.

Cyclea barbata Miers (Menispermaceae; voucher specimen 008-03/L.S-PB/UII/2013) was collected in the Bantul district of Yogyakarta and locally known as “Cincau rambat” (Javanese). *C. barbata* can be easily distinguished by its deltoidovate, hispid leaves with acuminate apex, finely mucronate acumen and long male and female inflorescences with dense capitate flowers.

Tinospora crispa (L.) (Menispermaceae; voucher specimen 009-03/S-PB/UII/2013) known by the vernacular name of “brotowali”, was collected from Yogyakarta. *T. crispa* is a woody tropical liana with shiny green leaves.

Arcangelisia flava (L.) Merr. (Menispermaceae; voucher specimen 010-04/S-PB/UII/2013), collected in South Borneo, is a large, woody, glabrous and dioecious liana, up to 20 m long; with a stem of up to 5 cm in diameter and with yellow wood exuding a yellow sap when cut.

Pycnarrhena cauliflora (Miers.) Diels (Menispermaceae; voucher specimen 011-01/S.R-PB/UII/2013) was collected in Sintang district, West Borneo. This plant can be found in primary or secondary forests and tend to grow in clusters. From 8 species in the genus *Pycnarrhena*, only 4 (*P. australiana*, *P. ozantha*, *P. manilensis*, *P. longifolia*) have been investigated in any detail.

2.2. Preparation of plant extract

For *T. diversifolia* (leaves), *C. barbata* (stem and leaves), *T. crispa* (stem), and *A. flava* (stem), powdered samples were extracted by maceration in 70% ethanol for 24 h, 300 g of plant powder with 1.5 L of solvent (Fig. 1). This process was repeated for two days consecutively using fresh 70% ethanol. Each ethanol extract was filtered and filtrates were evaporated to dryness using a rotary vacuum evaporator. Dried ethanol extracts were solubilized in ethanol to be fractionated by *n*-hexane/by liquid–liquid separation method which created two separate layers (*n*-hexane and first ethanol layer). The *n*-hexane layer gave the *n*-hexane fraction after evaporation. The fractionation process was continued by fractionation of the first ethanol layer using ethyl acetate (liquid–liquid separation) which produced ethyl acetate and second ethanol layers giving respectively after evaporation, the ethyl acetate fraction and ethanol fraction residue.

For *P. cauliflora* powdered radix part (Fig. 2) was directly treated by *n*-hexane to extract lipophilic compounds. After filtration the *n*-hexane extract was evaporated to dryness. The precipitate resulting of *n*-hexane extraction was further put back in suspension/solubilized using dichloromethane to obtain dichloromethane extract after filtration. The new precipitate was submitted to a methanol extraction followed by a filtration and an evaporation of the filtrate.

This extraction method was employed to investigate the possible active compounds responsible for plant activities. Particularly for *P.*

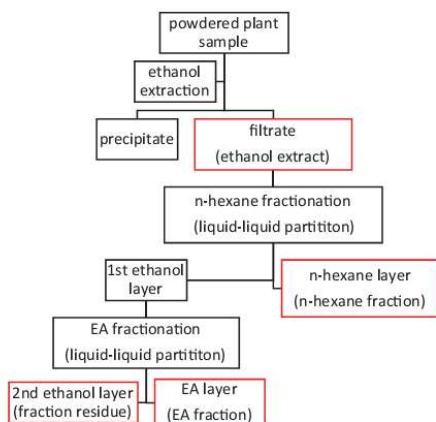


Fig. 1. Representative scheme of *T. diversifolia*, *C. barbata*, *T. crispa*, and *A. flava* extraction.

EA: ethyl acetate

Red boxes correspond to extracts used for antiparasitic activity investigations

cauliflora, dichloromethane was used to extract active compounds with semi-polar characteristics in order to explore new compounds other than those already published. For each part of leaves of *T. diversifolia*, stem and leaves of *C. barbata*, and stem of *T. crispa* 3 different extracts were obtained: ethanol, *n*-hexane and ethyl acetate fractions, and 1 fraction residue. For stem of *A. flava* only the ethanol, *n*-hexane and ethyl acetate fractions were obtained. For each part of stem and radix of *P. cauliflora* it was produced *n*-hexane, dichloromethane and methanol extracts. Finally, it yielded 25 extracts ready for antiparasitic activity investigations.

To evaluate the anti-parasite (against *Plasmodium*, *Babesia* and *Leishmania*) activities, stock solutions of the 25 crude and semi-purified extracts (1 mg/ml) were prepared in dimethylsulfoxide (DMSO, Sigma, France) and diluted for use in RPMI 1640 (Biowest, France). The dilutions were checked in order to confirm that the extracts did not precipitate under these conditions. The extracts were tested immediately after their dissolution in DMSO and RPMI, then kept at 4 °C for 3–8 days, and tested again to validate the conservation of mother solutions. The maximum percentage of residual DMSO in the assay was 2% and was evaluated alone on parasite cultures to check its absence of activity.

2.3. *In vitro* culture of *Plasmodium falciparum* and antiparasitic activity

The antiparasitic activity was assessed on the FcM29-Cameroon chloroquine-resistant strain of *Plasmodium falciparum*, cultured continuously according to the Trager and Jensen method (Trager and Jensen, 1976), in a 5% CO₂ atmosphere at 37 °C, taking into account the modifications previously described (Benoit-Vical et al., 2007). Briefly, the parasites were maintained *in vitro* in human red blood cells and diluted in RPMI 1640 medium, supplemented with 25 mM HEPES, L-glutamine (Biowest, France) and completed with 5 % human serum (French Blood Bank, EFS; Toulouse, France). The antiparasitic activity was assessed as previously reported by Desjardins et al. (Desjardins et al., 1979) and modified as follows. Extract dilutions and reference compounds were tested in triplicate at least 3 times independently, in 96-well plates with cultures at a parasitaemia of 1 % and a haematocrit of 1 %. For each test, the plates of parasite culture were incubated with extracts or reference compounds for 48 h and [³H]-hypoxanthine (Perkin Elmer, France) was added to the medium 24 h after the beginning of incubation (Benoit-Vical et al., 2007). Parasite growth was estimated by [³H]-hypoxanthine incorporation. The control parasite culture (without drug and with 2% DMSO) was referred to as 100 % growth. The IC₅₀ values (50 % inhibitory concentration) were graphically determined as concentrations versus percentage of parasite growth.

2.4. *In vitro* anti-*Babesia divergens* activity

Babesia divergens (Rouen 1986 strain) was cultured continuously according to Précigout et al. (Précigout et al., 1991). The parasites were maintained *in vitro* in human red blood cells (O⁺; EFS; Toulouse, France), diluted to 6 % haematocrit in RPMI 1640 medium (Biowest, France) supplemented with 30 nM NaHCO₃ and complemented with 10 % human AB⁺ serum (EFS).

The antibabesial activity of extracts was evaluated by the same radioactive micromethod as for *Plasmodium* in 96-well culture plates except for the incubation time, which was reduced to 24 h. The drug-free control parasite cultures were considered as 100 % growth, and an imidocarb sensitivity control was included in every plate. IC₅₀ were determined graphically from concentration versus percent inhibition curves.

2.5. Antileishmanial activity on promastigote stages

The effect of the compounds tested on the growth of *Leishmania infantum* (line MHOM/MA/67/ITMAP-263) promastigotes was assessed by luciferase assay. Briefly, promastigotes in log-phase in RPMI 1640

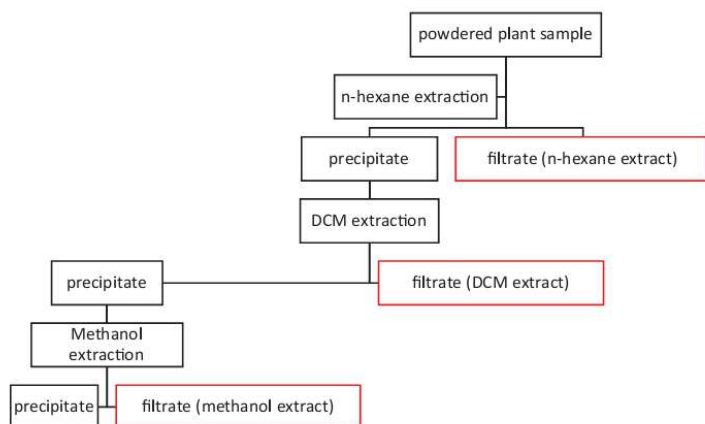


Fig. 2. Representative scheme of *P. cauliflora* extraction.

DCM: dichloromethane

Red boxes correspond to extracts used for antiparasitic activity investigations

medium (Biowest, France) supplemented with 10 % fetal calf serum (Hyclone, ThermoFischer, France), 2 mM L-glutamine and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL geneticin (Sigma, France)) were incubated at an average density of 10^6 parasites/mL in sterile 96-well plates with various concentrations of compounds dissolved in DMSO or MeOH (final concentration less than 0.5 % v/v), in duplicate. Appropriate controls treated by DMSO, MeOH and amphotericin B (reference drug purchased from Sigma Aldrich) were added to each set of experiments. After a 72 h incubation period at 24 °C, each plate well was then microscope-examined for any possible precipitate formation. To estimate the luciferase activity of promastigotes, 80 µl of each well were transferred to white 96-well plates, Steady Glow reagent (Promega) was added according to the manufacturer's instructions, and the plates were incubated for 2 min. Luminescence was measured in a Microbeta Luminescence Counter (PerkinElmer). Inhibitory concentration 50 % (IC₅₀) was defined as the concentration of drug required to inhibit 50 % of the metabolic activity of *Leishmania infantum* promastigotes compared to the control. IC₅₀ were calculated by non-linear regression analysis processed on dose-response curves, using TableCurve 2D V5[®] software. IC₅₀ values are the geometric mean values calculated from three independent experiments.

2.6. Cytotoxic evaluation on macrophages and VERO cell lines

The cytotoxicity tests were performed on macrophages and VERO cell lines for compounds showing the best antileishmanial and antiplasmodial activities, respectively. The evaluation of cytotoxicity by MTT assay on the J774A.1 cell line (mouse macrophage cell line, Sigma-Aldrich) was evaluated according to Mosmann (Mosmann, 1983) with slight modifications, and cytotoxicity on VERO cells (monkey epithelial cell line, Sigma-Aldrich) was assessed using [³H]-hypoxanthine incorporation. Briefly, cells ($5 \cdot 10^4$ cells/mL) in 100 µL of complete medium were seeded into each well of 96-well plates and incubated at 37 °C in a humidified 5 % CO₂ with 95 % air atmosphere. The complete medium consisted of RPMI 1640 supplemented with 10 % foetal calf serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) for J774A.1 cell line and MEM with 10 % foetal calf serum, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), NEAA 1X for VERO cell line. After 24 h incubation, 100 µL of medium with various product concentrations and appropriate controls (DMSO and doxorubicin) were added and the plates incubated for 72 h at 37 °C, 5 % CO₂. For cytotoxic evaluation on macrophages, each plate well was then microscope-examined to detect any precipitate formation before the medium was aspirated from the wells. One hundred µL of MTT solution (0.5 mg/mL in RPMI) was then added to each well and the cells incubated for 2 h at 37 °C. Then the MTT solution was removed and DMSO (100 µL) was added to dissolve the resulting formazan crystals. The plates were shaken vigorously for 5 min and absorbance was measured at 570 nm with a microplate spectrophotometer (EON).

For cytotoxic evaluation on VERO cells, the plates were incubated with the test compounds for 48 h and [³H]-hypoxanthine (Perkin Elmer, France) was added to the medium 24 h after the beginning of the incubation (Benoit-Vical et al., 2007) to estimate VERO cell growth. The control VERO cell culture (drug-free but with 2 % DMSO) was referred to as 100 % of growth. The IC₅₀ values (50 % inhibitory concentration) were graphically determined as concentrations versus percentage of parasite growth.

3. Results and discussion

Antiplasmodial activities of *T. diversifolia*, *C. barbata*, *T. crispa*, *A. flava* and *P. cauliflora* extracts collected from Indonesia are reported in Table 1. Seven out of the 25 crude extracts from these 5 plants showed very interesting activities against *P. falciparum* with IC₅₀ values less

than the threshold of 5 µg/ml (Willcox et al., 2011). These best antiplasmodial extracts were also tested on the VERO cell line in order to evaluate the specificity of their activity. *T. diversifolia* ethanol and ethyl acetate extracts were active against *P. falciparum* but their cytotoxic values were equivalent (selectivity index less than 1). The ether extract of aerial parts of this plant had already demonstrated *in vitro* antiplasmodial activity (IC₅₀: 0.75 µg/ml on the chloroquine-sensitive strain FCA). Those findings led to the extraction and identification of the active compound: taginin C, a sesquiterpene lactone with an IC₅₀ of 0.33 µg/ml against FCA strain (Goffin et al., 2002). The ethanol extract of *T. diversifolia* was active at 200 mg/kg *in vivo* in a mouse malaria model (Elufioye and Agbedahunsi, 2004). However, because of the low selectivity index of *T. diversifolia*, this plant presents a limited interest for further pharmacological investigations. *A. flava* ethanol, N-hexane and ethyl acetate (Fig. 3) extracts demonstrated a good antiplasmodial activity (IC₅₀ around 3 µg/ml) and interestingly a high selectivity regarding their activities against the VERO cell line (> 50 µg/ml). *A. flava* possesses alkaloid compounds having antioxidant and cytotoxic activities (Keawpradub et al., 2015). Other findings revealed that *A. flava* extracts have IC₅₀ ranging from 0.4 to 8.6 µg/mL against *P. falciparum* (Nguyen-Pouplin et al., 2007) and one of its active compounds, berberine, inhibits telomerase activity in *P. falciparum* (Sriwilaijareon et al., 2002). *P. cauliflora* showed the best antiplasmodial IC₅₀ values with dichloromethane and methanol extractions particularly of the radix part (3.3 µg/ml with both solvents) and the weakest cytotoxicity for the radix dichloromethane extract (> 50 µg/ml). Bisbenzylisoquinoline alkaloids of *P. cauliflora*, i.e. obaberine and pyncarrhine (Siwon et al., 1981a) have been reported to be responsible for various activities involving antioxidant and cytotoxic properties (Masriani and Adnyana, 2011) but no studies to date have revealed its antiplasmodial activity. This novel plant thus appears to be particularly promising and deserves further study.

Surprisingly and despite their traditional use for malaria treatment, no extracts from *C. barbata* (stem and leaf) or *T. crispa* (stem) showed antiplasmodial activity. Previous studies have reported alkaloid extraction from the root of *C. barbata* with both cytotoxic and antimalarial activities. Five bisbenzylisoquinoline alkaloids were isolated as active principals: tetrandrine, limacine, thalrugosine, homoaromaline, cyclealpetine (Lin et al., 1993). Some of these bisbenzylisoquinoline alkaloids were reported elsewhere for their high antiplasmodial activity (Fang and Jiang, 1986; Marshall et al., 1994). However, *T. crispa* extracts showed *in vivo* antiplasmodial activity of stems against *P. berghei* (Marthianti, 2006) and also *in vitro* but the IC₅₀ value of 0.8 mg/mL reported for methanol stem extract (Najib Nik et al., 1999) is far too high to have a pharmacological interest and is thus in accordance with our results.

Because *Babesia* is another protozoan apicomplexan parasite that infects erythrocytes and also causes haemolytic disease, it was interesting also to determine the activity of these plant extracts against *Babesia in vitro*. The 8 extracts showing the best antiplasmodial activity were tested on *Babesia divergens* culture (Table 2). With the exception of *T. diversifolia* ethanol extract, for which the standard deviation of the results was too large to conclude, all the extracts tested showed IC₅₀ values ranging from 1.2 to 14.1 µg/ml. These data are particularly interesting because they concern crude extracts and are not so far from the IC₅₀ of the control drug imidocarb (0.5 µg/ml) and better than those already reported with other Indonesian medicinal plant extracts (Murnigsih et al., 2005; Murnigsih et al., 2005; Subeki et al., 2007). Our results suggested a strong correlation between antiplasmodial and antibabesial activities and so the potential use of antibabesial compounds from the antiplasmodial drugs portfolio. Moreover, because *Babesia* is an erythrocyte parasite sharing numerous biochemical pathways with *Plasmodium* with the notable exception of the haemoglobin degradation pathways, we could hypothesise that these extracts act on parasitic pathways that do not involve hemozoin polymerisation.

The same 25 extracts tested against *P. falciparum* were also

Table 1
Antiplasmodial activity against *P. falciparum*, chloroquine-resistant strain (FCM29-Cameroon strain), and cytotoxicity on VERO cell line

Extract	IC ₅₀ , µg/mL; Mean ^a ± SD						
	Antiplasmodial activity/Cytotoxicity						
	(Selectivity Index) ^b						
	<i>T. diversifolia</i> (leaf)	<i>C. barbata</i> (stem)	<i>C. barbata</i> (leaf)	<i>T. crista</i> (stem)	<i>A. flava</i> (stem)	<i>P. cauliflora</i> (stem)	<i>P. cauliflora</i> (radix)
EtOH	3.9 ± 2/3.5 (0.9)	> 50/ND ^c	> 50/ND	50/ND	2.8 ± 0.9/ > 50 (> 18)	- ^d	-
n-hexane	16.7 ± 6/ND	13.7 ± 7/ND	41 ± 8/ND	18.7 ± 5/ND	2.8 ± 0.8/ > 50 (> 18)	> 50/ND	> 50/ND
Ethyl acetate	3.3 ± 0.5/3.1 ± 1.7 (0.9)	27 ± 5/ND	33 ± 13/ND	> 50/ND	3 ± 0.7/ > 50 (> 18)	-	-
Residue	> 50/ND	> 50/ND	> 50/ND	> 50/ND	-	-	-
Dichloromethane	-	-	-	-	-	10.5 ± 8/17.5 ± 2 (1.7)	3.3 ± 0.1/ > 50 (> 15)
Methanol	-	-	-	-	-	> 50/ND	3.3 ± 0.3/6.5 (2)
Chloroquine (drug control)	0.28 ± 0.03 ^d /69.3 ± 21 (245)						

^a Mean of at least 3 independent experiments.

^b Selectivity Index: Cytotoxicity/activity ratio.

^c Not done.

^d Corresponding to the IC₅₀ value of 0.51 ± 0.04 µM.

^e No corresponding extract.

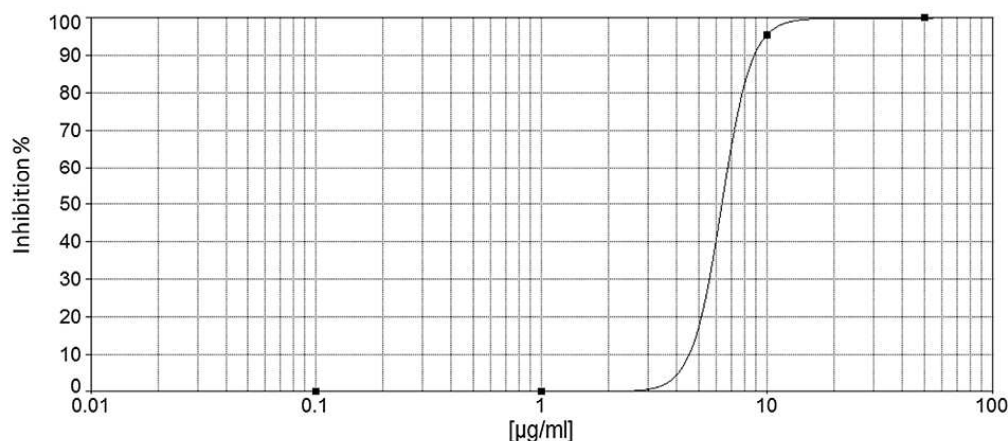


Fig. 3. Representative curve of percentage inhibition of *Plasmodium falciparum* growth *in vitro* by *A. flava* ethyl acetate extract.

Table 2
Anti-*Babesia* activity against *B. divergens*.

Extract	IC ₅₀ , µg/mL; Mean ^a ± SD
<i>T. diversifolia</i> ethanol extract	36.2 ± 23
<i>T. diversifolia</i> ethyl acetate extract	1.7 ± 0.7
<i>A. flava</i> ethanol extract	6.4 ± 2.8
<i>A. flava</i> n-hexane extract	4.8 ± 2.9
<i>A. flava</i> ethyl acetate extract	6.3 ± 3.2
<i>P. cauliflora</i> dichloromethane extract (stem)	1.5 ± 1
<i>P. cauliflora</i> dichloromethane extract (radix part)	14.1 ± 9
<i>P. cauliflora</i> methanol extract (radix part)	1.2 ± 0.7
Imidocarb (drug control)	0.5 ± 0.5 ^b

^a Mean of at least 3 independent experiments.

^b Corresponding to the IC₅₀ value of 1.4 µM.

evaluated against *L. infantum* and in parallel on its macrophage host cell (Table 3). Only 3 extracts out of 25 showed IC₅₀ values of less than 50 µg/ml. Dichloromethane (Fig. 4) and methanol fractions of *P. cauliflora* radix showed a good activity on *L. infantum* with IC₅₀ values of 1.7 and 4.4 µg/ml, respectively. However the selectivity indexes

Table 3
Antileishmanial activity against *L. infantum* promastigote and cytotoxicity on murine J774A.1 macrophage line.

Extract ^b	IC ₅₀ , µg/mL; Mean ^a ± SD Antileishmanial activity/Cytotoxicity (Selectivity Index)
<i>T. diversifolia</i> ethyl acetate extract	21.9 ± 1.2/3.3 ± 1.4 (0.03)
<i>P. cauliflora</i> dichloromethane extract (radix part)	1.7 ± 0.5/5.8 ± 0.9 (3.4)
<i>P. cauliflora</i> methanol extract (radix part)	4.4 ± 0.9/12.4 ± 1.5 (2.8)
Amphotericin B (drug control)	0.06 ± 0.01/3.3 ± 1.4 (55)

^a Mean of at least 3 independent experiments.

^b All extracts tested against *P. falciparum* (see Table 1) were also evaluated against *L. infantum* promastigote. With the exception of those reported here in Table 3, all the other extracts showed IC₅₀ greater than 50 µg/ml against *Leishmania* (data not shown) and were not tested for their cytotoxicity.

