Medizinische Universitätsklinik Tübingen
Innere Medizin VII – Institut für Tropenmedizin, Reisemedizin, Humanparasitologie

Rapid and specific action of methylene blue against *Plasmodium* transmission stages

Inaugural-Dissertation
zur Erlangung des Doktorgrades der Medizin

der Medizinischen Fakultät
der Eberhard-Karls-Universität
zu Tübingen

vorgelegt von

Saison, Nathanaël François Louis

2024

Dekan: Prof. Dr. Bernd Pichler

1. Berichterstatter: Prof. Dr. med. Steffen Borrmann
2. Berichterstatter: Prof. Dr. med. Matthias Schwab
3. Berichterstatter: Prof. Dr. med. Olaf Müller

Tag der Disputation: 08.10.2024

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List of abbreviations

ACT	Artemisinin combination therapies
AG	Arbeitsgruppe
AHA	Autoimmune haemolytic anaemia
An	Anopheles
BMM	Blood medium mixture
BSL	Biosafety Level
C	Vectorial capacity
CDC	Centers for disease control and prevention
CERMEL	Centre de Recherches Médicales de Lambaréné
CHU	Centre Hospitalier Universitaire
CML	CoMaL Identification
CoMaL	Plasmodium species co-infections in Anopheles mosquitoes: a pilot study of parasite-vector interactions that define transmission in Africa
DHA	Dihydroartemisinin
DMEM	Dulbecco's Modified Eagle Medium
DMFA	Direct membrane feeding assay
DPI	Day post infection
EIR	Entomological inoculation rate
G6PD	Glucose 6 phosphate deficiency
GFP	Green fluorescent protein
GR	Glutathione Reductase
GSH	Glutathione
HepG2	Human Hepatoma cell line
IC_{50}	The half maximal inhibitory concentration
ID	Identification
IPT	Intermittent preventive treatment
IRS	Indoor residual spraying
ITNs:	insecticide treated nets
l	litre
LLIN	Long-lasting insecticide-treated net

Luc	Luciferase
M	Molar
MB	Methylene blue
mg	Milligram
min	minutes
ml	Millilitre
mM	Millimolar
NADP/NADPH	Nicotinamide-adenine dinucleotide phosphate
nM	Nanomolar
P	Plasmodium
p	P- value
Pb	Plasmodium berghei
PBS	Phosphate-buffered saline
PQ	Primaquine
RBCs	Red blood cells
P	p-value
PR	Parasite rate
PRR	Parasite reduction ratio
RBC	Red blood cells
Ro	Basic reproduction number
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
TBVs	Transmission blocking vaccines
VIMTs	Vaccines that interrupt malaria transmission
WHO	World Health Organization
WS	Working solution
µl	Microliter
µM	Micromolar

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1 Introduction

1.1 Malaria in sub-Saharan countries

Malaria remains an important cause of under-five morbidity and mortality in sub-Saharan African countries. Regarding the last WHO Report, 229 million cases of malaria and 409.000 deaths related to malaria were estimated worldwide. Over 94% of the cases and deaths in 2019 occurred in the WHO African region, most of them caused by the most threatening human malaria parasites, *P. falciparum* [1]. Malaria occurs mostly in poor, tropical, and subtropical areas of the world and remains indeed an important a health and socio-economic issue. Purchasing of antimalarial drugs, treatment at dispensaries and clinics, mosquito nets expenses, lost days of work or absence from school are important costs to every individual and their families in endemic area. Malaria has an important impact on national economies and hinders economic development in countries where the burden of malaria is highest. Direct costs of malaria have been estimated to be at least US$ 12 billion per year [2].

The most important brunt of mortality burden appears in young children, because they most of time did not developed sufficient immunity to clear the infection. Other individuals frequently exposed to the parasite acquire a functional immune response and can clear infection without becoming ill and may in certain cases develop an overt clinical response and lead to severe complications. Severe complications require medical intervention and could result in death and may induced sequelae, such as blindness, epilepsy or cognitive impairment [3].

Other indirect effects can be linked to chronical infection and disease process. Malaria clinical manifestations are leading to chronical anaemia and increase the susceptibly to others pathogenic infection. Moreover, pregnant women are also vulnerable because of decreasing of her immunity during a pregnancy and asymptomatic infection in placenta reduces birthweights and infant survival rates [4].

Countries with indigenous cases in 2000 and their status by 2017 Countries with zero indigenous cases over at least the past 3 consecutive years are considered to be malaria free. All countries in the WHO European Region reported zero indigenous cases in 2016 and again in 2017. In 2017, both China and El Salvador reported zero indigenous cases. *Source: WHO database.*

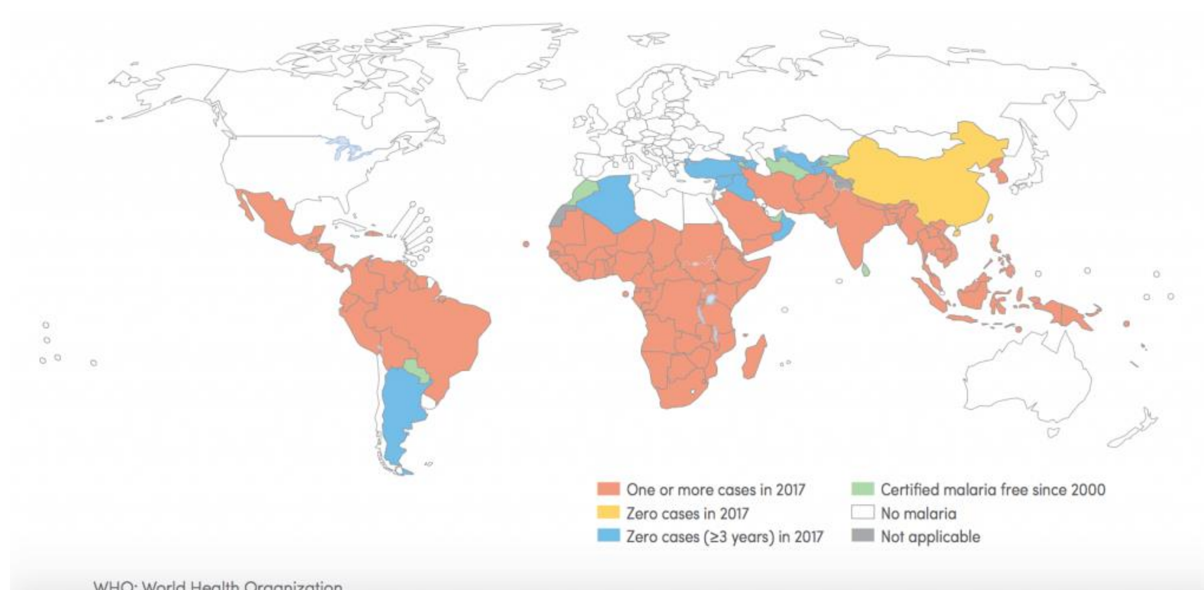

Fig. 1: Malaria distribution map. Countries and territories with indigenous malaria cases in 2000 and in 2016. Countries in green have been certified malaria-free since 2000. Countries in blue have had zero indigenous cases over at least the past 3 consecutive years and are eligible to request certification of malaria free status from WHO. Countries with one or more cases of malaria in 2017 are reported in red. Countries with zero cases in 2017 are reported in yellow. Reproduced from World malaria report 2017 [5].

Malaria is transmitted by bites of mosquitoes of the genus *Anopheles*, which are able to transmit protozoan parasites of the genus *Plasmodium* (Apicomplexa: Haemosporidae) during a blood meal on humans. Five *Plasmodium* species are considered able to invade humans: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* and *P. knowlesi*. *P. vivax* is the most widespread *Plasmodium* species over the world with a highest prevalence in Southeast Asia and Latin America [6]. *P. falciparum* is most dangerous and lethal species and by far responsible for most of malaria related deaths in Sub-Saharan Africa [3]. *P. knowlesi* is considered as a macaque malaria parasite that could infect humans in rare cases, which have been described in Southeast Asia [7]. The parasite is transmitted by female Anopheles mosquitoes, which commonly bite in the darkness. During the blood meal, the mosquito can also get infected and spread the parasite to another individual. However, the parasite cannot be transmitted directly from human to human and needs an intermediate host.

1.2 Overview of *Plasmodium* life cycle

Plasmodium parasite requires two different hosts to complete their multi-stage life cycle: human or vertebrates and female Anopheles mosquito.

Plasmodium parasites in human host were first described by Laveran [8], a French physician working in the military hospital of Constantine, Algeria. He observed pigmented parasites inside the red blood cells (erythrocytes) of malaria sufferers and noticed pigmented spherical bodies moving with great vivacity between the red blood cells, which was in fact the first observation of male gametocyte exflagellation. However, his observations raised scepticism among the medical community who thought that malaria was caused by a bacterium. In 1987, Ross confirmed Laveran's observations and described the transmission of malaria parasite and its complete life cycle [9].

1.2.1 Human host stage

1.2.1.1.1 Pre-erythrocytic stage

During a blood meal, an infected female *Anopheles* mosquito infect the human host through inoculation of sporozoites into the human host. Sporozoites infect liver cells (hepatocytes) during the exo-erythrocytic cycle, which rupture and release merozoites in the blood stream. Some species of *Plasmodium* can persist in the liver as dormant stage (hypnozoites) and cause relapses by invading bloodstream.

After being released in the blood stream (erythrocyte cycle), the parasites undergo asexual multiplication in the erythrocytes. First into ring trophozoites, which mature into schizonts and release merozoites in the blood stream. Rupture of schizonts induces fever and chills in malaria infected humans. Merozoites in turn infect red blood cells and replicate inside them. Replication cycle time differ from specie to specie but take approximately 48h for *P. falciparum* and *P. vivax*. In some cases, parasitized red blood cells may obstruct capillaries, leading to local hypoxia and inducing the release of local toxic molecules such as cytokines, which lead to severe manifestation of malaria.

Obstruction of the bloodstream can also reach the cerebral microcirculation inducing impaired consciousness and brain injury [10].

