

**Extending the spectrum of antimalarial treatment:
Artemisinin combinations for the treatment of rare
Plasmodium species infections and the development
of dyes as antimalarials**

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Abbreviation

ACT	Artemisinin-based Combination Therapies
CERMEL	Centre de Recherche Médicale de Lambaréné
DDT	Dichloro-Diphenyl-Trichloroethane
DNA	Desoxyribonucleic Acid
dNTPs	Desoxy Ribonucleotides
ELISA	Enzyme Linked Immunosorbent Assay
FU	Follow-Up
GIA	Growth Inhibition Assay
GMAP	Global Malaria Action Plan
GMEP	Global Malaria Eradication Program
HeLa	Named after the patient Henrietta Lacks
HRP2	Histidine-Rich Protein 2
HTS	High Throughput Screening
IC50	50% Inhibitory Concentration
i.p.	Intraperitoneal
i.v.	Intravenous
i.m	Intramuscular
NMCP	National Malaria Control Program
nmol	Nanomole
nM	Nanomolar
PCR	Polymerase Chain Reaction
<i>P.falciparum</i>	<i>Plasmodium falciparum</i>
RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
rRNA	ribosomal Ribonucleic Acid
TBS	Thick Blood Smear
UN	United Nation
WHO	World Health Organization
mL	milliliter
µg	microgram

Kurzfassung

Malaria ist eine Infektionskrankheit, welche durch einen Parasiten der Gattung *Plasmodium* verursacht wird. Im Jahr 2013 gab es geschätzte 198 Millionen Krankheits- und 584000 Todesfälle durch Malaria, von denen die meisten in Afrika auftraten.

Als Therapie der Wahl für die unkomplizierte Infektion mit *Plasmodium falciparum* hat sich die Verwendung von Artemisinin-basierten Kombinationstherapien (ACTs) etabliert. Die Wirksamkeit von ACT gegen *P. falciparum* ist sehr gut belegt, jedoch gibt es nur wenige Daten, welche die Wirksamkeit von ACT gegen andere humanpathogene Spezies des Malariaerregers zeigen, da hier weiterhin meistens das Medikament Chloroquin zur Anwendung kommt und bisher klinische Studien fehlen. Jedoch finden sich in hochendemischen Gebieten neben *P. falciparum* auch Infektionen durch andere Spezies von *Plasmodium*. In der Praxis ist eine exakte mikroskopische Speziesidentifikation seltenerer *Plasmodium*-Spezies bei Mono- und Mischinfektionen sowie der Nachweis von residualen Parasiten nach der Chemotherapie oftmals nicht möglich. Die Verfügbarkeit einer Breitbandmedikation würde die Behandlung von seltenen (non-*falciparum*) *Plasmodium*-Spezies und Mischinfektionen erleichtern.

Diese Arbeit zeigt Daten über die Wirksamkeit von Artemether-Lumefantrin (AL), ein häufig in Zentralafrika eingesetztes ACT, bei unkomplizierten non-*falciparum* oder Mischinfektionen in Lambaréné, Gabun. Vierzig Patienten mit durch *P. malariae* oder *P. ovale* verursachter unkomplizierter Malaria sowie Mischinfektionen mit *P. falciparum* wurden drei Tage lang mit AL behandelt und für 28 Tage beobachtet. Die Therapie führte bei allen teilnehmenden Patienten zu einer raschen Reduktion der Parasitämie und gutem klinischem Ansprechen. Das Spektrum unerwünschter Ereignisse (*adverse events*) war gering und transient. Alle Patienten waren am Ende der Studie gesund. Nach 28 Tagen lag auch die Reduktion der Parasitenlast bei 100 % bei guter Verträglichkeit der Medikation, was für die Verwendung von AL als Behandlung der unkomplizierten Malaria in Gabun bei non-*falciparum* oder Mischinfektionen spricht. Die Ergebnisse der mikroskopischen Identifikation von non-*falciparum* Spezies waren trotz etablierter Qualitätskontrolle nur unzureichend. Von 39 Proben, welche mikroskopisch als non-*falciparum* oder Mischinfektionen deklariert wurden, konnten nur 19 durch die PCR als solche bestätigt werden. Als Grund hierfür kann unter anderem die häufig niedrige Parasitämie der Proben benannt

werden. Dies wiederum unterstreicht den Nutzen einer breit wirksamen Malariatherapie.

Der zweite Teil dieser Arbeit befasst sich mit der *in vitro* Wirksamkeit von Fluoreszenzfarbstoffen gegen *P. falciparum* Parasiten. Manche heute verwendeten antimikrobiellen Wirkstoffe, welche eine Breitbandwirkung besitzen, stammen ursprünglich von molekular veränderten künstlichen Farbstoffen ab und aufgrund des zunehmenden Auftretens von Resistenzen gegen die aktuell verwendeten Malariamedikamente ist die Entwicklung von neuen Chemotherapeutika notwendiger denn je.

Die ursprüngliche Idee die Wirksamkeit von fluoreszierenden Farbstoffen gegen *P. falciparum* zu testen entwickelte sich aus *in vivo* Markierungsversuchen. Während dieser Versuche wurde eine bemerkenswerte antiparasitäre Wirksamkeit mancher Farbstoffe beobachtet. Durch diese Beobachtung motiviert, wurde die Wirksamkeit von vierzehn fluoreszierenden Farbstoffen in *in vitro* Wachstums-Tests für die zwei Laborstämme von *P. falciparum* 3D7 und Dd2 systematisch untersucht.

Fünf der verwendeten Farbstoffe (Rhodamine B, MitoTracker Red, and DiOC6, Hoechst33342, SYTO 9) hatten eine vergleichbare Wirksamkeit zur Kontrollgruppe mit Chloroquin und verhielten sich zudem nicht zytotoxisch bei Anwendung an menschliche HeLa Zellen. Daraufhin wurden die Farbstoffe Rhodamine B und DiOC6 im Maus-Modell untersucht, jedoch zeigte sich hier eine hohe Toxizität von DiOC6. Rhodamine B war weniger toxisch, führte aber nicht zu einer relevanten Reduktion der Parasitämie. Ungeachtet dessen zeigen diese Testversuche, dass die Untersuchung der antiparasitären Eigenschaften von Farbstoffen eine interessante Option darstellt um neue effiziente pathogen spezifische Substanzen zu finden. Die bereits erwähnte Notwendigkeit von breit wirksamen Malariawirkstoffen in Endemiegebieten ist unumstritten. Dabei sollte die Anforderung an das Wirkspektrum nicht nur die Elimination von verschiedenen *Plasmodium*spezies, sondern auch das jeweilige Entwicklungsstadium des *Plasmodium*s umfassen.

AL besitzt zwar eine sehr gute Wirksamkeit gegen verschiedene Parasitenspezies, wirkt jedoch nicht gegen die prä-erythrozytären Stadien des Parasiten (Sporoziten und infizierte Hepatozyten) und nur unzureichend gegen Gametozyten. Farbstoffe sind eine heterogene Stoffgruppe, die diesem erweiterten Wirksamkeitsspektrum entsprechen könnten. Es wurde gezeigt, dass Methylenblau tatsächlich diese

Anforderungen erfüllt. Die Untersuchung von größeren Farbstoff Datenbanken könnte zur Entwicklung von neuen breit wirksamen Wirkstoffen beitragen.

Summary

Malaria, an infectious disease caused by a parasite of the genus *Plasmodium*, was responsible for about 198 Million cases and 584 000 deaths in 2013, mainly in Sub-Saharan Africa.

The first line treatment for uncomplicated *Plasmodium falciparum* infections are artemisinin combination therapies (ACTs). Although the efficacy of ACTs against *P. falciparum* has been well documented, there is only anecdotal evidence of efficacy of ACTs against other human *Plasmodium* species because chloroquine is still recommended and commonly used. However, in highly endemic regions non-*falciparum* species can occur together with *P. falciparum* and, moreover, exact microscopic species identification and detection of residual parasites is usually not possible in those settings. Availability of broadly acting drugs would ease clinical management of suspected non-falciparum and mixed infections.

This thesis presents data on the efficacy of artemether-lumefantrine (AL), a widely used ACT, against uncomplicated non-*falciparum* or mixed-species malaria in Lambaréné, Gabon and assesses the performance of microscopy in an African routine laboratory setting. Forty patients presenting with uncomplicated malaria caused by *P. malariae*, *P. ovale* or a mixed infection (including *P. falciparum*) were treated with a three days AL regimen and were followed up to 28 days after start of the treatment. All evaluable patients presented an adequate clinical and parasitological response. All adverse events were mild or moderate and resolved by the end of the follow-up. The parasitological cure rate was 100% and the drug was well tolerated. These findings support the use of AL for the treatment of uncomplicated malaria in Gabon, even when non-falciparum or mixed infections are suspected. Microscopic identification of *Plasmodium* species other than *P. falciparum* did not perform well. From 39 samples microscopically determined as non-falciparum or mixed infection, only 19 were confirmed by PCR. This might be due to the very low parasitaemia found in most cases and underlines the need for the development of broadly acting antimalarials.

The second part of this thesis reports on the *in vitro* activity of fluorescent dyes against cultured malaria parasites. The emergence of resistance against most of the drugs in current use urges the development of new antimalarial compounds. Since

several antimicrobials were derived from molecular modification of synthetic dyes, which often have a broad spectrum of activity, screening of dyes may represent an interesting strategy to identify novel antimalarial lead structures. The original motivation to screen fluorescent dyes in *Plasmodium* were *in vivo* labeling experiments. Here, a remarkably high antiparasitic activity was noted for some compounds. Hence, the activity of fourteen fluorescent dyes was systematically investigated by *in vitro* growth assays using the two laboratory *P. falciparum* strains 3D7 and Dd2. Five had an activity comparable to the control drug chloroquine and were not cytotoxic against human HeLa cells (Rhodamine B, MitoTracker Red, and DiOC6, Hoechst33342, SYTO 9). Rhodamine B and DiOC6 were further tested in a mouse model but DiOC6 was toxic and Rhodamine B, although less toxic, did not lead to a notable reduction of parasitaemia. Despite of this, this pilot experiment shows that pre-screening for staining properties may be an interesting way to build highly efficient pathogen-specific compound libraries.

There is a need for broadly acting antimalarial drugs. This requirement includes multi-stage activity as well as activity against different parasite species. AL showed high *in vivo* activity against different *Plasmodium* species, but does not act against pre-erythrocytic stages (sporozoites and infected hepatocytes) and reduces gametocytes only modestly. Dyes are a group of chemically heterogeneous compounds that may extend the spectrum of activity. In fact, the dye methylene blue was shown to meet many of the requirements. Investigation of larger dye libraries may result in highly interesting novel antimalarial compounds and may lead to the development of novel, broadly acting drugs.

Résumé

Le paludisme est une maladie infectieuse causée par un parasite du genre *Plasmodium*. En 2013, cette maladie était responsable d'environ 584000 décès principalement en Afrique subsaharienne. Les traitements de première ligne pour les infections à *Plasmodium falciparum* (*P. falciparum*) sont les thérapies combinées à base d'artémisinine (ACT). Bien que l'efficacité des ACTs contre *P. falciparum* a été clairement démontrée, il n'existe que des preuves anecdotiques de l'efficacité des ACTs contre les autres espèces de paludisme humain et la chloroquine est toujours le traitement recommandé dans ces cas-là. Cependant, les espèces non-*falciparum* peuvent souvent apparaître de façon concomitante avec *P. falciparum* et de plus, l'identification des espèces s'avèrent toujours difficile. Ce serait ainsi un avantage important d'avoir un traitement unique pour toutes les espèces de paludisme. C'est pourquoi la première partie de cette thèse propose d'évaluer l'efficacité d'artemether-lumefantrine (AL), une ACT couramment utilisée contre les infection paludiques non compliquées, non-*falciparum* ou bien mixtes à Lambaréné, Gabon.