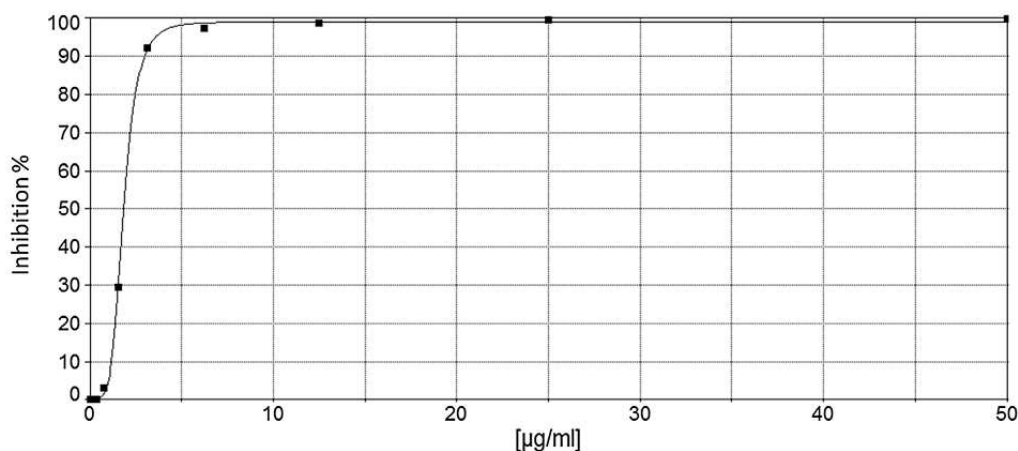


Fig. 4. Representative curve of percentage inhibition of *Leishmania infantum* promastigote growth *in vitro* by *P. cauliflora* dichloromethane extract (radix part).

obtained on macrophages are relatively low (around 3). The analysis of these data shows that both best extracts (*P. cauliflora*) on *Leishmania* were also active, at similar concentrations, on *Plasmodium*. It is particularly interesting since the anti-parasite activity of this plant (whether on *Plasmodium*, *Babesia*, or *Leishmania*) has thus far never been reported. Moreover Malaria and Leishmaniasis are co-endemic in Indonesia and the same plant extracts locally present could fight both pathogens.

4. Conclusions

Based on an ethnopharmacology approach, 7 plant extracts out of 25 tested displayed good antimalarial activities with $IC_{50} < 5 \mu\text{g/ml}$. Activities observed with ethanolic crude extracts, the extraction solvent studied here that was closest to the aqueous traditional extraction, could justify the traditional use of both plants *T. diversifolia* and *A. flava* for malaria treatment. However regarding their cytotoxicity against mammalian cells, *A. flava* appeared to be the most promising antiplasmodial plant of this study with the highest antiplasmodial activity (IC_{50} values less than $3 \mu\text{g/ml}$) and the lowest cytotoxicity. Moreover, for all extracts showing antiplasmodial activity a positive correlation was found with antibabesial activity, suggesting that antiplasmodial extracts can be a potential source of antibabesial compounds. In contrast, *A. flava* had no activity on *L. infantum*. Furthermore, only *P. cauliflora* radix, through its dichloromethane and methanol fractions also demonstrated a good activity against *L. infantum*, with IC_{50} values equivalent for the 3 pathogens; their selectivity index, especially on VERO cells, suggests a specific parasitocidal action more than a global cytotoxic effect.

These preliminary results are sufficiently interesting to pursue phytochemical investigations in order to identify the chemical series responsible for these activities and obtain standardized preparations. This is an absolute necessity before considering any therapeutic applications to malaria, leishmaniasis and/or babesiosis for either herbal preparations or pure molecules.

Conflict of interest

The authors declare no conflict of interest.

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B. Antiplasmodial Activity of Gold(I) Complexes

1. Results obtained

Synthetic molecules are part of another approach to discover new antimalarial drugs. Organometallic compounds containing metal complex that acts as pro-drugs and can have an enhanced efficacy (such as ferrocene with CQ leading not only to ferroquine but also gold and ruthenium complexes). NHCs, as the most important ligands in organometallic chemistry, are easy to prepare and able to stabilize the complexes. Biomedical applications of gold complexes based on NHCs are beginning to unfold. In particular, these ligands form strong Au-carbene bonds, giving stable Au^I-NHCs complexes that are insensitive to biologically important thiol groups. Au^I-NHC have shown potential medical applications [183], especially as anticancer [182, 184-187] and antimicrobial agents [189]. It has to be mentioned that gold-based compounds, including some gold(I)-NHCs, show anti-mitochondrial activity, a promising mode of action to fight cancer. Their antitumor activity may stem from the lipophilic and cationic properties, allowing their accumulation in mitochondria of tumor cells with great specificity [183]. It has also been demonstrated that the combination of metal complexes with NHCs can exhibit interesting biological activities against such parasitic infections as malaria and leishmaniasis [190, 204-206].

Our previous study proved that mononuclear cationic gold(I) complexes containing aromatic amino-functionalized NHC have *in vitro* antiplasmodial activity against FCM29-Cameroon [190]. This finding is exploited in this present study by further studies and fine tuning of the main structure of these organometallic molecules in relation to their antiplasmodial activity or against other parasites as well as studying the activity of new complexes.

To achieve the objective of investigating the influence of the different structure modulations on biological activities, synthesized proligand imidazolium salts (compounds 1-

11) and mononuclear gold(I) complexes (compounds **12-25**) were evaluated for its activity against CQ-resistant *P. falciparum* strains (FcM29-Cameroon and FcB1-Colombia) and put in comparison with CQ and artemisinin. These synthesized compounds (Fig. 12-14) were a series of N-, O- or S-functionalized proligands and their corresponding mononuclear cationic, anionic or neutral gold(I) bisNHC complexes. The most active compound would be investigated on its cytotoxic activity on Vero or McF7 cell lines.

Compound **1** (2-chloroquinoline) is an imidazolium precursor that is functionalized by nitrogen containing heterocycles quinoline. An aryl directly attached to its azolium ring generates compound **2** and **3**. As another imidazolium precursor, compound **4** is containing bipyridine. Meanwhile, compound **5 – 8** are the amine functionalized imidazolium salt combine with alkyl- (metyl for compound **5** and n-butyl for **6**) and arylimidazoles (benzyl for compound **7** and mesityl for **8**).

Using those imidazolium precursors, gold(I) complexes are developed. Compound **1** generates compound **12, 2 to 14, 3 to 15, 4 to 16, 5 to 18, 6 to 19, 7 to 20, and 8 to 21**. Meanwhile compound **13** and **17** are neutral form of the complexes. In addition, imidazolium salt compound **9, 10, 11** are precursor for compound **22 and 23; 24; and 25** respectively.

Assessed against *P. falciparum* strains FcM29-Cameroon and FcB1-Columbia, different compounds (**4, 5, 6, 18, 19**) indicated various results allowing relationship structure activity studies. As cationic molecule, the imidazolium salts (proligand **1** and **4-11**) were inactive while proligands **2** and **3** showed IC₅₀ value of 1.4 and 1.5 μ M respectively. Tested gold(I) complex compounds (**12, 15-16 and 19-22**) expressed improved activities compared to imidazolium salt demonstrating that gold cation plays an important role in antiplasmodial activity. A prior study suggested that one of the possible mechanisms of action of gold compounds is based on the reversible inhibition of the parasite enzyme falcipain 2 (Fp2)

involved in hemoglobin digestion. As a cysteine protease of *P. falciparum*, Fp2 will provide a significant quantity of peptides and amino acids from human hemoglobin degradation for parasite growth. Moreover, it is also characterized as a validated target for antimalarial drug discovery [191].

It has been demonstrated that cationic compounds can enhance antiplasmodial activities by positively facilitating the accumulation of molecules inside the parasite, the global charge thus become the key factor. It has been shown that the cationic Au(I) bis (NHC) species had the best results against *P. falciparum* with sub-micro molar IC_{50} s for some of them were 2.1 μ M (**19**), 0.48 μ M (**20**), 0.21 μ M (**21**), and 0.32 μ M (**22**). On the contrary, moderate activities were obtained with neutral complexes (compounds **13**, **17** and **23** with IC_{50} between 4.1 and 6.6 μ M) as well as anionic compounds **24** and **25** (IC_{50} 5.6 μ M and 3.7 μ M, respectively).

The NHC carbenic ligand not only acted as a transporter of metal center to its target but also affected antiplasmodial efficacy proven by the gold(I) series **18-21** reducing IC_{50} values from methyl- (**18**: 22 μ M) to mesityl – substitutes (**21**: 0.21 μ M). The complex **21**, the most active compound against *P. falciparum*, had lipophilic properties allowing to penetrate into the parasite through its different membranes.

Another compound with nitrogen-functionalized NHC revealed that a quinoline moiety in the complex had a lower antiplasmodial activity (compound **13**, IC_{50} : 5.2 μ M) than compounds **12** and **14** (IC_{50} 1.1 μ M) which had methyl and mesityl as non-functionalized substituents. Compound **15** (IC_{50} : 0.47 μ M) with a thioether group demonstrated an increased antiplasmodial activity, and a methylthiophenyl group was responsible for the antiplasmodial potency of compound **22** (IC_{50} : 0.32 μ M).

The most active compounds (**15**, **16**, **20**, **21** and **22**) were also tested for their specificity against mammalian cell lines. The selectivity index was ranked from 0.9 to 18 with the highest

selectivity index for compound **21**. These findings supported previous studies of cancer cell lines ($IC_{50} < 1\mu M$) and non-tumor cell lines ($IC_{50} > 5\mu M$) [204]. It means that complex **21** is particularly active against *P. falciparum* or several cancer cell lines but having limited effects on normal human cells.

It is noted that cationic lipophilic gold(I)-NHC complexes are the most active compounds against *P. falciparum* compared to both neutral and anionic compounds. The lipophilicity property and presence of nitrogen or sulfur atoms in the ligands were likely to be responsible for the antiplasmodial activity as detailed in the following published article.

2. Corresponding publication



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Antiplasmodial activities of gold(I) complexes involving functionalized *N*-heterocyclic carbenes



Catherine Hemmert^{a,b,*}, Arba Pramundita Ramadani^{a,b,c}, Luca Boselli^{a,b}, Álvaro Fernández Álvarez^{a,b}, Lucie Paloque^{a,b}, Jean-Michel Augereau^{a,b}, Heinz Gornitzka^{a,b,*}, Françoise Benoit-Vical^{a,b,*}

^a CNRS, LCC (Laboratoire de Chimie de Coordination), 205 route de Narbonne, BP 44099, F-31077 Toulouse Cedex 4, France

^b Université de Toulouse, UPS, INPT, F-31077 Toulouse Cedex 4, France

^c Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

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ABSTRACT

A series of twenty five molecules, including imidazolium salts functionalized by *N*-, *O*- or *S*-containing groups and their corresponding cationic, neutral or anionic gold(I) complexes were evaluated on *Plasmodium falciparum* in vitro and then on Vero cells to determine their selectivity. Among them, eight new compounds were synthesized and fully characterized by spectroscopic methods. The X-ray structures of three gold(I) complexes are presented. Except one complex (**18**), all the cationic gold(I) complexes show potent antiplasmodial activity with IC₅₀ in the micro- and submicromolar range, correlated with their lipophilicity. Structure–activity relationships enable to evidence a lead-complex (**21**) displaying a good activity (IC₅₀ = 210 nM) close to the value obtained with chloroquine (IC₅₀ = 514 nM) and a weak cytotoxicity.

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1. Introduction

According to the latest estimates, malaria led to about 500,000 deaths in 2015 with 70% of all deaths for children aged under 5 years.¹ Thanks to global fight against vectors and the large use of artemisinin-based combination therapies (ACT), estimated malaria mortality rates decreased by 60% worldwide between 2000 and 2015.¹ Unfortunately, ACT resistance is now reported in many South-east Asia countries whereas there are no other antiplasmodial drugs to replace them.¹ New drugs with alternative chemical structures are urgently needed.

The peculiar biological properties of some organometallic compounds have helped in the development of new drugs based on metal complexes for major medical human problems including mainly cancer along with bacterial, viral and parasitic infections.² Generally, metal complexes are subject to modifications (ligand substitution and/or redox reactions) during uptake and transport inside the human body; this will influence the reactivity of the metal, but also greatly affect the absorption and delivery of the complex, and sometimes even the specificity of target recognition so they are therefore almost always 'pro-dugs'. Metals may also be

used to enhance the efficacy of known organic drugs and the metal–drug synergism results in the enhancement of the activity of the parental organic drug due to binding to the metal ion. More specifically, numerous metal-containing compounds have been evaluated as antimalarial agents and a recent review overviewed the advancements in this field during the last three decades.³ Among metal coordination complexes in which the metal center is directly coordinated to a known organic drug, gold and ruthenium complexes containing chloroquine (CQ) or analogs were the most active (with IC₅₀ in the nM range), with improved efficacy, when compared to the drug, against CQ-sensitive and CQ-resistant strains, in vitro and in vivo.⁴ In particular, the principal mechanism proposed for the antimalarial action of [Au(CQ)(PPh₃)₂]⁺PF₆[−] against resistant strains of *Plasmodium falciparum* is the interaction with heme and the inhibition of β-hematin formation. Both the enhanced activity and the ability of this compound to lower CQ-resistance are related to the high lipophilicity of the metal complex and the important structural modification of the CQ structure imposed by the presence of the metal-containing fragment.^{4a} A great success was obtained by C. Biot and coll. with impressive biological results in the introduction of a ferrocenyl moiety into the lateral side chain of CQ, leading to the bioorganometallic drug Ferroquine (FQ) which is more active than CQ, and equally active against CQ-susceptible and CQ-resistant clones.⁵ The potent activity of FQ and the absence of cross-resistance with other

* Corresponding authors.

E-mail addresses: Catherine.Hemmert@cc-toulouse.fr (C. Hemmert), Heinz.Gornitzka@cc-toulouse.fr (H. Gornitzka), Francoise.Vical@inserm.fr (F. Benoit-Vical).<http://dx.doi.org/10.1016/j.bmc.2016.05.023>

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antimalarials were reported both in vitro on a large number of clones and field isolates of *P. falciparum*, and in vivo on rodent models that demonstrated the high bioavailability of the product.^{5d} FQ has been developed by Sanofi and is the only metallodrug that has entered clinical trials (Phase IIb) against malaria, currently evaluated in combination with Artefenomel (OZ439).^{5f} FQ has a multi-mode of action: a capacity to target lipids, an ability to inhibit the formation of hemozoin, and to generate reactive oxygen species.^{5e,6}

NHCs represent one of the most important classes of ligands in organometallic chemistry. The easy preparation of NHC-precursors has allowed an almost infinite access to new organometallic complexes, in which the nitrogen atoms of the azolium rings can be functionalized by organic groups, organometallic moieties and biologic entities. NHCs are extremely good σ -donors, making strong metal–carbene bonds and giving stable complexes in biological medium. Numerous NHC–metal complexes exhibit very interesting biological activities as reflected in several review articles.⁷ By varying the properties of the NHCs and the nature of the metals (mainly Cu, Ag, Au, Ni, Pd, Pt and Ru), different important diseases have been targeted, mainly fungi,^{7b,i} bacteria,^{7b-d,i} and cancer.^{7a-f,h-k} We focused our research on heteroditopic *N*-heterocyclic carbene (NHC) gold(I) complexes for their biomedical potential in anticancer and parasitic diseases such as malaria and leishmaniasis.⁸ New gold(I) complexes containing two 1-[2-(diethylamino)ethyl]imidazolylidene ligands have been synthesized and their anticancer activity reported in five representative human cancer cell lines (prostate, breast, brain, lung and liver).^{8a} Very recently, we have demonstrated that mononuclear cationic and especially neutral gold(I) NHC–quinoline proved to be promising metallo-drugs with potent and selective action against the pathological relevant form (intramacrophagic amastigote) of *Leishmania*.^{8d} Moreover, we have previously synthesized and tested against *Plasmodium falciparum* dinuclear dimeric and mononuclear gold(I) complexes and we have shown that mononuclear cationic gold(I) complexes containing aromatic amino-functionalized NHC exhibit in vitro potent antiparasitoid activity against the chloroquine-resistant strain FcM29–Cameroon with IC₅₀ values up to 330 nM.^{8c}

The rational design of new gold *N*-functionalized NHCs complexes with specific and/or selective parasite targets appears thus as a very useful avenue in the antiparasitoid drug research.

In this work, we aim to extend our preliminary work concerning gold(I) NHCs complexes against *Plasmodium falciparum* by fine tuning and studying the key building blocks of these organometallic molecules. For this purpose, we report the antiparasitoid activity of our gold(I) complexes already described for other biomedical applications.^{8c} In addition, we report the preparation, characterization and antimalarial potential of new mononuclear cationic, anionic and neutral gold(I) NHC or bis(NHC) complexes.

2. Results and discussion

2.1. Chemistry and structural characterization

The synthesis of proligands **1–8** was already described (Scheme 1).^{8a,c,d} The imidazolium salts **9** and **11** were simply obtained after a quaternization step of 1-arylimidazole, namely 1-(4-methylthiophenyl)-1*H*-imidazole and 1-(4-hydroxyphenyl)imidazole, respectively, with one equivalent of benzylchloride and KPF₆ at 80 °C in acetonitrile (Scheme 2). The zwitterionic proligand **10** was easily synthesized by heating stoichiometric amounts of benzylimidazole and bromoacetic acid at 130 °C, following by deprotonation of the acidic proton by addition of

Na₂CO₃ under reflux in water (Scheme 2). The most notable features in the ¹H and ¹³C NMR spectra of the imidazolium salts are the resonances for imidazolium protons (*H*₂) located at 9.97, 9.21 and 10.16 ppm and the corresponding imidazolium carbons (*C*₂) in the range of 135.6–164.0 ppm, for **9**, **10** and **11** respectively. The high resolution mass spectra (ES) of proligands **9** and **11** exhibit the classical peak corresponding to the cation [M–PF₆]⁺ and that of zwitterion **10** shows the cation [M+H]⁺.

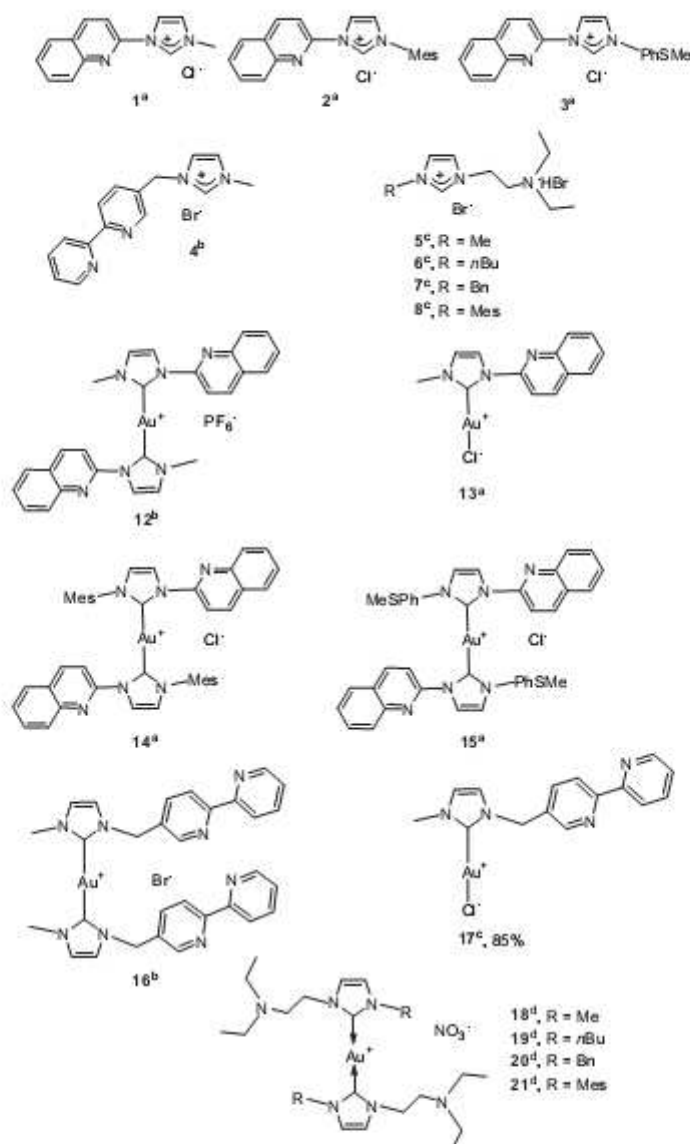
The synthesis of complexes **12–16** and **18–21** was previously reported in the literature (Scheme 1).^{8a,c,d} The mononuclear gold (I) bis(NHC) complexes **22** and **25** (Scheme 2) were prepared according a classical way, from the *N*-functionalized imidazolium salts **9** or **11**, respectively and one half equivalent of Au(SMe₂)Cl with sodium acetate as a mild base in hot *N,N*-dimethylformamide (120 °C) and were isolated as white or beige powders with good yields (66–87%). The neutral gold(I) NHC complexes **17** and **23** and the anionic complex gold(I) bis(NHC) **24** (Schemes 1 and 2) were synthesized via the convenient transmetalation route. Firstly, the silver precursor complexes were prepared by deprotonation of the imidazolium salts **4**, **9** or **10**, with one half equivalent of the mild base Ag₂O in a mixture of CH₂Cl₂–MeOH at room temperature or in MeOH at 50 °C. For **17**, the silver species Ag(4)₂Br^{8c} was isolated before the carbene transfer reaction while for compounds **23** and **24**, the transmetalation was carried out in situ, by adding one (for **17** and **23**) or one half equivalent (for **24**) of Au(SMe₂)Cl, with respect to the ligand. The three later compounds were obtained as gray solids with high yields (82–85%) and all complexes are stable towards air and moisture. NMR spectroscopy unequivocally demonstrates the formation of the gold(I) complexes; the ¹³C spectra show the resonance for the carbene carbon atoms between 181.6 and 184.5 ppm for the gold(I) bis (NHC) complexes **22**, **24** and **25**, and at 172.0 and 168.9 ppm for the neutral species **17** and **23**, respectively. These values are in the range of reported values for Au^I–NHC complexes having C–Au–X (X = halide) or C–Au–C motifs.⁹ It has to be mentioned that in the ¹H NMR of complex **24**, both protons of the CH₂COO[–] become inequivalent. This could be due to the interaction of the two carboxylate with a proton, which may impede the free rotation of the CH₂. Moreover, for complex **25** the phenol functions are deprotonated during the complexation process which is supported by the absence of the OH proton in the ¹H NMR spectrum (the OH proton of proligand **11** was located at 10.16 ppm) and by the ES–HRMS spectrum showing the molecular peak [M+H]⁺ at *m/z* 697.1874 with M = Au^Ibis(NHC–phenolato). The elemental analysis of the gold(I) complexes are in agreement with the proposed structures and the high resolution mass spectra (ES) exhibit the classical peaks corresponding to the cationic fragments [M+H]⁺ for the neutral and the anionic complexes **17**, **23–25** and [M–PF₆]⁺ for the cationic compound **22**.

Crystals of **17** suitable for X-ray diffraction analysis were obtained by slow evaporation of a chloroform solution of **17** (Fig. 1).

Slow evaporation of a MeOH solution of **22** gave crystals suitable for X-ray diffraction analysis (Fig. 2).

Crystals of **23** suitable for X-ray diffraction analysis were obtained by slow evaporation of an acetonitrile solution of **23** (Fig. 3).

All carbon–gold and chlorine–gold distances, as well as the carbon–gold–carbon and carbon–gold–chlorine angles are in the normal range for such compounds. In **22** the gold cation is located on an inversion center and both NHC ring systems are absolutely coplanar. Moreover, the benzyl substituents are in trans position.