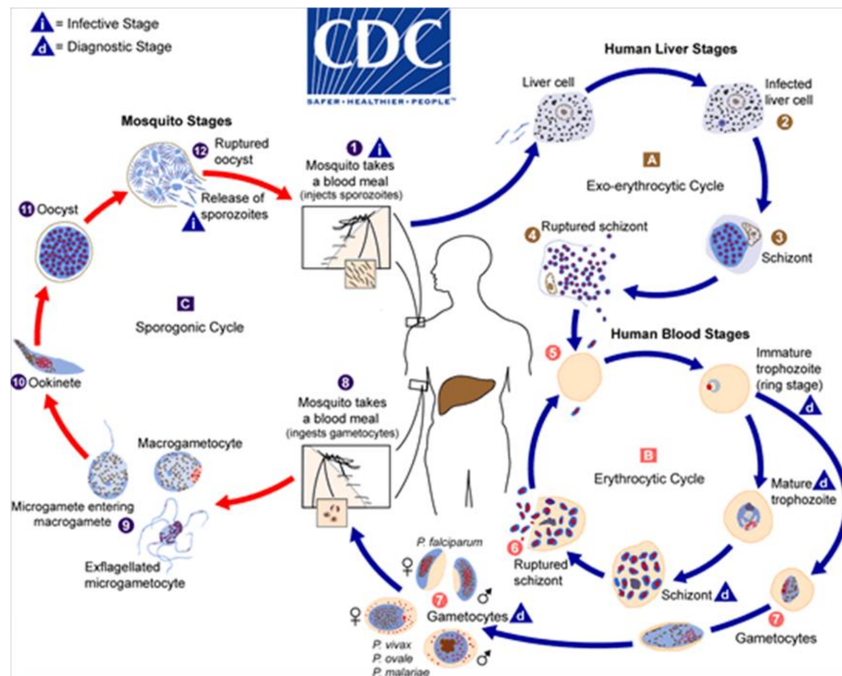

Fig. 2 :*Plasmodium* parasites require two hosts (vertebrate and mosquito host) to complete their life cycle. Asexual multiplication takes place in vertebrate, sexual multiplication takes place in the anophelines. Reproduced from the CDC website on malaria lifecycle

In red blood cells, some merozoites develop into sexual erythrocytic stages and differentiate in male and female gamete – gametocytes – which are highly specialized for the transmission of the parasite. The development of *P. falciparum* gametocytes is a long and complex process over a period of ~10 days in which they develop from stage I to V in the human body (mainly in bone marrow) [11-13]. After becoming mature (Stage V), gametocytes circulate in the peripheral blood stream. When a female *Anopheles* mosquito bites the host, gametocytes are then transmitted by blood ingestion.

1.2.2 Sporogonic cycle

Development of malaria parasites in *Anopheles* mosquitoes following ingesting of *plasmodium* gametocytes was first described by Ronald Ross in 1897 [14] and in 1898 he demonstrated that mosquitoes were intermediate hosts for *Plasmodium* parasites, and necessary for malaria transmission [15]. Sporogonic phase cycle of the parasite's life cycle lasts 8 to > 20 days in the tropics. Length of cycle differs between *Plasmodium* species and depends upon external factors such as temperature and humidity. The female and male gametocytes are infested by an Anopheles mosquito during a blood meal. Triggered by external factors including a drop-in temperature, pH changes or presence of xanthurenic acid (XA), male gametocytes exflagellate and develop into microgametocytes. [16, 17] The microgametes penetrate the macrogametes generating zygotes. About 24 hours post-infective blood meal, zygotes transform into a motile, banana-shaped ookinete [18]. Ookinete migrate from the midgut lumen to basal side, causing morphological changes and tissue damage, and transform into oocysts 24-48 hours post blood meal [19]. For 7 days or more, the oocysts grow and multiplicate. Sporozoites develop inside the oocysts [20].

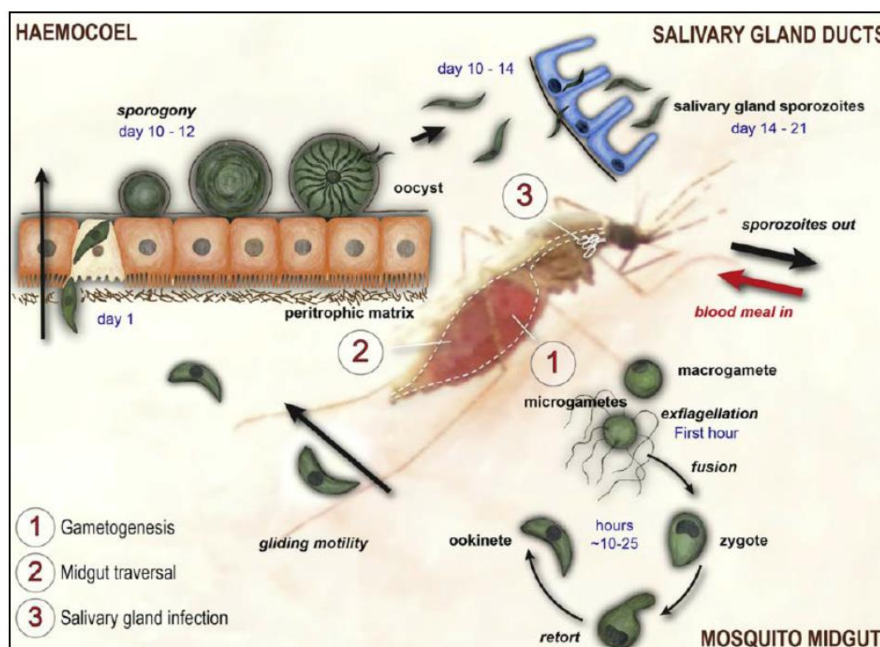

Fig. 3: *Plasmodium* sporogonic development in the mosquito starts when a female mosquito feeds on an infected vertebrate host. Key stages are (1) gametogenesis, (2) ookinete midgut traversal followed by the establishment of oocysts, and (3) sporozoite salivary. Reproduced from *Angrisano et al.* [21].

The last part of the sporogony cycle begins with oocysts bursting, releasing sporozoites into the hemocoel. The sporozoites migrate and invade the salivatory glands, within 10-16 days after infection. In order to perpetuate the malaria life cycle of the parasite, inoculation of the sporozoites into a new individual is required [22].

1.2.3 Clinical features of malaria

Infection with *P. falciparum* may result in a wide variety symptom, ranging from an asymptomatic infection or uncomplicated malaria infection to severe form and even death. Symptoms vary depending of levels acquired immunity to the parasite in patients in endemic areas, therefore, severe malaria mainly occurs in young children. The time elapsed (incubation period) between exposure to the infectious agent (bite of Anopheles) and the first clinical sign or symptom vary depending on the species of *Plasmodium* causing malaria. The average incubation period is 9-14 days for *P. falciparum*, 12 – 17 days for infections by *Plasmodium vivax* and 18-40 days for infections caused by *Plasmodium malariae* [23-25]. Symptoms result exclusively from the asexual erythrocytic stages or blood stages of *Plasmodium. P. falciparum* – infected erythrocytes adhere to the vascular endothelium of venular blood vessel walls and occurs in the vessels of the brain causing severe form as cerebral malaria, which is associated with high mortality. Commonly, malaria episode results in a combination of the following symptoms: fever, chills, sweats, headaches, nausea and vomiting, abdomen pain, fatigue. Symptoms before the typical fever are unspecific and can be described as flu-like.

Uncomplicated malaria can develop into severe malaria form within hours when infections are complicated by serious organ failures or abnormalities in the patient's blood, with impairment of consciousness, severe anaemia due to haemolysis, acute respiratory distress syndrome (ARDS), low blood pressure caused by cardiovascular collapse or acute kidney injury [26]. Severe malaria is a medical emergency and should be treated earlier as possible. Despite optimal treatment with intravenous artesunate, the mortality rate of patients with severe malaria remains between 8.5 – 15 %. If parenteral artesunate is not available, quinine can be used but the mortality rate of patients remains higher between 10.9 – 22 % [27, 28]

1.3 Vector and parasite distribution

1.3.1 Anopheles gambiae s. s.

An. gambiae is one of the most efficient vectors of malaria in the world [29]. *An. gambiae s. s.* is part of the Anopheles gambiae species complex which include six morphologically indistinguishable species: *A. gambiae sensu strico Giles, Anopheles arabiensis Patton, Anopheles bwambae White, Anopheles melas Theobald, Anopheles merus Dönitz, and Anopheles quadriannulatus Theobald* [30]. *An. gambiae s.s.* are distributed throughout sub-Saharan African regions, including Madagascar. The presence of *An. gambiae* was reported in 34 countries of Africa, which makes it the most widespread on this continent [31].

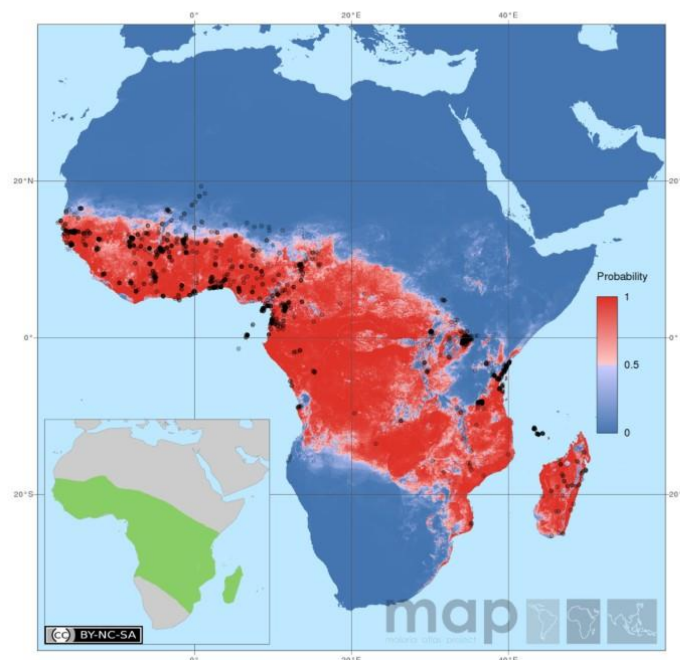

Fig. 4: The anticipated distribution of *An. gambiae* in Africa. The map was made using the Boosted Regression Tress technique and hybrid data. The black dots show spots were *An. gambiae* occurrence has been reported. This figure was taken from Sinka et al. (2010)

An. gambiae cycle is composed of four life stages: egg, larva, pupa and adult. Adult females lay their eggs on the surface of water, especially in sunlit pools of standing water [32]. Larvae hatch from egg and develop though four larval sizes before pupating within the aquatic habitat. (Foster and Walker). Larvae are very small in the first instar

and increase until reaching 5-6 mm before pupating once they acquire an appropriate amount of nourishment [32]. *An. gambiae* can develop from egg to adult in 10 – 11 days depending of temperature. Adult female *Anopheles* presents the palps (appendages found near the mouth) are as long as their proboscis (feeding tube). It's a key characteristic to differentiate them to others male mosquitoes. Male and female adult feed nectar from plants, but only female bloodfeds on vertebrates, where she obtains nutriments for her eggs [33]. Adult mosquitoes can survive for up to one month in a laboratory and they usually survive around one to two weeks in the wild (CDC 2010).

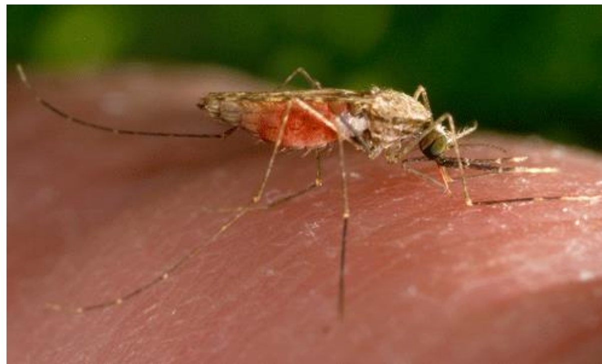

Fig. 5: Female *An. gambiae* during a blood meal. Reproduced from CDC Photographer James Gathany

Population of *An. gambiae* varies depending of the seasons and location, but generally the population decreases during the dry season and increases during the wet season [32, 34]. There are considered as effective vectors of human malaria due to their short development time and high preference to develop near human dwellings.