Quarante patients présentant un paludisme non compliqué provoqué par *P. malariae*, *P. ovale* ou bien mixtes (incluant *P. falciparum*) ont été traités avec un traitement à l'AL de trois jours et ont été suivi pendant 28 jours. Tous les patients évaluables présentaient une réponse clinique et parasitologique adéquat au jour 28. Tous les effets indésirables ont été d'intensité légère ou modérée et ont été résolus au jour 28. Le taux de guérison au niveau parasitologique a été de 100% et le traitement a été bien toléré ce qui supporte l'utilisation de AL pour le traitement de tous les cas de malaria non-complicés sans prendre en compte l'espèce. L'identification microscopique des espèces de *Plasmodium* autres que *P. falciparum* n'a pas été très performante. Sur 39 échantillons déterminés comme des infections non-*falciparum* ou mixtes par microscopie, seule 19 ont pu être confirmés par PCR. Cela peut être dû à la faible parasitémie et souligne le besoin pour des antipaludiques à large spectre d'action.

Dans la deuxième partie de cette thèse nous évaluerons l'activité de colorants fluorescents contre des souches de *P. falciparum* adaptées à la culture.

L'émergence de résistances à la plupart des médicaments en usage incite au développement rapide de nouvelles chimiothérapies. De nombreux médicaments antimicrobiens dérivent à l'origine de la modification moléculaire de colorants

synthétiques. Avec ce travail, nous voulons relancer l'utilisation de colorants comme critère de présélection pour la découverte de nouveaux composés actifs. Nous avons évalué l'activité de quatorze colorants fluorescents contre deux souches de laboratoire de *P. falciparum*, 3D7 et Dd2. Cinq colorants ont eu une activité comparable au contrôle (chloroquine) et n'étaient pas cytotoxiques contre les cellules humaines HeLa (Rhodamine B, MitoTracker Red, and DiOC6, Hoechst 33342, SYTO 9).

La Rhodamine B et DiOC6 ont été testés sur un modèle murin. DiOC6 était très toxique et Rhodamine B, bien que moins toxique, n'a pas réussi à entraîner une diminution de la parasitémie. Malgré cela, cette étude pilote montre que la présélection pour les propriétés colorantes des molécules peut être un moyen intéressant de construire des chimiothèques spécifiques du pathogène.

Il y a un besoin en médicaments antipaludiques à large spectre d'action. Ce besoin inclus une activité contre des stades multiples ainsi qu'une activité contre différentes espèces de parasites. AL a montré une très bonne activité contre différentes espèces mais n'agit pas contre les stades pré-érythrocytaires et ne réduit la gamétocytémie que de manière modeste. Les colorants sont un groupe de composés chimiques hétérogènes qui peuvent étendre le spectre d'activité. En fait, le colorant bleu de méthylène a montré qu'il répondait à un grand nombre de ces exigences. L'investigation de larges bibliothèques de colorants peut aboutir à de nouveaux composés antipaludiques très intéressants et peut conduire au développement de nouveaux médicaments à large spectre d'action.

List of publication

This work represents the summary of the two following publications:

- I) **Joanny F**, Held J, Mordmüller B. In vitro activity of fluorescent dyes against asexual blood stages of *Plasmodium falciparum*. *Antimicrob Agents Chemother*. 2012 Nov; 56(11):5982-5.
- II) Mombo-Ngoma G, Basra A, Würbel H, Diop DA, Capan M, Adegnika AA, Kurth F, Mordmüller B, **Joanny F**, Kreamsner PG, Ramharter M, B elard S. Prospective evaluation of artemether-lumefantrine for the treatment of non-*falciparum* and mixed-species malaria in Gabon. *Malar J*. 2012 Jul 10; 11:120.

In addition, three other articles have been published which will not be summarized in this work.

- III) **Joanny F**, L ohr SJZ, Engleitner T, Lell B and Mordm uller B. Limit of blank and limit of detection of *Plasmodium falciparum* thick blood smear microscopy in a routine setting in Central Africa. *Malar J*. 2014 Jun 14; 13(1):234.
- IV) Tschan S, Brouwer AJ, Werkhoven PR, Jonker AM, Wagner L, Knittel S, Aminake MN, Pradel G, **Joanny F**, Liskamp RM, Mordm uller B. Broad-spectrum antimalarial activity of peptido sulfonyl fluorides, a new class of proteasome inhibitors. *Antimicrob Agents Chemother*. 2013 Aug; 57(8):3576-84.
- V) Mordm uller B, Supan C, Sim KL, G omez-P erez GP, Ospina Salazar CL, Held J, Bolte S, Esen M, Tschan S, **Joanny F**, Lamsfus Calle C, L ohr SJ, Lalremruata A, Gunasekera A, James ER, Billingsley PF, Richman A, Chakravarty S, Legarda A, Mu oz J, Antonijoan RM, Ballester MR, Hoffman SL, Alonso PL, Kreamsner PG. Direct venous inoculation of *Plasmodium falciparum* sporozoites for controlled human malaria infection: a dose-finding trial in two centres. *Malar J*. 2015 Mar 18;14:117.

1. Introduction

1.1. Epidemiology and burden of the disease

Malaria is an infectious disease caused by an Apicomplexa parasite of the genus *Plasmodium*. It is transmitted by the bite of an infected female *Anopheles* mosquito. There are more than 200 different species of *Plasmodium* known, five of which cause naturally acquired malaria in humans: *Plasmodium falciparum* (*P. falciparum*), *Plasmodium vivax* (*P. vivax*), *Plasmodium malariae* (*P. malariae*), *Plasmodium ovale* (*P. ovale*) and *Plasmodium knowlesi* (*P. knowlesi*). A recent study based on genetic observation suggests that *P. ovale* can be differentiated in two species, a classic type (*P. ovale curtisi*) and a variant type (*P. ovale walikeri*) [1]. *P. falciparum* is the most deadly parasite and predominates in Sub-Saharan Africa but is also prevalent in Southeast Asia and South-America. Although *P. vivax* is considered as benign tertian malaria, it can be associated with significant morbidity and even mortality. A study conducted in Papua-New Guinea showed that 9% of children infected with *P. vivax* presented with severe malaria against 12% of children infected with *P. falciparum* [2]. Moreover *P. vivax* accounts for almost half of the cases outside Africa and can remain in the liver in a dormant stage as hypnozoite [3–5]. *P. ovale* is mainly distributed in Sub-Saharan Africa and in some islands of the Western Pacific and account for less than 5% of the malaria cases [6]. It is usually benign but can also develop hypnozoites, dormant liver stages. *P. malariae* is usually benign and has a low prevalence. Its distribution corresponds to *P. falciparum* to which it is often found as co-infection. However, *P. malariae* infections often remain undetected if no molecular diagnostic method is used.

P. knowlesi was first described in the 30's infecting *Macaca fascicularis* monkeys. It has been shown in 1932 that the parasite was also able to infect humans and it was even used to induce fever in the treatment of neurosyphilis [7]. Very few natural cases have been reported until 2004 [8], when it was shown that active transmission of the parasite from macaques to humans is present in Southeast Asia [7]. Although no naturally occurring human-to-human transmission has been confirmed so far, a host-switch of *P. knowlesi* from macaques to humans is not impossible and should be

closely monitored [9]. *P. knowlesi* can also lead to severe illness with symptoms similar to *P. falciparum* severe malaria [11]

Malaria is one of the major health problems in tropical and subtropical regions. In 2013, 198 million cases were reported and the number of deaths was estimated to be 584 000 according to WHO, 90% were in the African region and 78% in children under 5 years. [12]. These figures are estimates and slightly changed assumptions lead to significantly higher numbers [13]. Malaria accounts for 5% of deaths of children under the age of five globally and, with 16% in Africa, is a major contributor to the disease burden of highly endemic regions.

Beside this, malaria has a huge socioeconomic impact on the country affected by the disease. It has been estimated that the disease slows the economic growth of African countries by 1.3% a year. This is a consequence of the morbidity of the disease, which reduces the productivity of the population. It also discourages economic investment because of the risk of low productivity of the labor forces. Moreover, it challenges the health care system through additional expenses, this is especially relevant for economically weak countries with a poorly developed health sector [15].

1.2. Pathology of the disease

The life cycle of the parasite includes a sexual phase (sporogony) in the female mosquito and an asexual phase (schizogony) in the human host (Figure 1). An infected female mosquito feeds on a human host and inoculates sporozoites, which directly migrate to the liver. There they bind to heparin sulfate proteoglycans and glide along the surface of the sinusoidal endothelium and finally cross it through Kupffer cells. They transmigrate through several hepatocytes before invasion and becoming sessile. Within the host cell sporozoites divide and develop into schizonts, which form daughter cells called merozoites. This phase is called hepatic, pre-erythrocytic or exo-erythrocytic and is clinically silent (asymptomatic). It ends with the release of merozoites (merozoites containing vesicles) into the blood stream. Merozoites egress from the merozoite and invade red blood cells (RBC) where the parasites develop into trophozoites and subsequently schizonts. After completion of maturation, merozoites are released in the blood stream, which invade new erythrocytes. Some parasites can differentiate in female or male gametocytes and, if ingested by a female mosquito, mate, develop into new sporozoites and start the cycle again.

The symptoms of malaria occur during the erythrocytic phase of the cycle. Typically, symptoms are cyclic and peak shortly after merozoites are released into the blood stream. The symptoms are not specific and include fever, chills, headache, vomiting, fatigue, abdominal discomfort, muscle and joint ache, anemia, splenomegaly. In *P. falciparum* malaria, severe symptoms can occur: coma (cerebral malaria), prostration, convulsions, metabolic acidosis, severe anemia, hypoglycemia, acute renal failure or acute pulmonary oedema. Even with treatment, the case fatality in severe malaria can reach 20% [16].

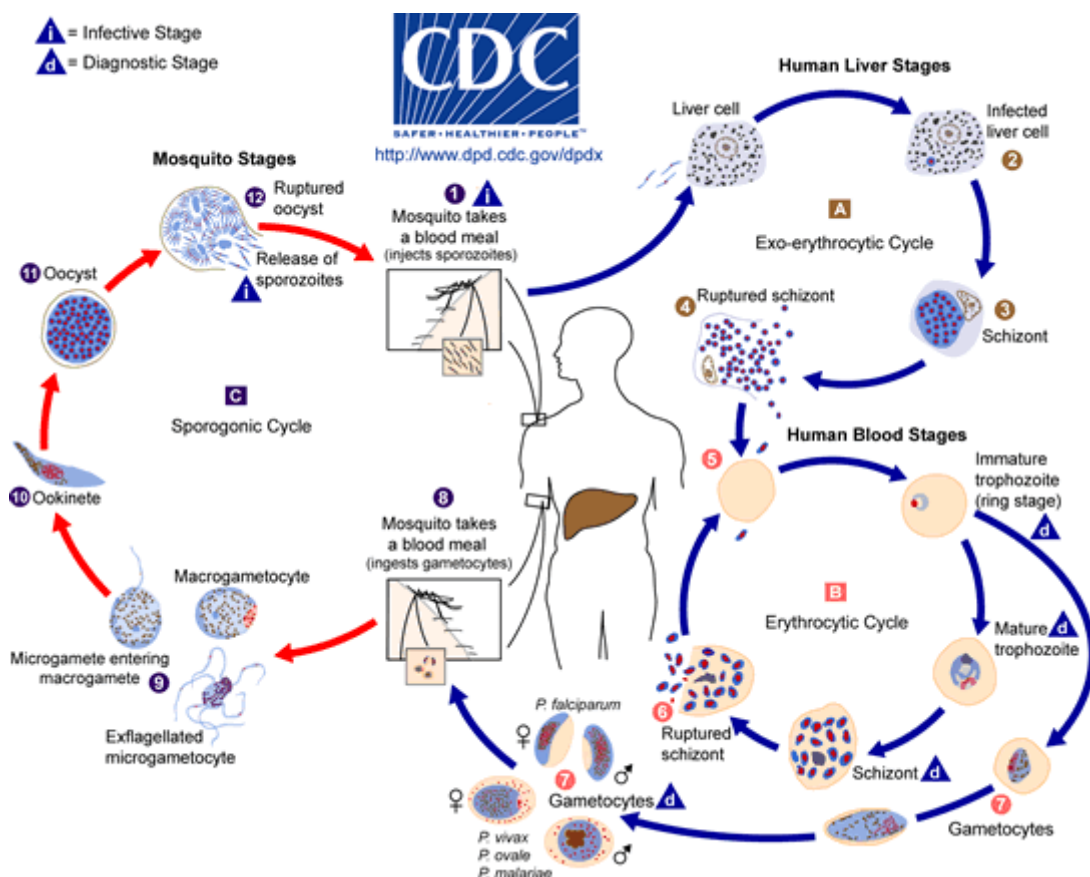


Figure 1: Life cycle of the malaria parasite [17]