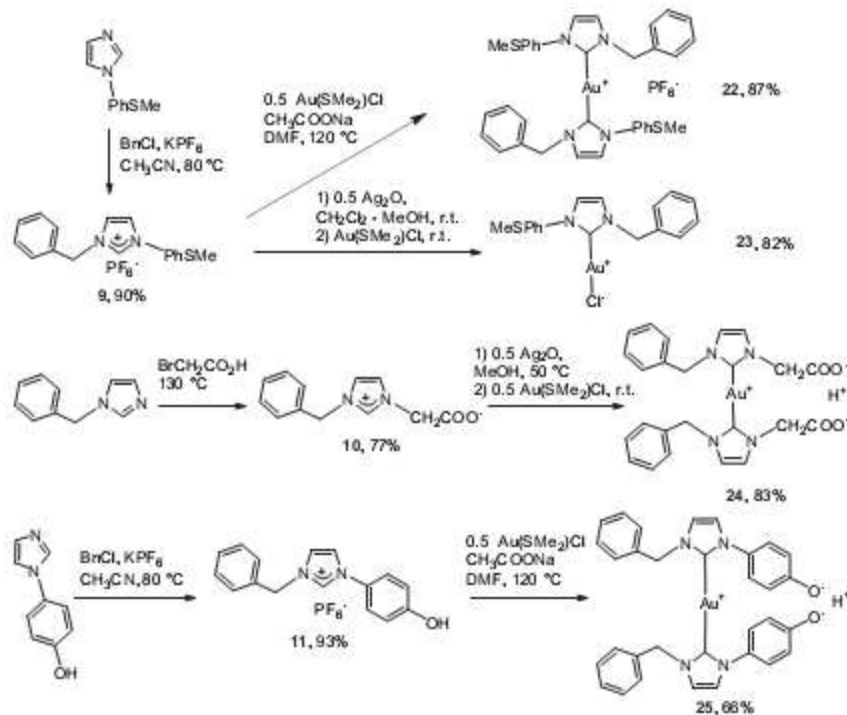


Scheme 1. Proligands and gold(I) complexes used in this study. ^aRef. 3d, ^bRef. 8c, ^cthis work and ^dRef. 8a.

2.2. Anti-malarial activity and selectivity

Twenty five molecules, involving imidazolium salts 1–11 and mononuclear gold(I) complexes 12–25 were screened *in vitro* against *P. falciparum* strains (Table 1) by determining their 50% inhibitory concentrations (IC₅₀) and compared to the antimalarial reference-drugs chloroquine and artemisinin. As expected, proligands 1 and 4–11 showed weak or no activity with IC₅₀ values ranging from 6.4 to >230 μM, the worst value being obtained for the neutral zwitterionic species 10, all other imidazolium salts being cationic. More surprising, the two proligands 2 and 3 con-

taining both a quinoline entity and an aryl directly branched on the azolium ring exhibit interesting antiparasitodal potency with IC₅₀ values of 1.4 and 1.5 μM, respectively. As expected, an enhanced activity was observed for the tested gold(I) complexes 12, 15–16 and 19–22 when compared to the corresponding starting proligands, except for the couple 2/14 showing similar activity. This set of results corroborates in most cases the key role of the gold cation in antiprotozoal activity against *P. falciparum*. The three neutral complexes 13, 17 and 23 showed moderate activity with IC₅₀ comprised between 4.1 and 6.6 μM and the IC₅₀ values of the two anionic compounds 24 and 25 are in the same order of



Scheme 2. Synthesis of imidazolium salts 9–11 and gold(I) bis(NHC) complexes 22–25.

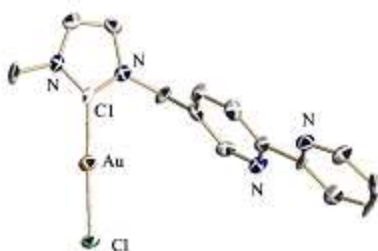
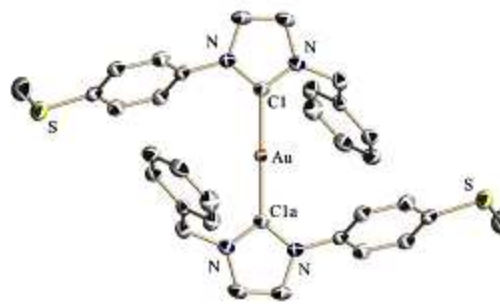


Figure 1. Structure of 17 in the solid state, thermal ellipsoids are depicted at a 50% level. Hydrogen atoms have been omitted for clarity. Selected bond lengths [Å] and angles [°]: C1–Au = 1.99(2), Au–Cl = 2.30(1), Cl–Au–Cl = 177.7(4).

magnitude, 5.6 μM and 3.7 μM , respectively. With the exception of complex 18, the best values on *P. falciparum* were obtained for the cationic Au(I)bis(NHC) species with IC_{50} in the micromolar and submicromolar range, with 2.1 μM (19), 0.48 μM (20), 0.21 μM (21), and 0.32 μM (22). A first insight can emerge from these results and confirm our preliminary published results (concerning 12 and 16^{3c}): the global charge of the complex constitutes an important factor and cationic compounds are correlated with an increase antiplasmodial activity. The same antiplasmodial properties were reported with albitiazolium, a bis cationic compound which reached the clinical 2b step for antimalarial drug development. Albitiazolium had shown its entry facilitated by a poly-specific cation transporter present in the parasite membrane.¹⁰ The cationic property could facilitate the accumula-

tion of the molecules inside the parasite where the pH of the cytoplasm is approximately 7.15 but certainly not in the malaria digestive vacuole where pH is about 5.¹¹ Moreover, even if the carbenic ligand acts mainly as a carrier for the metal center towards its target(s), as it is generally the case for gold complexes with biological potential, the two substituents on the NHC plays a crucial role with regard to the antiparasitic efficiency. This is well illustrated for the gold(I) series 18–21 where the antiplasmodial activity dramatically increases from methyl- (18: 22 μM) to mesityl-substituted (21: 0.21 nM) systems. By raising the steric hindrance, the antimalarial activity was highly improved, reflecting an

Figure 2. Structure of the cationic part of 22 in the solid state, thermal ellipsoids are depicted at a 50% level. Hydrogen atoms and the uncoordinated PF_6^- anion have been omitted for clarity. Selected bond lengths [Å] and angles [°]: C1–Au = 2.013(2), Au–Cl1a = 2.013(2), Cl1–Au–Cl1a = 180.

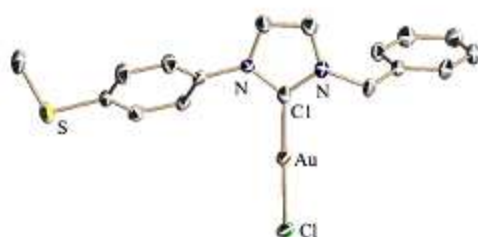


Figure 3. Structure of **23** in the solid state, thermal ellipsoids are depicted at a 50% level. Hydrogen atoms have been omitted for clarity. Selected bond lengths [Å] and angles [°]: C1–Au = 1.972(5); Au–Cl = 2.287(2); C1–Au–Cl = 178.2(2).

increase of the protective effect of the ligand around the metal and probably this allows to avoid releasing of the gold cation before reaching its target.

We have previously reported that the selective cytotoxic activity of this family was directly correlated with the lipophilic properties²⁴ and this trend is also verified here: the highest lipophilic complex **21** is the most active against *P. falciparum*. We can hypothesize that lipophilic properties could permit to compounds to better cross the different membranes (red blood

Table 1
Antiplasmodial and cytotoxicity of imidazolium salts and their gold(I) *N*-heterocyclic compounds

Compounds	Antiplasmodial activity IC ₅₀ (μM) on <i>P. falciparum</i> ^a	Cytotoxicity activity IC ₅₀ (μM) on Vero cell line ^b	Selectivity index
1	179 ± 1.3		
2	1.4 ± 0.2	>140	>100
3	1.5 ± 0.1	69.7 ± 5.6	46
4	73.2 ± 4.5 ^c		
5	>145 ^c		
6	>130 ^c		
7	596 ± 1.4		
8	11.3 ± 0.6		
9	6.4 ± 0.1		
10	>230		
11	9.3 ± 0.3		
12^d	1.1 ± 0.1	<1 ^e	<0.9
13	5.2 ± 2.0		
14	1.1 ± 0.2	6.6 ± 0.4	6
15	0.47 ± 0.1	3.6 ± 0.3	8
16^f	0.33 ± 0.03	1 ^g	3
17	6.6 ± 0		
18	2.2 ± 3.8 ^h		
19	2.1 ± 0.3 ^h		
20	0.48 ± 0.06	1.9 ± 0	4
21	0.21 ± 0.04	3.7 ± 0.1 ^h	18
22	0.32 ± 0.08	3.6 ± 0.7	11
23	4.1 ± 0.4		
24	5.6 ± 0.8		
25	3.7 ± 0.7		
Chloroquine ^g	0.514 ± 0.02	134 ± 20	261
Artemisinin ^g	0.187 ± 0.02 ^h	ND	717 ^g
Artemisinin ^g	0.010 ± 0.7	ND	

^a Antiplasmodial activity of the compounds were evaluated against the *P. falciparum* strain FcM29-Cameroon, except compounds noted (*) evaluated against the *P. falciparum* strain FcB1-Columbia.

^b Data represented the mean of 2–5 independent experiments.

^c Ref. 26.

^d Cytotoxicity activity of the compounds were evaluated against Vero cell lines, except compounds noted (*) evaluated on the MCF7 line.

^e Compound **21** was also tested against other cell lines and showed the following results: 0.4 μM (PC-3 cell line); 0.8 μM (MCF-7 cell line); 0.8 μM (U87 cell line); 0.9 μM (A549 cell line); 1 μM (Hep3B cell line); >5 μM (Huvec cell line).²⁴

^f Both antiplasmodial control drugs artemisinin and chloroquine were routinely tested.

cell membrane, parasitophorous vacuole membrane, parasite plasma membrane and eventually digestive vacuole membrane) to target the parasite. Concerning the aromatic nitrogen-containing *N*-functionalized NHC complexes **12**, **14** and **15**, the two quinoline moieties are positioned in trans-arrangement with respect to each other and thus provide already a good protection to the gold(I) center, in contrast to the previous complexes. That could also explain the less antiplasmodial activity with only one quinoline moiety (**13**: 5.2 μM). This feature can account for the same antiplasmodial activity for **12** (1.1 μM) and **14** (1.1 μM), differing in their non-functionalized substituents, methyl and mesityl, respectively. Introducing a second functionalized entity involving a thioether group (**15**) leads to an improvement of the antiproliferative activity with an IC₅₀ of 0.47 μM. Moreover, it should be noted that the use of the methylthiophenyl group as the only functionalized group is also very interesting as evidenced by the antimalarial potency of complex **22** (IC₅₀ = 0.32 μM).

Compounds **15**, **16**, **20**, **21** and **22** showed thus very high antiplasmodial activity with IC₅₀ below the micromolar range. Compounds with the most promising antimalarial properties were also evaluated on culture cell lines in order to determine the specificity of their activity. The selectivity index are ranging from <0.9 to 18. The best selectivity index was found with the compound **21**. These results confirmed those already reported with various cell lines²⁴ with IC₅₀ values below 1 μM for all cancer cell lines tested whereas the value was superior to 5 μM on non-tumor cells (primary cultures of human umbilical vein endothelial cells (HUVEC)). These data highlight that complex **21** is specifically active against pathogen cells such as *P. falciparum* or multiple cancer cell lines but shows restricted effects on normal human cells.

The potential of organometallic gold complexes without incorporation of known antimalarial organic drugs against parasitic diseases has been little explored until very recently.^{1,12} Messori and coll. used a panel of structurally diverse gold(I) and (III) complexes involving N, O or P heterocyclic ligands and the clinically established gold-based antiarthritic drug auranofin, to evaluate them as possible inhibitors of the parasite enzyme Falcipain 2 (Fp2) which is an important protease of *P. falciparum* and represents one of the most promising targets for antimalarial drug discovery.¹³ This protein is mainly involved in the degradation of host's hemoglobin (thus providing a substantial amount of peptides and amino acids to sustain parasite growth) and its proteolytic activity is more pronounced at the early trophozoite stage of parasite development. Remarkably, most tested gold compounds caused pronounced but reversible inhibition of Fp2 and important parasite growth inhibition of *P. falciparum* was observed in vitro. However, any direct correlation between enzyme inhibition and reduction of *P. falciparum* growth was established, suggesting that Fp2 inhibition represents just one of the various mechanisms through which gold compounds effectively antagonize *P. falciparum* replication. Chibale and coll. reported gold(I) and gold(III) thiosemicarbazone (TSCs) compounds which exhibit moderate in vitro activity against two *P. falciparum* strains, CQ-sensitive D10 (IC₅₀ of CQ = 0.0173 μM) and CQ-resistant W2 (IC₅₀ of CQ = 0.095 μM) with IC₅₀ ranging from 1.36 to 6.92 μM for the gold(I) compounds and IC₅₀ ranging from 3.04 to >20 μM for the gold(III) complexes; the gold(I) TSCs complexes also inhibit the Fp2 but no correlation was found between their antiplasmodial activity and the ability to inhibit Fp2.¹⁴ Mohr and coll. reported gold(I) complexes involving functionalized alkynes that have shown low in vitro activity against falciparum malaria parasite strains CQ-sensitive 3D7 (IC₅₀ of CQ = 0.01 μM) and CQ-resistant K1 (IC₅₀ of CQ = 0.3 μM) with IC₅₀ values between 7.2 and 23 μM.¹⁵ The same authors described also a series of mono- and dinuclear gold(I) phosphine complexes

containing mono-anionic seleno- and thiosemicarbazones ligands; the IC_{50} results showed that two sulfur containing mononuclear compounds exhibit activity similar to CQ against the 3D7 strain with IC_{50} of 7.06 and 10.7 nM (IC_{50} of CQ = 8.84 nM).¹⁶ Unfortunately, neither the IC_{50} of the thiosemicarbazones ligands nor the toxicity of the corresponding complexes were evaluated. Previous studies realized by Lambros and co-workers revealed that the two involved ligands themselves already exhibit high in vitro antimalarial activity (CQ-resistant *P. falciparum* Smith strain with IC_{50} below 10 ng/mL (ID_{50} of CQ = 60 ng/mL) and moreover they are toxic in vivo (on *P. berghei* at doses >20 or 40 mg/kg).¹⁷ Cronje and coll. reported preliminary in vitro antimalarial activities of *N*-heterocyclic ylideneamine gold(I) against *P. falciparum*; the coordination of ancillary phosphine or NHC ligands to these complexes results in better activities with an IC_{50} value of 5.1 μ g/ml for the NHC derivative.¹⁸ In light of the available literature concerning gold compounds, some of our Au(I)-NHC complexes display improved efficacy.

3. Conclusion

In summary, a series of *N*-, *O*- or *S*-functionalized proligands and their corresponding mononuclear cationic, anionic or neutral gold(I) bisNHC complexes, including eight new fully characterized compounds, were tested in vitro for their antimalarial potency against chloroquine-resistant *P. falciparum* strains. Moreover, cytotoxicity of the most active compounds was assessed, in order to determine their selectivity of action. From this screening, the importance of the charge of the molecules tested in the antiplasmodial activity was observed. Cationic lipophilic gold (I)-NHC complexes are more active than similar neutral and anionic complexes. Moreover, an active complex has to be highly lipophilic and the presence of nitrogen or sulfur atoms in the ligands seems to have a positive influence on the antiplasmodial activity.

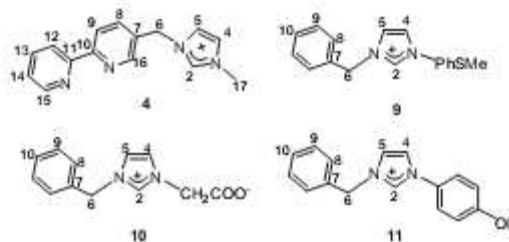
The pharmacomodulation, by fine tuning either the global charge of the complexes and the key substituents on the NHC ligands, permitted to increase the antiplasmodial activity with promising selectivity indexes and highlighted the interest for gold cationic compounds which can be considered as a good scaffold for the optimization of a new antimalarial chemistry.

4. Experimental

4.1. Chemistry

4.1.1. General methods

All manipulations were performed under an inert atmosphere of dry nitrogen by using standard vacuum line and Schlenk tube techniques. 3-Methyl-1-[6-(pyridin-2-yl)pyridin-3-yl]methyl-1*H*-imidazol-3-ium bromide (**4**), Ag(4)₂Br⁺ and 1-(4-methylthiophenyl)-1*H*-imidazole³¹ were synthesized according to literature procedures. All reagents were used as received from commercial suppliers. Reactions involving silver compounds were performed with the exclusion of the light. ¹H (300 or 400 MHz) and ¹³C NMR spectra (75 or 101 MHz) were recorded at 298 K on Bruker AV300 or Bruker AV400 spectrometers in CDCl₃, CD₂CN, DMSO-*d*₆ and D₂O as solvents. Elemental analyses were carried out by the "Service de Microanalyse du Laboratoire de Chimie de Coordination" (Toulouse). High Resolution Mass Spectrometry (HRMS) analysis were performed with a Thermo Finnigan MAT 95 XL spectrometer using electrospray ionization (ESI) or with a TOF mass analyzer by desorption chemical ionization with methane gas (DCI) by the "Service de Spectrométrie de Masse de Chimie UPS-CNRS" (Toulouse).



Numbering of H (¹H NMR) and C (¹³C NMR). These notations are used in the following section.

4.1.2. 1-(4-Methylthiophenyl)-3-benzylimidazolium chloride (**9**)

1-(4-Methylthiophenyl)-1*H*-imidazole (1.00 g, 5.26 mmol), benzylchloride (605 μ L, 5.26 mmol) and KPF₆ (967 mg, 5.26 mmol) were stirred in CH₃CN (20 mL) at 80 °C for 3 days. After cooling to room temperature, the solvent was evaporated and the yellow solid obtained was washed with CH₂Cl₂ and diethylether and dried under vacuum (2.018 g, 90% yield). Anal. Calcd for C₁₇H₁₇N₂SPF₆: C, 47.89; H, 4.02; N, 6.57. Found: C, 47.93; H, 4.06; N, 6.55. ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.44 (s, 1H, H₂), 8.38 (d, *J* = 1.6 Hz, 1H, H₅), 8.11 (d, *J* = 1.6 Hz, 1H, H₄), 7.79 (d, *J* = 8.6 Hz, 2H, H_{MSMe}), 7.99 (d, *J* = 6.6 Hz, 2H, H_B), 7.49 (d, *J* = 8.6 Hz, 2H, H_{MSMe}), 7.45–7.37 (m, 3H, H_B, H₁₀), 5.57 (s, 2H, H₆), 2.54 (s, 3H, H_{MSMe}). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 141.37 (1C, C_{MSMe}), 135.73 (1C, C₂), 134.79 (1C, C₇), 131.93 (1C, C_{MSMe}), 129.47 (2C, C₉), 129.34 (1C, C₁₀), 128.95 (2C, C₈), 127.09 (2C, C_{MSMe}), 123.58 (1C, C₃), 122.86 (2C, C_{MSMe}), 122.12 (1C, C₄), 52.93 (1C, C₆), 14.92 (1C, C_{MSMe}). HRMS (ES⁺) calculated for C₁₇H₁₇N₂S (M–PF₆)⁺ 281.1112, found 281.1120.

4.1.3. 1-(Carboxymethyl)-3-benzylimidazolium (**10**)

Benzylimidazole (419 mg, 2.65 mmol) and bromoacetic acid (368 mg, 2.65 mmol) were heated at 130 °C for 24 h. After the mixture was cooled to room temperature, the oily product was dissolved in 20 mL H₂O and Na₂CO₃ was added (281 mg, 2.65 mmol). The mixture was refluxed at 100 °C for 30 min. After evaporation of the solvent, the resulting solid was partially dissolved in MeOH, filtered and the filtrate was evaporated to dryness. The crude product was washed with CH₂Cl₂ and dried under vacuum to afford a white solid (0.44 g, 77% yield). Anal. Calcd for C₁₂H₁₂N₂O₂: C, 66.65; H, 5.59; N, 12.96. Found: C, 66.58; H, 5.66; N, 12.90. ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.21 (s, 1H, H₂), 7.69 (s, 1H, H₅), 7.60 (s, 1H, H₄), 7.40 (m, 5H, H_B, H₉, H₁₀), 5.43 (s, 2H, H₆), 4.45 (s, 2H, H_{CH2COO}). ¹³C NMR (75 MHz, D₂O) δ 172.10 (1C, C_{COO}), 163.97 (1C, C₂), 133.44 (1C, C₇), 129.36 (2C, C₉), 129.31 (1C, C₁₀), 128.70 (2C, C₈), 123.71 (1C, C₃), 121.94 (1C, C₄), 52.89 (1C, C₆), 52.01 (1C, C_{CH2COO}). HRMS (ES⁺) calculated for C₁₂H₁₂N₂O₂ (M + H)⁺ 217.0977, found 217.0978.

4.1.4. 1-(4-Hydroxyphenyl)-3-benzylimidazolium hexafluorophosphate (**11**)

1-(4-Hydroxyphenyl)imidazole (1.642 g, 9.760 mmol), benzylchloride (1.15 mL, 9.760 mmol) and KPF₆ (1.800 g, 9.760 mmol) were heated in acetonitrile (20 mL) at 80 °C for 3 days. The white solid obtained was filtered and dried under vacuum (3.595 g, 93% yield). Anal. Calcd for C₁₆H₁₅N₂OPF₆: C, 48.50; H, 3.82; N, 7.07. Found: C, 48.47; H, 3.95; N, 7.01. ¹H NMR (300 MHz, DMSO-*d*₆) δ 10.31 (s, 1H, H_{MSOH}), 9.74 (t, *J* = 1.8 Hz, 1H, H₂), 8.11 (t, *J* = 1.9 Hz, 1H, H₅), 7.90 (t, *J* = 1.9 Hz, 1H, H₄), 7.56 (d, *J* = 8.9 Hz, 2H, H_{MSOH}), 7.53–7.36 (m, 5H, H_B, H₉, H₁₀), 6.98 (d, *J* = 8.9 Hz, 2H, H_{MSOH}), 5.47 (s, 2H, H₆). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 159.09 (1C, C_{MSOH}),

135.58 (1C, C₂), 135.01 (1C, C₇), 129.49 (2C, C₆), 129.32 (1C, C₁₀), 128.89 (2C, C₈), 126.94 (1C, C_{10H}), 124.23 (2C, C_{10H}), 123.43 (1C, C₃), 122.52 (1C, C₄), 116.74 (2C, C_{10H}), 52.83 (1C, C₆). HRMS (ES⁺) calculated for C₁₆H₁₅N₂O (M⁺PF₆⁻) 251.1184, found 251.1189.