1.3.2 Vector control

Despite progress in malaria vector control, malaria cases do not decrease significantly and has stalled on a plateau. Vector control is a key strategy to prevent infection and reducing disease transmission. Current malaria vector tools are based on insecticide through long-lasting impregnated bed nets (LLINs) and indoor residual spraying (IRS). Use of bed nets impregnated with insecticides have drastically reduced malaria. By reducing the number of infectious bites and the survival probability of infected mosquitoes, bed nets led to save 7 million people between 2000 and 2015. [35]

However, mosquito resistances are emerging to insecticides [36, 37] and evidence showed shifts in vector biting behaviour from night biting to early evening [38]. Both are leading to recrudescence of malaria infection by increasing the number of infectious bites. In addition, first line malaria vector tools target exclusively indoor-resting and indoor-biting mosquitoes and do not protect humans from outdoor-biting mosquitoes.

Those considerations are leading scientists to pursue the research on additional implementation of interventions and other possible malaria transmission blocking strategies. High effective transmission blocking drugs or vaccines could be a breakthrough in malaria elimination efforts in Africa.

1.4 Malaria transmission

Understanding malaria transmission is a key component of intervention strategies that target the spread of malaria parasites from human to mosquito. However, malaria transmission involves several epidemiological parameters which permit to estimate her intensity and are necessary to interpret and compare innervations in a given population in a geographic area. They are key tools in malaria transmission control.

The intensity of malaria transmission is usually estimated through interconnected variables. Basic examples remain the incidence and prevalence rate, defined respectively as a number of new infections in a given population unit as well as the prevalence rate which corresponds as a fraction of a given population infected at a given point of time.

Another key variable for estimating and reporting malaria transmission intensity in endemic areas is the entomological inoculation rate (EIR), which is defined as the number of infectious bites received by an individual per time unit, over a time period. The EIR is defined by multiplying the sporozoite rate, the density of the vectors in a given population and the number of blood meals taken by vectors per time unit [39]. It is often estimated by catching mosquitoes most commonly using light-traps and then dissecting these mosquitoes to estimate the sporozoite (infectious) rate in field studies. The EIR remains the most standard measurement for assessing malaria parasite transmission and can be implemented to evaluate impact of vector control and anti-vector actions. [40-42].

The last significant variable, called vectorial capacity (VC), describes the vector's capacity to transmit malaria in terms of the number of infectious bites that result from a single infected person in a day. It was initially described by Garrett – Jones in 1964 [43] and demonstrates that malaria transmission is highly sensitive to adult mosquito survival. This finding may help to explain early insecticide intervention's success in the late 1940s and 1950s and justified expansion of use of IRS in the 1960s. [44] The prevalence of parasitaemia in the human population is charted against the average annual VC. At low levels of VC, small increases result in a rapid rise in parasitaemia prevalence rate. [45]

1.4.1 The Ross-Macdonald model

Mathematical and epidemiological models have been established to facilitate the quantification and monitoring malaria transmission levels and a given population. The main epidemiological model, known as the Ross-Macdonald model, provides a quantitative description of the malaria host life cycle and vector feeding cycle. The primary purpose of this model is to estimate the expected number of infectious mosquitoes from a single infectious mosquito after one parasite generation. The quantitative outcome of this model has been modeled by estimating the basic reproduction number R_0, defines as the potential total number of secondary cases of infections produced by one primary infection in a given population, assuming that a population is, and remains susceptible. If $R_0 > 1$ the infection will be able to spread in a population, but if $R_0 < 1$ it may become extinct.

Based on Macdonald's expression (Ref) $R_0$is described as:

$$\boldsymbol{R_0} = \frac{\boldsymbol{ma^2bp^nc}}{\boldsymbol{r(-log_e p)}}$$

m = density of vectors
a = number of blood meals taken on humans per vector per day
p = daily survival probability
b = vector competence, transmission probability from an infective mosquito to human
c = transmission probability from an infectious human to mosquitoes
r = recovery rate of man from infection

Estimating the R_0 plays an important role for malaria control and helps determine where interventions in reducing malaria transmission are needed to eradicate malaria in endemic regions. The basic reproduction number is an index of malaria transmission intensity and is an important metric to measure the effort and investments required to eliminate malaria in endemic areas.

1.4.2 Prevalence and intensity of infected mosquitoes

To evaluate infectiousness and transmission of malaria parasites from human to mosquitoes, the following indices are used for reporting infection intensity:

- Oocyst prevalence: Number of mosquitoes with oocysts, i.e. Number of infected mosquitoes
- Sporozoite rate: Number of mosquitoes with sporozoites
- Oocyst density: mean number of oocysts per midgut

There are especially essential to assess effectiveness of control programs or evaluate transmission blocking drugs potency. Both indices are essential for comparing a malaria control intervention. Moreover, the relationship between prevalence and intensity can be described by a negative binomial distribution, with a high degree of heterogeneity due to parasite-related and mosquito-related factors.

1.5 Malaria transmission blocking strategies

Malaria transmission-blocking strategies focuses on interventions in a population, in order to reduce the malaria transmission intensity and ability of the parasite to spread in a population. Transmission-blocking strategies targets the different stages of *plasmodium* parasites to disrupt the development of the parasite within the human host and the vector with the goal to reduce the transmission in a mosquito and infectious bites.

1.5.1 Antimalarial drugs

Artemisinin-based combination therapy (ACT) has been adopted as first-line treatment for uncomplicated malaria throughout. Despite emergence of chloroquine resistances [46], ACTs continue to be high effective to treat symptomatic forms of malaria. However, ACTs are always not sufficient to sufficient to eradicate all the forms of the parasite. Indeed, active metabolites of artemisinin and dihydroartemisinin (DHA) are highly active against stage I-III gametocytes but show incomplete activity against stage IV and V gametocytes [47, 48]. Although, impact of ACT reduced considerably asexual parasitaemia prevalence following ACT introduction, the reduction of gametocytaemia prevalence in the human population was very modest [49]. An obvious reduction of human infectiousness after introduction of ACT for malaria case management in the high endemic regions is not evident.

Moreover, gametocytes are most commonly detected in children but the proportion of asexual parasites that is committed to develop into gametocytes may increase with age. Adult are not considered as semi-immune and often not treated and may play an important role as human infectious reservoir for malaria [50]. Only gametocytocidal and sporontocidal antimalarial drugs prevent parasite development in the mosquito and reduce the transmission intensity.

1.5.2 Gametocidal and sporontocidal drugs

In order to completely eliminate malaria transmission in endemic areas, drugs targeting all stages of the parasite's life cycle are required, especially drugs effective on the sexual stages of *Plasmodium spp.* Development of sexual parasite stage is complex and induces metabolic ad morphological changes. Immature gametocytes, considered as gametocytes between stage I and III, have a comparable metabolism to asexual blood stage, which make them sensitive to almost antimalarial drugs. Gametocytes at stage IV-V, considered as mature, are relatively insensitive to almost antimalarial drugs, which complicated their elimination [51, 52].

Several antimalarials (antifolates and hydroxynaphtoquinones) show activity against transmissible parasite stages and interfere with parasite development in the mosquito (sporontocidal activity) but only the 8-aminoquinolines and MB have confirmed to kill mature *P. falciparum* gametocytes. Since October 2012, WHO recommended and considered safe to add a single low dose of primaquine (0.75 mg base/kg) in combination with ACT treatment of uncomplicated malaria [53]. Use of a single dose a PQ is well known to be efficient to reduce malaria transmission, including drug-resistant parasites. However, the implementation of PQ in endemic areas remains to be difficult because of concerns on the risk of haemolytic anaemia (AHA) in individuals who have phosphate dehydrogenase (G6PD) deficiency, an inherited X-linked abnormality. The prevalence of G6PD deficiency varies between 5 and 32.5 % in malaria endemic areas of Asia and Africa [54].

Sporontocidal drugs are described as candidates which interrupt or prevent the development of the parasite in the mosquito. Various approaches have been developed leading to identify mechanisms interrupting transmission after gametocytogenesis to formation of sporozoites [55]. Combination of pyrimethamine and proguanil have been shown interfering the sporogony development in damaging ookinetes and inhibiting dihydrofolate reductase. Ivermectin has been recently reviewed as a potential candidate blocking *P. falciparum* transmission [56] and modestly inhibiting *P. vivax* oocyst development [57].

1.5.3 Transmission blocking vaccines (TBVs)

In line with the research strategy, technological innovations like vaccines are currently in development against the parasite to reduce its transmission. Transmission blocking vaccine candidates aim to generate an antibody-mediated response or activation of the complement lysis cascade against gametocytes in the mosquito blood meal to interfere with parasite survival and sexual development. [12, 21] Arrest of the life cycle at this stage acts to reduce transmission in the mosquito midgut. First experiments for TBVs in chickens were reported in 1958 in turkey [58] and revisited in 1976 with an immunization regime of formalin-fixed *P. gallinaceum* gametocytes in chickens, achieving to transmission-blocking immunity (TBI). [59] Analogous reports demonstrated similar induction of TBI in mice with *P. yoelii* [60] and in primates with *P. knowlesi* [61]. Improvements in the *in vitro* culture of sexual stage of *P. falciparum* lead to identification and production of transmission-blocking monoclonal antibodies (mAbs) in 1983, which could arrest parasite development in the mosquito midgut. [62, 63]

These studies demonstrated the role of antibodies against surface antigens of the malaria parasite and identified the first list of TBI targets by precipitation with transmission blocking mAbs. [63] Following antigens were named Pfs25, Pfs48/45 and Pfs230 due to their size migration by SDS-Page and remain the most studied and advanced in the clinical pipeline. TBV candidates (Pfs25, Pfs 48/45 and Pfs230) as recombinant proteins are currently best-characterized TBV candidates and have shown the most progress. At this time, there is no safe and effective transmission blocking vaccine, which lead of a durable antibody response.

1.6 Methylene blue

1.6.1 Methylene blue as a treatment of malaria

MB, also known as methylthioninium chloride, is the oldest synthetic anti-infective. First prepared in 1876 by German chemist Heinrich Caro, its first uses in the treatment of malaria were reported by Paul Guttman and Paul Ehrlich in 1891 [64].

MB is well absorbed from the gastrointestinal tract and reach a maximal plasma concentration after two hours with a half-life of around 20 hours. In many countries, MB is indicated for treatment of methemoglobinemia, treatment of septic shock, prevention of urogenital infections or visualization of organ structures during surgery [65, 66]. MB shows a high affinity for *Plasmodium* parasites [67] – especially against quinine resistant parasites [68] - and low toxicity for human cells. It disappeared during the Pacific war in the tropics because of prominent reversible side effects: urine and sclera discoloration.