1.3. Malaria control policy

In 1955, WHO started a first Global Malaria Eradication Program (GMEP) aiming to eradicate malaria in all endemic country except Sub-Saharan Africa and Madagascar. The strategy was to eliminate the vector through spraying with dichloro-diphenyl-trichloroethane (DDT) in order to prevent transmission. Chloroquine was used as first line treatment and because of its efficacy and its low price, it was given

without laboratory confirmation of malaria. Other control strategies such as the use of bed nets or the draining of mosquitoes breeding marshes were abandoned [18]. However, DDT was found to be toxic and resistance started to develop in the mosquito population. Although it is still used in some tropical countries in the absence of a good alternative, its use is limited to indoor residual spraying. Additionally, *P. falciparum* chloroquine resistance emerged from independent foci and spread in the 60s and 70s to an almost universal level. Nowadays, chloroquine cannot be used to treat *P. falciparum* malaria in almost all epidemiological relevant areas anymore [19]. Despite some success and advances, WHO recognized that eradication was not feasible in many countries and that a strategy of control with the aim to reduce morbidity and mortality would be more appropriate. As a consequence, the program was stopped in 1969. In the 70s and 80s, economic restrictions and a decrease in funding for malaria control, resulted in a severe increase of the burden of malaria in some countries [18]. Meanwhile, the WHO focused on research and development of new tools for malaria control and thus created a program in the mid 1970s; the Program for Research and Training in Tropical Diseases (TDR) [18]. In 1998, the WHO launched the Roll Back Malaria initiative with its strategy to reduce malaria morbidity and mortality by implementing global coverage and strengthening local health system [20]. Among the targets of WHO is the reduction of global malaria death to zero and a reduction of global malaria cases by 75% from 2010 to 2015. WHO's strategy comprises two main approaches: malaria prevention through malaria vector control and better diagnosis and treatment. The fight against malaria is also one of the eight Millennium Development Goals from the United Nation which were established in 2000 with the objective to halt and begin to reverse the incidence of malaria by 2015 [21].

1.4. Malaria treatment

During the campaign for malaria eradication in the 60s, chloroquine was the first line treatment because of its efficacy and its low price. Today, the drug has almost vanished from the market in endemic countries and is no more effective against *P. falciparum* infections in all relevant endemic areas [19].

In the late 60s, the Chinese government launched a large research program in order to find an alternative to chloroquine. Plants and their extracts used in traditional medicine were screened for their activity. Among them were extracts of *Artemisia*

annua, a plant used for centuries in traditional medicine that contains the endoperoxide artemisinin as the antimalarial principle. However it took a long time before artemisinin arrived on the global market, notably because it had to go through a full clinical development program to ensure that it was efficacious and not toxic. Moreover it was expensive to produce and thus not suitable for developing countries [22]. But thanks to a decrease in production cost its use has widely extended and since 2006 the first line treatments for *P. falciparum* uncomplicated malaria recommended by the WHO are Artemisinin based combination therapies (ACTs): artemether plus lumefantrine, artesunate plus amodiaquine, artesunate plus mefloquine and artesunate plus sulfadoxine-pyrimethamine (AS-SP). Because of its short half-life and to prevent development of resistance it is recommended to always use artemisinin and its derivatives in combination with partner drugs. Second line treatments consist of *ad-hoc* combinations of artesunate or quinine in combination with an antibiotic such as tetracycline, doxycycline or clindamycin. In case of pregnancy, quinine plus clindamycin is recommended during the first trimester and ACTs are recommended for the second and third trimester. For travelers returning to non-endemic countries, the following treatments are recommended: atovaquone-proguanil, artemether-lumefantrine, quinine plus doxycycline or clindamycin. For the treatment of severe malaria, it is recommended to use artesunate intravenously (i.v.) or intramuscularly (i.m.) or, if not available, the recommended alternative is artemether or quinine i.v. or i.m. [16].

For the treatment of *P. vivax*, chloroquine in combination with primaquine is still the treatment of choice. ACTs can also be used in combination with primaquine, which is the only licensed and available drug acting on hypnozoites. However due the development of resistance to pyrimethamine, the combination artesunate plus sulfadoxine-pyrimethamine (AS-SP) might be less effective against *P. vivax* in many places [16].

The recommended treatment of *P. ovale* and *P. malariae* infection is the same as for *P. vivax*, namely chloroquine but without primaquine in the case of *P. malariae* as this species does not produce hypnozoites [16].

Despite the recommendation of the WHO for the treatment of non-*falciparum* species, ACTs are often used for the treatment of all forms of malaria. This is due to the fact that non-*falciparum* species and especially *P. malariae* and *P. ovale* often occur concomitantly with *P. falciparum*. Moreover they are often difficult to identify in

absence of molecular diagnostic test such as Polymerase Chain Reaction (PCR) and low-grade infection with *P. falciparum* cannot be ruled out. Nevertheless, only anecdotal evidence is present to show that ACTs are also efficient against *P. malariae*, *P. ovale* and mixed-species infection.

1.5. The problem of resistance

Resistance is defined as the ability for a parasite strain to survive or develop despite the administration of a drug at a dose equal or higher than the normally recommended dose. In the case of malaria, it has to be clarified that the active drug must be able to reach the parasite within the infected cell, for the duration of the time necessary for its normal action [23]. If the efficacy of a drug is decreased because the active substance did not reach the parasite, we talk about medicament failure. This is for example the case if some individuals metabolize the drug too quickly to ensure a sufficient concentration or because the RBC membrane becomes impermeable to the drug because of an increase pH due to parasite metabolites.

P. falciparum resistance to chloroquine is spread worldwide and, except in very few areas, chloroquine should not be used for the treatment of *P. falciparum* infections anymore. Artemether-lumefantrine remains highly effective worldwide except Cambodia, where resistance against many ACTs have already been reported [24]. In addition, high treatment failure rates have been reported in six African countries who adopted artesunate-amodiaquine as first line treatment, most likely due to resistance against the partner drug. Artesunate-mefloquine has lower efficacy in the Mekong region where mefloquine resistance is already widespread. Moreover, several *in vitro* and *in vivo* studies showed a decreased sensitivity of *P. falciparum* to artesunate, notably in Bangladesh, Thailand and Cambodia [25–27].

Chloroquine treatment failure against *P. vivax* has been reported since 1989 from Indonesia, Myanmar, India and South-America. Moreover higher dosage of primaquine are now often required to clear hypnozoite in Southeast Asia, South-America and South-Pacific, which suggests the presence of primaquine tolerant parasites [19]. *P. vivax* resistance to sulfadoxine-pyrimethamine (SP) is frequent, hence SP-treatment is not recommended for *P. vivax* infections [16].

No case of resistance has been reported for *P. ovale* but some cases of treatment failure with chloroquine and quinine have been reported for *P. malariae* [28–30]. However in absence of reliable study, the existence or level of drug resistance in these two parasite species is questionable.

1.6. Rational for the development of new anti-malarial drugs

Antimalarial drugs belong to only few classes of chemical scaffolds. Nowadays, mainly eight different chemotypes are in clinical use, which act on five ways of action (see Figure 2) [31]. For example, chloroquine, piperaquine and amodiaquine are 4-aminoquinoline molecules; mefloquine as well as halofantrine and lumefantrine are analogues of quinine. In fact, all these molecules are related to the active compound of the cinchona bark [32]. Even if the mode of action of many of these drugs is not completely understood, it is assumed, and in some cases experimentally shown, that resistance develops faster if molecules are similar. Indeed, resistance against many of these drugs developed quickly after they have been introduced. For example resistance to mefloquine arose only a few years after its introduction at the border of Cambodia and Thailand, partly because of its long half-life, which leads to extended exposure of parasites to sub-therapeutic mefloquine concentrations, but also of preexisting quinine resistance [33].

Therefore, it is commonly accepted that there is a constant need for new drugs to circumvent this resistance problem. E.g. the UN millennium report recommends: *“It is important to maintain a sufficiently large, high-quality pipeline of antimalarial compounds to ensure sustainability in medicine discovery and development.”* [21]

The important challenges in the fight against malaria are that the parasite exist under different forms (asexual and gametocytes), can stay dormant for several years in the case of *P. vivax* and *P. ovale* (hypnozoites) or can remain at low level in asymptomatic carriers and that five different species can infect humans. To address these challenges, an ideal drug should be broadly acting which means that it could eliminate all five species of *Plasmodium* as well as the gametocytes and the liver forms.

It is often assumed from case report that a drug effective against *P. falciparum* is also effective against *P. malariae* and that a drug effective against *P. vivax* is also effective against *P. ovale* but usually no hard evidence is present. This can be a

problem as the strategy to eliminate malaria often focus on *P. falciparum* and *P. vivax* but if the prevalence of these both species is reduced, we do not know how the transmission of *P. malariae* and *P. ovale* will evolve [34]. Therefore more clinical studies are required about these neglected species and they should be considered when developing new drugs.

A broadly acting drug should dramatically reduce the transmission of the parasites. It should be radically effective to kill all replicating asexual parasites as well as the gametocytes and the hypnozoites. It should moreover be safe and well tolerated to allow administration to vulnerable groups and asymptomatic carriers (e.g. in mass administration campaigns). Such a drug would also prevent the development of resistance [35]. Currently no drug combines all these characteristics, therefore research and development of new drugs is important. Moreover, for drugs already in clinical use, evidence through clinical trials is needed to assess their efficacy against all malaria species, including *P. ovale* and *P. malariae*.

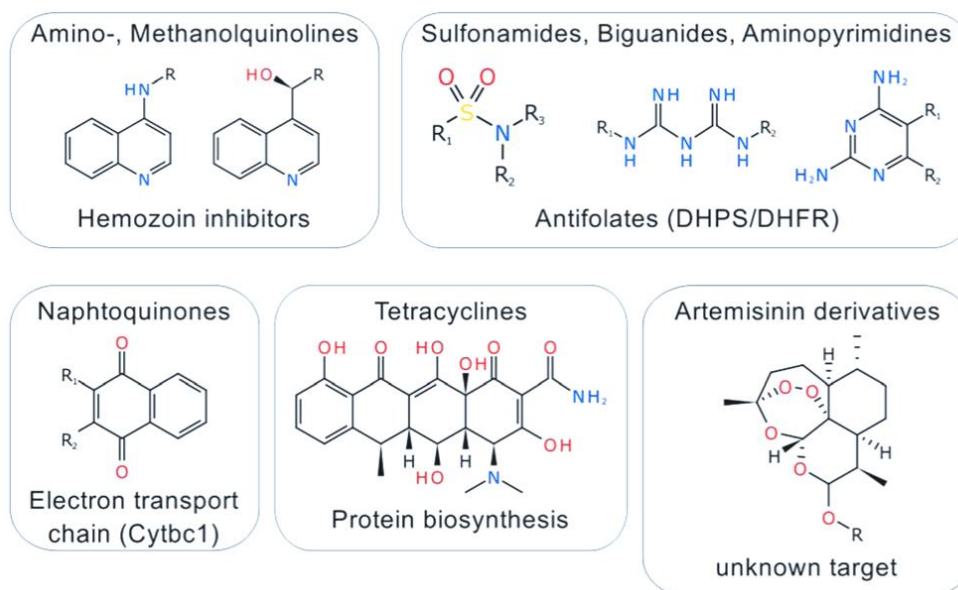


Figure 2: Clinically used antimalarial chemotypes and their way of action. (From Spitzmüller A et al. [31])

1.7. Screening of dyes for their antiplasmodial activity

The history of malaria chemotherapy and the history of synthetic dyes are heavily intertwined. In fact, one could claim that the dye industry is founded in malaria research. The first synthetic dye, mauvein, was discovered in 1856 by William Henry

Perkin when he was trying to synthesize quinine. This was a commercial success and Perkin developed many other dyes used in the industry [36]. Synthetic dyes revolutionized the textile industry but also modern chemotherapy. Paul Ehrlich was using dyes to differentially stain tissue, cells or microbes and developed the concept that a dye staining specifically a microorganism could also have a specific toxic effect by interacting with it. Thus, when he noticed that methylene blue stains specifically *P. falciparum*, he tried to treat two malaria patients with this compound and successfully cured them [37]. He also had success in the treatment of trypanosomiasis with trypan red [36].