4.1.5. {1-[(2,2'-Bipyridin)-5-yl]methyl}-3-methylimidazol-2-ylidene(chloro)gold(I) (17)

Complex 17 was obtained by transmetalation of Ag(4)₂Br (81 mg, 0.118 mmol) with Au(SMe₂)Cl (69.2 mg, 0.235 mmol) in a mixture of CH₂Cl₂-MeOH (4 mL / 2 mL) at rt. After stirring for 1 h, the solution was filtered through a pad of celite and the solvent removed under reduced pressure to give a gray solid (0.72 g, 63% yield). Anal. Calcd for C₁₅H₁₄N₄AuCl: C, 37.32; H, 2.92; N, 11.61. Found: C, 37.27; H, 3.06; N, 11.57. ¹H NMR (400 MHz, CDCl₃) δ 8.73–8.67 (m, 2H, H₁₅, H₁₆), 8.48 (d, J = 8.2 Hz, 1H, H₁₂), 8.43 (d, J = 8.0 Hz, 1H, H₆), 7.95–7.80 (m, 2H, H₈, H₁₃), 7.39 (ddd, J = 7.5, 4.8, 1.1 Hz, 1H, H₄), 7.00 (d, J = 1.9 Hz, 1H, H₅), 6.96 (d, J = 1.9 Hz, 1H, H₄), 5.49 (s, 2H, H₆), 3.90 (s, 3H, H₁₇). ¹³C NMR (101 MHz, CDCl₃) δ 171.99 (1C, C₂), 156.78 (1C, C₁₀), 155.25 (1C, C₁₁), 149.25 (1C, C₁₃), 148.50 (1C, C₁₆), 137.05 (1C, C₁₃), 136.68 (1C, C₈), 130.61 (1C, C₇), 124.11 (1C, C₁₄), 122.70 (1C, C₃), 121.31 (1C, C₉), 121.25 (1C, C₁₂), 120.21 (1C, C₄), 52.41 (1C, C₆), 38.41 (1C, C₁₇). HRMS (ES⁺) calculated for C₁₅H₁₄N₄Au (M-Cl)⁺ 447.0884, found 447.0887.

4.1.6. Bis(1-benzyl-3-[4-(methylsulfanyl)phenyl]imidazol-2-ylidene)gold(I) hexafluorophosphate (22)

Under a nitrogen atmosphere, sodium acetate (69.3 mg, 0.844 mmol) was added to a mixture of 9 (300 mg, 0.704 mmol) and Au(SMe₂)Cl (104 mg, 0.352 mmol) in dry DMF (6 mL) at 100 °C. The mixture was then heated to 120 °C and this temperature was maintained for 2 h. After cooling to room temperature, the solution was filtered through a pad of celite and the solvent removed under reduced pressure to give a white solid (0.277 g, 87% yield). Anal. Calcd for C₃₄H₃₂N₄S₂PF₆Au: C, 45.24; H, 3.57; N, 6.21. Found: C, 45.29; H, 3.62; N, 6.24. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.88 (d, J = 1.9 Hz, 2H, H₃), 7.82 (d, J = 1.9 Hz, 2H, H₄), 7.63 (d, 4H, J = 8.6 Hz, H₁₀), 7.37–7.28 (m, 6H, H₉, H₁₀), 7.26 (d, 4H, J = 8.6 Hz, H₁₀), 7.24–7.17 (m, 4H, H₈), 5.35 (s, 4H, H₆), 2.45 (s, 6H, H₁₅). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 181.57 (2C, C₂), 140.26 (2C, C₁₅), 136.62 (2C, C₇), 135.92 (2C, C₁₅), 129.21 (4C, C₆), 128.66 (2C, C₁₀), 127.94 (4C, C₈), 126.64 (4C, C₁₅), 125.71 (4C, C₁₅), 123.93 (2C, C₃), 123.61 (2C, C₄), 54.35 (2C, C₆), 14.95 (2C, C₁₅). HRMS (ES⁺) calculated for C₃₄H₃₂N₄S₂Au (M-PF₆)⁺ 757.1734, found 757.1744.

4.1.7. {1-Benzyl-3-[4-(methylsulfanyl)phenyl]imidazol-2-ylidene(chloro)gold(I) (23)

Under a nitrogen atmosphere and protection of the light, a Schlenk tube was charged with 9 (261 mg, 0.612 mmol) in a mixture of CH₂Cl₂-MeOH (5 mL/1 mL), Ag₂O (71 mg, 0.306 mmol) and KCl (68 mg, 0.918 mmol). The reaction mixture was stirred at rt for 6 h. After that Au(SMe₂)Cl (180 mg, 0.612 mmol) was added and the mixture was stirred for 4 h. The solution was filtered through a pad of celite and the solvent removed under reduced pressure to give a gray solid (0.267 g, 82% yield). Anal. Calcd for C₁₇H₁₆N₂SAuCl: C, 39.82; H, 3.15; N, 5.46. Found: C, 39.76; H, 3.22; N, 5.48. ¹H NMR (300 MHz, CD₃CN) δ 7.63 (d, J = 8.8 Hz, 2H, H₁₅), 7.48–7.38 (m, 8H, H₁₅, H₈, H₉, H₁₀), 7.36 (d, J = 2.0 Hz, 1H, H₄), 5.48 (s, 2H, H₆), 2.56 (s, 3H, H₁₇). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 168.90 (1C, C₂), 140.10 (1C, C₁₅), 136.84 (1C, C₇), 136.18 (1C, C₁₅), 129.30 (2C, C₆), 128.69 (1C, C₁₀), 128.23 (2C, C₈), 126.73 (2C, C₁₅), 125.93 (2C, C₁₅), 123.57 (1C, C₃), 123.01 (1C, C₄), 54.62 (1C, C₆), 15.00 (1C, C₁₅). HRMS (DCI) calculated for C₁₇H₁₆N₂SAu (M-Cl)⁺ 477.0700, found 477.0710.

4.1.8. Hydrogen bis[1-benzyl-3-(carboxylatomethyl)imidazol-2-ylidene]gold(I) (24)

Under a nitrogen atmosphere and protection of the light, a Schlenk tube was charged with 10 (338 mg, 1.563 mmol) in 10 mL of dry MeOH and Ag₂O (181 mg, 0.782 mmol). The mixture was stirred for 12 h at 50 °C. After cooling to rt, Au(SMe₂)Cl (230 mg, 0.782 mmol) was added and the mixture was stirred at rt for 2 h. The solution was filtered through a pad of celite and the solvent removed under reduced pressure to give a gray solid (0.407 g, 83% yield). Anal. Calcd for C₂₄H₂₃N₄O₄Au: C, 45.87; H, 3.69; N, 8.92. Found: C, 45.90; H, 3.90; N, 8.93. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.45 (s, 2H, H₃), 7.39–7.28 (m, 12H, H₄, H₈, H₉, H₁₀), 5.32 (s, 4H, H₆), 4.50 (s, 2H, H₁₀), 4.42 (s, 2H, H₁₀). ¹³C NMR (75 MHz, D₂O) δ 184.51 (2C, C₂), 174.02 (2C, C₁₀), 136.53 (2C, C₇), 128.83 (4C, C₆), 128.15 (2C, C₁₀), 127.40 (4C, C₈), 123.05 (2C, C₃), 121.74 (2C, C₄), 53.91 (2C, C₆), 53.85 (2C, C₁₀). HRMS (ES⁺) calculated for C₂₄H₂₄N₄O₄Au (M+H)⁺ 629.1463, found 629.1478.

4.1.9. Hydrogen bis[1-benzyl-3-(4-oxidophenyl)imidazol-2-ylidene]gold(I) (25)

Under a nitrogen atmosphere, sodium acetate (74.5 mg, 0.908 mmol) was added to a mixture of 11 (300 mg, 0.757 mmol) and Au(SMe₂)Cl (112 mg, 0.379 mmol) in dry DMF (5 mL) at 100 °C. The mixture was then heated to 120 °C and this temperature was maintained for 2 h. After cooling to room temperature, CH₂Cl₂ (5 mL) and hexane (10 mL) were added to the solution to afford a beige solid, which was filtered and dried under vacuum (0.175 g, 66% yield). Anal. Calcd for C₃₂H₂₇N₄O₂Au: C, 55.18; H, 3.91; N, 8.04. Found: C, 55.23; H, 3.96; N, 8.10. ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.77–7.76 (m, 4H, H₄, H₅), 7.37 (d, J = 8.8 Hz, 4H, H₁₀), 7.31–7.29 (m, 6H, H₉, H₁₀), 7.19 (dd, J = 6.7, 2.9 Hz, 4H, H₈), 6.82 (d, J = 8.8 Hz, 4H, H₁₀), 5.31 (s, 4H, H₆). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 181.55 (2C, C₂), 158.36 (2C, C₁₀), 136.66 (2C, C₇), 130.71 (2C, C₁₀), 129.22 (4C, C₆), 128.69 (2C, C₁₀), 127.94 (4C, C₈), 126.43 (4C, C₁₀), 123.88 (2C, C₃), 123.12 (2C, C₄), 116.24 (4C, C₁₀), 54.19 (2C, C₆). HRMS (ES⁺) calculated for C₃₂H₂₈N₄O₂Au (M+H)⁺ 697.1878, found 697.1874.

4.2. Crystallographic data for 17, 22 and 23

All data were collected at low temperature using oil-coated shock-cooled crystals on a Bruker-AXS APEX II diffractometer with MoK α radiation ($\lambda = 0.71073$ Å). The structures were solved by direct methods³⁹ and all non hydrogen atoms were refined anisotropically using the least-squares method on I^2 .²⁰

CCDC-1441512 (17), CCDC-1441513 (22) and CCDC-1441514 (23) contain the supplementary crystallographic data. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

17: C₁₅H₁₄AuClN₄, Mr = 482.72, crystal size = 0.30 × 0.30 × 0.10 mm³, monoclinic, space group *P2₁/c*, *a* = 5.6934(3) Å, *b* = 11.8070(6) Å, *c* = 21.8435(12) Å, β = 92.646(3)°, *V* = 1466.8 (2) Å³, *Z* = 4, *T* = 173(2) K, 2506 unique reflections, *R*₁ = 0.0554, *wR*₂ = 0.1535 [*I* > 2 σ (*I*)], *R*₂ = 0.0759, *wR*₂ = 0.1659 (all data), residual electron density = 2.400 e Å⁻³.

22: C₃₄H₃₂AuF₆N₄S₂, Mr = 902.69, crystal size = 0.30 × 0.30 × 0.10 mm³, monoclinic, space group *P2₁/n*, *a* = 11.7142(5) Å, *b* = 11.6965(5) Å, *c* = 12.7877(6) Å, β = 108.408(1)°, *V* = 1662.5 (2) Å³, *Z* = 2, *T* = 173(2) K, 24252 reflections collected, 6058 unique reflections (*R*_{int} = 0.0409), *R*₁ = 0.0228, *wR*₂ = 0.0551 [*I* > 2 σ (*I*)], *R*₂ = 0.0324, *wR*₂ = 0.0613 (all data), residual electron density = 1.811 e Å⁻³.

23: C₁₇H₁₆AuClN₂S, Mr = 512.79, crystal size = 0.30 × 0.20 × 0.05 mm³, triclinic, space group *P*, *a* = 9.545(1) Å, *b* = 9.931(1) Å, *c* = 10.044(1) Å, α = 103.547(4)°, β = 112.197(4)°,

$\gamma = 97.717(4)^\circ$, $V = 830.4(2)\text{Å}^3$, $Z = 2$, $T = 173(2)\text{K}$, 11653 reflections collected, 4054 unique reflections ($R_{\text{int}} = 0.0366$), $R_1 = 0.0276$, $wR_2 = 0.0679$ [$I > 2\sigma(I)$], $R_1 = 0.0320$, $wR_2 = 0.0702$ (all data), residual electron density = 2.108 e Å^{-3} .

4.3. Biological material and methods

4.3.1. In vitro antimalarial activities

The in vitro antimalarial activities of the 25 synthesized compounds were tested comparatively to their ligands in order to determine the influence of the different structure modulations on the biological activities. Both chloroquine-resistant *P. falciparum* strains FcM29-Cameroon and FcB1-Colombia, were cultured continuously according to the modified Trager and Jensen's method.²¹ The antimalarial activity was evaluated by the radioactive micro-method described by Desjardins et al.²² with modifications as reported.²³

Drug testing was performed from 2 to 5 times in triplicate in 96-well culture plates on D-sorbitol synchronized cultures. Compounds were dissolved in dimethylsulfoxide (DMSO) (stock solution: 10 mg/mL) and further diluted in culture medium so that the final DMSO concentration never exceeded 2%. For each assay, it was verified that the 2% concentration of DMSO did not affect parasite growth. Parasite growth was estimated by [³H]hypoxanthine incorporation (Perkin Elmer, France).

The control parasite culture (culture medium with or without 2% DMSO) was referred to as 100% growth. The IC₅₀ values were determined graphically by plotting the log of the drug concentration versus the percentage inhibition of parasite growth. Results are summarized in Table 1.

4.3.2. Cytotoxicity assays

Evaluation of the cytotoxicity of selected compounds was performed on Vero cell lines (Monkey epithelial cell line, Sigma-Aldrich) or on McF7 cell line with the same dilution protocol used for antiplasmodial assays. Cell lines were distributed in 96-well plates in 100 μL MEM medium with 10% fetal calf serum at 37 °C under a humidified atmosphere (5% CO₂). After 24 h-culture, the cells were supplemented with 100 μL of the compounds dilutions and [³H]hypoxanthine was also added for 48 h more. Cell growth was estimated by [³H]hypoxanthine incorporation read by β counter (1450-Microbeta Triflux, Wallac-Perkin Elmer) as reported for antiplasmodial assays.²⁴ Selectivity index was then calculated by the cytotoxicity/activity ratio.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc.2016.05.023>.

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C. Gene *Pfk13* and Artemisinin Resistance

a. Results

Based on the identification of a candidate marker of artemisinin resistance from the highly artemisinin resistant laboratory strain F32-ART, a study conducted by Ariey *et al.* led to precision of the molecular marker of this resistance in isolated resistant parasites from patients in Cambodia. It was demonstrated that specific mutations in the K13-propeller are strongly correlated with artemisinin resistance, both *in vitro* and *in vivo*. Three-high frequency mutant alleles with high survival rates determined by RSA_{0-3h} were Y493H, R539T, and C580Y [24] and long parasite clearance half-lives (>5 hours) in patients [207]. On the other hand, first K13 mutant allele that have been observed was M476I. It was found in Tanzanian F32 parasites that experienced long-term artemisinin pressure *in vitro* for over 5 years, yielding the F32-ART line [22].

Therefore, the molecular mechanism of this resistance would be defined by determining the impact of *Pfk13* gene mutations on the levels of artemisinin resistance in clinical isolates in lab strains and assessing the role of individual polymorphism in certain types of genetic backgrounds. The study was a collaborative work between CNRS-LCC- (Dr F. Benoit-Vical, Toulouse, France), Columbia University College of Physicians and Surgeons (Dr D. Fidock, New York, USA), Institut Pasteur Paris (Dr O. Mercereau-Puijalon, France), and Institut Pasteur du Cambodge (Dr D. Ménard, Cambodia).

Zinc-finger nucleases (ZFNs) were successfully used to modify K13 genetically culture-adapted Cambodian isolates and the well-known reference lines of *P. falciparum* (Dd2, FcB, V1/S, F32-ART). ZFNs promoted double-stranded break in K13 genomic target locus followed by DNA resection and repair, allowing to capture the mutations transferred by pZFN^{K13}-*hdhfr* plasmids.

Assessment of mutations insertion or removal was conducted through RSA_{0-3h} assay on parenteral and edited parasites. The 0-3 hours of post-invasion ring (1% parasitemia, 2% hematocrit) were exposed to 700 nM Dihydroartemisinin (DHA) for 6 hours. To remove drug, a washing step was employed by adding culture medium and followed by returning fresh medium without drug to culture conditions for 66 hours. The survival rate was determined by assessing parasite viability using microscopic examination of Giemsa-stained thin blood smears.

Higher survival rates were obtained from Cambodian isolates (Cam3.I^{R539T} and Cam3.II^{R539T}) containing the R539T mutation. In contrast, significantly lower survival rates were found when the R539T mutation was removed from the isolates, restoring therefore the wild type allele (Cam3.I^{rev} and Cam3.II^{rev}). Same results were observed with I543T mutation removal (43% survival rates in Cam5^{I5435T} versus 0.3% in Cam5^{rev}) and C580Y mutation removal (13% survival rates in Cam2^{C580Y} versus 2.4% in Cam2^{rev}).

Moreover, survival rates increased when K13 mutation was introduced to wild type carrying parasite (CamWT, Cam3.II^{rev}, V1/S, F32-TEM and FcB). In details, C580Y to CamWT (0.6% survival rate in CamWT to 8.9% in CamWT^{C580Y}), Cam3.II^{rev} and C580Y (0.7% in Cam3.II^{rev} and 24.1% in Cam3.II^{C580Y}), R539T to V1/S (0.3% in V1/S versus 20.7% in V1/S^{R539T}) showed significant increase, while it was moderate with M476I for F32-TEM and C580Y for FcB.

To examine the differences of K13 mutations in ART resistance level, M476I, R539T, I543T were inserted in the Dd2 reference line resulting in higher survival rates than when Y493H and C580Y were introduced. Although C580Y mutation was predominant in Cambodia, Laos and Vietnam [28], the associated survival rates increased modestly. The variety of survival rates obtained after the insertion of the mutation C580Y into different clones

like Dd2, FcB, and three Cambodian isolates indicated the influence of the parasite genetic background. This phenomenon was also found in other Asian countries, but not in the most endemic malaria region, Africa, because the Cambodian parasite has restricted genetic admixture that contains distinctive K13 mutation as opposed to the one in Africa.

Latest publications determined that non-synonymous K13 mutations were located in Southeast Asia to China with proportion ranging from moderate (10-20%) to fixed (> 95%), and were confirmed to be associated with artemisinin resistance. Based on geographical localization, there are two areas harboring specific individual mutations including Cambodia, Vietnam, and Laos for C580Y, R539T, Y493H, and I543T and Thailand, Myanmar, and China for F446I, N458Y, P574L, and R561H. K13 mutations were uncommon in Africa, but were thought to be found in few African countries (the proportion ranging from 3.0% to 8.3%). Further identifications showed that there was no Asian K13 mutation allele in African samples. Moreover, the fourth most frequent mutant allele in Africa observed in one sample from Thailand, A578S, was clearly defined as not correlated to artemisinin-resistance. It is suggested that for the moment, no ART-resistance was reported in Africa. Furthermore, no Asian ART-resistant alleles were found in Africa isolates [28].

It has been demonstrated that K13-propeller mutations lead to ART resistance *in vitro* and are a relevant marker of this resistance in Asia. Hence, it is worth considering that global sequencing effort for K13 is conducted to trace the distribution of ART resistance and to initiate the impact mitigation for supporting world malaria elimination program, especially in highest incidence level area in Africa. Furthermore, K13 mutations monitoring is included in the WHO criteria to follow ART resistance worldwide.

b. Corresponding publication

RESEARCH | REPORTS

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SUPPORTING ONLINE MATERIAL

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Materials and Methods

Figs. S1 to S16
Tables S1 to S3
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DRUG RESISTANCE

K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates

Judith Straimer,¹ Nina F. Gnädig,¹ Benoît Witkowski,^{2,3*} Chanaki Amaratunga,^{3,4*} Valentine Duru,^{2,5*} Arba Pramundita Ramadani,^{4,5,6,†} Mélanie Dacheux,¹ Nimol Khim,² Lei Zhang,⁶ Stephen Lam,⁶ Philip D. Gregory,⁶ Fyodor D. Urnov,⁶ Odile Mercereau-Pujalon,⁷ Françoise Benoit-Vical,^{4,5,†} Rick M. Fairhurst,^{3,†} Didier Ménard,^{2,†} David A. Fidock^{1,8,9‡}

The emergence of artemisinin resistance in Southeast Asia imperils efforts to reduce the global malaria burden. We genetically modified the *Plasmodium falciparum* K13 locus using zinc-finger nucleases and measured ring-stage survival rates after drug exposure in vitro; these rates correlate with parasite clearance half-lives in artemisinin-treated patients. With isolates from Cambodia, where resistance first emerged, survival rates decreased from 13 to 49% to 0.3 to 2.4% after the removal of K13 mutations. Conversely, survival rates in wild-type parasites increased from $\leq 0.6\%$ to 2 to 29% after the insertion of K13 mutations. These mutations conferred elevated resistance to recent Cambodian isolates compared with that of reference lines, suggesting a contemporary contribution of additional genetic factors. Our data provide a conclusive rationale for worldwide K13-propeller sequencing to identify and eliminate artemisinin-resistant parasites.

The worldwide use of artemisinin (ART)-based combination therapies (ACTs) for the treatment of *Plasmodium falciparum* malaria is the foundation of renewed efforts to eradicate this leading cause of childhood mortality (1, 2). The pharmacodynamic properties of clinically used ART derivatives [artesunate, artemether, and dihydroartemisinin (DHA)] can reduce the biomass of drug-sensitive parasites by four orders of magnitude every 48 hours (3), corresponding to a single cycle of asexual blood-stage *P. falciparum* development. The short half-life (typically <1 hour) of ART derivatives in plasma necessitates the use of longer-lasting partner drugs that can eliminate residual parasites once the ART component has dropped to subtherapeutic concentrations (4). The use of ACTs in expanded malaria control and elimination programs has yielded notable successes in recent years, contributing to an estimated 30%

reduction in global mortality rates in the past decade (5).

These impressive gains, however, are now threatened by the emergence of ART resistance, first detected in western Cambodia and now observed in Thailand, Vietnam, and Myanmar (6, 7). The severity of this situation is underscored by the fact that resistance to piperazine, an ACT partner drug, is emerging in western Cambodia (8, 9). No alternative, fully effective first-line therapy is currently available to replace ACTs, should ART fail globally. Clinically, ART resistance is defined as a long parasite clearance half-life (the time it takes for the peripheral blood parasite density to decrease by 50%) after treatment with ART monotherapy or an ACT (6, 10, 11). This metric correlates with the percentage of early “ring-stage” parasites (0 to 3 hours after invasion of human erythrocytes) that survive a pharmacologically relevant exposure to DHA (the active metabolite

of all ARTs), as measured in the in vitro Ring-stage Survival Assay (RSA_{0-3h}) (12).

Recently, mutations in the propeller domain of the K13 gene were identified as candidate molecular markers of ART resistance (13). This gene resides on chromosome 13 of the *P. falciparum* genome, near regions earlier associated with slow parasite clearance rates (14–16). K13 belongs to the kelch superfamily of proteins, whose propeller domain harbors multiple protein-protein interaction sites and mediates diverse cellular functions, including ubiquitin-regulated protein degradation and oxidative stress responses (17). The K13 M476I mutation was first observed in Tanzanian F32 parasites that were exposed in vitro to escalating concentrations of ART over 5 years, yielding the F32-ART line (13, 18). [Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. In the mutants, other amino acids were substituted at certain locations; for example, M476I indicates that methionine at position 476 was replaced by isoleucine.] Subsequent genomic analysis of Cambodian isolates identified four prevalent K13-propeller mutations (Y493H, R539T, I543T, and C580Y) that were associated with elevated RSA_{0-3h} survival rates in vitro and long parasite clearance half-lives (>5 hours) in patients (13, 19).

¹Department of Microbiology and Immunology, Columbia University College of Physicians and Surgeons, New York, NY, USA. ²Malaria Molecular Epidemiology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia. ³Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA. ⁴Centre National de la Recherche Scientifique (CNRS), Laboratoire de Chimie de Coordination UPR824L, Toulouse, France. ⁵Université de Toulouse, UPS, Institut National Polytechnique de Toulouse, Toulouse, France. ⁶Sangamo BioSciences, Richmond, CA, USA. ⁷Institut Pasteur, Parasite Molecular Immunology Unit, Paris, France. ⁸Division of Infectious Diseases, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY, USA.