Investigations on MB efficacy against malaria got reactivated in the last decades, especially in the context of spreading resistance of P. falciparum to antimalarial drugs like chloroquine and sulfadoxine-pyrimethamine [69, 70]. Based on *in vitro*, MB demonstrated its high antimalarial potency (IC_{50} = 4 nM) without cross-resistance with chloroquine [65].

Antimalarial activity of MB consists of inhibition of parasite growth by inhibiting (met)haemoglobin degradation. Evidences show that methaemoglobin is the substrate of the parasite's proteases and may play a key role in riboflavin reduction [71]. Methaemoglobin formation appears in severe malaria contributing to tissue hypoxia [72] and MB may revert the oxidation of haemoglobin to methaemoglobin in the parasite's digestive organelles through oxidation of NADPH to NAPD. The reverting results to haemoglobin formation [71].

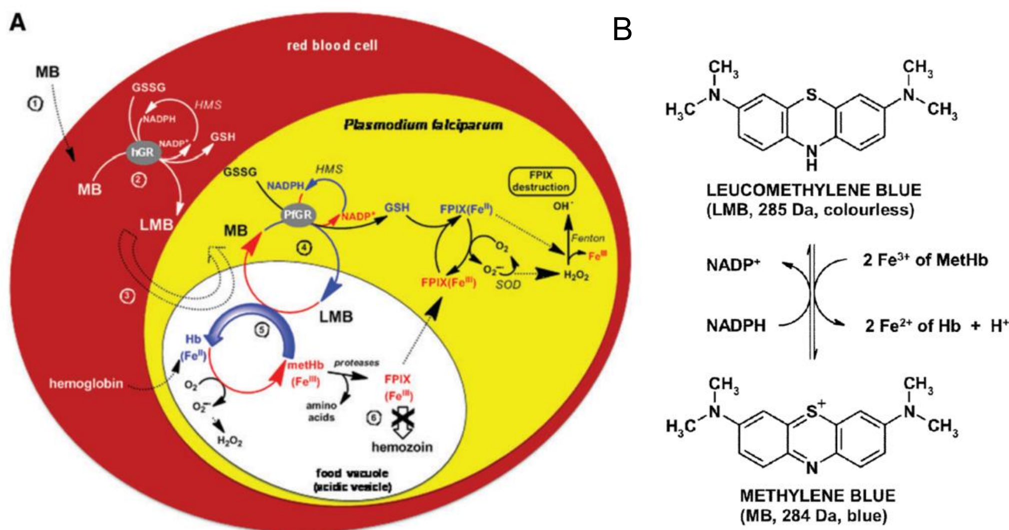

Fig. 6: 6a Schematic overview of redox-cycling and trafficking processes of MB in *P. falciparum*. 6b Oxidoreduction reaction of MB and NADPH resulting in haemoglobin formation. Reprinted from Blank et al. [73].

Moreover, MB is a non-competitive inhibitor of *P. falciparum* glutathione reductase which catalyses the reduction of glutathione disulphide using NADPH as a source of reducing equivalents [65].

Glutathione plays a key role in the defence of *Plasmodium* parasites against oxidative damage, regulation of enzyme activity and maintains the redox balance of the parasite and host-parasite interaction [74]. MB stimulated the oxidation of glutathione and lead indirectly to glutathione depletion in the cytosol [75].

1.6.2 Gametocidal activity

MB has been shown to be effective against *Plasmodium* spp. mature gametocytes and has shown *in vitro* a powerful early transmission blocking activity [76, 77]. Indeed, previous studies reported a morphological deformation on *P. falciparum* gametocytes after MB treatment [78] and a sterilizing effect [79] with a preferential clearance for male gametocytes [80], which reduce the gametocyte infectivity and block the transmission of *P. falciparum* from the human to the mosquito.
A last important clinical study conducted in Mali [80] has shown that oral MB treatment combined with ACTs is safe and effective in the treatment of uncomplicated malaria

and lead to a complete human to mosquito transmission inhibition day 2 post treatment.

1.6.3 Safety issue

Blue discoloration of urine is often associated with treatment with MB and remains the most known adverse event. Others adverse events such as mild urogenital symptoms or mild gastrointestinal symptoms were also reported, but more frequently in treatment with higher doses of MB [68]. MB is listed as a potentially dangerous drugs for patients with G6PD deficiency [81], but until now there is no evidence that MB treatment causes an excess of severe haemolytic anaemia [82]. However, MB treatment is often associated with a slightly reduction of haemoglobin level, but this is also often observed in treated patients with ACTs [83].

1.7 Rationale

In the face of unacceptably high global morbidity and mortality and general lack of progress in malaria control, the emergence and spread of artemisinin-tolerant lineages represents a major global health challenge. The likelihood of resistance emergence is increased with the spread of poor-quality drugs. With the shift in global malaria management programs from control to eradication of the disease, the transmissible, asymptomatic, intra-erythrocytic sexual gametocyte stages of *Plasmodium* parasites have been identified as a priority target for transmission-blocking efforts. In the search of a novel transmission-blocking lead, the research on potency of MB against the transmissible late-stage gametocytes is justified.

Directly targeting the stages of the malaria parasites responsible for transmission from the human host to the mosquito vector could be a key pharmacological strategy for malaria control. In the recent recommendations of WHO [53], primaquine has been identified helpful to contain malaria transmission in hyperendemic area. WHO recommend to add a single 0.25 mg base/kg primaquine to all patients with confirmed *P. falciparum* malaria in addition with ACTs. However, clinical use of primaquine is

limited due to the risk of developing haemolytic anaemia (AHA) in individuals who have phosphate dehydrogenase (G6PD) deficiency.

WHO even called for more research on the effects of MB on the transmission of the parasite in order to find among others a candidate to replace primaquine which poses many safety issues [54]. Added in a triple combination with artesunate and amodiaquine, MB has been shown to be high effective against the gametocytes of *P. falciparum*. Moreover, a plethora of *in vivo* and *in vitro* experiments have shown evidence of transmission blocking potency of MB in a standard membrane feeding assay (SMFA) and in murine model using *P. yoelii* [76, 77].
However, MB activity against oocyst and sporozoite development has not been evaluated compared to others transmission blocking candidates such as Ivermectin [84] or Atovaquone [85].

1.8 Aims of the presented work and objectives

In order to better characterize the transmission blocking activity of MB and identify at what point in the mosquito and sporozoite stages MB is active, this study aims, therefore:

- To assess the *ex vivo P. falciparum* transmission blocking activity in *Anopheles gambiae* of MB using field isolates in Lambaréné, Gabon.

- To assess the *ex vivo P. berghei* sporontocidal activity of MB in a secondary feeding procedure at CIMI Paris, France.

- To assess the *in vitro* anti-sporozoite activity of MB using freshly dissected *P. berghei* GFP sporozoites at CIMI Paris, France.

- To re-evaluate the *in vitro* anti-liver stage activity of MB using freshly dissected *P. berghei* GFP sporozoites at CIMI Paris, France.

- To assess if MB can block the hepatocyte invasion and interfere the liver stage development *in vivo* using *P. yoelii* GFP-Luc and *P. berghei* GFP.

2 Material and Methods:

2.1 Material

2.1.1 Colony rearing

Name	Manufacturer
BugDorm-4F3030 insect rearing cage (32,5cm x 32,5cm x 32,5cm)	MegaView Science Co., Ltd.
Collapsible cages (30,5cm x 30,5cm x 30,5cm)	BioQuip Products, Inc.
Fisherbrand™ Plastic Petri Dishes	Thermo Fisher Scientific Inc.
labell® 10 Sterile gauze sponges (40x40cm), individual packing	Labell®
neoLab melamine instrument tray (35cm x 24cm x 4cm)	NeoLab Migge GmbH
Pipette Standard Non-Graduated Non-Sterile Pastette®, bulb draw 3.2mL	Alpha laboratories
Qualitative filter paper, 413 (150mm)	VWR International, LLC.
Stérilux® soin, 100% hydrophile cotton (250g)	Paul Hartmann AG
Sugar	Princesse TATY (in Franceville)
Vitakraft® Premium VITA Flake-Mix	Vitakraft pet care GmbH & Co. KG

2.1.2 Sampling

Name	Manufacturer
BD Vacutainer™ Heparin Plasma Tubes (10mL)	Becton Dickinson GmbH
BD Sentry™ Lancet (1,8mm x 23G (0,63mm))	Becton Dickinson GmbH
Disposable needle SUPRA (0,90x100mm), 100pcs., sterile	Misawa Medical Industry Co., Ltd.

2.1.3 Membrane feeding Assay

Name	Manufacturer
Cryo-Babies®, white (33 x 13mm)	Diversified Biotech, Inc.
CO2 Incubator 160 K	mytron Bio- und Solartechnick GmbH
Dental Dam (152mm x 5.5m)	COLTENE Group
Eppendorf™ Autoclavable safe lock micropestle, for 1,5ml and 2ml vessels	Eppendorf AG
Eppendorf Tubes®, Safe-Lock Tubes (2,0mL)	Eppendorf AG
Eppendorfpipette Research® plus, variable 0,5-10µL	Eppendorf AG
Eppendorfpipette Research® plus, variable 100-1000µL	Eppendorf AG

Eppendorfpipette Research® plus, variable 20-200µL	Eppendorf AG
Eppendorfpipette Research® plus, variable 2-20µL	Eppendorf AG
epT.I.P.S.® standard, Eppendorf Quality™, 0,1 – 20µL, 40mm, white tips	Eppendorf AG
epT.I.P.S.® standard, Eppendorf Quality™, 2 – 200µL, 53mm, yellow tips	Eppendorf AG
epT.I.P.S.® standard, Eppendorf Quality™, 50 – 1.000µL, 7 mm, white tips	Eppendorf AG
Falcon™ 15mL conical centrifuge tubes	Thermo Fisher Scientific Inc.
Falcon™ 50mL conical centrifuge tubes	Thermo Fisher Scientific Inc.
HE-4 heating circulator, stainless-steel bath tank (4,5L)	JULABO GmbH
Heraeus™ Centrifuge Biofuge primo R	Thermo Fisher Scientific Inc.
Human bloodserum, type AB	Provided by the blood bank of University of Tübingen
Injekt® Single-use syringes, 2-piece (20mL), sterile	B. Braun Melsungen AG
Millex®- GS, filter unit, MF-Millipore™ Membrane, Ø33mm, MCE 0,22µm	Tullagreen, Carrigtwohill, Co. Cork

Mosquito feeder, membrane style, glass only, 50mm feed diameter	Zitt-Thoma GmbH
Mouth aspirator	Self-made (see supplement Fig. 22)
Parafilm®, Laboratory film (4 in. x 125 ft., roll)	Bemis Company, Inc.
Pipette Graduated 1ml Sterile Pastette®, 0.25ml graduations, bulb draw 3.4mL	Alpha laboratories
Pipette tips 100-1000µL, blue, loose packed, 250 tips/package	Greiner Bio One International GmbH
Pipette tips 10-200µL, yellow, loose packed, 500 tips/package	Greiner Bio One International GmbH
Pipetting aid accu-jet® pro	BRAND GmbH + Co. KG
Prodigy® insulin syringes with needles	Prodigy Diabetes Care, LLC.
Roswell Park Memorial Institute medium (RPMI) 1640 without L-Glutamine	Sigma Aldrich, USA
Thermo Haake SC150/S14P heated bath circulator	Thermo Fisher Scientific Inc.
Thermo Scientific™ HERAsafe™ KSP12, Class 2 biological safety cabinet	Thermo Fisher Scientific Inc.