Generally speaking, the pharmaceutical industry developed mainly from the dye industry after World War I. For example the pharmaceutical company Bayer derived from the German dye company Ig Farben. The sulfonamide drugs, precursors of sulfadoxine, were developed in this company from a textile dye [38]. After WWI, the German government funded a search for quinine substitute in order to supply the troops. The research was performed by Ig Farben, where thousands of compounds were tested. One of the most promising was mepacrine derived from the dye acridine [22]. In 1934 Resochin was synthesized and tested but because of its presumed toxicity it was not developed further. It was only during World War II that the American army obtained the compound and developed it as chloroquine.

Nowadays, the strategy to develop new drugs is not anymore based on staining properties but still follows Paul Ehrlich's concept:

"We must search for magic bullets. We must strike the parasites and the parasites only, if possible, and to do this, we must learn to aim with chemical substances!"

The conventional approach is high throughput screening of large chemical libraries in order to find compounds acting on a specific target. It is also possible to pre-select compounds by multiple steps e.g. through the modifications of known pharmacophores. This latter approach is not the best in the context of malaria because of the previously mentioned resistance and cross-resistance problems.

In this work, the use of staining properties as a pre-selection criterion in order to discover new anti-malarial compounds was successfully revived.

1.8. Objective of the thesis

The thesis is divided in two parts:

1.8.1. Evaluation of the antiplasmodial activity of fluorescent dyes

The objectives of this work were:

- 1) to identify novel antimalarial structures with a broad spectrum of activity.
- 2) to investigate specific staining as a pre-selection criterion to discover new anti-malarial compounds.

To prove the concept, fourteen commercially available dyes were assessed: MitoTracker red, MitoTracker green, Daspei, DiOC₆, Rhodamine 123, Rhodamine B, JC1, and SYTO 18, all of which stain specifically mitochondria, Hoechst 33342, Acridine Orange, SYBR Green I, SYTO 9 which stain nucleic acids and carboxyfluorescein diacetate [CFDA] and CFDA-succinimidyl ester [CFDA-SE], two molecules binding proteins. Their anti-plasmodial activity was tested with an *in vitro* test against two laboratory strains of *P. falciparum*, the chloroquine sensitive 3D7 and the chloroquine resistant Dd2. In a second step, the toxicity against human cells was evaluated for the dyes with good antiplasmodial activity.

1.8.2. Prospective evaluation of artemether-lumefantrine for the treatment of non-falciparum and mixed-species malaria in Gabon

The efficacy of ACT therapy has been shown against *P. falciparum* and *P. vivax* but no study has been done so far to investigate their efficacy against *P. malariae* and *P. ovale*. However, because these parasites often occur as co-infection together with *P. falciparum* and frequently remain undetected or miss-classified because they are difficult to distinguish from other species by classical diagnostics methods, these two species are often treated with ACTs. To develop recommendations for the treatment of non-falciparum and mixed infections, it is important to know the efficacy of ACT treatment against these two rare and neglected species. Artemether-lumefantrine is the most widely used ACT.

The objectives of this work were:

- 1) The primary objective of this work was to determine the activity of Artemether-lumefantrine against non-falciparum or mixed-species malaria infection in Gabon.
- 2) The secondary objective was to investigate the reliability of microscopic determination of *Plasmodium* species under routine conditions was assessed.

2. Results

2.1. *In vitro* activity of fluorescent dyes against asexual blood stages of *Plasmodium falciparum* (publication 1)

In order to find new classes of antimalarial drugs, I tried to revive the use of staining property as pre-selection criterion in the discovery of new drugs. Fourteen commercially available fluorescent dyes which stain specifically mitochondria (MitoTracker red, MitoTracker green, Daspei, DiOC₆, Rhodamine 123, Rhodamine B, JC1, and SYTO 18), nucleic acids (Hoechst 33342, Acridine Orange, SYBR Green I, SYTO 9) or proteins (carboxyfluorescein diacetate [CFDA], CFDA-succinimidyl ester [CFDA-SE]) were selected. The antiplasmodial activity of the dyes were tested against two laboratory strains of *P. falciparum*, the chloroquine sensitive 3D7 and the multi-resistant Dd2 strain, and their activity was compared with those of two reference drugs, chloroquine and methylene blue. Parasite multiplication in the presence of serial dilutions of candidate dyes or controls was measured by the Histidine-Rich-Protein 2 assay as described previously [39] and an 50% Inhibitory concentration (IC₅₀) was calculated by regression analysis of log concentration response curve. Two different incubation times of either three or six days have been tested. The six days assay has been performed as described previously in order to see if the substance acted on the subsequent cycle (delayed death phenomenon) [40]. An ideal drug should act specifically against the parasite and not against human cells. Therefore cytotoxicity of dyes with the highest antiplasmodial activity was evaluated in human HeLa cells by a neutral red assay [41]. The dyes with the highest activity against *P. falciparum* strains 3D7 and Dd2 were Hoechst 33342 (7.2 nM and 16.3 nM), MitoTracker red (8.2 nM and 15.9 nM), DiOC₆ (11.4 nM and 20.8 nM), SYTO 9 (20.5 nM and 36.1 nM) and Rhodamine B (20.6 nM and 26.1 nM, respectively). CFDA and CFDA-SE had very low or no activity against both strains (IC₅₀ above 333µM and 55µM respectively) (table 1). All dyes with high activity were at least 50 times less toxic against human HeLa cells than against *P. falciparum*.

Dyes	IC ₅₀ ±SD		IC ₉₀ ±SD	
	3 days	6 days	3 days	6 days
Hoechst 33342	7.2±3.1	18.9±11.9	14.0±8.8	25.8±17.3

	16.3±11.2	28.0±2.6	72.7±107.9	36.5±0.8
MitoRed	8.2±9.6	10.6±6.1	14.1±19.5	15.3±8.4
	15.9±10.9	6.3±7.3	44.8±24.0	9.0±0.2
DiOC ₆	11.4±8.1	17.3±17.1	17.4±9.4	24.1±23.9
	20.8±12.2	168.3±301.8	55.7±31.5	229.0±401.4
Rhodamine B	20.6±12.6	19.2±10.8	40.7±12.4	29.5±8.9
	26.1±15.3	69.5±113.2	101.1±34.6	117.2±194.8
SYTO 9	20.5±20.0	23.1±44.9	101.5±105.	41.0±64.2
	36.1±17.7	4.9±0.73	261.7±114.	10.8±3.9
JC1	107.8±66.3	177.0±55.0	170.6±84.8	279.8±95.36
	100.1±96.9	121.0±128.2	269.9±252.	162.0±156.3
MitoGreen	115.5±149.9	187.1±197.2	331.9±407.	245.2±215.0
	148.9±102.7	103.8±58.0	1521±2335	150.8±87.9
Rhodamine 123	387.7±208.6	189.2±235.1	870.3±567.	490.3±584.0
	140.7±117.4	287.2±316.0	2335±3893	414.2±450.7
Acridine Orange	465.7±274.9	691.9±391.8	1772±1057	691.9±391.8
	166.0±75.9	208.2±22.5	620.4±379.	278.8±31.4
Daspei	327.2±327.2	502.7±797.2	2026±2364	834.7±1298
	591.3±405.8	270.6±267.2	55046±798	831.3±1315
SYTO 18	1751±969.8	873.1±482.1	5016±3186	1916±1144
	387.1±207.6	636.3±413.5	1865±381.1	1141±880.2
SYBR Green I	2585±4693	11497±17785	4669±8532	17897±28028
	2100±1024	1218±583.2	14062±173	1792±872.5
CFDA-SE	> 55 µM	> 55 µM	> 55 µM	> 55 µM
	> 55 µM	> 55 µM	> 55 µM	> 55 µM
CFDA	> 333 µM	> 333 µM	> 333 µM	> 333 µM
	> 333 µM	> 333 µM	> 333 µM	> 333 µM
Methylene blue	7.8±3.2	14.3±10.7	13.3±1.5	20.3±13.4
	14.3±4.1	17.2±12.4	29.2±7.8	27.3±20.2
Chloroquine	4.1±1.8	6.2±1.8	6.8±3.3	7.8±2.1
	63.8±30.1	84.6±35.6	99.3±45.3	131.8±33.6

Table 1: Inhibitory concentrations IC₅₀ and IC₉₀ (in nM) of selected dyes and chloroquine against the culture-adapted strain of *P. falciparum* 3D7 or Dd2 (grey highlight). Each value is the geometric mean IC of at least 3 independent experiments.

2.2. Activity of DiOC6 and Rhodamine B against *P. berghei* in mice

In the previous experiments, five dyes with good antiplasmodial activity and a good selectivity index were identified. The next step was to test their activity *in vitro* in a mouse model. It was decided not to test SYTO 9 and Hoechst 33342 because of the risk of mutation as they target nucleic acids. Since MitoTracker Red and Rhodamine B are both Rhodamine dyes, it was decided to pilot the experiment with Rhodamine B, a compound which is still being used in cosmetics in some countries although it has been removed from the FDA color additive status list since 1983 [42]. In addition, DiOC6 was tested as well.

2.2.1. Evaluation of the toxicity of DiOC6 and Rhodamine B

Three female CD1 *swiss* mice received one dose of 40mg/kg of DiOC6 i.p. Two mice died within 2h after receiving DiOC6. The third one died 24h after the injection. The three mice who received 15mg/kg of Rhodamine B were monitored for four days. There was no significant change in the weight and mice behaved normally.

2.2.2. Evaluation of the efficacy *in vivo*

Two groups of nine mice were infected i.v. with $2 \cdot 10^7$ *P. berghei* infected erythrocytes. Two hours later, the mice received either 15mg/kg of Rhodamine B in 0.3mL or the same volume of injection solution (7% Tween 80/3% Ethanol). Mice continued to receive the same treatment every day for three days and their parasitaemia was monitored until four days after infection. Parasitaemia was determined by thin blood smear microscopy. Three days after infection, two mice who received Rhodamine B died, four days after infection another four mice died. In comparison, all the mice from the control group survived until the end of the experiment (4 days after the infection). There was no reduction in parasitaemia in the Rhodamine B group compared to the control group (Figure 3).

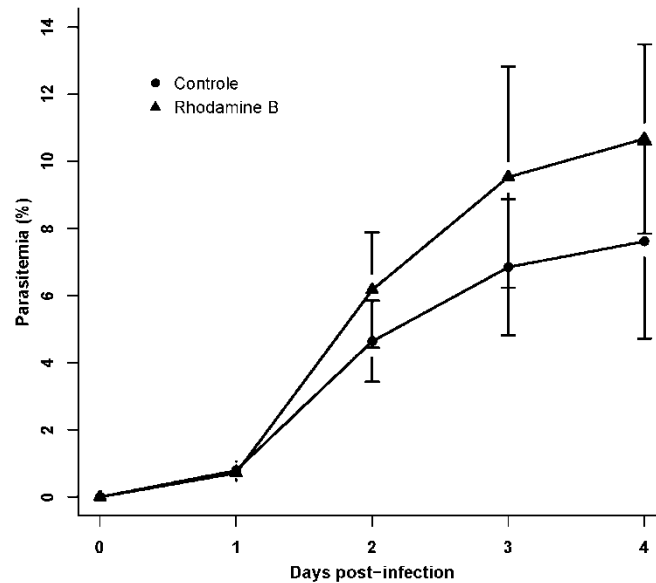


Figure 3: Parasitaemia counted by thin blood smear microscopy over time in the group of mice treated with Rhodamine B compared to the control group.

2.3. Prospective evaluation of artemether-lumefantrine for the treatment of non-falciparum and mixed-species malaria in Gabon (publication 2)

2.3.1. Published results

The study was conducted at the Centre de Recherches Médicales de Lambaréné (CERMEL) and the hospital of Fougamou in Gabon. Subjects were recruited after giving their written informed consent if they presented fever or history of fever in the last four days and if non-*falciparum* or mixed-species (including *P. falciparum*) *Plasmodium* infection was confirmed by microscopy of thick blood smears (TBS). Subjects were excluded if they required hospitalization, in case of previous hypersensitivity to artemether- lumefantrine, pregnancy or breastfeeding or prior use of other antimalarial medication. A total of forty subjects were included in the study between July 2008 and July 2010. One subject withdrew his consent before the initiation and one subject was not included in the final analysis because of concomitant use of chloroquine. Subjects received an oral treatment consisting of six doses of artemether-lumefantrine (Coartem®) over three consecutive days. TBS and blood dried on filter paper were prepared 0, 8, 24, 36, 48 and 60 hours following start of treatment and subsequently during the follow-up visits at Days 7, 14 and 28 after inclusion.