*These authors contributed equally to this work. †Present address: Department of Pharmacology and Therapy, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. ‡To whom correspondence should be addressed. E-mail: df2260@columbia.edu

Determining whether K13-propeller mutations confer ART resistance in clinical isolates and assessing the contributions of individual polymorphisms in distinct genetic backgrounds is essential to defining the underlying molecular mechanisms.

We developed zinc-finger nucleases (ZFNs) (20) to enable targeted genetic engineering of *K13* in newly culture-adapted Cambodian isolates and older established reference lines of *P. falciparum* (tables S1 and S2). ZFNs were introduced into cultured intra-erythrocytic parasites via electroporation with plasmids containing *K13* donor templates. ZFNs triggered double-stranded breaks in the *K13* genomic target locus of this haploid organism, leading to DNA resection and repair events that captured mutations delivered by pZFN^{K13}-hdhfr plasmids (fig. S1). Donor plasmids contained additional synonymous mutations that preclude ZFN binding while preserving the K13-translated amino acid sequence across that same stretch of DNA base pairs. These silent ZFN binding-site mutations protected the donor sequence and prevented the edited recombinant locus from being recleaved by the nucleases. Plasmids contained either the wild-type *K13* allele or one of several mutations (present in the six-blade K13-propeller domain) found in ART-resistant Cambodian isolates or F32-ART. This strategy successfully introduced or removed mutations in a set of *P. falciparum* clinical isolates from Cambodia, the epicenter of emerging ART resistance, as well as reference laboratory lines from distinct geographic origins (Fig. 1 and table S3). Of note, RSA_{0-3h} assays comparing parental and edited control parasites showed no difference if only the binding-site mutations were introduced into the K13-propeller domain, indicating that these synonymous mutations were phenotypically silent

(Fig. 1 and fig. S2). Independent assays with the same parasite lines tested by our different groups yielded consistent survival rates between laboratories (fig. S3).

Using donor plasmids containing a wild-type *K13*-propeller sequence and silent binding-site mutations, we generated a series of clones in which individual K13 mutations were removed from ART-resistant Cambodian isolates. One of these isolates (Cam3.II) showed slow clearance after ART monotherapy (in vivo half-life 6.0 hours) (table S2). Parental Cam3.I^{R539T} and Cam3.II^{R539T} isolates harboring the R539T mutation showed 40 to 49% RSA_{0-3h} survival, whereas edited Cam3.I^{WT} and Cam3.II^{WT} clones carrying the reverted wild-type allele showed only 0.3 to 0.7% survival (Fig. 2, A and B, and table S4). These highly significant differences in the survival rates of ring-stage parasites exposed to elevated DHA concentrations confirm the importance of R539T in mediating in vitro ART resistance in Cambodian isolates. Significant reductions in RSA_{0-3h} survival rates were also observed upon removal of I543T (4.3% in Cam5^{I543T} versus 0.3% in Cam5^{WT}) (Fig. 2C) and C580Y (1.3% in Cam2^{C580Y} versus 2.4% in Cam2^{WT}) (Fig. 2D).

We also assessed the impact of introducing K13 mutations into a fast-clearing Cambodian isolate (CamWT; in vivo half-life 3.7 hours) (table S2), the Cam3.II^{WT} clone, and three reference lines (V1/S, F32-TEM, and FCB). CamWT and Cam3.II^{WT} parasites harboring wild-type *K13* alleles showed 0.6 to 0.7% RSA_{0-3h} survival, whereas the corresponding C580Y-edited clones yielded 9 and 24% survival, respectively (Fig. 2, E and F). Introducing R539T into V1/S caused a similar increase in RSA_{0-3h} survival (0.3 to 21%) (Fig. 2G and table S4). Editing F32-TEM to express M476I

caused a moderate increase in RSA_{0-3h} survival (<0.2% in F32-TEM to 1.7% in F32-TEM^{M476I}) (Fig. 2H). We also observed modest in vitro resistance in FCB parasites edited to express C580Y, with RSA_{0-3h} survival increasing from 0.3% in the parental line to 1.9% in FCB^{C580Y} parasites (Fig. 2I). This result differs from a recent study of the use of Cas9 in *P. falciparum*, which reported a greater increase in RSA_{0-3h} survival (11 to 15%) in two clones engineered to express K13 C580Y (21). That report used the drug-sensitive NF54 strain—which was isolated decades before ART use and the emergence of resistance (22)—and did not examine additional mutations or assess the impact of removing K13 mutations from ART-resistant clinical isolates.

In contrast to the substantial changes we observed in the RSA_{0-3h}, standard in vitro dose-response measurements by use of parental and *K13*-edited V1/S and Cam3.II parasites revealed no effect of R539T or C580Y on DHA or artesunate median inhibitory concentration (IC₅₀) values (fig. S4). This finding is consistent with earlier studies that showed no correlation between IC₅₀ values and clinical ART resistance (6, 10, 12).

We subsequently investigated whether individual mutations confer different levels of ART resistance in the RSA_{0-3h}. In the Dd2 reference line, the introduction of M476I, R539T, or I543T mutations conferred considerably higher degrees of resistance than those of Y493H and C580Y (10 to 30% versus 2 to 4% survival, respectively) (Fig. 2J and table S4). These data corroborate the recent observation of higher levels of in vitro resistance in Cambodian isolates containing the R539T mutation as compared with Y493H or C580Y (23).

The relatively modest increase in survival of C580Y-expressing Dd2 parasites compared with

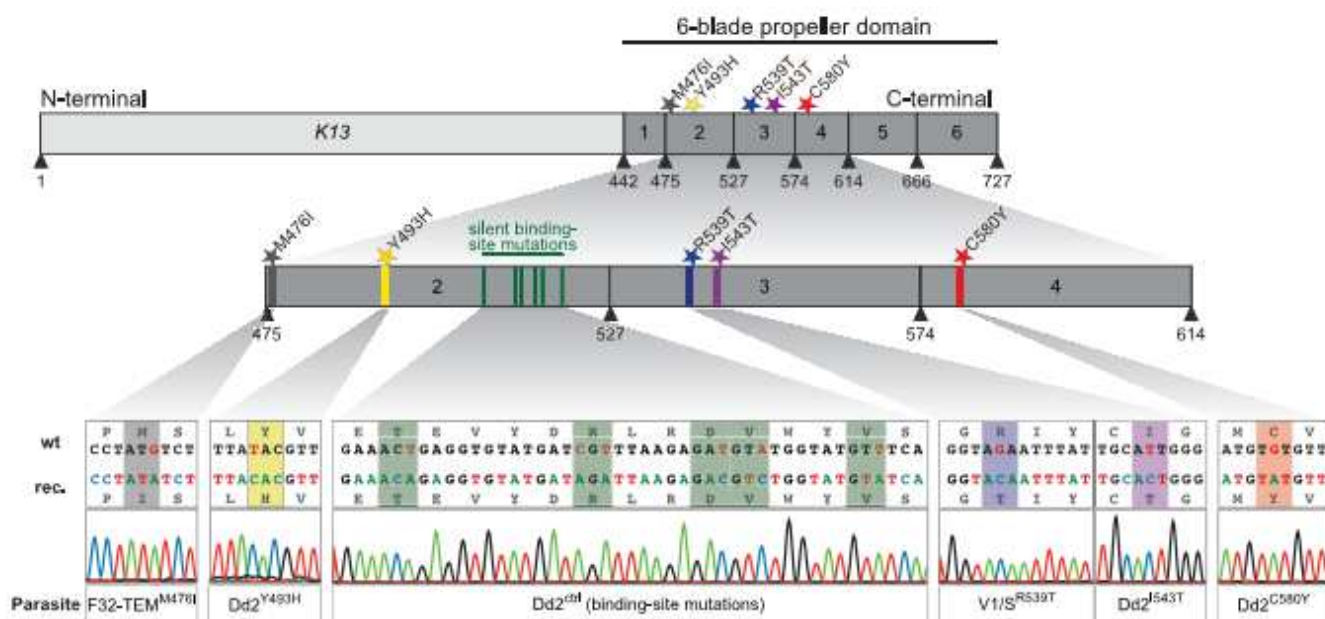


Fig. 1. Genetic modification of the K13-propeller domain. Location of K13-propeller mutations and sequencing results showing the insertion of individual mutations into recombinant parasites used in the RSA_{0-3h}. Dd2^{WT} parasites contain only synonymous, phenotypically silent binding-site mutations and showed 0.7% survival rates, which is equivalent to those of parental Dd2 parasites (fig. S2).

R539T- and I543T-expressing clinical isolates and edited clones was quite unexpected, given that C580Y has rapidly become the predominant mutant allele in western Cambodia (7, 13). We thus explored the impact of C580Y in different genetic backgrounds. Introducing C580Y conferred greater levels of resistance in three Cambodian isolates as compared with Dd2 and FCB parasites (Fig. 2K), suggesting a role for additional parasite factors in augmenting K13-mediated resistance in these contemporary field isolates. The disparity between relatively low in vitro resistance conferred by C580Y and its widespread dissemination in Cambodia might be explained by a lower fitness cost or increased transmission potential of C580Y-expressing parasites, or by the parasite genetic background.

Cambodian parasites are specifically characterized by sympatric subpopulations that show only limited genetic admixture and that generally harbor distinct K13 mutations (16). These findings suggest that K13 mutations might have arisen preferentially on backgrounds with favorable genetic factors. In this context, recent comprehensive analyses of K13 mutations across multiple sites in Southeast Asia have documented a series of additional mutations associated with slow-clearance rates in Cambodia, Thailand, Myanmar, Laos, and Vietnam (7, 24). K13 mutations have also been observed in African isolates (7, 25, 26), although none of these correspond to the most prevalent mutations in Cambodia, and ART or ACT treatments in African sites continue to show a

high level of efficacy (7). A recent deep-sequencing study of the K13-propeller domain in more than 1110 *P. falciparum* infections collected from 14 sites across sub-Saharan Africa identified a large reservoir of naturally occurring K13-propeller variation, whose impact on artemisinin susceptibility is unknown and requires further investigation. These polymorphisms include one rare mutation previously observed in Cambodia (P553L) and several others (including A578S) close to known resistance-causing mutations in the propeller domain (26). Our gene-editing system can now be used to comprehensively dissect K13 polymorphisms across malaria-endemic regions and identify those that confer ring-stage ART resistance.

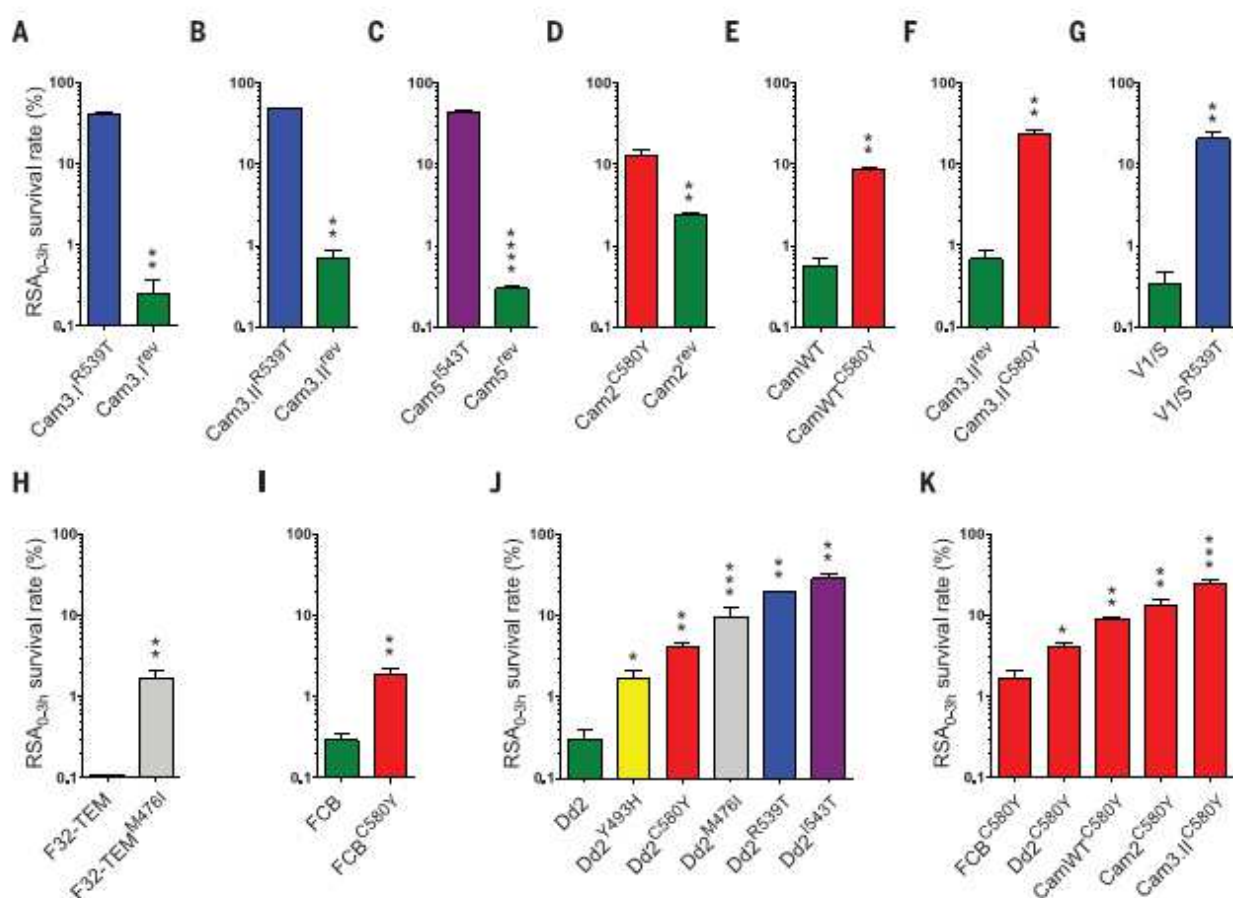


Fig. 2. K13-propeller mutations confer artemisinin resistance in clinical isolates and reference lines in vitro, as defined in the RSA_{0-3h}. Results show the percentage of early ring-stage parasites (0 to 3 hours after invasion of human erythrocytes) that survived a 6-hour pulse of 700 nM DHA (a pharmacologically relevant concentration of the active metabolite of ARTs), as measured by microscopy 66 hours later. Data show mean \pm SEM percent survival compared with control dimethyl sulfoxide-treated parasites processed in parallel. (A to D) RSA_{0-3h} survival for Cambodian isolates harboring native K13 mutations (shown in superscript) and ZFN-edited isogenic clones carrying wild-type K13 alleles (superscript "rev"). (E to I) RSA_{0-3h} survival for Cambodian isolates and reference lines harboring wild-type K13 alleles and ZFN-edited isogenic clones carrying individual K13 mutations (shown in superscripts). (J) Impact of different K13 mutations on RSA_{0-3h} survival in the Dd2 reference line, showing that I543T and R539T confer

the highest levels of resistance. (K) Introduction of C580Y into multiple Cambodian clinical isolates and reference lines, showing that this mutation confers varying degrees of in vitro resistance depending on the parasite genetic background. The geographic origins and known drug-resistance genotypes of these isolates and lines are provided in table S2. Results were obtained from 3 or 4 independent assays performed in duplicate (values provided in table S4; F32-TEM showed <0.2% RSA_{0-3h} survival). Two-sample *t* tests with unequal variances (performed with the STATA package) were used to assess for statistically significant differences between K13-edited clones and their comparator lines—the parental isolates listed on the left in (A) to (J) and the FCB^{C580Y} clone in (K) (**P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001). Statistical outputs (including calculations of the SE of the difference between the means of samples being compared and the *P* values) are listed in the supplementary materials.

Mode-of-action studies have shown that ARTs are active against all asexual blood stages of parasite development. In the more mature trophozoite stages, ARTs are activated after hemoglobin degradation and liberation of reactive heme whose iron moiety can cleave the endoperoxide linkage of these sesquiterpene lactone drugs (27). Activation generates free radicals that are thought to trigger oxidative stress and damage cellular macromolecules, including parasite membrane components, proteins, and neutral lipids (28, 29). Recent evidence suggests that hemoglobin degradation begins early after merozoite invasion, potentially providing a source of ART activator in ring-stage parasites (30). Our *RS_{AS}* data support earlier evidence that reduced ring-stage susceptibility accounts for the clinical phenotype of slow parasite clearance after ART treatment (12, 31). K13 mutations might achieve this by protecting parasites from the lethal effects of ART-induced oxidative damage, potentially via a cellular pathway similar to antioxidant transcriptional responses regulated by the mammalian ortholog Keap1 (32). Our set of K13-modified isogenic parasites with different levels of ART resistance on distinct genetic backgrounds now enables a search for K13-interacting partners and delivers tools to interrogate the underlying mechanism.

Our data demonstrate a central, causal role for K13-propeller mutations in conferring ART resistance in vitro and provide a molecular explanation for slow parasite clearance rates in patients (6, 7, 10). By exposing greater parasite biomasses to ACTs in vivo, K13-propeller mutations may promote the evolution of partner drug resistance (8, 9) and higher-grade ART resistance. Our study thus offers a conclusive rationale for a global K13 sequencing effort to track the spread of ART resistance and mitigate its impact on malaria treatment and control programs, particularly in hyperendemic regions in Africa.

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(also known as IPC 3445); MRA-1254, Cam2.Rev; MRA-1250, Cam1WT; MRA-1251, Cam1WTCS80Y; MRA-150, Dd2; MRA-1255, Dd2R539T. Parasite lines generated for this study will also be provided upon request from D.A.F. Requests for ZFNs should be directed to F.D.U. (Rimov@Sangamo.com); a materials transfer agreement is required. We extend our gratitude to F. Anley (Institut Pasteur, Paris) for his important contribution to initiating this study, I. McKeague and O. Lieberman (Columbia University Medical Center) for their statistical and scientific input, and E. Rebar and the Production Group at Sangamo BioSciences for ZFN assembly and validation. L.Z., S.L., P.D.G., and F.D.U. declare that they are full-time employees of Sangamo, which designed, validated, and provided the ZFNs used in this study. B.W., Q.M.-P., F.B.-V., and D.M., are co-inventors on the pending patents US61/504651 and US62/062439 and N.K. is a co-inventor on the pending patent US62/062439. Both patents are filed by Institut Pasteur. These patents cover the use of K13 mutations as a molecular marker of *P. falciparum* ART resistance. Sangamo holds patents on engineered DNA-binding proteins and the use thereof in targeted genome engineering and gene-specific regulation. All other authors declare no competing financial interests.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/347/6220/428/suppl/DC1
Materials and Methods
Figs. S1 to S4
Tables S1 to S5
Results of two-sample *t* tests with unequal variances
References (33–36)

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DRUG RESISTANCE

Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance

Sachel Mok,¹ Elizabeth A. Ashley,^{2,3} Pedro E. Ferreira,¹ Lei Zhu,¹ Zhaoting Lin,¹ Tomas Yeo,¹ Kesinee Chotivanich,⁴ Mallika Imwong,⁵ Sasithon Pukrittayakamee,⁴ Mehul Dhorda,^{3,6,7} Chea Ngoun,⁸ Phurath Lim,^{8,9} Chanaki Amaralunga,⁹ Seila Suon,⁸ Tran Tinh Hien,¹⁰ Ye Htut,¹¹ M. Abul Faiz,¹² Marie A. Onyamboko,¹³ Mayfong Mayxay,^{14,15} Paul N. Newton,^{2,3,16} Rupam Tripura,² Charles J. Woodrow,^{2,3} Olivo Miotto,^{2,16,17} Dominic P. Kwiatkowski,^{16,17} François Nosten,^{2,18} Nicholas P. J. Day,^{2,3} Peter R. Preiser,¹ Nicholas J. White,^{2,3} Arjen M. Dondorp,^{2,3} Rick M. Fairhurst,^{2,3} Zbynek Bozdech^{1*}

Artemisinin resistance in *Plasmodium falciparum* threatens global efforts to control and eliminate malaria. Polymorphisms in the kelch domain-carrying protein K13 are associated with artemisinin resistance, but the underlying molecular mechanisms are unknown. We analyzed the in vivo transcriptomes of 1043 *P. falciparum* isolates from patients with acute malaria and found that artemisinin resistance is associated with increased expression of unfolded protein response (UPR) pathways involving the major PROSC and TRIC chaperone complexes. Artemisinin-resistant parasites also exhibit decelerated progression through the first part of the asexual intraerythrocytic development cycle. These findings suggest that artemisinin-resistant parasites remain in a state of decelerated development at the young ring stage, whereas their up-regulated UPR pathways mitigate protein damage caused by artemisinin. The expression profiles of UPR-related genes also associate with the geographical origin of parasite isolates, further suggesting their role in emerging artemisinin resistance in the Greater Mekong Subregion.

Artemisinin resistance in *Plasmodium falciparum* is spreading rapidly throughout Southeast Asia. Since it was first detected 7 years ago in Pailin, western Cambodia (1), artemisinin resistance has

become prevalent in other Cambodian provinces (2, 3), Thailand-Myanmar border areas (4, 5), and southern Vietnam (4, 6) and is emerging in southern Laos and central Myanmar (4). Artemisinin resistance threatens the

D. Dihydroartemisinin-Piperaquine Resistance in Kupang

Regarding the emergence of ART resistance and its spread in Asia, it is important to evaluate the level of ACTs resistance in Indonesia. Monitoring of K13 mutations is part of the WHO criteria to investigate ART resistance worldwide. Consequently, this gene is important to be examined during malaria clinical trials in Kupang to evaluate the possible ART resistance emergence in Indonesia.

Even though there was no report of any treatment failure yet after ACTs treatment and linked to ART resistance in Indonesia, this study was undertaken on isolates coming from areas where *P. falciparum* has been recorded as strongly present to assess whether ACTs resistance can be detected through *ex-vivo* RSA assay.

Ethics approval submission was the first step of the research parallel to a study of the population to define the most appropriate area among the endemic areas of malaria in Indonesia. Patients were eligible if they were aged 2-65 years, diagnosed for non-complicated malaria caused by particularly *P. falciparum* (slide-confirmed malaria), having fever (a tympanic temperature $\geq 37.5^{\circ}\text{C}$) or history of fever 48 h preceding presentation and parasite density no more than 200,000 parasites per μL with minimum 0.5% parasitemia, seeking treatment in primary health center or regency referral hospital, and agreeing to sign the informed consent and follow the study. Patients were excluded if they were pregnant or lactating women, children under 5 kg, already taking malaria treatment for 4 weeks in advance, diagnosed for complicated malaria (co-infection), infected by other *Plasmodium* species than *P. falciparum*, and having signs of severe malaria, severe malnutrition, serious underlying disease (renal, cardiac, or hepatic), or known allergy to the study drugs.