2.1.4 Sporozoite assay

Name	Manufacturer
Primary cryopreserved Monkey hepatocytes (M. fascicularis)	CEA, Fontenay aux roses
96- well plates	Falcon by Becton-Dickinson Labware Europe, France
Collagen I	BD Bioscience, USA
William's medium	Gibco, USA
Fetal clone III serum	FCS, Hyclone
Penicillin-streptomycin	Gibco, USA
Human insulin	Sigma Aldrich, USA
Hydrocortisone	Upjohn Laboratories SERB, France

2.1.5 Microscopy

Name	Manufacturer
Filter papers MN 615 ¼, Ø240mm	MACHEREY-NAGEL GmbH & Co. KG
Giemsa	Sigma Aldrich, USA
Microscope Leica DM750	Leica Microsystems IR GmbH

Microscope slides, Ground edges with Frosted end (Clear glass 26x76mm – 1,0/1,2mm thick), 50pcs	MasterGlass

2.1.6 Midgut Dissection

Name	Manufacturer
Cover glasses (18 x 18mm)	Langenbrinck GmbH
DURAN® laboratory bottles, round, borosilicate glass 3.3, clear, with PP screw cap (500mL)	DWK Life Sciences
Mercury dibromofluorescein disodium salt (practical grade, crystalline)	Sigma-Aldrich, Inc.
Gibco™ phosphate-buffered saline (PBS) tablets	Fisher scientific
Ethanol	Medilab (in Libreville)
Stereomicroscope Stemi 508	Carl Zeiss AG

2.1.7 Software

Name	Manufacturer
Microsoft Office 2017	Microsoft
Prism 7	GraphPad Software Inc
SPSS	IBM corp.
ImageJ	Public Domain

2.2 Methods

2.2.1 Mosquito colony rearing

2.2.1.1 Introduction

Two species of Anopheles were used in this study:

1. *P. falciparum* transmission experiments were performed using female mosquitoes from a laboratory colony of *An. gambiae* Kisumu strain in Lambaréné*.* They were originally obtained from the Kisumu strain from Yaoundé, Cameroon. Adult mosquitoes were kept at standard insectary conditions (26 ± 0,5°C and 80% ± 10 relative humidity) in heating cabinets with a 12:12 hours light and dark cycle. They were fed with a 10 % sugar solution. The larvae were also reared in warmed climatic chambers at 28-30°C and 90% ± 2% relative humidity and a 12:12 hours light and dark cycle.

2. *P. berghei* transmission investigations were performed with a Laboratory colony of *An. stephensi* strain, reared in insectarium of University Pierre et Marie Curie (CIMI) under similar conditions to *An. gambiae*. Female mosquitoes 3 - 7 days old will be used for transmission blocking assays from rearing cages.

2.2.1.2 Collection of anophelines eggs

After subsequent blood feeding (48h), anophelines laid eggs on one or two petri dishes, preliminary placed into the cage that was fed before. The petri dishes were fully filled with cotton and wetted with distilled water. Most eggs are laid at night, and egg dishes were typically removed the day following their insertion. The filter papers with the eggs were folded twice in the middle and put in a clean and dry petri dish, which was covered. They were left in the heating cabinet of the adults to incubate overnight.

2.2.1.3 Egg hatching

One day after, mature eggs were removed from the cage and hatched by submerging them with distilled water in a neoLab melamine instrument tray (35cm x 24cm x 4cm). Tray was filled up until it was half full and labelled with the date of the hatching using Cryo-Babies®. Care was taken to move tray as little as possible to avoid eggs from sticking to the side of the tray.

A very small amount of Vitakraft® Premium VITA Flake-Mix fish food was also added to the water. The fish food was crushed before adding it and kept in a spice shaker in order to be able to dose it better.

2.2.1.4 Larvae culture

One day after hatching, the tray containing the eggs was split in 3 others trays to get between 150 and 200 larvae (L1) per plastic tray to avoid over-crowding. Over larval density lead to high mortality and distort sex ratios by favouring males over females [86]. A reasonable density for most L3-4 anophelines is 1 larva per ml with the water level 0.5 – 1 cm in depth. Trays were filled up with distilled water, which is crucial to avoid culture fails. Tap water does often contain high levels of toxic chemicals, which kill the larvae.

Larvae were fed with a fish food mixture, added daily to each tray. Three days per week, the trays were cleaned by removing sediment and dead larvae and changing the water. Development and mortality of the larvae were examined to estimate the mosquitoes' density in the following days. The amount of food provided daily increased as the larvae develop and as their density increased. However, care was taken to not overfeed the trays which lead to larval death.

2.2.1.5 Sorting Larvae and pupae

About seven days after the hatching, pupae were manually separated from larvae by removing them with a Pasteur Pastette®. Pupation duration is about 48 hours and Pupae emerged often over the night. Pupae were transferred into a small plastic container and transferred to an adult rearing cage. Number of pupae collected was reported to follow the size of the colony.

2.2.1.6 Anopheles adult caging and feeding

Adult mosquitoes were reared in heating cabinets using either BugDorm insect rearing cages (32,5cm x 32,5cm x 32,5cm) made from plastic and fabric net or collapsible cages (30,5cm x 30,5cm x 30,5cm) out of metal. Young mosquitoes were used for ex vivo membrane feeding assay and the older mosquitoes were used for maintenance of the colony.

Mosquitoes were daily fed by providing a 10 % sucrose solution to the adults through a feeder as the carbohydrate source. The sucrose solution was poured in a 50ml Falcon. The cages were provided with a 10% sugar solution, which was poured in a 20cm long cotton strand wrapped in gauze. The strand was put into a 50ml falcon tube through tuning. The sucrose solution was changed completely three times per week to avoid drying out and fungus proliferation.

2.2.1.7 Blood feeding of adults

Females anophelines require to be blood fed on mammalian blood to produce eggs. Mammalian blood brings all the nutrient, serving as a source of protein for the eggs production. Small mammals are usually employed as a blood source for the maintenance of the colony.

From two to four times per week, adult mosquitoes were blood fed on a male rabbit. Adults were preliminary starved for almost 6 hours by removing sugar source. A male rabbit was shaved on its flank and tied down on a wooden panel. Into a square hole in

the middle of the panel of the size of the rabbits' abdomen, mosquitoes were allowed to feed. The panel was placed on top of the cage during approximately 30 min in the dark. After the feeding, rabbits returned to the vivarium.

2.2.2 Mosquito dissections

2.2.2.1 Dissection of *An. spp.* infected midguts

An. gambiae and *An. stephensi* midguts were dissected following the same procedure performed respectively in Lambaréné and in Paris. Dissections were performed in a BSL-3 (Lambaréné) or BSL-2 (Paris) to not pose a direct health hazard for laboratory personnel and avoid spreading of infected mosquitoes in the area.

Mosquito midguts were examined 6-9 days post infection (*P. falciparum*) and 10-12 days post infection (*P. berghei*). Statistically, day 8 (*P. falciparum*) and day 10 (*P. berghei*) were the best since earlier days have smaller oocysts making identification and enumeration difficult, and on later days oocysts may already have ruptured. All survived mosquitoes were dissected per cup. To ensure viability of the results, the dissector was blinded.

Mosquitoes were first anesthetized by placing them in a -20°C freezer for 10 min. They were then transferred to a petri dish placed on ice to avoid mosquitoes to wake up and fly in laboratory room. An anesthetized mosquito was placed in a drop of PBS on a microscope slide under a stereomicroscope. Using an insulin needle and a pair of forceps, the midgut was removed by pulling the posterior tip of the abdomen from the remainder of the body. Care was taken to not perforate the midgut leading to oocysts release. The midgut was then completely separated by cutting the oesophagus with the insulin needle. Malpighian tubules and debris of the cuticulin were as well removed from the midgut.

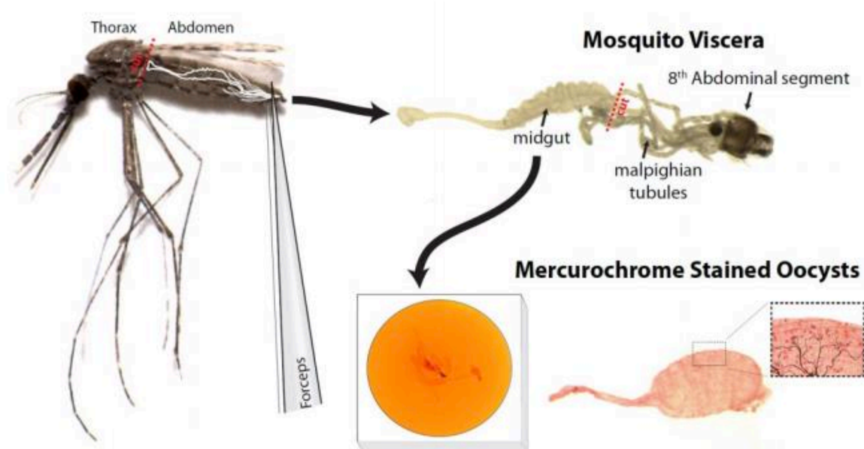

Fig. 7 : Outline of the procedure for detecting and counting *Plasmodium* oocysts in the mosquito midgut. Reproduced from Ouedraogo et al. [87]

2.2.2.2 Staining of *An. gambiae* midguts

Dissected midgut with then placed in a small drop of 1.2 % mercurochrome (Pied) on a new microscope slide. They were left to stain for 10 min and transferred in a drop of PBS on a new slide. Ten stained midguts were mounted on a glass slide covered with a cover slip.

2.2.2.3 Counting of oocysts of *An. gambiae* midguts and calculation of infection rate

Stained midguts were placed and visualized under a light microscope with a 10x objective (Leica DM750). Each midgut was examined, and the number of oocysts was recorded. Oocysts prevalence or infection rate was calculated by dividing the number of infected mosquitoes by the total number of dissected mosquitoes.

2.2.2.4 Dissection of *An. stephensi* infected midguts

An. stephensi midguts were dissected following the same procedure performed on *An. gambiae*. Care was taken to dissect one by one so as not to let the stomach dry out. The oocyst burden was analysed just afterwards.