To validate thick blood smear readings and confirm the presence of the reported malaria species, DNA was extracted from the filter papers and used to amplify a species specific fragment of the 18S rRNA gene by nested PCR according to standard procedures [1,43,44]. For some samples nested PCR failed to amplify a fragment of the 18S rRNA gene. In this case a nested PCR was carried out to amplify a fragment of the genes coding for caseinolytic protease C (*clpC*) and cytochrome B (*cytB*). Subsequently, PCR products were sequenced by the Sanger method to identify the parasite species [45]. Only 19 samples out of 39 were confirmed as non-*falciparum* or mixed infection. The remaining 20 samples contained only *P. falciparum* DNA. Out of the 19 samples positive for non-*falciparum* species, one was a *P. malariae* mono-infection. The rest contained mixed infection with *P. falciparum* and *P. ovale* (12 samples, 63%), *P. falciparum* and *P. malariae* (3 samples, 16%) and *P. falciparum*, *P. malariae* and *P. ovale* occurring simultaneously (3 samples, 16%). *P. ovale curtisi* and *P. ovale walikeri* were also detected in nine and ten samples, respectively and appeared concurrently in four samples.

The median parasite clearance time, evaluated by microscopy of TBS, was 24h after initiation of treatment and all patients were microscopically negative after 48h. All patients who presented with fever (n=5) cleared fever within 36h. The overall cure rate was 100% (95% CI: 91-100%). No serious adverse events occurred during the study and eight patients experienced adverse events from which five were of mild severity and three were of moderate severity. Only one adverse event was considered as possible drug related (vomiting at Day 1 after beginning of treatment).

2.3.2. Non-published results

For three samples, the sequencing of the product of the 1st step of the nested PCR of the 18S rRNA gene showed presence of an additional *Plasmodium* species with the best sequence alignment to the 18S rRNA gene from *Plasmodium berghei* (*P. berghei*) with 95 to 99% identity (See Figure 4). Sequencing of the *clpC* and the *cytB* gene from these samples showed the presence of *P. falciparum* and *P. ovale*. This finding is currently investigated using next generation sequencing methodology as well as additional sampling in the same region as the original study.

3. Discussion

3.1. *In vitro* activity of fluorescent dyes against asexual blood stages of *Plasmodium falciparum* (publication 1)

Synthetic dyes represent a valuable source of chemotherapeutics as Paul Guttman and Paul Ehrlich showed more than one century ago when they successfully cured two malaria patients with methylene blue [37]. In the present proof of principle study five out of 14 compounds showed high antiplasmodial activity. Three of them stain specifically the mitochondrion (MitoTracker Red, Rhodamine B, DiOC6) and two stain specifically nucleic acids (Hoechst 33342, SYTO 9). MitoTracker Red is a Rhodamine based dye such as MitoTracker Green. Although antiparasitic activity of other Rhodamine dyes has been shown previously, this is the first time that these two dyes are studied. Contrary to what was previously reported, it was found that Rhodamine B was more active than Rhodamine 123 which could be explained by the fact that a different derivative (a hexyl ester) of the Rhodamine B was used in this study [46]. The molecule is positively charged and therefore able to accumulate specifically in the mitochondrion [47]. Unfortunately, when tested in a mouse model, Rhodamine B was not active and showed signs of toxicity. DiOC6 is a lipophilic green fluorescent dye that accumulates in metabolically active mitochondria. It can reversibly impair mitochondrion functions in yeast [48], which could explain the higher IC₅₀ in the 6-day experiment performed with Dd2. In the 6-day experiment, a fraction of the drug was removed without replacement after two days of incubation, allowing the recovery and growth of the parasites until Day 6. Although it has a good selectivity index, it was shown to be highly toxic in mice. Hoechst 33342 and SYTO 9 are two nucleic acids stains. Hoechst 33342 is a non-intercalating agent with a preference for AT rich region which could explain its specificity for the AT rich *Plasmodium* genome. Moreover, Hoechst 33258, a related dye, was previously shown to be antiparasitic as well [49]. Hoechst 33342 contains an additional ethyl group, rendering it more lipophilic and thus more membrane permeable than Hoechst 33258, which may explain the better antiplasmodial activity of Hoechst 33342.

With this study five molecules with good antiplasmodial activity which do not belong to chemotypes currently in current clinical use were identified. Although the activity of two of these compounds was not further confirmed *in vivo* in a mouse model, it

shows that prescreening for staining properties may be an interesting way to build restricted pathogen-specific compound libraries.

JC1, MitoTracker Green, Rhodamine 123, and Daspei had low antiplasmodial activity and are therefore unlikely to be further developed as antimalarial drugs. SYTO 18, acridine orange, SYBR green I, CFDA, and CFDA-SE showed very low or no antiplasmodial activity compared to chloroquine or methylene blue. This observation may however have interesting implications for the development of new methods to track parasites with minimal interference with their viability.

In conclusion, five dyes, Hoechst 33342, MitoTracker Red, DiOC6, SYTO 9, and Rhodamine B, show high antiplasmodial activity *in vitro* and low cytotoxicity against human HeLa cells. However, when further tested in a mouse model, Rhodamine B and DiOC6 were found to be toxic. On the other side of the spectrum, dyes with low toxicity against *P. falciparum* can be used to stain parasites without interfering with cell growth or metabolism.

3.2. A prospective evaluation of artemether-lumefantrine for the treatment of non-falciparum and mixed-species malaria in Gabon (publication 2)

This study is the first systematic and prospective evaluation of the efficacy of artemether-lumefantrine against *P. malariae*, *P. ovale* or mixed species malaria infection. Treatment resulted in rapid parasite clearance and a 100% cure rate after 28 days, which confirms the primary hypothesis of an adequate clinical and parasitological response rate above 90%.

One of the limitations of the study is the poor performance of microscopy against molecular diagnostic to identify non-*falciparum* species, which led to an over-estimate of the number of patient infected with non-*falciparum* or mixed species malaria. This is however not completely unexpected because of the difficult assessment of parasites species by thick blood smear microscopy. This may be partly due to the fact that non-*falciparum* species often present with low parasitaemia and *P. falciparum* predominates in mixed species infection. In our context the laboratory undergoes internal and external quality control, moreover a senior expert microscopist was available to decide on ambiguous samples. Despite this, the discrepancy with the PCR results were still unexpectedly high. This shows that the misclassification of malaria species is high in an African routine setting and may be

even higher in laboratories without adequate quality management. This emphasizes the need for a single treatment to cure all malaria species. In Gabon, where the prevalence of chloroquine resistance is almost 100% [50], misclassification of malaria species is likely to lead to treatment failure and can be dangerous for the patient. The present study is the first to systematically show the efficacy of artemether-lumefantrine in non-*falciparum* and mixed-species malaria and encourage the use of this drug as single therapy for the treatment of all cases of malaria. Further studies are still needed notably with a bigger samples size and with the comparison with a control group between chloroquine and artemether-lumefantrine for the treatment of mono-infection with non-*falciparum* species.

3.3. Non published data

In three samples for which the nested PCR amplicon of the ssurRNA was sequenced, I found that the best alignment was with a sequence of the *P. berghei* 18S rRNA gene. *P. berghei* is a murine malaria parasite which is not known to infect human. These results are difficult to explain however it is unlikely to be a contamination as we do not work with *P. berghei* in the laboratory. Moreover, only viable parasites are usually detected in the blood by PCR. Therefore it is suggestive that the result is the consequence of a zoonotic infection. Further work is being conducted to reproduce and confirm these results. Besides detailed analysis using next generation sequencing methods to identify other samples with similar parasites, a new study on non-*falciparum* parasites in malaria patients and potential animal reservoirs (small ruminants and bats) has been initiated. These investigations are still ongoing and results are expected by the end of 2016.

4. Conclusion

There is a need for broadly acting antimalarial drugs. This requirement includes multi-stage activity as well as activity against different parasite species. AL showed very good activity against different species, but does not act against pre-erythrocytic stages and reduces gametocytes only modestly. Dyes are a group of chemically heterogeneous compounds that may extent the spectrum of activity. In fact, the dye methylene blue was shown to meet many of the requirements. Investigation of larger

dye libraries may result in highly interesting novel antimalarial compounds and may lead to the development of novel, broadly acting drugs. The use of such drug would not only reduce dramatically the transmission of the parasite but also prevent the development of resistance and would therefore have a huge impact of malaria control and elimination.

5. Material and Method for unpublished data

5.1. Biological material

I used the *P. berghei* ANKA cl15cy1 strain (Malaria Research and Reference Reagents Resource center, ATCC) and female mouse (*mus musculus*) from the strain Swiss (CD1) purchased from *Charles River* (Sulzfeld). The protocol has been approved by the Regierungspräsidium Tübingen.

5.2. Evaluation of the toxicity of DiOC6 and Rhodamine B

Three mice were injected intraperitoneally (i.p.) with either 15mg/kg of Rhodamine B or 40mg/kg of DiOC6. The compounds were diluted in a sterile solution of 7% tween 80 and 3% ethanol. The health status of the mice was monitored 30 minutes and 2h post injections then once a day for four days and notably weight was recorded every day. In case of severe degradation of health status and weight loss >30%, mice were euthanatized before day 4 by CO₂ asphyxia and neck dislocation.

5.3. Evaluation of the activity *in vitro* of DiOC6 and Rhodamine B

I used the method described by Fidock et al [51]. Briefly, nine mice per group were injected intravenously with $2 \cdot 10^7$ *P.berghei* ANKA cl15cy1 parasites. After 2-4h, they received a single dose of either Rhodamine B (Hexyl ester, Invitrogen), DiOC6 (Invitrogen) i.p. (15mg/kg and 40mg/kg respectively) or an equivalent volume of injection solution (7% tween 80 and 3% ethanol). The mice received the same treatment every day for four days. The weight as well as the general health status was monitored. Moreover, about 10µL of blood were taken every day from the end of the tail in order to prepare thin blood smear. Thin blood smear were stained for 20 minutes in 5% Giemsa solution after methanol fixation. Parasitaemia was estimated by counting at least 1000 erythrocytes on a Giemsa-stained thin blood film. To

calculate treatment efficacy, the area under the curve (AUC) until Day 4 was calculated using the trapezoidal rule. The ratio of sums of AUCs was used to calculate anti-parasitic efficacy of the compounds in percent.

6. Personal contribution

Publication “In Vitro Activity of Fluorescent Dyes against Asexual Blood Stages of Plasmodium falciparum”

- *In vitro* culture of Dd2 and 3D7 *P. falciparum* parasites
- Drug inhibition assay
- HRP2 ELISA
- Neutral red assay
- Statistical analysis
- Preparation of the manuscript

Activity of Rhodamine B and DiOC6 against P. berghei in mice

- Toxicity test on mice
- *P. berghei* rodent malaria 4-days suppressive test
- Statistical analysis

Publication “A prospective evaluation of artemether-lumefantrine for the treatment of non-falciparum and mixed-species malaria in Gabon”

- DNA extraction from dried blood spot
- Establishing PCR for species identification
- Species identification by PCR
- Species identification by sequencing
- Drafting of the manuscript

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8. Publication

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In Vitro Activity of Fluorescent Dyes against Asexual Blood Stages of *Plasmodium falciparum*

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Many successful antimicrobial drugs originate from synthetic dyes. This paper reports the *in vitro* activity of 14 fluorescent dyes against *Plasmodium falciparum*. Five of these dyes (Hoechst 33342, MitoRed, DiOC₆, SYTO 9, and rhodamine B) show activity at a low nanomolar concentration against two *P. falciparum* strains in the histidine-rich protein 2 drug sensitivity assay, while toxicity in HeLa cells is low. These dyes may be a starting point for developing new drugs against *P. falciparum*.