Dihydroartemisinin-Piperaquine (Darplex[®]) was administered during the first 3 days of the study and a follow-up was conducted until day 42. To perform *ex-vivo* RSA and K13 gene

identification, parasites were isolated from 3 mL of patient's venous blood placed in ACD coagulant tube just prior to taking the drug. A couple drops of blood were examined for its parasitemia (thin smear), $\pm 100 \mu\text{L}$ was dropped into filter paper (Whatman® paper, Human ID Bloodstain card) for DNA extraction, and the remaining was prepared for RSA *ex-vivo*.



Figure 13. Position of Kupang (circled) in the map of Indonesia [208].

After several evaluations based on the number of patients, laboratory facilities, and other factors, the most relevant area to conduct the research was Kupang Regency, East Nusa Tenggara, one of the areas with high malaria incidence in Indonesia. Furthermore, according to the Department of Health of Kupang (internal epidemiology data, 2015), there were 64,953

malaria cases in East Nusa Tenggara province and 831 positive cases of *P. falciparum* malaria found in Kupang Regency during 2014, and it was noted that there were 50 *P. falciparum* malaria patients seeking treatment in primary health-care center of Kupang Regency from January to June 2015 (based on a direct observation at the primary health-care center of Kupang Regency). The distance between Kupang Regency and the laboratory of BPOM Kupang as the laboratory center for conducting RSA assay also contributed to the choice of this population area. Since the patients' blood withdrawal had limited time to be tested for its ACT resistance, the distance between the primary health-care center and laboratory became important. In the meantime, the ethics approval (ref. number: KE/FK/222/EC) from the Medical and Health Research Ethics Committee of the Faculty of Medicine, Gadjah Mada University, and research permits from each primary health-care center in Kupang Regency, referral hospital and BPOM Kupang were obtained prior to the beginning of the study.

After one year of study, only 6 patients of *P. falciparum* malaria enrolled among whom one was developing severe malaria and one refused to continue the study after a week of follow-up. As a result, only four patients met the criteria and were followed up for the study. The small number of *P. falciparum* malaria patients that had been found was contrary to the previous data collected from direct observation at the primary health-care centers of Kupang Regency (2015). It might be so because there was a decrease in the malaria prevalence in this area. Based on the report of the Department of Health, there were 36,128 malaria cases reported in this province during 2015 [209]. This number indicated a significant decline in malaria prevalence from 13 API level in 2014 to 7 in 2015. Additionally, the national annual parasite also reduced by 0.99 to 0.85 in 2015 [4]. This finding was likely to represent the limited number of cases in Indonesia, particularly during our study in Kupang. Furthermore, this phenomenon occurs not only in Indonesia but also in other countries. A recent publication mentioned that malaria cases were declining by 54 % for last 3 years in Greater Mekong Sub-region (GMS) [210]. Several

factors that might influence the decrease number of malaria cases were climatic and non-climatic.

Climatic factors include temperature, rainfall, and relative humidity. The optimum temperature for parasite development is 27°C. Temperature raise delays the parasite development in the gut of mosquito (more than 10 days). In addition, when the rain is delayed, the breed of mosquitos decreases. Moreover, lower relative humidity reduces the survival time of mosquito to transmit malaria. Based on the data from Metrological, Climatological, and Geophysical Agency (BMKG), the temperature in Kupang reached 31.07°C with 65-90% humidity and 362 mm average rainfall. This condition was highly likely to affect malaria transmission.

Non-climatic factors including urbanization and human host can have an impact on the transmission. The incidence of malaria is lower in urban area than in rural area, but the development in rural area may also influence malaria distribution. Moreover, there is a possibility that people already have immunity which makes better tolerance to the effects of malaria, and non-immune people believe that being infected by malaria is the same as common cold and does not need serious treatment in healthcare center. This makes many malaria cases under reported.

In addition, malaria elimination program run by the government also takes part in case diminution. Specific strategies have been implemented by the government to expedite the elimination program. Since Kupang is one of the high malaria endemic areas, the acceleration strategies have been employed through mass ITNs campaign, early malaria detection and appropriate treatment [4]. The evidence of the number of malaria cases reduction may indicate that the elimination program runs well.

From the six eligible patients, bloods were taken and prepared for *ex-vivo* RSA. Thin blood smears were made to confirm the diagnosis and determine the parasitemia ratio. Unfortunately, all the blood samples which were delivered to the laboratory could not allow the detection of parasite presence although it has been previously held as positive *P. falciparum* malaria. We did not have access to the original smears in order to re-confirm whether the initial parasitemia met the eligible criteria ($> 0.5\%$). Therefore, the *ex-vivo* RSA could not be carried out though the analysis of K13 mutation is currently in progress. Some possibilities are offered to assess the causes and provide the solutions.

Theoretically, the blood must be processed right after having been taken since delayed processing can potentially undermine the parasite condition [211]. If the distance between the site of blood withdrawal and the laboratory to conduct the RSA assay is quite long, the blood should be transported in a cold temperature. It took approximately two hours from Kupang Regency to BPOM Kupang in Kupang city, therefore the patient's blood was stored in an ice box during transportation. However, this did not work. A sudden temperature change from the patient's body to the storage condition might contribute to this problem. Then, an approach was made by modifying the storage method. The blood was stored in a cold temperature (2-10°C) during the transport to the laboratory and placed in a room temperature during the preparation in laboratory. Yet, it did not work either. Moreover, since the authorized working hours in the laboratory were limited, the blood preparation was not possible to process on the same day.

Another problem was related to the diagnosis process. The diagnosis was made by physicians based on patient's blood smears prepared by technicians. Not all of the technicians were certified giving an impact on smear evaluation. There was a possibility that false positive diagnosis occurred.

These findings lead us to the following perspectives. Looking for another feasible study area which has a large number of *P. falciparum* cases, adequate laboratory facility and more flexible access to conduct the study outside working hours are strongly suggested. Furthermore, in order to perform RSA assay, it is important to understand the Indonesian parasite characteristic by modification of blood transport and storage conditions if a field laboratory is not available. Also, as K13 has a great impact on malaria control in Cambodia, it is interesting to work on K13 screening not only in one endemic district but also in several other endemic regions in Indonesia to get whole a map of K13 existence in Indonesia.

CONCLUSION AND PERSPECTIVES

A. CONCLUSION

Various efforts and studies have been undertaken to reduce malaria prevalence all around the world. This study points out several strategies for combating malaria particularly in antimalarial drug resistance. The approaches to solve antimalarial resistance problem include discovering new drugs, understanding the resistance mechanism, and investigating the occurrence of ART resistance in endemic area through identification of K13 gene mutation.

The first approach is discovering new drug by assessing selected medicinal plants in Indonesia, namely *T. diversifolia*, *C. barbata*, *T. crispa*, *A. flava* and *P. cauliflora*. Those plants represent not only good antiplasmodial activity but also potential antibabesial and antileishmanial with *P. cauliflora* as the most promising medicinal plant for further investigation.

Potential new drugs are also assessed by synthesizing organometallic compounds. Gold complex combined with NHCs is prospective as a new antimalarial synthetic drug since it exerts good antiplasmodial activity against CQ-resistant strain, especially in the cationic form, and has lipophilic properties.

The second approach is done by understanding the role of K13-propeller mutation in artemisinin resistance. K13-propeller mutation mediates the occurrence of artemisinin resistance *in vitro* on both Cambodian clinical isolates and laboratory lines.

The last approach is identification of K13-gene mutation in Kupang, one of the endemic regions in Indonesia. Unfortunately, it could not be established due to the limited number of samples.

B. PERSPECTIVES

As Indonesia government intends to achieve malaria-free state by 2030, several schemes on antimalarial drug resistance should be dealt with in advance. Some of the schemes were explored in this study resulting in several outlooks as follows.

First, selected plants from Indonesia have been proved to possess good antiplasmodial activity. Therefore, further phytochemical investigation is essential to explore the active compound of promising plants in order to discover new antimalarial drug. Additionally, a study on their mode of action will be more beneficial for the drug development.

Another drug source, chemical synthesis from gold compound combined with NHC as a ligand, also presents a good activity against CQ-resistant parasite. Hence, immediate investigation on advanced pharmacology assays is required, including exploration of pharmacokinetics profile, stability study, and toxicity study before continuing to a clinical level.

Third, exploring ART resistance mechanism has resulted in confirmation of K13-propeller mutation role as a mediator in ART resistance. Indeed, global attempts for K13 gene sequencing are worth making to figure out the distribution in Southeast Asian countries, particularly in several endemic areas in Indonesia. Moreover, K13 mutation monitoring is included in the WHO criteria to follow ART resistance worldwide.

Following the third perspective, the study on K13-propeller mutation in Kupang, one of the malaria endemic areas in Indonesia, has yet to be clearly conclusive. Follow-up attempts to investigate its occurrence are strongly suggested to look for another feasible study area which has a large number of *P. falciparum* cases, adequate laboratory facility and more flexible access to conduct the study outside working hours. Still, in order to perform RSA assay, it is

important to understand the Indonesian parasite characteristics by modification of blood transport and storage conditions if a field laboratory is not available. Also, as K13 has a great impact on malaria control in Cambodia, it is interesting to work on K13 screening not only in one endemic district but also in several other regions in Indonesia to obtain the whole map of K13 existence in Indonesia.

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ANNEXES

Annex 1. Plant determination certificate: *T. diversifolia*

LABORATORY OF PHARMACEUTICAL BIOLOGY
DEPARTMENT OF PHARMACY
UNIVERSITAS ISLAM INDONESIA
Jl. Kaliurang km 14,5 Ngemplak Sleman, Yogyakarta
Phone: (0274)895920 ext 4033, 4034

CERTIFICATE

Number: 007-02/L-PB/UII/2013

Customer name : Arba Pramundita Ramadani
Address : Dept of Pharmacology, Faculty of Medicine, Gadjah Mada
University, Yogyakarta, Indonesia
Phone : +62-81804209884
Date of determination : August 1st 2013

The undersigned explained that the plant determination was performed using aerial part of *kembang bulan* in the Laboratory of Pharmaceutical Biology.

The result was :

Thithonia diversifolia

I certify that this letter is a true and accurate record of the certificate of plant determination.

Yogyakarta, August 7th 2013
Head of Pharmaceutical Biology Laboratory



Hady Anshory T, M.Sc., Apt

Annex 2. Plant determination certificate: *C. barbata*

LABORATORY OF PHARMACEUTICAL BIOLOGY
DEPARTMENT OF PHARMACY
UNIVERSITAS ISLAM INDONESIA
Jl. Kaliurang km 14,5 Ngemplak Sleman, Yogyakarta
Phone: (0274)895920 ext 4033, 4034

CERTIFICATE

Number: 008-03/L.S-PB/UII/2013

Customer name : Arba Pramundita Ramadani
Address : Dept of Pharmacology, Faculty of Medicine, Gadjah Mada
University, Yogyakarta, Indonesia
Phone : +62-81804209884
Date of determination : August 1st 2013

The undersigned explained that the plant determination was performed using aerial part of *Cincau* in the Laboratory of Pharmaceutical Biology.

The result was :

Cyclea barbata

I certify that this letter is a true and accurate record of the certificate of plant determination.

Yogyakarta, August 7th 2013
Head of Pharmaceutical Biology Laboratory

Hady Anshory T, M.Sc., Apt

Annex 3. Plant determination certificate: *T. crispa*

LABORATORY OF PHARMACEUTICAL BIOLOGY
DEPARTMENT OF PHARMACY
UNIVERSITAS ISLAM INDONESIA
Jl. Kaliurang km 14,5 Ngemplak Sleman, Yogyakarta
Phone: (0274)895920 ext 4033, 4034

CERTIFICATE

Number: 009-03/S-PB/UII/2013

Customer name : Arba Pramundita Ramadani
Address : Dept of Pharmacology, Faculty of Medicine, Gadjah Mada
University, Yogyakarta, Indonesia
Phone : +62-81804209884
Date of determination : August 3rd 2013

The undersigned explained that the plant determination was performed using aerial part of *Brotowali* in the Laboratory of Pharmaceutical Biology.

The result was :

***Tinospora crispa* (L.) Miers**

I certify that this letter is a true and accurate record of the certificate of plant determination.

Yogyakarta, August 7th 2013
Head of Pharmaceutical Biology Laboratory

Hady Anshory T, M.Sc., Apt

Annex 4. Plant determination certificate: *A. flava*

LABORATORY OF PHARMACEUTICAL BIOLOGY
DEPARTMENT OF PHARMACY
UNIVERSITAS ISLAM INDONESIA

Jl. Kaliurang km 14,5 Ngemplak Sleman, Yogyakarta
Phone: (0274)895920 ext 4033, 4034

CERTIFICATE

Number: 010-04/S-PB/UII/2013

Customer name : Arba Pramundita Ramadani
Address : Dept of Pharmacology, Faculty of Medicine, Gadjah Mada
University, Yogyakarta, Indonesia
Phone : +62-81804209884
Date of determination : August 3rd 2013

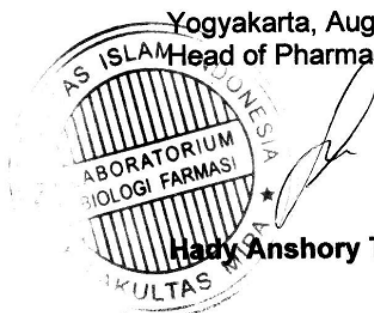
The undersigned explained that the plant determination was performed using lignum part of *Kayu kuning* in the Laboratory of Pharmaceutical Biology.

The result was :

Arcangelisia flava

I certify that this letter is a true and accurate record of the certificate of plant determination.

Yogyakarta, August 7th 2013
Head of Pharmaceutical Biology Laboratory



Hardy Anshory T, M.Sc., Apt

Annex 5. Plant determination certificate: *P. cauliflora*

LABORATORY OF PHARMACEUTICAL BIOLOGY
DEPARTMENT OF PHARMACY
UNIVERSITAS ISLAM INDONESIA
Jl. Kaliurang km 14,5 Ngemplak Sleman, Yogyakarta
Phone: (0274)895920 ext 4033, 4034

CERTIFICATE

Number: 011-01/S.R-PB/UII/2013

Customer name : Arba Pramundita Ramadani
Address : Dept of Pharmacology, Faculty of Medicine, Gadjah Mada
University, Yogyakarta, Indonesia
Phone : +62-81804209884
Date of determination : August 3rd 2013

The undersigned explained that the plant determination was performed using aerial part of *Sengkubak* in the Laboratory of Pharmaceutical Biology.

The result was :

***Pycnarrhena cauliflora* (Miers.) Diels**

I certify that this letter is a true and accurate record of the certificate of plant determination.

Yogyakarta, August 7th 2013
Head of Pharmaceutical Biology Laboratory

Hady Anshory T, M.Sc., Apt

Annex 6. Supplementary materials for publication of K13

www.sciencemag.org/cgi/content/full/science.1260867/DC1

Supplementary Materials for

K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates

Judith Straimer, Nina F. Gnädig, Benoit Witkowski, Chanaki Amaratunga, Valentine Duru, Arba P. Ramadani, Mélanie Dacheux, Nimol Khim, Lei Zhang, Stephen Lam, Philip D. Gregory, Fyodor D. Urnov, Odile Mercereau-Puijalon, Françoise Benoit-Vical, Rick M. Fairhurst, Didier Ménard, David A. Fidock*

*Corresponding author. E-mail: df2260@columbia.edu

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This PDF file includes:

Materials and Methods
Figs. S1 to S4
Tables S1 to S5
Statistical data
References

MATERIALS AND METHODS

Zinc-finger nuclease (ZFN) engineering. ZFNs directed against *K13* (PlasmoDB ID PF3D7_1343700; also referred to as Kelch13) were designed and assembled using an archive of validated modules (33). These were screened for activity using an episomal reporter system, yielding ZFN pairs 18/19 and 18/20 (Table S1).

Plasmid construction. Left and right ZFNs were first linked with the 2A peptide to ensure equal levels of expression driven by a single promoter (20). This ZFN L-2A-ZFN R sequence was cloned into the AvrII and XhoI restriction sites in pDC2 (34). This placed the fused sequence under the regulatory control of the 5' *calmodulin* (PF3D7_1434200) promoter and the 3' *hsp86* (PF3D7_0708500) terminator. This plasmid also contained the human *dhfr* selectable marker (Fig. S1).

We generated six different donor plasmids to introduce either the wild-type *K13* allele or one of five mutant alleles: M476I (allele 1), C580Y (allele 2), R539T (allele 3), Y493H (allele 4) or I543T (allele 5). In a first step we amplified a 1.5-kb donor sequence of the *K13*-propeller domain using the primer pair p1/p2 (Table S5) and cloned this into the pGEM[®]-T vector (Promega). At the zinc-finger binding site we introduced six silent mutations by site-directed mutagenesis using the primer pair p3/p4. Allele-specific mutations were introduced via mutagenesis with specific primer pairs: p5/p6 for M476I, p7/p8 for C580Y, p9/p10 for R539T, p11/p12 for Y493H and p13/p14 for I543T. *K13* donor sequences were excised from the pGEM-T vector with the restriction enzymes SacII and PstI while the ZFN-containing plasmid was digested with SalI and PstI. The two incompatible sites SacII and SalI from the donor fragment and the vector, respectively, were previously filled in using Klenow DNA polymerase. *K13* templates were ligated into the pDC2-based plasmids described above (with primer pairs 18/19 or 18/20, both showing similar efficiency; Table S1) using the compatible PstI site and blunt-end cloning, yielding the final pZFN^{K13}-*dhfr* transfection plasmids.

Parasite cultures, transfections and DNA analysis. Asexual blood-stage parasites were propagated in human erythrocytes in RPMI-1640 medium containing 2 mM L-glutamine, 50 mg/L hypoxanthine, 25 mM HEPES, 0.225% NaHCO₃, 10 mg/L gentamycin and 0.5% (w/v) Albumax II (Invitrogen) for the Dd2, FCB and V1/S reference lines. Cambodian isolates and F32 parasites were further supplemented with 5% human AB⁺ serum (Interstate Blood Bank). Parasites were maintained at 37°C in an atmosphere

of 5% O₂, 5% CO₂ and 90% N₂, and electroporated with purified circular plasmid DNA as described (35). One day after electroporation, parasites were exposed to 2.5 nM or 10 nM WR99210 for 6 days (the latter concentration was reserved for quadruple *dhfr* mutant parasites; Table S3) to select for transformed parasites (Fig. S1A). Parasites generally became microscopically detectable 15–25 days post-electroporation. *K13* editing was assessed by PCR analysis of bulk cultures, and cultures showing the highest level of editing were selected for 96-well cloning by limiting dilution (Table S2). Clones were identified after 17–28 days by staining with 2× SYBR Green I and 1.6 μM MitoTracker Deep Red (Invitrogen) and assaying for growth by flow cytometry on an Accuri C6 cytometer (36). To test for *K13* editing, *P. falciparum* trophozoite-infected erythrocytes were harvested and saponin-lysed. Parasite genomic DNA was extracted and purified using DNeasy Blood kits (Qiagen). *K13* sequences (Fig. 1) were examined by PCR-amplifying the genomic locus with primers p15/p16 flanking the *K13* plasmid donor sequence. These products were amplified from bulk cultures or parasite clones. Sequencing was performed with the internal p17 primer (Table S5). For select clones, expression of the mutant or wild-type *K13* alleles was confirmed by RT-PCR and sequencing.

Ring-stage survival assays (RSA0-3h). These assays were carried out as previously described, with minor modifications (12). A detailed protocol is available on the WorldWide Antimalarial Resistance Network's website at <http://www.wwarn.org/toolkit/procedures/ring-stage-survival-assays-rsa-evaluate-susceptibility-p-falciparum>. In summary, 10–15 mL parasite cultures were synchronized 1–2 times using 5% sorbitol (Sigma-Aldrich). Synchronous multinucleated schizonts were incubated in RPMI-1640 containing 15 units/ml sodium heparin for 15 min at 37°C to disrupt agglutinated erythrocytes, concentrated over a gradient of 75% Percoll (Sigma-Aldrich), washed once in RPMI-1640, and incubated for 3 hours with fresh erythrocytes to allow time for merozoite invasion. Cultures were then subjected again to sorbitol treatment to eliminate remaining schizonts. The 0–3 hours post-invasion rings were adjusted to 1% parasitemia and 2% hematocrit in 1 mL volumes (in 48-well plates), and exposed to 700 nM DHA or 0.1% DMSO (solvent control) for 6 hours. Duplicate wells were established for each parasite line ± drug. One-mL cultures were then transferred to 15 mL conical tubes, centrifuged at 800xg for 5 min to pellet the cells, and the supernatants carefully removed. As a washing step to remove drug, 9 mL culture medium were added to each tube and the cells resuspended, centrifuged, and the medium aspirated. Fresh medium lacking drug was then added to cultures, which were returned to standard culture conditions for 66 hours.

Parasite viability was assessed by microscopic examination of Giemsa-stained thin blood smears by counting parasites that developed into second-generation rings or trophozoites with normal morphology. To obtain a homogenous smear for all slides, the cultures were resuspended, transferred into an Eppendorf tube, and briefly centrifuged. 2 μ L of the pellet were then used for each smear. Parasitemias were calculated from a total of at least 40,000 erythrocytes per assay. Slides were read from the two duplicate wells per assay by two separate microscopists, each of whom examined at least 10,000 erythrocytes per slide. In instances of >20% discrepancy in parasite counts, slides were further examined by a third microscopist. The mean \pm SEM numbers of erythrocytes counted per parasite line and per assay were 44,160 \pm 970. Our mean \pm SEM parasitemia for non DHA-treated cultures was 5.1 \pm 0.3%. For assays where \leq 5 infected erythrocytes were counted from the initial examination of 40,000 erythrocytes (corresponding to a calculated parasitemia of \leq 0.0125%), the total number of erythrocytes was expanded to a mean \pm SEM of 58,360 \pm 3,460.

Percent survival was calculated as the parasitemia in the drug-treated sample divided by the parasitemia in the untreated sample \times 100. As an example, a 20% RSA_{0-3h} survival value corresponded to a 1% parasitemia in drug-treated parasites compared to a 5% parasitemia in the untreated control (the mean parasitemia in our untreated controls). Assuming a total cell count of 40,000 erythrocytes, this produced a 95% confidence interval (CI) of 0.9–1.1% parasitemia (corresponding to 360–440 infected erythrocytes for that particular line in one assay). A 2% RSA_{0-3h} survival value corresponded to a 0.1% parasitemia in the drug-treated sample (95% CI 0.07–0.13%; i.e. 28–52 infected erythrocytes) assuming a count of 40,000 erythrocytes. Given a 0.01% parasitemia (corresponding to a 0.2% RSA_{0-3h} survival value) and an average count of 58,360 erythrocytes, the 95% CI was 0.002–0.018% (i.e. 1–10 infected erythrocytes). The limit of assay sensitivity was therefore set to 0.2% survival. For each parent and corresponding *K13*-edited clones, individual groups were designated to perform the RSA_{0-3h} assays, whose results are presented in Fig. 2 and summarized in Table S4.

***In vitro* 50% inhibitory concentration (IC₅₀) assays.** *In vitro* IC₅₀ values were determined by incubating parasites for 72 hours across a range of concentrations of DHA or artesunate (0.25–24 nM for both). Proliferation was determined by staining parasites with SYBR Green I and MitoTracker Deep Red, and measuring parasitemia by flow cytometry (see above). *In vitro* IC₅₀ values were calculated by nonlinear regression analysis, and Student *t* tests were used for statistical analysis.