2.2.2.5 Counting of oocysts of *An. stephensi* midguts and calculation of infection rate

Fluorescent *P. berghei* oocysts were visualized using a Leica DMI4000 inverted fluorescent microscope. Infected midguts were observed using a 40x magnitude objective and a GFP excitation filter BP470/40 (bandpass filter). Images were captured in both field and fluorescence modes using Leica microscope camera. Oocyst burden was determined by counting the number of oocysts using ImageJ. [88]

2.2.2.6 Dissection of *An. stephensi* salivary glands

18-21 days post-infection, salivary glands of infected mosquitoes were dissected. Mosquitoes were first aspirated into a small tube and euthanized by spraying ethanol inside the tube. Mosquitoes were then transferred in Leibovitz's L-15 Medium supplemented with 10 % human serum and were washed twice in another medium bad. 15 mosquitoes were then placed on a sterile microscopic slide. The head of a mosquito was first pulled away from the thorax by applying enough pressure with a curved needle on the thorax. Salivary glands which are attached to the base of the head came out and were separated by cutting the threadlike salivary ducts with the curved needle. They were then transferred into a prepared Eppendorf tube and left on ice until the dissection was completed. All dissected glands from the same treatment group were put in a same labelled Eppendorf tube.

2.2.2.7 Counting the sporozoites

Salivary glands were crushed with an Eppendorf micropestle and sporozoites were released and conserved in L15 medium. A drop of 15 µl of the medium containing the sporozoites was then transferred in one chamber of a quick read precision slide. The slide was then put in humid covered petri dish for 10 min to allow the sporozoites to settle down. By placing it under a light microscope, sporozoites were counted and examined with a 40x objective. The number of sporozoites was counted in 27 of the 96 quadrants.

2.2.3 *Ex vivo* transmission blocking assessment of MB

2.2.3.1 Study site

The first part of the study was conducted from September 2019 to March 2020 at the Centre de Recherches Médicales de Lambaréné (***Fig. 8***) in the Province Moyen-Ogooué in Gabon. Moyen-Ogooué is one of Gabon's nine provinces. Covering an area of 18,535 km^2, the total population approaches 70, 000 people. The provincial capital is Lambaréné which is a small city located 250 km southeast of Libreville (capital of the country). Lambaréné is characterized by a rural setting with a population of approximately 35,000. Malaria transmission in Lambaréné is perennial [89, 90].

13 villages around Lambaréné along the road to Fougamou and the road to Libreville were screened. Those villages often do not have names as they sometimes only have several houses next to the main road. Therefore, they are being referred to as kilometre points (in French point kilomètre = PK) starting from Lambaréné which is kilometre point zero. Screened were households in Moussamoukougou which is a suburb of Lambaréné, PK8, PK18, Massika (PK30), PK34, Tchad (PK48), Saint Martin, Siat and Mamiengue, Benguie, Mbolani and Bindo.

Fig. 8 : Centre de Recherche Médicale de Lambaréné. Left = CHU, Right = CERMEL Insectary.

2.2.3.2 COMAL Study

This short study was an ancillary study of the CoMal project: "*Plasmodium* species co-infections in *Anopheles* mosquitoes: A pilot study of parasite-vector interactions that define transmission in Africa" (CoMal, study ID number: DFG BO 2494/3-1). CoMal is an international study conducted in different part of Africa (Benin, Cameroon, Congo and Gabon), which aims to better characterize the biology of *P. malariae* transmission.

2.2.3.3 Study design

This study was conducted in an experimental evaluation of the *ex vivo* transmission blocking activity of MB. Following the same CoMal participants recruitment procedure, *P. falciparum* gametocytes positives blood samples were collected. The extended study protocol was submitted to and approved by the CERMEL institutional ethics committee.

2.2.3.4 Field screening

From September 2019 to March 2020, two field screening were carried out per week. 13 villages around Lambaréné along the road to Fougamou and the road to Libreville were screened.

In order to identify *P. falciparum* gametocytes presence, thick and thin smears were made for microscopic examination. About 10 µL of blood for the thick smear and 5 µL for the smear were distributed on a microscope slide with a micropipette. Each participant was identified with identification number ID (CML xxxx) related to the CoMal study. Weight and age of the participants were also registered. Slides were stained with Giemsa (20 % Giemsa, pH 7.2) 20 min and double read by professional microscopists the clinical laboratory of CERMEL.

2.2.3.5 Determination of parasite density in the laboratory

Microscopic examination of a thick and thin blood film was essential to quantify parasitaemia. Parasite density was expressed using Lambaréné method. The Lambaréné method of counting slides is a variant of WHO method for counting thick films and a rapid, simple and accurate method of quantifying parasitaemia.

The parasitaemia per microliter is calculated by an appropriate multiplication factor that depends on the magnification and the area of the microscopic field.

$$\textbf{\textit{Parasite density (per µl)}} = \frac{\textbf{\textit{Number of parasites (or gametocytes) counted}}}{\textbf{\textit{Number of HPFs read}}}\ \textbf{\textit{x microscopic factor}}$$

The microscopic factor for the Leica DM750 was 580.

2.2.3.6 Inclusion and exclusion criteria

To assess transmission blocking activity of MB through direct membrane feeding assay (DMFA), *P. falciparum* gametocytes from naturally infected humans were used. Asymptomatic and symptomatic participants with *P. falciparum* gametocytes and negative for other *Plasmodium* species were selected as blood donors for the direct membrane feeding assay (DMFA) scheduled for the following day.
Volunteers participating on the field screening of the COMAL project positive with *P. falciparum* gametocytes or CERMEL ongoing studies were included at the age of at least 3 year. The participants had to sign an informed consent sheet to be enrolled in the study. For children under the age of 11 a legal representative had to sign the consent sheet. Children aged from 11 to 17 had to give their assent in addition to the signature of their legal representative.

2.2.3.7 Treatment of the participants

All participants with confirmed malaria infection were treated with a combination of artemether (4 mg/kg body weight)/lumefantrine (10 mg/kg body weight), two time daily for 3 days according to the guidelines of the National Malaria Control Programme.

Participants selected as gametocyte donors for the DMFA were treated the following morning after venous blood collection at the laboratory, those not recruited for the study were given the treatment the same evening by the village health workers.

2.2.3.8 Transmission blocking design

The infective blood collected from participants was supplemented with MB to obtain two concentrations of 5 µM and 10 µM. Concomitantly without prior incubation time (< 15 min), mosquitoes were blood fed at day zero. For each feeding procedure, there was two MB treatment (5 and 10 µM) and control group. Mosquitoes from the single feed experiment were dissected 7 days post parasite ingestion to enumerate oocysts. Experimental infections were carried out using wild *An. gambiae s.l.* in direct membrane feeding assays following established protocols [87, 91], using venous blood samples obtained from the local gametocytaemic volunteers.

2.2.3.9 Preparation of methylene blue

The stock solution of MB was prepared by dissolving 2,5 mg MB (Sigma Aldrich) in 1 ml of sterile distilled water in an Eppendorf tube of 1,5 ml. Care was taken that MB powder was completely dissolved. The stock solution was stored at 4°C. Every month a new stock solution was prepared. Working solution was prepared by appropriate dilution of the stock solution.

2.2.3.10 Mosquito selection

2-7-days old female Anopheles gambiae mosquitoes were selected. Young mosquitoes were selected because of their naïve immune system and ability to survive after experimental infections to avoid large variations in mosquito survival rates between experiments.

Males differ from females by having feathery antennae and mouthparts not suitable for piercing skin. Males mosquitoes feed mainly on nectar or others glucose sources. Female mosquitoes have antennae with fewer, shorter hairs and their mouthparts (in female mosquitoes) form a long piercing sucking proboscis.

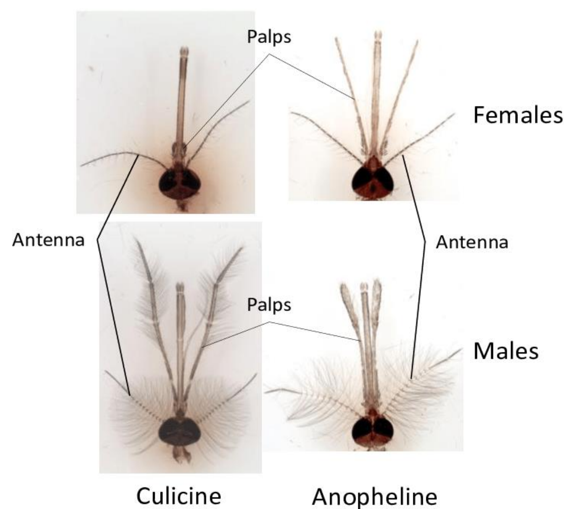

Fig. 9: Differences in the head of male and female anopheline mosquitoes. Reprinted from Training Manual on Malaria Entomology; Jacob et al. 2012 [92].

Before collecting female Anopheles mosquitoes, mosquitoes were starved by removing glucose sources. Usually, mosquitoes were starved from early morning (5-6 am) for experiments conducted after 10 am. One hand was applied to one side of the cage, preferably opposite the opening. Females that are seeking a blood meal were attracted to the heat and were trying to pick the glove. Female that were trying to pick to the glove, were gently aspirate with a mouth aspirator and transferred to a cup.

50 selected female *An. gambiae* mosquitoes were transferred to GreenBox© Premium-universal-cups (800ml, Ø 115mm). Into the cup, a small rectangular hole was carved out to transfer the mosquito through mouth aspiration. To prevent mosquitoes from getting out of the cup, a double layer of black tulle net was put over the cup and held with rubber bands.

2.2.3.11 Field isolate preparation and serum replacement

While the membrane feeding apparatus was running, 5 ml of venous blood was collected in a heparin tube. Care was taken that heparin tube was correctly labelled. Once the blood has been drawn, the blood was kept was at 37-38 °C in the incubator to avoid exflagellation of male gametocytes. The blood collected from participants was centrifuged for 5 min at 1500 g at 37° C. The plasma was removed and packed red

blood cells (RBCs) were washed with RPMI 1640 medium, repeated twice. RPMI 1640 Medium and non-immune human AB serum were kept at 37-38 °C to minimize drop of temperature. 600 µL of non-immune AB serum and 400 µL RBCs were transferred in 1.5 ml Eppendorf tubes preliminary labelled to reconstitute a 40 % haematocrit.

2.2.3.12 Mosquito membrane feeding

Membrane feeders were attached to a water pump related to a water bath to reach 37-38 °C in the feeders. Each Eppendorf tubes containing serum replaced samples supplemented with MB concentrations were given through a safety window to the BSL3 laboratory. They were put first into the water bath to keep them at 37°C and injected inside the pre-warmed glass membrane feeders using pre-warmed 20 ml syringes. The samples were released gently inside the tube of the glass membrane feeders. By tapping on the Parafilm® membrane with one finger the blood was spread out evenly and air bubbles were removed. Removing the air bubbles was crucial to avoid the rupture of the membrane. Mosquitoes were placed under the glass feeder, adjusted so that the membrane touched the net of the cup. This procedure had to be conducted quickly to avoid a temperature drop leading to a failing of the experimental infection. Mosquitoes were allowed to blood feed for about 30 – 60 min in the dark.