The increasing resistance of *Plasmodium falciparum* to most drugs in clinical use and first reports of reduced sensitivity to artemisinin derivatives, the latest first-line drugs, made the development of new antimalarial compounds a research priority of utmost importance (20). Since existing drugs belong to only a few chemical classes, there is a high probability of the development of cross-resistance, which may shorten the product life of new drugs. In the history of drug discovery, many drugs were derived from synthetic dyes. Indeed, the pharmaceutical industry developed from the dye industry before World War II (22), and the antiparasitological effect of dyes was shown more than a century ago by Guttman and Ehrlich (10), who cured two malaria patients with methylene blue. They were inspired to use methylene blue as an antimalarial drug after observing that it stains *P. falciparum* particularly well. Later, the antimalarial activity of other dyes such as rhodamine 123 (19), rhodamine B, Janus green (7), and eosin B (17) was discovered.

With this work, we attempt to revive the use of staining properties as a preselection criterion for new antimalarial drugs. The use of dyes has several advantages: they accumulate in the target organism, they are easily screened for, and they belong to a large family of chemical entities not restricted to known pharmacophores. For this proof-of-concept study, we selected 14 different commercially available dyes staining mitochondria (MitoRed, MitoGreen, Daspei, DiOC₆, rhodamine 123, rhodamine B, JC1, SYTO 18), nucleic acids (Hoechst 33342, acridine orange, SYBR green I, SYTO 9), or proteins (carboxyfluorescein diacetate [CFDA], CFDA-succinimidyl ester [CFDA-SE]). Dyes were obtained from the following sources. A yeast mitochondrial stain sampler kit containing DiOC₆ (CAS no. 53213-82-4), rhodamine B (hexyl ester perchlorate; CAS number not available), MitoGreen (MitoTracker Green FM; CAS no. 201860-17-5), and SYTO 18 (the structure is proprietary) was obtained from Invitrogen. The mitochondrial stains MitoRed (MitoTracker Red CMXRos; CAS no. 167095-09-2) and JC1 (CAS no. 47729-63-5) as well as the nucleic acid stains SYTO 9 (SYTO 9 green fluorescent nucleic acid stain; the structure is proprietary), Hoechst 33342 (CAS no. 23491-52-3), and acridine orange (CAS no. 65-61-2) were also from Invitrogen. Daspei (CAS no. 3785-01-1) was obtained from Biotium, CFDA (CAS no. 79955-27-4), CFDA-SE (CAS no. 150347-59-4), SYBR green I (CAS no. 163795-75-3), and chloroquine diphosphate (CAS no. 50-63-5) (molecular weight [MW], 515.86) from Sigma, and methylene blue (CAS no. 61-73-4) (MW, 319.86) from AppliChem. Stock solutions were prepared accord-

ing to the manufacturer's instructions. Further dilutions were prepared in complete culture medium. The chemical structures of the dyes are given in Fig. 1.

The antiparasitological activity of the dyes and two reference drugs (chloroquine and methylene blue) were tested against two laboratory strains of *P. falciparum*, the chloroquine-sensitive 3D7 strain and the multiresistant Dd2 strain, by using the histidine-rich protein 2 (HRP2) assay as described previously (18). In brief, parasites were added at the ring stage to a 96-well plate in complete culture medium (RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 50 µg/ml gentamicin, and 0.5% [wt/vol] AlbuMAX) at a parasitemia of 0.05% and a hematocrit of 1.5% and incubated for 3 or 6 days in the presence of a 3-fold serial dilution of the dyes. The 6-day assay was done to assess if the compounds exert their action in the subsequent cycle (delayed death) and was performed as described previously (12) with medium changes on day 2 and day 4 without replacement of the dye. After cultivation, plates were stored at -20°C until HRP2 measurement was performed with an enzyme-linked immunosorbent assay (ELISA). The assay was done in duplicate in at least three independent experiments for each dye.

The activity of MitoRed was additionally evaluated in clinical isolates from patients with malaria in Lambaréné, Gabon, between March and May 2009. Details of the study are described elsewhere (13). The experiments were approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréné. The assay was performed as described for the laboratory strains except that parasites were cultivated at 37°C in a candle jar.

The 50% inhibitory concentration (IC₅₀) and IC₉₀ were determined by nonlinear regression analysis of log concentration-response curves using drc package v0.9.0 of R v2.6.1 (18a).

Of the 14 dyes tested, Hoechst 33342 and MitoRed showed the

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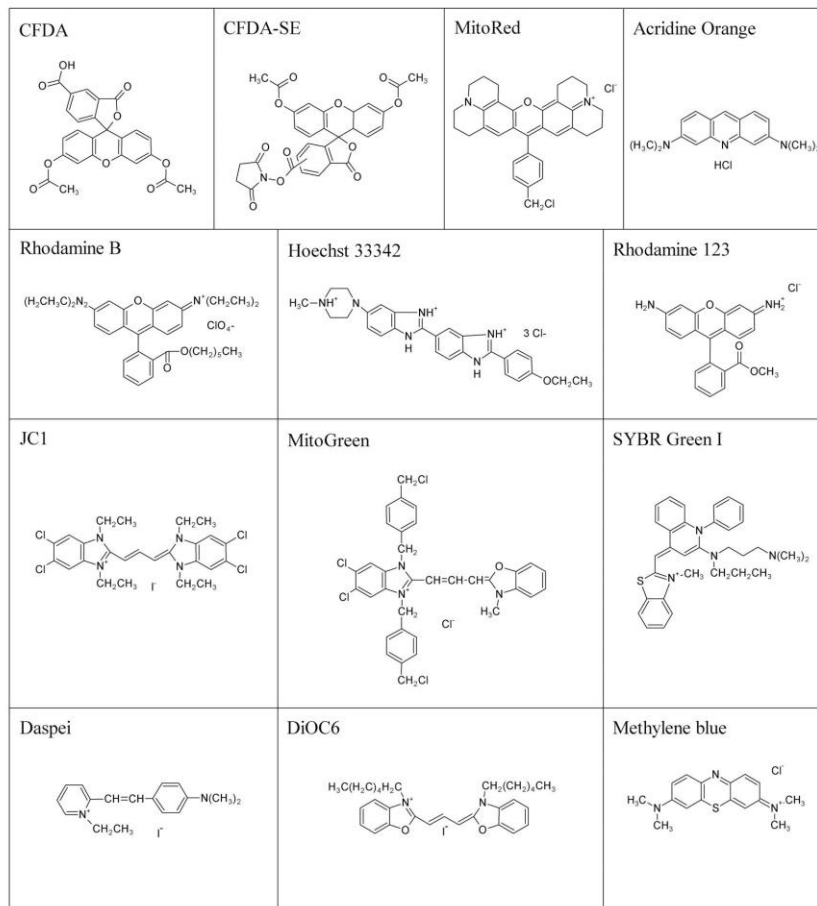


FIG 1 Chemical structure of dyes used in the present study (the structures of SYTO 9 and SYTO 18 are proprietary).

highest antiplasmodial activity against both laboratory strains after 3 days (Table 1). MitoRed was also highly active against clinical isolates ($n = 19$) from Gabon (IC_{50} geometric mean [range], 15.5 nM [3.4 to 35.9]; IC_{90} geometric mean, 26.3 nM [13.3 to 59.6]). The levels of activity were similar against clinical and laboratory isolates, and IC_{50} s fell within a narrow range for the genetically diverse clinical isolates from Gabon, which is an area of high endemicity for *P. falciparum* malaria with high-grade chloroquine resistance (2). DiOC₆, SYTO 9, and rhodamine B showed high activities as well, with IC_{50} s similar to those of chloroquine and methylene blue (Table 1). Most stains were active in a low nanomolar concentration against the two laboratory strains. Delayed death, a typical feature of some antibiotics, which is characterized by cell death one cycle after drug exposure due to specific action on cell organelles (corresponding to 6 days in our assay), was not observed for the tested dyes. Indeed, some drugs had even lower

activity after 6 days. This may be explained by the replacement of medium after 2 days without the replacement of the dye. Thus, if the dye does not have a delayed death effect, the surviving parasites can recover, leading to a higher IC_{50} . JC1, MitoGreen, rhodamine 123, acridine orange, and Daspei showed moderate antiplasmodial activity against both 3D7 and Dd2. SYTO 18 and SYBR green I had only low antiplasmodial activity. CFDA and CFDA-SE had no measurable antiparasitic activity at the highest concentrations tested (333 μ M and 55 μ M, respectively), showing that *P. falciparum* is not sensitive to their effects.

An ideal drug should act specifically against the parasite and not against the human host (or cell). Therefore, we evaluated the HeLa cell cytotoxicity of the dyes with the highest antiplasmodial activities using the neutral red assay (1). Each dye was tested at least three times.

To assess the safety of a compound, a selectivity index (SI) was

TABLE 1 IC₅₀ and IC₉₀ of selected dyes and chloroquine against the culture-adapted strain of *P. falciparum* 3D7 or Dd2^a

Dye and <i>P. falciparum</i> strain	IC ₅₀ ± SD (nM)		IC ₉₀ ± SD (nM)	
	Day 3	Day 6	Day 3	Day 6
Hoechst 33342				
3D7	7.2 ± 3.1	18.9 ± 11.9	14.0 ± 8.8	25.8 ± 17.3
Dd2	16.3 ± 11.2	28.0 ± 2.6	72.7 ± 107.9	36.5 ± 0.8
MitoRed				
3D7	8.2 ± 9.6	10.6 ± 6.1	14.1 ± 19.5	15.3 ± 8.4
Dd2	15.9 ± 10.9	6.3 ± 7.3	44.8 ± 24.0	9.0 ± 0.2
DiOC₆				
3D7	11.4 ± 8.1	17.3 ± 17.1	17.4 ± 9.4	24.1 ± 23.9
Dd2	20.8 ± 12.2	168.3 ± 301.8	55.7 ± 31.5	229.0 ± 401.4
Rhodamine B				
3D7	20.6 ± 12.6	19.2 ± 10.8	40.7 ± 12.4	29.5 ± 8.9
Dd2	26.1 ± 15.3	69.5 ± 113.2	101.1 ± 34.6	117.2 ± 194.8
SYTO 9				
3D7	20.5 ± 20.0	23.1 ± 44.9	101.5 ± 105.7	41.0 ± 64.2
Dd2	36.1 ± 17.7	4.9 ± 0.73	261.7 ± 114.1	10.8 ± 3.9
JC1				
3D7	107.8 ± 66.3	177.0 ± 55.0	170.6 ± 84.8	279.8 ± 95.36
Dd2	100.1 ± 96.9	121.0 ± 128.2	269.9 ± 252.2	162.0 ± 156.3
MitoGreen				
3D7	115.5 ± 149.9	187.1 ± 197.2	331.9 ± 407.5	245.2 ± 215.0
Dd2	148.9 ± 102.7	103.8 ± 58.0	1,521 ± 2,335	150.8 ± 87.9
Rhodamine 123				
3D7	387.7 ± 208.6	189.2 ± 235.1	870.3 ± 567.6	490.3 ± 584.0
Dd2	140.7 ± 117.4	287.2 ± 316.0	2,335 ± 3,893	414.2 ± 450.7
Acridine orange				
3D7	465.7 ± 274.9	691.9 ± 391.8	1,772 ± 1,057	691.9 ± 391.8
Dd2	166.0 ± 75.9	208.2 ± 22.5	620.4 ± 379.6	278.8 ± 31.4
Daspei				
3D7	327.2 ± 327.2	502.7 ± 797.2	2,026 ± 2,364	834.7 ± 1298
Dd2	591.3 ± 405.8	270.6 ± 267.2	55,046 ± 79,810	831.3 ± 1315
SYTO 18				
3D7	1,751 ± 969.8	873.1 ± 482.1	5,016 ± 3,186	1,916 ± 1,144
Dd2	387.1 ± 207.6	636.3 ± 413.5	1,865 ± 381.1	1,141 ± 880.2
SYBR green 1				
3D7	2,585 ± 4,693	11,497 ± 17,785	4,669 ± 8,532	17,897 ± 28,028
Dd2	2,100 ± 1,024	1,218 ± 583.2	14,062 ± 17,391	1,792 ± 872.5
CFDA-SE				
3D7	>55 μM	>55 μM	>55 μM	>55 μM
Dd2	>55 μM	>55 μM	>55 μM	>55 μM
CFDA				
3D7	>333 μM	>333 μM	>333 μM	>333 μM
Dd2	>333 μM	>333 μM	>333 μM	>333 μM
Methylene blue				
3D7	7.8 ± 3.2	14.3 ± 10.7	13.3 ± 1.5	20.3 ± 13.4
Dd2	14.3 ± 4.1	17.2 ± 12.4	29.2 ± 7.8	27.3 ± 20.2
Chloroquine				
3D7	4.1 ± 1.8	6.2 ± 1.8	6.8 ± 3.3	7.8 ± 2.1
Dd2	63.8 ± 30.1	84.6 ± 35.6	99.3 ± 45.3	131.8 ± 33.6

^a Each value represents the geometric mean IC determined from the results of at least 3 independent experiments. Data in columns 2 to 5 represent nanomoles except where otherwise indicated.