Fig. S1

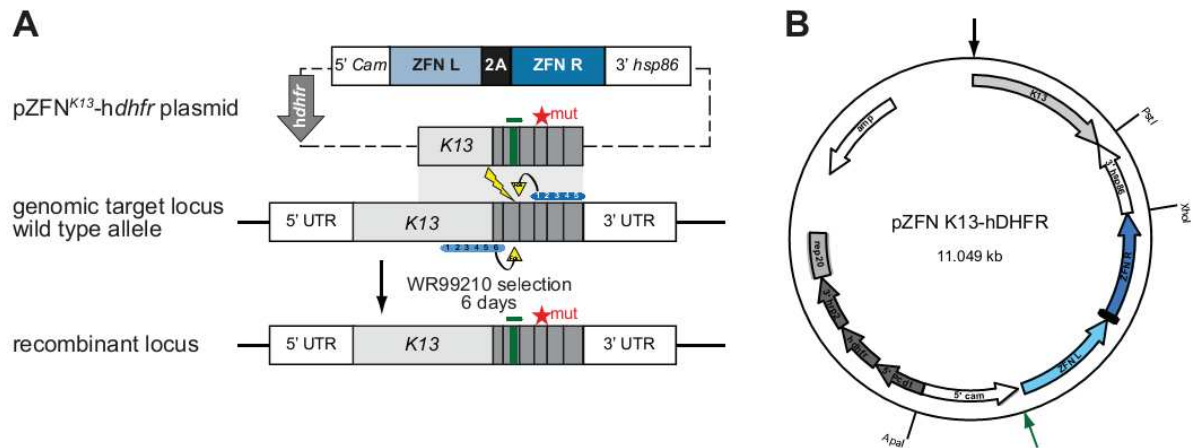


Fig. S1. *K13*-propeller editing strategy and plasmid map. (A) SNPs were inserted into wild-type or removed from mutant *K13*-propeller alleles using zinc-finger nucleases (ZFNs; Table S1). In this schematic, ZFNs introduce a double-stranded break (thunderbolt) in the 2nd propeller domain of the wild-type genomic target locus, thus triggering DNA resection. The pZFN^{K13}-*hdhfr* donor plasmid sequence provides a DNA template for homology-directed repair processes. Green bars indicate the presence of silent binding-site mutations that prevent the plasmid and the edited recombinant locus from being cleaved by ZFNs. Red stars indicate the presence of a non-synonymous propeller mutation in the plasmid and edited recombinant locus. (B) pZFN^{K13}-*hdhfr* plasmid map. Plasmids contained either the ZFN pairs 18/20 or 19/20 (Table S1). Black and green arrows indicate SacII/SalI and AvrII/NheI restriction sites that were ablated during plasmid construction (see supplementary text).

Fig. S2

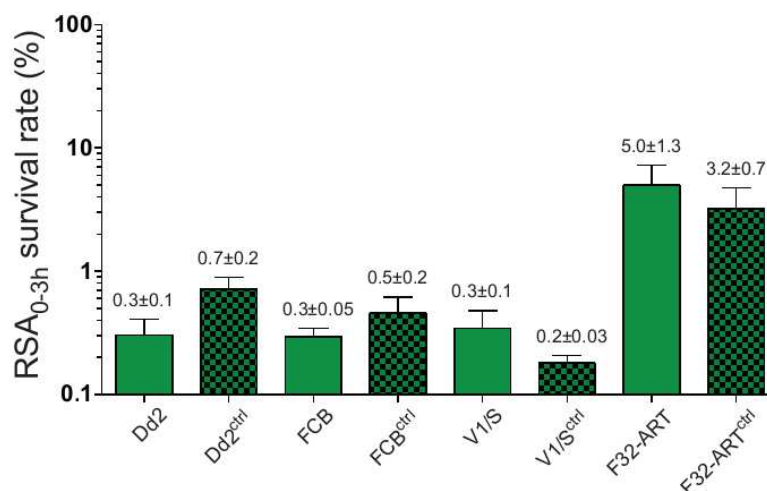


Fig. S2. Synonymous *K13* binding-site mutations do not significantly alter ring-stage susceptibility to artemisinins. Parasite lines were transfected with pZFN^{K13}-*hdhfr* plasmids harboring only silent binding-site mutations in the *K13* donor template (Fig. 1), and edited clones were obtained. RSA_{0-3h} assays were performed on 3–5 separate occasions in duplicate, and percent survival was calculated as described in Methods. We note that Dd2, FCB and V1/S harbor the wild-type *K13* allele, whereas F32-ART harbors the *K13*-propeller mutation M476I that was acquired during several years of artemisinin pressure *in vitro*. The F32-ART line and the edited F32-ART^{ctrl} line carrying silent binding-site mutations (in addition to M476I), showed survival rates in the RSA_{0-3h} that were 2–3 fold lower than those originally reported for F32-ART, suggesting that the resistance phenotype had attenuated over time. Mean±SEM values are represented above each column. Student *t* tests revealed no significant differences between the edited clones harboring only the synonymous binding-site mutations (designated with superscript ctrl) and the isogenic parental lines ($p>0.05$ in all cases).

Fig. S3

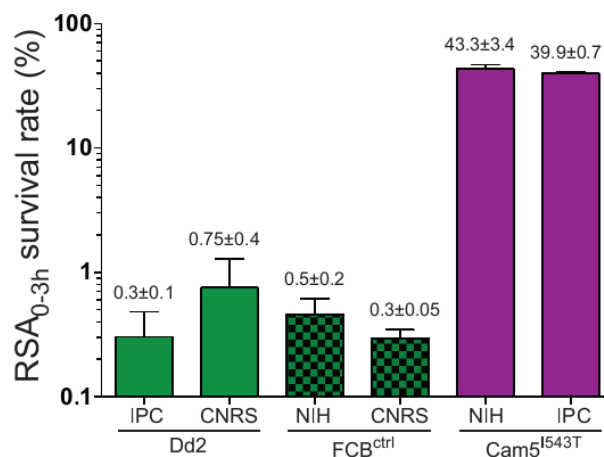


Fig. S3. Demonstration of consistent RSA_{0-3h} values between test sites. To test for consistency between research groups, several parasite lines were shipped to separate laboratories for independent analysis. Results showed equivalent survival rates among the three sites, namely those led by Dr. Didier Ménard at the Institut Pasteur du Cambodge (IPC), Dr. Françoise Benoit-Vical at the Université de Toulouse, Centre National de Recherche Scientifique UPR8241 (CNRS), and Dr. Rick Fairhurst at the National Institutes of Health (NIH). Assays were performed on 2–3 separate occasions in duplicate, and results are shown as mean±SEM above each column. Dd2 and FCB^{ctrl} parasites harbor the wild-type *K13* allele (the latter parasite line also contains synonymous binding-site mutations), whereas Cam5 parasites harbor the *K13* I543T mutation that confers elevated survival rates in the RSA_{0-3h}, indicative of high-level *in vitro* resistance. *t* tests showed no significant difference between RSA_{0-3h} results of different laboratories when testing the same parasite lines ($p>0.05$ in all cases).

Fig. S4

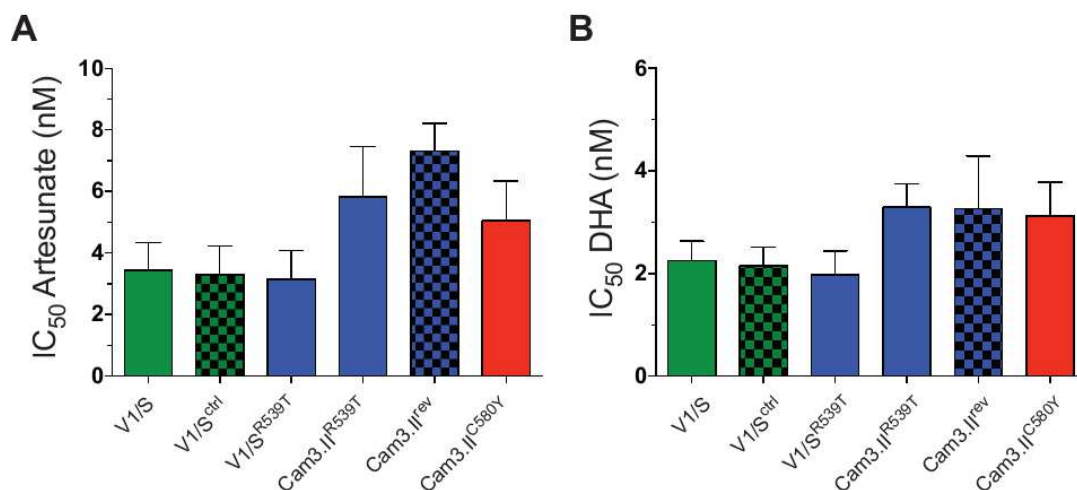


Fig. S4. *In vitro* proliferation assays show no significant differences in IC₅₀ values between K13 wild-type and mutant parasites. IC₅₀ values (mean±SEM) were measured in 72-h proliferation assays with final parasitemias determined using flow cytometry with SYBR Green I and MitroTracker Deep Red-stained parasites. Assays were performed on three separate occasions in duplicate. DHA, dihydroartemisinin.

Table S1. Nucleotide sequences of *K13*-specific ZFN binding sites and amino acid sequences of zinc-finger proteins.

<i>K13</i> ZFN Binding Sequence (underlined)	ZFN	Finger 1	Finger 2	Finger 3	Finger 4	Finger 5	Finger 6
CTTT <u>GACTCCACACTAGCAA</u> ATTCTCTACATACATACAAAGT	43418-ELD ^a	RSDNLSV	IRSTLRD	RSADLTR	TNQNRIT	TSGNLTR	DSSNLAT
GAAACTGAGGTGTATGATCGTTTAAAGAGATGATGGTATGTTTCA	43419-KKR	TSGSLTR	TSSNRKT	RPTRMQ	QSGSLTR	TSANLSR	-
GAAACTGAGGTGTATGATCGTTTAAAGAGATGATGGTATGTTTCA	43420-KKR	TSGLSLR	TSSNRKH	RSAHLT	QSGSLTR	TSANLSR	-

Amino acid sequences of ZFNs used in this study (individual fingers are underlined).

43418-ELD (414 amino acids)

MDYKDHGDYKDHIDYKDDDDKMAPKKRKGVIHGVPAAEAERPFQCRICMRNFSRSDNLSVHIRTHTGKPFACDICGRKFAIRSTLRDHTKIHTGSQKPFQCRICMRNFSRSDLTRHIRTHTGKPFACDICGRKFATNQNRITHTKIHTGSQKPFQCRICMRNFSRSGNLTRHIRTHTGKPFACDICGRKFADSSNLATHTKIHLRGSQLVKSELEKKSSELRHKLKYPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGRGKHLGSSRKPDAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMERYVEENQTRDKHLNPNNEWKVPSSVTEFKFLFVSGHFKGNKYAQLTRLNHNITNCNGAVLSVEELIIGGEMIKAGTLTLEEVRRKFNNGEINFRSXX

43419-KKR (382 amino acids)

MDYKDHGDYKDHIDYKDDDDKMAPKKRKGVIHGVPAAEAERPFQCRICMRNFATSGSLRHTKIHTGKPFQCRICMRNFSRSTSNRKHIRTHTGKPFACDICGRKFARPTRMQHTKIHTGSQKPFQCRICMRNFSQSGSLTRHIRTHTGKPFACDICGRKFATSNLSRHTKIHLRGSQLVKSELEKKSSELRHKLKYPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGRGKHLGSSRKPDAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRVYKVENQTRNKHINPNNEWKVPSSVTEFKFLFVSGHFKGNKYAQLTRLNHNITNCNGAVLSVEELIIGGEMIKAGTLTLEEVRRKFNNGEINFRSXX

43420-KKR (383 amino acids)

MDYKDHGDYKDHIDYKDDDDKMAPKKRKGVIHGVPAAEAERPFQCRICMRNFSRSTSGLSRHRHIRTHTGKPFACDICGRKFATSNRHHHTKIHTGSQKPFQCRICMRNFARSALHTTHTKIHTGKPFQCRICMRNFSQSGSLTRHIRTHTGKPFACDICGRKFATSNLSRHTKIHLRGSQLVKSELEKKSSELRHKLKYPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGRGKHLGSSRKPDAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRVYKVENQTRNKHINPNNEWKVPSSVTEFKFLFVSGHFKGNKYAQLTRLNHNITNCNGAVLSVEELIIGGEMIKAGTLTLEEVRRKFNNGEINFRSXX

^a The left ZFN 43418-ELD contains six zinc fingers, whereas the two right ZFNs 43419-KKR and 43420-KKR each contain five. Each finger recognizes a 3-nucleotide *K13* sequence. 43418-ELD binds to the *K13* non-coding (-) strand, whereas 43419-KKR and 43420-KKR bind to the same sequence on the coding (+) strand. 43418-ELD / 43419-KKR, and 43418-ELD / 43420-KKR constitute the ZFN pairs 18/19 and 18/20 respectively (Table S3).

Table S2. Geographic origin, native K13 allele and drug-resistance genotypes of *Plasmodium falciparum* clinical isolates and reference lines.

Parasite	Original ID	Sanger ID	Provider	Geographic origin (year)	PCT _{1/2} (h)	KH group	K13-propeller	PMID #	<i>pfcr1</i>	<i>pfmdr1</i>	<i>pfmdr1</i> CN	<i>dhfr</i>	<i>dhps</i>
Cam3.I	IPC 5202	N/A	D. Ménard	Battambang, W. Cambodia (2011)	ND	ND	R539T (allele 3)	24352242	Dd2 ^a	184F	4	Triple ^b	436A/540E
Cam3.II	RF 967	PH0306-C	R. Fairhurst	Pursat, W. Cambodia (2010)	6.0	3	R539T (allele 3)	22940027	Dd2	184F	1	Triple	436A/540E
Cam5	IPC 4912	N/A	D. Ménard	Mondulkiri, E. Cambodia (2011)	ND	ND	I543T (allele 5)	24352242	Dd2	WT	1	Quadruple	436F/540E/613S
Cam2	IPC 3445	N/A	D. Ménard	Pailin, W. Cambodia (2010)	ND	ND	C580Y (allele 2)	24352242	Dd2	WT	2	Triple	436A/540E
CamWT	RF 915	PH0164-C	R. Fairhurst	Pursat, W. Cambodia (2010)	3.7	1	Wild-type	22940027	Dd2	WT	1	Triple	436A/540E
F32-TEM	F32	N/A	F. Benoit-Vical	Tanzania (1982)	N/A	N/A	Wild-type	20160056	WT	WT	1	WT	436A
V1/S	V1/S	N/A	MR4	Vietnam (1976)	N/A	N/A	Wild-type	12124623	Dd2	86Y	1	Quadruple	613T
FCB	FCB	N/A	MR4	Asia (1980)	N/A	N/A	Wild-type	12124623	FCB	86Y	2	16V/108T	437A
Dd2	Dd2	N/A	MR4	Indochina (1980)	N/A	N/A	Wild-type	12124623	Dd2	86Y	3	Triple	436F/613S

^a Dd2: 74I/75D/76T/220S/271E/326S/356T/371I; FCB: 74I/75E/76T/220S/271E/326S/371I.

^b Triple, 51I/59R/108N; Quadruple, 51I/59R/108N/164L.

PC_{1/2}, parasite clearance half-life; PMID #, PubMed identification number; *pfmdr1* CN, *pfmdr1* copy number; MR4, Malaria Research and Reference Reagent Resource, Manassas, VA; N/A, not available; ND, not determined.

Table S3. K13 transfection outcomes with *Plasmodium falciparum* clinical isolates and reference lines.

Parasite	ZFN pair	Binding site mutations only		M476I (allele 1)		C580Y (allele 2)		R539T (allele 3)		Y493H (allele 4)		I543T (allele 5)	
		Edited cultures (%)	Edited clones (%)	Edited cultures (%)	Edited clones (%)	Edited cultures (%)	Edited clones (%)	Edited cultures (%)	Edited clones (%)	Edited cultures (%)	Edited clones (%)	Edited cultures (%)	Edited clones (%)
Cam3.I	18/19	1 of 1 (100%)	ND	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
Cam3.I	18/20	1 of 1 (100%)	2 of 7 (29%) ^a	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
Cam3.II	18/19	1 of 2 (50%)	4 of 16 (25%)	ND	N/A	0 of 1 (0%)	ND	ND	N/A	ND	N/A	ND	N/A
Cam3.II	18/20	1 of 2 (50%)	ND	ND	N/A	1 of 1 (100%)	1 of 4 (25%)	ND	N/A	ND	N/A	ND	N/A
Cam5	18/19	1 of 4 (25%)	ND	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
Cam5	18/20	1 of 4 (25%)	6 of 11 (55%)	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
Cam2	18/19	1 of 2 (50%)	1 of 6 (17%)	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
Cam2	18/20	1 of 2 (50%)	ND	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
CamWT	18/19	0 of 1 (0%)	ND	ND	N/A	1 of 2 (50%)	1 of 2 (50%)	0 of 1 (0%)	ND	0 of 2 (0%)	ND	1 of 2 (50%)	0 of 3 (0%)
CamWT	18/20	ND	N/A	ND	N/A	0 of 2 (0%)	ND	0 of 1 (0%)	ND	1 of 2 (50%)	ND	ND	N/A
F32-TEM	18/19	ND	N/A	1 of 2 (50%)	ND	ND	N/A	ND	N/A	ND	ND	ND	N/A
F32-TEM	18/20	ND	N/A	1 of 2 (50%)	2 of 19 (11%)	ND	N/A	ND	N/A	ND	N/A	ND	N/A
F32-ART	18/19	1 of 2 (50%)	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
F32-ART	18/20	1 of 2 (50%)	0 of 3 (0%) ^b	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
V1/S	18/19	0 of 1 (0%)	N/A	0 of 2 (0%)	ND	1 of 1 (100%)	0 of 18 (0%)	0 of 1 (0%)	ND	1 of 2 (50%)	ND	0 of 1 (0%)	ND
V1/S	18/20	1 of 1 (100%)	ND ^c	0 of 2 (0%)	ND	0 of 1 (0%)	ND	1 of 1 (100%)	1 of 8 (13%)	0 of 1 (0%)	ND	0 of 1 (0%)	ND
FCB	18/19	0 of 1 (0%)	N/A	0 of 1 (0%)	ND	1 of 2 (50%)	2 of 25 (8%)	0 of 3 (0%)	ND	0 of 2 (0%)	ND	0 of 2 (0%)	ND
FCB	18/20	0 of 1 (0%)	N/A	0 of 1 (0%)	ND	0 of 2 (0%)	ND	0 of 3 (0%)	ND	0 of 3 (0%)	ND	0 of 2 (0%)	ND
Dd2	18/19	0 of 1 (0%)	N/A	0 of 3 (0%)	ND	1 of 1 (100%)	1 of 10 (10%)	0 of 6 (0%)	ND	1 of 1 (100%)	1 of 17 (6%)	0 of 3 (0%)	ND
Dd2	18/20	1 of 3 (33%)	ND	1 of 3 (33%)	1 of 8 (13%)	0 of 1 (0%)	ND	1 of 6 (0%)	1 of 8 (13%)	0 of 1 (0%)	ND	1 of 3 (33%)	3 of 21 (14%)
Positive events		12 of 30 (40%) ^d	13 of 43 (30%)	3 of 16 (19%)	3 or 27 (11%)	5 of 14 (36%) ^e	5 of 59 (9%)	2 of 22 (9%)	2 of 16 (13%)	3 of 14 (21%)	1 of 17 (6%)	2 of 14 (14%)	3 of 24 (13%)

^a Clones were generated from individual bulk cultures with the highest percentage of editing events for a given mutation and parasite. The percentages of edited clones are listed only for those that captured the desired nucleotide changes (either insertion or removal of a point mutation of interest). Clones that captured binding site mutations but not *K13* mutations were not included in this analysis; therefore, the percentages listed underestimate the percentage of editing events.

^b One clone (F32-ART^{ctrl} in Fig. S3) captured the binding site mutations, but did not revert M476I to wild-type. This clone was therefore not listed as being edited at the mutation of interest (see definition in footnote a).

^c This culture was not cloned because the desired binding site control line (V1/S^{ctrl}) was obtained from the transfection with the R539T plasmid, resulting in incorporation of the binding site mutations but not R539T. This was one of several instances where a binding site mutant control was obtained from transfections with mutant donor sequences that captured only the binding site mutations, but not the *K13* mutations more distant from the ZFN cleavage site. Binding site control clones are shown in Fig. S3.

^d These data suggest that removing *K13* mutations (achieved using the plasmid containing the binding site mutations only) was generally more efficient than introducing them, possibly due to a fitness cost associated with their presence.

^e These data suggest that C580Y editing into *K13* wild-type parasites was more efficient than editing of other mutations, possibly because the C580Y mutation might have a lesser fitness cost. These results are consistent with C580Y being highly prevalent in western Cambodia, despite our evidence that C580Y mediates a lower level of *in vitro* resistance than R539T or I543T (see Fig. 2).
N/A, not applicable; ND, not done.

Table S4. Data from ring-stage survival assays (RSAs).

Parasite	Original isolate/line	K13 sequence	Binding-site mutations	RSA values mean \pm SEM	Assays	Primary site of RSA data
Cam3.I ^{R539T}	IPC 5202	R539T (parent allele 3)	No	40.2 \pm 3.0 ^{a,b}	3 ^c	IPC, Cambodia ^d
Cam3.I ^{rev}	IPC 5202	wild-type (revertant)	Yes	0.3 \pm 0.1	3	IPC, Cambodia
Cam3.II ^{R539T}	RF 967	R539T (parent allele 3)	No	48.9 \pm 0.8	3	NIH, USA
Cam3.II ^{rev}	RF 967	wild-type (revertant)	Yes	0.7 \pm 0.2	3	NIH, USA
Cam3.II ^{C580Y}	RF 967	C580Y (allele 2)	Yes	24.1 \pm 2.9	3	NIH, USA
Cam5 ^{I543T}	IPC 4912	I543T (parent allele 5)	No	43.4 \pm 3.4	3	NIH, USA
Cam5 ^{rev}	IPC 4912	wild-type (revertant)	Yes	0.3 \pm 0.02	3	NIH, USA
Cam2 ^{C580Y}	IPC 3445	C580Y (parent allele 2)	No	13.0 \pm 2.6	3	IPC, Cambodia
Cam2 ^{rev}	IPC 3445	wild-type (revertant)	Yes	2.4 \pm 0.1	3	IPC, Cambodia
CamWT	RF 915	wild-type (parent)	No	0.6 \pm 0.1	3	NIH, USA
CamWT ^{C580Y}	RF 915	C580Y (allele 2)	Yes	8.9 \pm 0.5	3	NIH, USA
F32-TEM	F32-TEM	wild-type (parent)	No	<0.2 ^e	4	CNRS, France
F32-TEM ^{M476I}	F32-TEM	M476I (allele 1)	Yes	1.7 \pm 0.4	4	CNRS, France
V1/S	V1/S	wild-type (parent)	No	0.3 \pm 0.1	3	NIH, USA
V1/S ^{R539T}	V1/S	R539T (allele 3)	Yes	20.7 \pm 4.0	4	NIH, USA
FCB	FCB	wild-type (parent)	No	0.3 \pm 0.1	3	NIH, USA
FCB ^{C580Y}	FCB	C580Y (allele 2)	Yes	1.9 \pm 0.3	4	NIH, USA
Dd2	Dd2	wild-type (parent)	No	0.3 \pm 0.1	3	IPC, Cambodia
Dd2 ^{Y493H}	Dd2	Y493H (allele 4)	Yes	1.7 \pm 0.4	3	IPC, Cambodia
Dd2 ^{C580Y}	Dd2	C580Y (allele 2)	Yes	4.1 \pm 0.4	3	IPC, Cambodia
Dd2 ^{M476I}	Dd2	M476I (allele 1)	Yes	9.8 \pm 2.6	4	CNRS, France
Dd2 ^{R539T}	Dd2	R539T (allele 3)	Yes	19.4 \pm 0.8	3	NIH, USA
Dd2 ^{I543T}	Dd2	I543T (allele 5)	Yes	29.1 \pm 3.9	3	IPC, Cambodia

^a RSA values are the percentage of parasites that survived a 6-h pulse of 700 nM dihydroartemisinin, compared to DMSO-treated parasites. Parasitemias were determined 66 h after ending the drug pulse.