Fig. 10 : Direct membrane feeding assay using *P. falciparum* field isolate. The system is connected to a 37°C water circulation to avoid gametocytes ex-flagellation and allow the parasite transmission to the mosquitoes in a cup. The blood was gently put into the

glass tube and a parafilm membrane was placed on the bottom to mimic an animal skin.

2.2.3.13 Selection and follow-up of fully fed mosquitoes

At the end of membrane feeding, the cups were removed from under the glass feeders and mosquitoes were allowed to settle for 5 -10 min. Unfed mosquitoes were identified by visually checking their abdomen and removed with a mouth aspirator. Unfed mosquitoes were discarded by killing them by cold (-20°C overnight).

Each cups preliminary labelled with the ID of the participants, the MB Concentration (0, 5 and 10 µM) and the date of the infection were transferred into a heating cabinet inside the BSL3 at standard insectary conditions (26±0,5°C, 70-80% relative humidity). To provide sugar and distilled water, two cotton balls soaked in 10 % sugar solution and a gauze soaked with distilled water were placed on top of the cups and changed daily to avoid fungus infection. Number of dead mosquitoes was counted and reported daily for every cup.

2.2.4 Assessment of anti-mosquito stage activity of MB by secondary *in vivo* feeding

All the following experiments were carried out at Centre d’immunologie et des maladies infectieuses (CIMI) thanks to our partnership between ITM and Sorbonne Paris.

2.2.4.1 Mice infection with *P. berghei* parasites

P. berghei was the first rodent malaria parasite to be identified [93], cultivated [94] and has been used into multiple investigations preliminary to develop antimalarials and vaccine candidates. Genetic manipulation on *P. berghei* leading to expression of a fusion GFP has been generated to be detected by fluorescent microscopy, which considerably helped development of drug assay on *P. berghei* blood stage. *P. berghei* ANKA GFP line was maintained in BALB/c mice (18–22 g), obtained from Janvier (Le

Genest-Saint-Isle, France). Mice were infected by intra-peritoneal (i.p.) inoculation of 10^7 erythrocytes parasitized with *P. berghei* from cryopreserved parasite stocks.

2.2.4.2 Microgametes exflagellation

Before mosquitoes' blood meal on an infected mouse, a drop of blood was collected from the tail of an infected mouse between D3-D4 post ip inoculation of *PbGFP* and placed on a microscopic glass slide and covered with a small cover slip. After 10 min at room temperature, exflagellation of male gametocytes was examined by light microscopy. Mice with several spots with exflagellating male gametocytes were selected to perform the mosquito infection. Exflagellation of gametocytes is usually triggered by temperature decrease of 2-5 °C [95] .Other factors like change in pH value [96] or presence of xanthurenic acid [97] lead to exflagellation too.

2.2.4.3 *An. stephensi* infection

Mice were anesthetized and placed according to the treatment on the top of individual cages containing glucose-starved *An. stephensi* female mosquitoes, which were allowed to feed for 30 min. Afterwards, mice were returned to the vivarium. Unfed mosquitoes were removed from the cages. Engorged mosquitoes were provided with moistened cotton and a sucrose source (saccharose 10 %), and maintained at 26 °C.

2.2.4.4 Animal euthanasia and ethical considerations

After completion of all experiments, mice were turned over to the prone position and the neck was manually broken by applying pressure to the cervical vertebrae with a thumb and index finger and dislocating the spinal column from the skull or brain. The carcasses were discarded in a designated biohazard plastic bag. Surfaces were disinfected after completion of all animal procedures.
All animal procedures were conducted in accordance with the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes and were approved by the Charles Darwin Ethics Committee of the University Pierre et Marie Curie, Paris, France.

2.2.5 *In vitro* sporozoite and liver stage development assay

2.2.5.1 Parasites

Two species of *Plasmodium* were used in these *in vitro* experiments:

1. *Plasmodium berghei* ANKA GFP, a parasite line constitutively expressing GFP as previously described.

2. *Plasmodium falciparum* (NF54 strain) sporozoites were obtained from infected salivary glands of *A. stephensi* 14-21 days after an infective blood meal (Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, The Netherlands).

2.2.5.2 Hepatocytes culture

To assess sporozoites infectivity after pre-treatment with MB, three type of cellular models were used.

- Primary simian hepatocytes were isolated from liver of a 3 of 10 segments collected from healthy Macaca fascicularis (CEA, Fontenay aux Roses, France) using collagenase perfusion as previously described. Cells were immediately cryopreserved with a Nicool-Freezal (Air liquid Santé, Marne la Vallée, France) and then used generally 1 day before infection after fast thawing at 37°C. Simian hepatocytes were cultured in DMEM supplemented with 10% FCS (Biowest, Nuaillé, France), 2 mM glutamine, 50 lg/ml penicillin, 50 lg/ml streptomycin and 100 lg/ml neomycin (Invitrogen).

- Primary human hepatocytes, preliminary cryopreserved (Biopredic International and Lonza), were seeded generally 4 days before infection and maintained at 37 °C in 5% CO_2 in William's E medium (Gibco) supplemented with 10% fetal clone III serum (FCS, Hyclone); 1% penicillin-streptomycin (Gibco), 5 x 10^{-3} g/L human insulin (Sigma Aldrich, USA), 5 x 10^{-5} M hydrocortisone (Upjohn Laboratories SERB, France) and Matrigel (Corning, Ref. 354234)

- HepG2 cells expressing CD81 were also used and cultured in same medium supplemented as above. Hepatocytes were cultured in culture flasks coated with rat tail collagen I (Becton Dickinson, Le Pont de Claix, France). 24-48h prior *P. berghei* sporozoites inoculation, Cells were trypsinised, washed and incubated in fresh medium.

2.2.5.3 *In vitro* sporozoite invasion assay

Primary simian and human hepatocytes (90,000 cells per well) were seeded into collagen-coated black 96-wells plates and maintained at 37°C in 5% CO_2 in complete medium (William's E medium supplemented with 10% of fetal clone III serum, 1% penicillin-streptomycin, 5×10^{-3} g/l human insulin, 5×10^{-5} M hydrocortisone, 1/70 Matrigel). *P. falciparum* and *P. berghei* sporozoites were used for the invasion assays. Between 18 to 21 days post infection, salivary glands of infected mosquitoes were isolated by hand dissection, sporozoites were counted, resuspended and aliquoted into Eppendorf tube to a final concentration of 20.000 spz/50µL in phosphate buffered saline (Gibco, Life Technologies). MB, dissolved in distilled water, was added to the sporozoite suspensions and mixed gently. Final MB concentrations ranged between 1.25 to 50 µM. Aliquots were incubated at room temperature for 1 hour and then centrifuged for 5 min at 4000g to remove MB from the suspension. The sporozoites were resuspended in complete medium. This washing step was repeated to remove residual MB concentrations. Sporozoites were then added to the hepatocyte cultures (20,000 sporozoites/well). Each MB concentration was tested in quadruplicates. The infected hepatocyte culture plates were centrifuged for 10 min at 750g at room temperature allowing fast parasite sedimentation and then incubated for 3h at 37°C and 5% CO_2 to promote parasite invasion of hepatocytes. Extracellular sporozoites were then washed away and plates returned to the incubator with fresh media. Cells were fixed with cold methanol at 48h post-infection (pi) for *P. berghei* and at 6 days pi for *P. falciparum. P. falciparum* exo-erythrocytic forms (EEFs) were immune-stained using a mouse polyclonal serum raised against the PfHSP70, and an Alexa-Fluor 488 conjugate anti-mouse antibody. All nuclei were stained with DAPI. Anti-malarial activity was evaluated by counting and sizing EEFs (***Fig. 11***) using a Cell-Insight High Content Screening platform equipped with the Studio HCS software (ThermoFisher Scientific,

USA) at Paris Brain Institute. Cell cytotoxicity was evaluated by assessing cell confluence and by comparing the numbers of DAPI-positive hepatocytes before *vs.* after drug treatment[1].

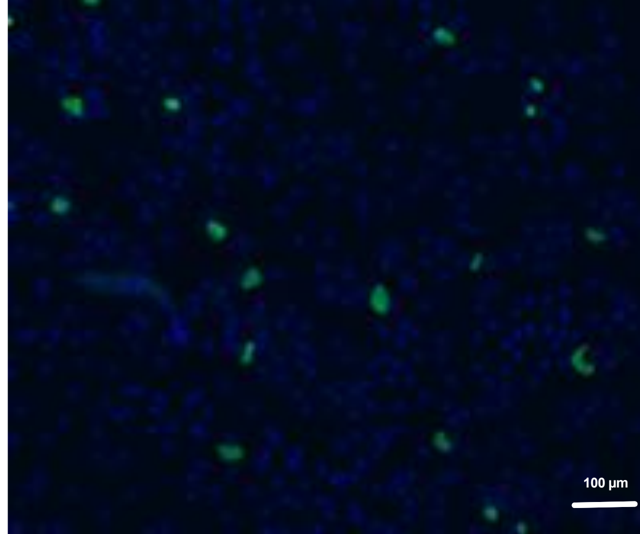

Fig. 11 : Representative image of *P. berghei* liver stage parasites in simian hepatocytes day 2 post sporozoite infection visualized with DAPI (blue), GFP expression and immunofluorescent detection (green). Scale bars indicate 100 µm[2].

2.2.5.4 *In vitro* liver stage development assay

Primary simian hepatocytes (90,000 cells per well) seeded into collagen-coated 96-wells plates were maintained at 37°C in 5% CO_2 in a complete medium. 3×10^4 freshly dissected sporozoites of *P. berghei* were resuspended in complete medium and added to each well. Infected hepatocytes were centrifuged for 10 min at 750g and further incubated for 2h as above. MB treatment of infected cultures was initiated at different time points, simultaneously, i.e., during the infection, 2h pi and 12h pi and renewed for all cultures at 24h pi. Final MB concentrations ranged between 1.25 to 100 µM. Parasite numbers and sizes were determined using the same procedure as describe above for the *in vitro* sporozoite invasion assay[2].

[1] Reproduced from Saison, N., et al., *Rapid and Specific Action of Methylene Blue against Plasmodium Transmission Stages.* Pharmaceutics, 2022. **14**(12).

2.2.5.5 Schizont counting using high-content imaging

Hepatocyte invasion rate and intensity were analysed by fluorescent microscopy. Anti-malarial activity was evaluated by counting EEFs using CellInsight High Content Screening platform equipped with the Studio HCS software (TermoFisher Scientifc, USA) at Paris Brain Institute. Cell cytotoxicity was evaluated by assessing cell confluence and number of DAPI-positive hepatocytes after drug treatment.