TABLE 2 Geometric mean determined from the results of at least 3 independent experiments for the 50% inhibitory concentration (IC₅₀) of the five dyes presenting the best antiplasmodial activity against human HeLa cells

Dye	Cytotoxicity IC ₅₀ ± SD (nM) on HeLa cells	SI ^a			
		HC/3D7 (day 3)	HC/3D7 (day 6)	HC/Dd2 (day 3)	HC/Dd2 (day 6)
Hoechst 33342	1,357 ± 184.8	188	72	83	48
MitoRed	2,666 ± 1,238	325	251	168	423
DiOC ₆	2,727 ± 1,831	239	158	131	16
SYTO 9	3,287 ± 1,141	160	142	91	671
Rhodamine B	4,372 ± 710.0	212	228	168	63

^a SI, selectivity index (calculated as the ratio of the IC₅₀ against HeLa cells [HC] to the IC₅₀ against culture-adapted strains of *P. falciparum* 3D7 and Dd2).

calculated as the fractional ratio between the IC₅₀s for HeLa cells and *P. falciparum*. All five compounds with strong activity had high selectivity indices (Table 2), indicating that they are at least 50 times more toxic for *P. falciparum* than for HeLa cells. MitoRed had the highest selectivity, with indices between 168 and 423.

In this report, we demonstrate the high *in vitro* antiplasmodial activity of three mitochondrial stains, MitoRed, DiOC₆, and rhodamine B, and two nucleic acid stains, Hoechst 33342 and SYTO 9. MitoRed, like MitoGreen, rhodamine B, and rhodamine 123, is a rhodamine-based dye (Fig. 1). The activity of rhodamine dyes against plasmodia has already been tested in previous studies, but this is the first report of antiplasmodial activity of MitoRed and MitoGreen (14). In contrast to other reports (14), we found that rhodamine B was more active than rhodamine 123 against 3D7 and Dd2 after 3 days. This could be explained by the fact that we used the hexyl ester of rhodamine B, rendering the molecule positively charged and able to accumulate specifically in the mitochondrion (11), in contrast to other studies using the neutral molecule of rhodamine B (15).

DiOC₆ is a lipophilic green fluorescent dye that accumulates in mitochondria of living cells and is also able to stain the endoplasmic reticulum when used at higher concentrations (>1 μM). It was previously shown that it can reversibly impair mitochondrial functions in yeast (16), which could explain the higher IC₅₀ in the 6-day experiment performed with Dd2. Indeed, in the 6-day experiment, a fraction of the drug was removed after 2 days of incubation, allowing the recovery and growth of the parasites until day 6.

The two other dyes with strong antiplasmodial activity were Hoechst 33342 and SYTO 9, both able to stain nucleic acids. Hoechst 33342 did not exhibit intercalation while staining double-stranded DNA with a preference for AT-rich regions. The rather high selectivity index may be explained by the higher AT content of *P. falciparum* compared to human DNA. Hoechst 33258, a related dye, was previously shown to be antiparasitic as well (8). Hoechst 33342 contains an additional ethyl group, rendering it more lipophilic and thus more membrane permeable than Hoechst 33258, which may explain the better antiplasmodial activity of Hoechst 33342. Since Hoechst 33342 was previously shown to inhibit topoisomerase I (3) and increase the mutation rate (5), it is not a preferred candidate for clinical development.

However, this pilot experiment shows that prescreening for staining properties may be an interesting way to build highly efficient pathogen-specific compound libraries. It is estimated that currently used antimalarials belong to only 10 chemotypes (9).

Simply by screening for staining properties, we showed the anti-malarial activity of five molecules which do not belong to these chemotypes and thus could lead to the development of new classes of antimalarial molecules that are not hampered by preexisting resistance mechanisms (6). A next step could be the testing of these dyes for *in vivo* activity and toxicity in mouse models. The most promising candidate from our experiments is MitoRed, as it showed high activity against laboratory strains and clinical isolates and low toxicity against HeLa cells. Since some rhodamine derivatives are even used in cosmetics and have little toxicity in humans (4), further development may be interesting.

JC1, MitoGreen, rhodamine 123, and Daspei presented lower antiplasmodial activity and are therefore unlikely to be further developed as antimalarial drugs. SYTO 18, acridine orange, SYBR green I, CFDA, and CFDA-SE showed very low or no antiplasmodial activity compared to chloroquine or methylene blue. This observation may have interesting implications for the development of new methods to track parasites with minimal interference with their viability.

In conclusion, five dyes, Hoechst 33342, MitoRed, DiOC₆, SYTO 9, and rhodamine B, show high antiplasmodial activity and low cytotoxicity against human HeLa cells and could be further developed as antimalarial drugs. On the other side of the spectrum, dyes with low toxicity against *P. falciparum* can be used to stain parasites without interfering with cell growth or metabolism.

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RESEARCH

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Prospective evaluation of artemether-lumefantrine for the treatment of non-falciparum and mixed-species malaria in Gabon

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Abstract

Background: The recommendation of artemisinin combination therapy (ACT) as first-line treatment for uncomplicated falciparum malaria is supported by a plethora of high quality clinical trials. However, their recommendation for the treatment of mixed-species malaria and the large-scale use for the treatment of non-falciparum malaria in endemic regions is based on anecdotal rather than systematic clinical evidence.

Methods: This study prospectively observed the efficacy of artemether-lumefantrine for the treatment of uncomplicated non-falciparum or mixed-species malaria in two routine district hospitals in the Central African country of Gabon.

Results: Forty patients suffering from uncomplicated *Plasmodium malariae*, *Plasmodium ovale* or mixed-species malaria (including *Plasmodium falciparum*) presenting at the hospital received artemether-lumefantrine treatment and were followed up. All evaluable patients (n = 38) showed an adequate clinical and parasitological response on Day 28 after oral treatment with artemether-lumefantrine (95% confidence interval: 0.91,1). All adverse events were of mild to moderate intensity and completely resolved by the end of study.

Conclusions: This first systematic assessment of artemether-lumefantrine treatment for *P. malariae*, *P. ovale* and mixed-species malaria demonstrated a high cure rate of 100% and a favourable tolerability profile, and thus lends support to the practice of treating non-falciparum or mixed-species malaria, or all cases of malaria without definite species differentiation, with artemether-lumefantrine in Gabon.

Trial Registration: ClinicalTrials.gov Identifier: NCT00725777

Keywords: Malaria, Ovale, Malariae, Artemisinin-combination-therapy, Artemether-lumefantrine, Non-falciparum

Background

Effective treatment of malaria is one of the main tools to control and eventually eradicate malaria. A plethora of clinical trials demonstrating high efficacy, satisfying effectiveness, and good tolerability and safety of artemisinin combination therapy (ACT) – the current first-line treatment of falciparum malaria – has been conducted and published over the past decade [1,2]. Treatment recommendations for non-falciparum malaria have however

remained virtually unchanged since the introduction of chloroquine more than five decades ago [3].

Plasmodium falciparum and to a lesser extent *Plasmodium vivax* have attracted more scientific interest compared to the other human plasmodial species due to their high incidence, virulence and the emergence of drug resistant isolates. Today *Plasmodium ovale* and *Plasmodium malariae* are among the most neglected tropical diseases particularly in sub-Saharan Africa. This fact is illustrated by an analysis of clinical trials on *P. ovale* and *P. malariae* malaria resulting in less than five interventional clinical trials over the past 10 years, compared to 930 reports of clinical trials for *P. falciparum* malaria [4]. More research activities

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are, therefore, needed to boost the development of novel and improved control tools for these plasmodial infections.

Today chloroquine remains the standard of care for *P. malariae* and *P. ovale* malaria due to its low cost and sustained efficacy. ACT is recommended as first-line treatment for *P. falciparum* malaria and for mixed plasmodial infections [3]. Despite these official recommendations, ACT is used in many areas for the treatment of all forms of malaria including non-falciparum infections. This may be explained by the fact that non-falciparum species often occur concurrently with *P. falciparum* as mixed infections in sub-Saharan Africa [5-7], and that clinicians prefer the prescription of an ACT for malaria cases due to lacking or unreliable species differentiation. Finally, chloroquine treatment is unpopular in many parts of sub-Saharan Africa due frequently experienced adverse effects, e.g. induced pruritus.

At present, artemether-lumefantrine is the most widely used ACT worldwide with 130 million treatment courses procured in 2010 [8]. Anecdotal evidence indicates that artemether-lumefantrine may be highly efficacious against *P. malariae*, *P. ovale* and mixed-species malaria, however, a prospective evaluation is still lacking. Since artemether-lumefantrine is used on a large scale for these indications in sub-Saharan Africa we assessed the efficacy of artemether-lumefantrine in patients suffering from uncomplicated malaria due to non-falciparum species or mixed malaria species infections presenting at two district hospitals in the Central African country of Gabon.

Methods

This prospective study was performed in Gabon at the Medical Research Unit of the Albert Schweitzer Hospital in Lambaréné [9] and the Regional Hospital of Fougamou. This Central African region is characterized by perennial transmission of *P. falciparum* [10] and co-endemicity of *P. ovale* and *P. malariae* [5]. Young children and pregnant women are at highest risk for malaria associated morbidity and mortality in this region [11-13]. *Plasmodium falciparum* isolates are highly resistant to chloroquine and sensitivity to antifolate anti-malarials has decreased [14-16], hence ACT has become the first-line treatment of malaria [17]. The study followed the principles of the Declaration of Helsinki (5th revision) and was approved by the regional ethics committee (CERIL). Written informed consent was obtained prior to inclusion in the study from adult participants or from a legal representative of children below 21 years of age, and assent was obtained where applicable.

Participants older than six months presenting with fever or with a history of fever during the previous four days and for whom malaria was confirmed by a thick blood smear were considered for inclusion. Thick and thin blood smears were performed and patients positive for *P. ovale*, *P. malariae* or a mixed infection with more

than one plasmodial species (including *P. falciparum*) at a density of below 200,000/ μ l blood were considered eligible for enrolment.

Patients were excluded from this study when presenting with a clinical condition requiring hospitalization, haemoglobin <7 g/dl, history of hypersensitivity to artemether-lumefantrine, prior intake of any anti-malarial medication, or current pregnancy or breastfeeding. Demographic data, medical history, physical examination, and axillary temperature were recorded at inclusion. Blood was obtained for haemoglobin measurement (HemoCue AB, Ångelholm, Sweden), malaria diagnostics, and storage on filter papers for molecular analysis. Axillary temperature and blood films were sequentially taken twice daily (at hours 0, 8, 24, 36, 48 and 60) during the treatment period. Follow up visits were scheduled for clinical examination, haemoglobin measurement, and malaria diagnostics on Days 7, 14 and 28 after the start of treatment.

Oral treatment with the standard six doses artemether-lumefantrine regimen (Coartem[®] and Coartem Dispersible[®]) was administered on three consecutive days. Artemether-lumefantrine was given as tablets or dispersible tablets under supervision with a small amount of liquid and patients were encouraged to consume simultaneously fatty food following current recommendations by the manufacturer.