^b For each RSA value, the relative standard error (RSE) was calculated as the SEM/mean x100. The mean RSE for all assays was 17.0%, indicating an average SEM of 17.0% of the mean.

^c Assays were performed on separate occasions as enumerated, with each assay performed in duplicate.

^d IPC, Institut Pasteur du Cambodge (Ménard lab); CNRS, Centre National de Recherche Scientifique UPR8241 (Benoit-Vical lab); NIH, National Institutes of Health (Fairhurst lab).

^e RSA values for F32-TEM were calculated to be 0.02 \pm 0.02. Based on our limit of sensitivity (as discussed in Supplementary Materials) we have indicated this survival value to be <0.2%.

Table S5. Oligonucleotides used in this study.

Name	Nucleotide sequence (5'-3') ^a	Description	Lab name
p1	GTGACGTCGATTGATATTAATGTTGGTGGAGC	K13 donor fwd	p3984
p2	CCGCATATGGTGCAAACGGAGTGACCAAATCTGGG	K13 donor rev	p3986
p3	GGTGATGATaGaTTAAGAGAcGTcTGGTATGTaTCAAGTAATTTAAATATACC	SDM ZFN binding site mutation fwd	p4169
p4	CCAgACgTCTCTTAAtCiATCATAcACCTCiGTTTCAAATAAAGCC	SDM ZFN binding site mutation rev	p4170
p5	GCTGGCGTATGTGTACACCTATaTCTACCAAAAAGCTTATTTGGAAGTGC	SDM allele 1 M476I fwd	p4173
p6	GGTAGAAtAGGTGTACACATACGCCAGCATTGTTGACTAATATCTAATAATTCC	SDM allele 1 M476I rev	p4174
p7	CATCAGCTATGTaTgTTGCTTTTGATAATAAAATTTATGTCATTGG	SDM allele 2 C580Y fwd	p4003
p8	ATTATCAAAAGCAACAtACATAGCTGATGATCTAGGGGTATTCAAAGG	SDM allele 2 C580Y rev	p4004
p9	CGTCAAATGGTAcAATTTATTGTATTGGGGGATATGATGGCTCTTC	SDM allele 3 R539T fwd	p4005
p10	CCAATACAATAAATTgTACCATTTGACGTAACACCACAATTATTTTC	SDM allele 3 R539T rev	p4006
p11	GAATAATTTCTTAcACGTTTTGGTGGTAATAACTATGATTATAAGGC	SDM allele 4 Y493H fwd	p4188
p12	CCAAAACGTgTAAGAAATTTCAATACAGCACTTCCAAAATAAGC	SDM allele 4 Y493H rev	p4189
p13	GGTAGAATTTATTGTAcTGGGGGATATGATGGCTCTTCTATTATACC	SDM allele 5 I543T fwd	p4007
p14	CATATCCCCAgTACAATAAATTCTACCATTTGACGTAACACCAC	SDM allele 5 I543T rev	p4008
p15	GCAAATCTTATAAATGATGATTCTGG	K13 5' for screening fwd	p4433
p16	GCTAATAAGTAATATCAATATAAGGG	K13 3' for screening rev	p4434
p17	GGTATTAATTTTACCATTCCATTAGTATTTGTATAGG	Sequencing primer	p4186

^a Lower-case nucleotides show the position that was subjected to mutagenesis.
fwd, forward; rev, reverse; SDM, site directed mutagenesis.

Statistical data: Results of two-sample t-tests with unequal variances

Cam3.I^{R539T} vs. Cam3.I^{rev} (groups 1 vs. 2 respectively; $p < 0.01$; Fig. 2A)

. ttest logCam3I, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	3.688271	.0768044	.133029	3.357809	4.018734
2	3	-1.546587	.4405906	.7631253	-3.442296	.3491212
combined	6	1.070842	1.187515	2.908805	-1.981761	4.123445
diff		5.234858	.4472348		3.412362	7.057355

diff = mean(1) - mean(2) t = **11.7049**
 Ho: diff = 0 Satterthwaite's degrees of freedom = **2.12144**

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = **0.9971** Pr(|T| > |t|) = **0.0058** Pr(T > t) = **0.0029**

Cam3.II^{R539T} vs. Cam3.II^{rev} (groups 1 vs. 2 respectively; $p < 0.01$; Fig. 2B)

. ttest logCam3II, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	3.89019	.016374	.0283606	3.819738	3.960642
2	3	-.5074539	.3730999	.646228	-2.112773	1.097866
combined	6	1.691368	.9974256	2.443184	-.8725962	4.255332
diff		4.397644	.3734591		2.796674	5.998614

diff = mean(1) - mean(2) t = **11.7754**
 Ho: diff = 0 Satterthwaite's degrees of freedom = **2.0077**

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = **0.9965** Pr(|T| > |t|) = **0.0070** Pr(T > t) = **0.0035**

Cam5^{I543T} vs. Cam5^{rev} (groups 1 vs. 2 respectively; $p < 0.0001$; Fig. 2C)

. ttest logCam5, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	3.763479	.0753753	.1305539	3.439165	4.087793
2	3	-1.215887	.0725464	.125654	-1.528029	-.9037451
combined	6	1.273796	1.114403	2.729718	-1.590867	4.138459
diff		4.979366	.1046155		4.688739	5.269993

diff = mean(1) - mean(2) t = 47.5968
 Ho: diff = 0 Satterthwaite's degrees of freedom = 3.99416

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 1.0000 Pr(|T| > |t|) = 0.0000 Pr(T > t) = 0.0000

Cam2^{C580Y} vs. Cam2^{rev} (groups 1 vs. 2 respectively; $p < 0.01$; Fig. 2D)

. ttest logCam2, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	2.525393	.1843995	.3193893	1.731986	3.3188
2	3	.867163	.0518709	.0898431	.6439804	1.090346
combined	6	1.696278	.3805589	.9321752	.7180201	2.674536
diff		1.65823	.1915562		.9325863	2.383874

diff = mean(1) - mean(2) t = 8.6566
 Ho: diff = 0 Satterthwaite's degrees of freedom = 2.31454

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.9959 Pr(|T| > |t|) = 0.0082 Pr(T > t) = 0.0041

CamWT vs. CamWT^{C580Y} (groups 1 vs. 2 respectively; $p < 0.01$; Fig. 2E)

. ttest logCamWT, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-.5885569	.1905804	.330095	-1.408558	.2314445
2	3	2.184728	.0602016	.1042722	1.925701	2.443754
combined	6	.7980854	.6265337	1.534688	-.8124706	2.408642
diff		-2.773285	.1998628		-3.510595	-2.035975

diff = mean(1) - mean(2) t = -13.8759
 Ho: diff = 0 Satterthwaite's degrees of freedom = 2.3952

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0012 Pr(|T| > |t|) = 0.0024 Pr(T > t) = 0.9988

Cam3.II^{rev} vs. Cam3.II^{C580Y} (groups 1 vs. 2 respectively; $p < 0.01$; Fig. 2F)

. ttest logCam3IIC580Y, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-.5074539	.3730999	.646228	-2.112773	1.097866
2	3	3.167337	.1149281	.1990612	2.672841	3.661832
combined	6	1.329941	.8400516	2.057698	-.8294799	3.489363
diff		-3.67479	.3903998		-5.123775	-2.225806

diff = mean(1) - mean(2) t = -9.4129
 Ho: diff = 0 Satterthwaite's degrees of freedom = 2.37616

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0031 Pr(|T| > |t|) = 0.0062 Pr(T > t) = 0.9969

V1/S vs. V1/S^{R539T} (groups 1 vs. 2 respectively; $p < 0.01$; Fig. 2G)

. ttest logV1S, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-1.208234	.3612046	.6256247	-2.762372	.3459035
2	4	2.972716	.1999613	.3999226	2.33635	3.609082
combined	7	1.18088	.8622912	2.281408	-.9290704	3.290831
diff		-4.18095	.4128599		-5.447017	-2.914884

diff = mean(1) - mean(2) t = -10.1268
 Ho: diff = 0 Satterthwaite's degrees of freedom = 3.21256

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0008 Pr(|T| > |t|) = 0.0015 Pr(T > t) = 0.9992

F32-TEM vs. F32-TEM^{M476I} (groups 1 vs. 2 respectively; $p < 0.01$; Fig. 2H)

. ttest logF32TEM, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-1.609438	0	0	-1.609438	-1.609438
2	4	.3982619	.3075268	.6150537	-.5804257	1.37695
combined	7	-.4621809	.4376593	1.157938	-1.533095	.6087328
diff		-2.0077	.3075268		-2.986388	-1.029012

diff = mean(1) - mean(2) t = -6.5285
 Ho: diff = 0 Satterthwaite's degrees of freedom = 3

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0037 Pr(|T| > |t|) = 0.0073 Pr(T > t) = 0.9963

FCB vs. FCB^{C580Y} (groups 1 vs. 2 respectively; $p < 0.01$; Fig. 2I)

. ttest logFCB, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-1.260332	.1874178	.3246172	-2.066726	-.4539377
2	4	.5756699	.2217322	.4434645	-.129981	1.281321
combined	7	-.2111879	.3957941	1.047173	-1.179661	.7572853
diff		-1.836002	.2903285		-2.582576	-1.089427

diff = mean(1) - mean(2) t = -6.3239
 Ho: diff = 0 Satterthwaite's degrees of freedom = 4.99418

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0007 Pr(|T| > |t|) = 0.0015 Pr(T > t) = 0.9993

Dd2 vs. Dd2^{Y493H} (groups 1 vs. 2 respectively; $p < 0.05$; Fig. 2J)

. ttest logDd2Y493H, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-1.304008	.3314896	.5741568	-2.730292	.1222767
2	3	.4804795	.2148663	.3721593	-.4440154	1.404975
combined	6	-.4117641	.4363832	1.068916	-1.533523	.7099947
diff		-1.784487	.3950352		-2.957299	-.6116756

diff = mean(1) - mean(2) t = -4.5173
 Ho: diff = 0 Satterthwaite's degrees of freedom = 3.42843

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0076 Pr(|T| > |t|) = 0.0152 Pr(T > t) = 0.9924

Dd2 vs. Dd2^{C580Y} (groups 1 vs. 2 respectively; $p < 0.01$; Fig. 2J)

. ttest logDd2C580Y, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-1.304008	.3314896	.5741568	-2.730292	.1222767
2	3	1.39194	.1087222	.1883124	.9241457	1.859734
combined	6	.043966	.622694	1.525282	-1.55672	1.644652
diff		-2.695947	.3488637		-3.970905	-1.42099

diff = mean(1) - mean(2) t = -7.7278
 Ho: diff = 0 Satterthwaite's degrees of freedom = 2.42536

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0046 Pr(|T| > |t|) = 0.0092 Pr(T > t) = 0.9954

Dd2 vs. Dd2^{M476I} (groups 1 vs. 2 respectively; $p < 0.001$; Fig. 2J)

. ttest logDd2M476I, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-1.304008	.3314896	.5741568	-2.730292	.1222767
2	4	2.162859	.2838199	.5676399	1.259617	3.0661
combined	7	.6770588	.7275241	1.924848	-1.103129	2.457246
diff		-3.466866	.4363933		-4.634165	-2.299568

diff = mean(1) - mean(2) t = -7.9444
 Ho: diff = 0 Satterthwaite's degrees of freedom = 4.42262

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0004 Pr(|T| > |t|) = 0.0009 Pr(T > t) = 0.9996

Dd2 vs. Dd2^{R539T} (groups 1 vs. 2 respectively; $p < 0.01$; Fig. 2J)

. ttest logDd2R539T, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-1.304008	.3314896	.5741568	-2.730292	.1222767
2	3	2.96476	.0417134	.0722497	2.785281	3.144238
combined	6	.830376	.966149	2.366572	-1.653189	3.313941
diff		-4.268767	.3341038		-5.664839	-2.872696

diff = mean(1) - mean(2) t = -12.7768
 Ho: diff = 0 Satterthwaite's degrees of freedom = 2.06332
 Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0027 Pr(|T| > |t|) = 0.0054 Pr(T > t) = 0.9973

Dd2 vs. Dd2^{I543T} (groups 1 vs. 2 respectively; $p < 0.01$; Fig. 2J)

. ttest logDd2I543T, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-1.304008	.3314896	.5741568	-2.730292	.1222767
2	3	3.353108	.1395851	.2417684	2.752522	3.953694
combined	6	1.02455	1.053713	2.581058	-1.684104	3.733204
diff		-4.657116	.3596795		-5.880846	-3.433385

diff = mean(1) - mean(2) t = -12.9480
 Ho: diff = 0 Satterthwaite's degrees of freedom = 2.68763
 Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0008 Pr(|T| > |t|) = 0.0017 Pr(T > t) = 0.9992

FCB^{C580Y} vs. Dd2^{C580Y} (groups 1 vs. 2 respectively; $p < 0.05$; Fig. 2K)

. ttest logDd2C580Y, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	4	.5756699	.2217322	.4434645	-.129981	1.281321
2	3	1.39194	.1087222	.1883124	.9241457	1.859734
combined	7	.9254998	.2071994	.5481982	.418501	1.432499
diff		-.8162698	.2469528		-1.486439	-.1461009

diff = mean(1) - mean(2) t = -3.3054
 Ho: diff = 0 Satterthwaite's degrees of freedom = 4.24766

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0136 Pr(|T| > |t|) = 0.0272 Pr(T > t) = 0.9864

FCB^{C580Y} vs. CamWT^{C580Y} (groups 1 vs. 2 respectively; $p < 0.01$; Fig. 2K)

. ttest logCamWTC580Y, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	4	.5756699	.2217322	.4434645	-.129981	1.281321
2	3	2.184728	.0602016	.1042722	1.925701	2.443754
combined	7	1.265266	.3467581	.9174356	.4167797	2.113753
diff		-1.609058	.2297595		-2.290976	-.9271395

diff = mean(1) - mean(2) t = -7.0032
 Ho: diff = 0 Satterthwaite's degrees of freedom = 3.43063

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0019 Pr(|T| > |t|) = 0.0038 Pr(T > t) = 0.9981

FCB^{C580Y} vs. Cam2^{C580Y} (groups 1 vs. 2 respectively; $p < 0.01$; Fig. 2K)

. ttest logCam2C580Y, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	4	.5756699	.2217322	.4434645	-.129981	1.281321
2	3	2.525393	.1843995	.3193893	1.731986	3.3188
combined	7	1.411266	.4172107	1.103836	.3903876	2.432143
diff		-1.949723	.2883892		-2.691124	-1.208322

diff = mean(1) - mean(2) t = -6.7607
 Ho: diff = 0 Satterthwaite's degrees of freedom = 4.99837
 Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0005 Pr(|T| > |t|) = 0.0011 Pr(T > t) = 0.9995

FCB^{C580Y} vs. Cam3.II^{C580Y} (groups 1 vs. 2 respectively; $p < 0.001$; Fig. 2K)

. ttest logCam3IIC580Y, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	4	.5756699	.2217322	.4434645	-.129981	1.281321
2	3	3.167337	.1149281	.1990612	2.672841	3.661832
combined	7	1.686384	.5385969	1.424993	.3684851	3.004283
diff		-2.591667	.2497471		-3.263246	-1.920088

diff = mean(1) - mean(2) t = -10.3772
 Ho: diff = 0 Satterthwaite's degrees of freedom = 4.35678
 Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0002 Pr(|T| > |t|) = 0.0003 Pr(T > t) = 0.9998

Dd2 vs. Dd2^{ctrl} (groups 1 vs. 2 respectively; p = not significant; Fig. S2)

. ttest logDd2, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-1.304008	.3314896	.5741568	-2.730292	.1222767
2	3	-.3955087	.2486298	.4306395	-1.465276	.6742591
combined	6	-.8497582	.2749708	.6735382	-1.556593	-.1429232
diff		-.908499	.4143695		-2.095421	.2784229

diff = mean(1) - mean(2) t = -2.1925
 Ho: diff = 0 Satterthwaite's degrees of freedom = 3.70929

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.0494 Pr(|T| > |t|) = 0.0988 Pr(T > t) = 0.9506

FCB vs. FCB^{ctrl} (groups 1 vs. 2 respectively; p = not significant; Fig. S2)

. ttest logFCB, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-1.260332	.1874178	.3246172	-2.066726	-.4539377
2	3	-.8974407	.3438403	.5955488	-2.376866	.5819846
combined	6	-1.078886	.193015	.4727884	-1.575047	-.5827252
diff		-.3628909	.3916013		-1.588419	.8626372

diff = mean(1) - mean(2) t = -0.9267
 Ho: diff = 0 Satterthwaite's degrees of freedom = 3.09202

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.2103 Pr(|T| > |t|) = 0.4206 Pr(T > t) = 0.7897

V1/S vs. V1/S^{ctrl} (groups 1 vs. 2 respectively; p = not significant; Fig. S2)

. ttest logV1S, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-1.208234	.3612046	.6256247	-2.762372	.3459035
2	3	-1.735915	.1444376	.2501733	-2.35738	-1.11445
combined	6	-1.472075	.2102107	.514909	-2.012439	-.931711
diff		.5276807	.3890128		-.8171156	1.872477

diff = mean(1) - mean(2) t = 1.3565
 Ho: diff = 0 Satterthwaite's degrees of freedom = 2.62366

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.8601 Pr(|T| > |t|) = 0.2799 Pr(T > t) = 0.1399

F32-ART vs. F32-ART^{ctrl} (groups 1 vs. 2 respectively; p = not significant; Fig. S2)

. ttest logF32ART, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	1.54359	.2493203	.4318355	.470851	2.616329
2	5	1.056817	.2388532	.5340919	.3936547	1.71998
combined	8	1.239357	.1869997	.5289149	.7971732	1.681541
diff		.4867725	.3452701		-.3917757	1.365321

diff = mean(1) - mean(2) t = 1.4098
 Ho: diff = 0 Satterthwaite's degrees of freedom = 5.17592

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.8921 Pr(|T| > |t|) = 0.2158 Pr(T > t) = 0.1079

Dd2 comparison between IPC and CNRS (groups 1 vs. 2 respectively; $p =$ not significant; Fig. S3)

. ttest logDd2, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-1.304008	.3314896	.5741568	-2.730292	.1222767
2	2	-.4315231	.5493061	.7768362	-7.411119	6.548073
combined	5	-.9550139	.3298683	.7376079	-1.870875	-.0391527
diff		-.8724846	.6415782		-4.057465	2.312496

diff = mean(1) - mean(2) t = -1.3599
 Ho: diff = 0 Satterthwaite's degrees of freedom = 1.74524

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.1615 Pr(|T| > |t|) = 0.3230 Pr(T > t) = 0.8385

FCB^{ctrl} comparison between NIH and CNRS (groups 1 vs. 2 respectively; $p =$ not significant; Fig. S3)

. ttest logFCB, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	-.8974407	.3438403	.5955488	-2.376866	.5819846
2	3	-1.260332	.1874178	.3246172	-2.066726	-.4539377
combined	6	-1.078886	.193015	.4727884	-1.575047	-.5827252
diff		.3628909	.3916013		-.8626372	1.588419

diff = mean(1) - mean(2) t = 0.9267
 Ho: diff = 0 Satterthwaite's degrees of freedom = 3.09202

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = 0.7897 Pr(|T| > |t|) = 0.4206 Pr(T > t) = 0.2103

Cam5^{1543T} comparison between NIH and IPC (groups 1 vs. 2 respectively; p = not significant; Fig. S3)

. ttest logCam5, by(group) unequal

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
1	3	3.763479	.0753753	.1305539	3.439165	4.087793
2	2	3.685584	.0181838	.0257158	3.454537	3.916632
combined	5	3.732321	.0458427	.1025074	3.605041	3.859601
diff		.077895	.0775377		-.2254468	.3812368

diff = mean(1) - mean(2) t = **1.0046**
 Ho: diff = 0 Satterthwaite's degrees of freedom = **2.2245**

Ha: diff < 0 Ha: diff != 0 Ha: diff > 0
 Pr(T < t) = **0.7942** Pr(|T| > |t|) = **0.4116** Pr(T > t) = **0.2058**

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Annex 7. Ethic Committee Approval for K13 mutation study in Kupang



MEDICAL AND HEALTH RESEARCH ETHICS COMMITTEE (MHREC)
FACULTY OF MEDICINE GADJAH MADA UNIVERSITY
– DR. SARDJITO GENERAL HOSPITAL



ETHICS COMMITTEE APPROVAL

Ref : KE/FK/ 222 /EC

Title of the Research Protocol : Is There Emergence of Act (Artemisinin based Combination Therapy) Resistance in Indonesia

Documents Approved : 1. Study Protocol versi 01 2015
2. Information for Subjects versi 01 2015
3. Informed consent form versi 01 2015

Principle Investigator : Dr. dr. Mahardika Agus Wijayanti, DTM&H, M.Kes

Participating Investigator(s) : Dr. dr. Eti Nurwening Sholikhah, M.Kes

Date of Approval : **23 FEB 2015**


Institution(s)/place(s) of research : (Valid for one year beginning from the date of approval)
Lab Pharmacology & Therapy UGM, Laboratoire de Chimie de Coordination du CNRS


The Medical and Health Research Ethics Committee (MHREC) states that the above protocol meets the ethical principle outlined in the Declaration of Helsinki 2008 and therefore can be carried out.

The Medical and Health Research Ethics Committee (MHREC) has the right to monitor the research activities at any time.

The investigator(s) is/are obliged to submit:

- Progress report as a continuing review : Annually
- Report of any serious adverse events (SAE)
- Final report upon the completion of the study


Prof. Dr. dr. Sri Sutarni, Sp.S(K)
Vice Chairperson


dr. Madarina Julia, Sp.A(K), MPH., Ph.D
Secretary

Attachments:

- Continuing review submission form (AF 4.3.01-014.2013-03)
- Serious adverse events (SAE) report form (AF 6.1.01- 019.2013-03)