Due to the well-known limitations of microscopic malaria species differentiation in routine laboratories in sub-Saharan Africa, PCR analysis based on the sequence of the small subunit ribosomal RNA (ssrRNA) gene and optional sequencing were performed for final plasmodial species classification. Capillary blood was collected on filter paper (Whatmann[®]) for subsequent PCR analysis. DNA was extracted with a commercial extraction kit (QIAamp DNA Blood Mini Kit, Qiagen) following manufacturer instructions and stored at -20°C for further analysis. Species-specific nested PCR for *P. falciparum*, *P. malariae*, *P. ovale curtisi* and *P. ovale wallikeri* was carried out as previously described [18-21]. In samples not showing an amplicon for further species differentiation, a fragment of caseinolytic protease C gene (clpc) or cytochrome b gene was amplified by nested PCR [22] and subsequently sequenced on an ABI3100 Genetic Analyzer.

The primary objective was to assess the efficacy of artemether-lumefantrine for the treatment of non-falciparum and mixed plasmodial infections. The primary efficacy endpoint was the adequate clinical and parasitological response on Day 28. Re-appearing parasitaemia was further classified by PCR genotyping as treatment failure or newly acquired infection [19] except for *P. ovale* infections where potential relapse from liver hypozoites precludes such a distinction. Secondary endpoints included descriptive assessment of safety, tolerability, parasite clearance time (PCT) and fever clearance time (FCT).

A sample size of 40 patients was shown to be sufficient to demonstrate a lower 90% confidence interval of adequate clinical and parasitological response of at least 90% with a power of 0.8 assuming more than 98% efficacy on day 28. The intention to treat population (ITT) was defined as primary outcome for tolerability and safety analysis and comprised of all patients included in the study. Per protocol population (PP) was defined as primary efficacy outcome and consisted of all patients having received a full course of study medication who were not prematurely withdrawn from follow up.

Data were captured on paper case record forms and transcribed to an electronic database. All data were checked manually and further statistical analysis was performed using JMP 5.0 (SAS Institute Inc., NC, USA). Descriptive statistics of baseline characteristics and outcome measures were computed.

Results

Between July 2008 and July 2010, 40 patients were included in this study. One patient decided to withdraw consent before treatment initiation and another participant was excluded from the final analysis because of reported concomitant chloroquine intake. All 39 patients completed 28 day follow up and final analysis was done for 38 patients (15 female participants, 39%). The age of patients ranged from two to 50 years, with eight patients aged less than five years and 19 between five and 12 years of age. Baseline parasitaemia ranged from 31 – 100,680 trophozoites per microliter capillary blood (median 777/ μ l) (Table 1). Based on microscopy thirty-two patients had

mixed-species infections and 7 patients presented with a non-falciparum mono-infection.

Eight hours after initiation of anti-malarial treatment 12 (32%) patients had cleared parasites in peripheral blood. Further 19 patients (50%) showed parasite clearance at 24 hours and all patients (100%) had cleared parasites within 36 hours. Median parasite clearance time was 24 hours. Median parasite clearance time was comparable in children below 5 years of age and adults (Table 2). Patients with higher parasite densities on inclusion had longer parasite clearance times. Only a minority of patients presented with fever at inclusion (n = 5, 13%) and all cleared fever within 36 hours after initiation of treatment. All evaluable patients had an adequate clinical and parasitological response on Day 28 and the overall cure rate was therefore 100% (95% CI: 91-100%).

Tolerability and safety of the artemether-lumefantrine was assessed for all patients from inclusion to Day 28. No serious adverse event was recorded in the course of this clinical trial and eight patients experienced adverse events (diarrhoea, urinary tract infection, convulsion, vomiting, cephalgia, abdominal pain, fever, and lymphadenopathy). Five adverse events were of mild severity (urinary tract infection, vomiting, cephalgia, abdominal pain), the other three adverse events were of moderate severity, and all adverse events completely resolved by the end of follow-up. The only adverse event considered as possibly study drug related was the occurrence of one episode of vomiting on day 1 after first drug administration.

Capillary blood was obtained and further tested by PCR for molecular species differentiation. In this analysis 19 out of 39 samples were confirmed as non-falciparum or mixed species infection. In the remaining 20 samples PCR analysis demonstrated amplification of *P. falciparum* isolates only and no amplification of non-falciparum parasite-specific amplicons. Presence of *P. malariae* mono-

Table 1 Patient characteristics

Study flow		
Patients included	40	100%
Evaluable patients	38	95%
Baseline characteristics		
Females	15	39%
Age ¹	8	5-14
Children < 5 years of age	8	21%
Systolic blood pressure (mmHg) ¹	100	90-110
Heart rate (/min) ¹	88	68-104
Haemoglobin (g/dl) ¹	11.3	10.1-12.4
Haemoglobin in children < 5 years (g/dl) ¹	11.5	10.0-12.6
Haemoglobin in patients > 5 years (g/dl) ¹	11.3	10.2-12.6
Asexual parasitaemia ¹	777	266-3053
Asexual parasitaemia in children < 5 years ¹	480	278-742
Asexual parasitaemia in patients > 5 years ¹	849	225-6400
Participants presenting with fever	5	13%

¹ median, interquartile range.
 ACPR adequate clinical and parasitological response.
 95% CI 95% confidence intervals.

Table 2 Patient outcomes

Outcome Parameters		
Patients with ACPR (n, 95% CI)	38	91-100%
Parasite clearance time (hours) ¹	24	8-24
Parasite clearance time in children < 5 years (hours) ¹	24	24-33
Parasite clearance time in patients > 5 years (hours) ¹	24	8-24
Fever Clearance Time (n = 5, hours) ¹	8	8-30
Patients experiencing any serious adverse event	0	0%
Patients experiencing any adverse event	8	20%
Haemoglobin D28 (g/dl) ¹	11.6	10.6-12.3
Haemoglobin D28 in children < 5 years (g/dl) ¹	11.1	10.6-12.5
Haemoglobin D28 in patients > 5 years (g/dl) ¹	11.7	10.5-12.2

¹ median, interquartile range.
 ACPR adequate clinical and parasitological response.
 95% CI 95% confidence intervals.

infection and mixed species infection was demonstrated in one and 18 samples, respectively. Mixed infections were confirmed by PCR analysis indicating co-infection with *P. falciparum* and *P. ovale* (n = 12, 63%), *P. falciparum* and *P. malariae* (n = 3, 16%) and concurrent occurrence of all three species in three samples (n = 3, 16%). *P. ovale curtisi* (n = 9) as well as *P. ovale wallikeri* (n = 10) isolates were present in the samples, concurrent presence of *P. ovale curtisi* and *P. ovale wallikeri* was found in four patients.

Discussion

This is the first prospective assessment of artemether-lumefantrine in the treatment of *P. ovale*, *P. malariae* and mixed-species infection with *P. falciparum*. The results demonstrate rapid parasite and fever clearance when assessed twice daily and a reassuringly high cure rate of 100% on Day 28. No clinical or parasitological treatment failure occurred during the 28 day follow up period. The primary hypothesis of an adequate clinical and parasitological response rate above 90% could, therefore, be confirmed. Although – at least in theory – the occurrence of recrudescence parasitaemia after Day 28 cannot be ruled out, a longer follow-up period was precluded due to the potential for relapse of *P. ovale* infections and associated problems in classification as treatment failure or reinfection in such circumstances. Anti-relapse therapy with primaquine in *P. ovale* infection is not commonly prescribed in Gabon due to the high risk for reinfection and safety concerns in G6PD deficient individuals.

A limitation of the study design may be seen in the lack of a control group. However, due to frequent occurrence of mixed infections including *P. falciparum*, a randomized controlled trial design comparing standard chloroquine treatment with artemether-lumefantrine was precluded due to concerns for patients' safety. Future randomized clinical trials evaluating different forms of ACT versus chloroquine in the treatment of *P. malariae* or *P. ovale* mono-infections would however be highly desirable. Despite recent reports of delayed parasite clearance of *P. malariae* and *P. ovale* to chloroquine the curative efficacy of this drug appears to be maintained [23]. In analogy to mixed malaria infections of *P. falciparum* with *P. malariae* and *P. ovale* in Africa, co-endemicity of *P. vivax* and *P. falciparum* in South East Asia requires more rigorous assessment of the use of ACT in general, and artemether-lumefantrine in particular, for the treatment of *P. vivax* malaria [24,25]. Solid evidence of the efficacy of ACT in the treatment of non-falciparum malaria is essential before removal of chloroquine from the market as a cheap over the counter drug taken for undiagnosed and undifferentiated malaria cases.

The discrepancy between microscopic and molecular species classification as observed in this study is striking

but not unexpected. Firstly, microscopic determination of mixed infections is prone to misclassification due to the relative predominance of *P. falciparum* trophozoites and often low parasitaemia of the non-falciparum species. Microscopic assessment may, therefore, under- or over- estimate mixed species infections. To minimize this source of misclassification internal and external quality control systems for microscopic malaria diagnosis are present in most African malaria research institutions, but not in smaller hospitals or other primary care facilities. In this study, microscopists were under external quality assessment and an expert senior microscopist was available to decide on discrepant results. However, despite these measures a significant proportion of patients showed discrepant results in microscopy and PCR. This discrepancy is likely to be even more important in routine laboratories without the additional quality control mechanisms. This fact further underlines potential advantages of a single treatment algorithm for all plasmodial species since misclassification may lead to the prescription of inadequate and potentially ineffective anti-malarials. Molecular determination of non-falciparum and mixed species infection may also lead to misclassification. The phenomenon of low sensitivity for the detection of mixed species and non-falciparum infections by PCR was previously described and may be explained by multiple factors [26]. DNA recovery of low-level parasitaemia in mixed infections may be too low, potentially leading to an underestimation of mixed infections. Use of frozen blood samples instead of filter paper sampling may improve the yield of DNA extraction and should be compared to dried blood samples in future studies of mixed infections. Most importantly, no difference in the clinical or parasitological response was noticed for PCR confirmed and un-confirmed cases. Hence, artemether-lumefantrine is as an excellent option for the treatment of patients with non-falciparum and mixed species infection. Since the prevalence of chloroquine resistance in Gabon is almost 100% [27], chloroquine treatment of those patients with sub-microscopic *P. falciparum* and microscopically detected non-falciparum mixed infection (n = 6 (15%) in this study) is likely to fail.

This data support the current practice of using artemisinin-combination therapy for the treatment of non-falciparum and mixed plasmodium infections or indeed clinically suspected malaria without prior species diagnosis in Central Africa. Besides high efficacy, proven safety and tolerability of artemether-lumefantrine, the use of this combination may also reduce the risk of treatment failure due to misdiagnosis of plasmodial species. However, contrary to our findings, two recent reports of clinical failures of artemether-lumefantrine treatment of *P. falciparum* and *P. malariae* mixed infections challenge this assumption [28,29]. Whether these reports are the rare

exception or whether late recurrence of *P. malariae* constitutes a significant problem needs further evaluation.

Non-falciparum infections and mixed plasmodial species infections constitute a common infectious disease entity in tropical regions. In the setting of this study these infections account for an estimated 5-10% of malaria cases indicating the importance of non-falciparum and mixed species infections. With the exception of the recently conducted clinical development program of pyronaridine-artesunate combination therapy for the treatment of vivax malaria [30], non-falciparum malaria has been utterly neglected in anti-malarial drug development until these days accounting for less than 1% of published anti-malarial trials [4]. The conduct of clinical trials providing more evidence on the appropriate treatment of such infections therefore seems mandatory.

Conclusions

In conclusion, this report constitutes the first prospective assessment of artemether-lumefantrine in the treatment of *P. ovale*, *P. malariae*, and mixed-species malaria. The demonstrated high cure rate of 100% and the proven favourable tolerability profile of artemether-lumefantrine lend support to the approach of treating non-falciparum or mixed species malaria or indeed all cases of malaria without the possibility of species differentiation with artemether-lumefantrine combination therapy in the Central African country of Gabon.

Competing interests

The authors declare that they have no competing interests.

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Authors contributions

GMN contributed to data collection, interpretation of results, production and revision of the manuscript. CK contributed to data collection, production and revision of the manuscript. AB contributed to data collection, production and revision of the manuscript. HW contributed to data collection, production and revision of the manuscript. DA contributed to data collection, production and revision of the manuscript. MC contributed to data collection, production and revision of the manuscript. AAA contributed to conception and design, interpretation of data and revision of manuscript. FK contributed to conception and design, interpretation of data and revision of manuscript. BM contributed to data collection, interpretation of results, production and revision of manuscript. FJ contributed to data collection, interpretation of results, production and revision of manuscript. PGK

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Appendix

Publication not mentioned in the thesis

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