2.2.6 *In vivo* sporozoite invasion/development assay

Six weeks old female BALB/C mice were randomly allotted in four groups of 5 mice. Two groups were treated with MB at 50 mg/kg by intra-peritoneal (ip) injection. Untreated mice (2 groups of 5 mice) were used as negative control. MB was administrated 30-40 min before mosquito feeding or sporozoite inoculation in order to reach the plasma peak of MB during the short intra-vascular travel of sporozoites to the liver. One MB treated group and one control group were challenged by retro-orbital injection of 5,000 *P. yoelii* (GFP-luc strain) sporozoites (17XNL strain), a parasite line expressing both GFP and Luciferase reporters (GFP-luc) as previously described [99] while the last two groups were infected by the bite of 20 *P. berghei*-infected mosquitoes per mice. Liver and blood stage development was monitored 44h post-infection by bioluminescent imaging. Luciferase activity was monitored using an intensified charged-coupled device video camera of the In Vivo Imaging System (IVIS, Caliper Life Science, Hanover, MD, USA). 10 min before bioluminescence signal acquisition, 100 µl of luciferin sodium salt dissolved in phosphate-buffered saline (100 mg/kg) was ip administered and mice were anesthetized with isoflurane. They were then placed into the camera chamber and bioluminescence imaging was acquired. Images were acquired and analysed using the living Image 3.0 software (Caliper Life Science, Hanover, MD, USA). To assess blood stage development in *P. berghei* infected mice, presence of parasites and parasitaemia were determined by counting *P. berghei* on blood smears at days 6 and 7 pi[2].

[2] Reproduced from Saison, N., et al., *Rapid and Specific Action of Methylene Blue against Plasmodium Transmission Stages.* Pharmaceutics, 2022. **14**(12).

2.2.7 Microscopy

2.2.7.1 Slide preparation

2 microscope slides were prepared per participant, tagged with the study ID, the date and Slide A and B. Those slides were placed on a template with a 1x1,8cm square for the thick blood smear and point where the drop of blood for the thin blood smear was placed. 10µl of blood from the heparinized tube were taken for the thick smear and 5µl for the thin smear. With an Eppendorf pipette (2-20µl) firstly the 10µl were taken and placed on the slide where it was spread with the pipette tip to fill out the square evenly. For the thin blood smear, the 5µl of blood were placed on the tag point and spread out with another slide in one fast movement to the end of the slide. Those slides were put in the incubator until they were completely dry.

2.2.7.2 Slide staining

The dry slides were stained with 10% Giemsa stain, which was prepared as described above. Firstly, the thin blood smear was fixed in methanol by placing them in a slide stand filled with methanol for a few seconds. Care was taken to ensure that the methanol did not reach the thick blood smear. The slides were taken out and placed on paper towels to dry. After that the Giemsa stain solution was placed on the thin blood smear with a Pastette ®, again by taking care that the thick blood smear was not touched with it. Then a timer was set to 30 min. When those 30 min were up the thick blood smears were also covered with the Giemsa stain. Another 15 min were timed so that the thin blood smear stained for 45 min and the thick blood smear for 15. When the time was up the stain was dumped into the drain and the slides were gently washed in a plastic container filled with distilled water. Excess water was tapped of on paper towels and the slides were then left to dry.

2.2.7.3 Slide reading

The slides were read according to WHO Method with a 100X objective under a Leica DM750 microscope. With this method asexual and sexual parasite stages, and the number of WBCs, were counted. First, 200 WBCs were read. If by then 50 or more asexual parasites were counted the reading was done. If less than 50 were counted, 30 HPFs were read. To end the reading here, 5 or more asexual parasite stages had to be counted. If by then less than 5 have been found, 100 HPFs needed - 41 - to be counted. To declare that a slide was negative 100 HPFs needed to be read without finding a single parasite. The thin blood smear was used to identify the parasite species and to determine the gametocytaemia by counting gametocytes with the same method as described above.

2.2.7.4 Calculation of parasitaemia and gametocytaemia

The parasitaemia (P) and gametocytaemia (G) were calculated as follows and given in parasites/µl and gametocytes/µl:

$$Parasite\ density\ (per\ \mu l) = \frac{Number\ of\ gametocytes\ counted\ \times\ 8{,}000}{Number\ of\ leukocytes\ counted}$$

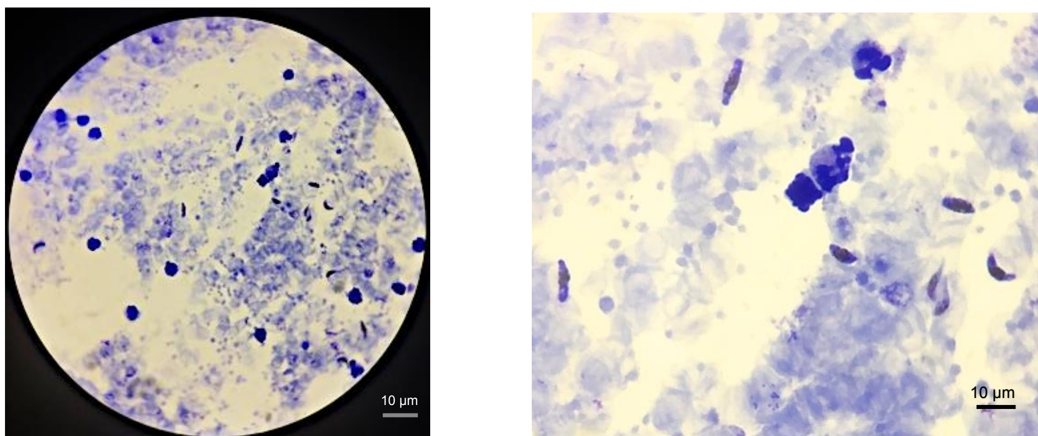

Fig. 12: *Plasmodium falciparum* gametocytes (CML 0897)

2.2.8 Statistical analysis

The transmission blocking activity of MB will be estimated by determining both oocyst prevalence (infection rate) and oocyst density. Data was entered into an Excel spreadsheet (Microsoft Office 2018) before being analysed with Prism 7.03. (GraphPad, USA). Differences in oocyst prevalence (number of oocyst positive mosquitoes per group) were examined using a binomial distribution for the number of positive mosquitoes. A zero-inflated negative binomial distribution was adjusted to compare the proportion of oocysts per mosquito (including negative insects) [100]. The bootstrapping process was used to get 95% confidence interval values. We set the maximal inhibition to 100% and the minimum inhibition (zero drug concentration) and the drug concentration producing 50% of maximum effect (IC_{50}) was estimated. Statistical significance was defined as a p-value of 0.05 or less.

3 Results

The following results have been published and reprinted from Saison et al. 2022 [98].

3.1 *Ex vivo P. falciparum* transmission blocking activity assessment of MB.

3.1.1 Field screening

The transmission blocking activity of MB was investigated in a series of 8 membrane feeding experiments using *P. falciparum* gametocyte positive blood from different donors. Out of 743 participants screened in 189 Households between December 2019 and March 2020 throughout the study area. Gametocyte densities varied among the donors from 80 to 716 sexual forms per microliter of blood to sexual forms per microliter of blood (***Table 1***). The average age of the participants was 9 years old. Infection rate and intensity of control mosquitoes varied considerably between the experiments: infection rate (oocyst prevalence) ranged between 40 and 80 % (mean, CI) and intensity (mean number of oocysts per midgut) between 0,88 and 10,8.

ID	Age	T (°C)	Parasitaemia	Gametocytaemia	Infection (%)	Mean Oocyst
CML0570	8	36,8	589	288	55	1,15
CML 0897	3	36,3	1072	716	60	10,8
CML 1036	10	36,5	0	212	67	3,14
CHU-16	20	36,6	0	637	80	5,55
CML 1099	5	37,1	0	208	40	4,15
CHU-55	30	36,7	0	474	57	0,88
CML1215	25	36,1	792	148	54	1,23
CML 1284	47	36,7	256	342	53	2,12

Table 1 : Characteristics of the participants who were successfully included in the *P. falciparum* transmission experiments.

3.1.2 Membrane feeding assay (*P. falciparum*)

3.1.3 Short-term exposure of fresh *P. falciparum* isolates to MB efficiently blocks mosquito infection

Using DMFAs with 5 fresh *P. falciparum* gametocyte-containing patient isolates, we 238 established a robust inhibition of *P. falciparum* infection after only short-term incubation 239 with MB (<5 min before feeding) in laboratory reared *An. gambiae* Kisumu strain colonies (***Fig. 13***). Gametocytes densities of *P. falciparum* 241 in the 5 isolates ranged from 212/µL to 716/µL (median 288) (***Table 1***). We evaluated the effect of 2 concentrations of MB (5 and 10 µM) on *P. falciparum* infection prevalence, oocyst density and *An. gambiae* mortality. There were significant differences among MB treatment groups for infection prevalence (F (2,351) = 98,966, p<0.0001) compared to the control group. Mean *P. falciparum* infection prevalence was significantly reduced in mosquitoes that ingested MB at 10 µM (90.3%; 95% CI 86.0-94.1; p<0.0001 compared to control), and at 5 µM (78.7%; 95% CI 74.1-82.0; p<0.0001 compared to control). Mean infection intensity (i.e., number of oocysts per mosquito) was reduced in the groups of mosquitoes that ingested MB-exposed infective blood meals by 98.8 (95% CI 96.2–99.9 250 p<0.001) for 10 µM vs control and by 96.0 (95% CI 85.4–100 p<0.0001) for 5 µM vs control (***Fig. 13***).

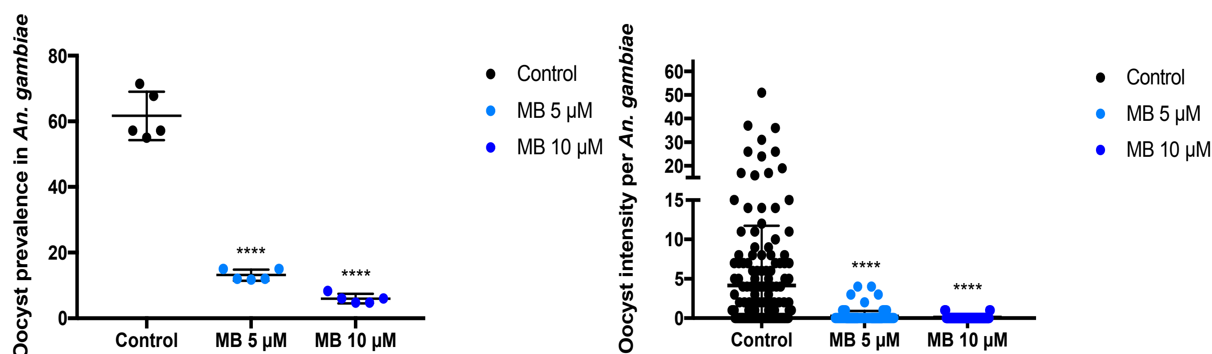

Fig. 13 : MB inhibits *P. falciparum* transmission in *An. gambiae*. (a) Prevalence of the infection is presented as the percentage of infected mosquitoes as measured by the presence of oocysts. (b) The infection intensity is presented as the number of oocysts per a single midgut (black dots). The black lines represent the mean and

standard deviation. Data from 5 independent experiments are presented in A-B corresponding to 5 different isolates. ****p =0.0001 when compared to control by t-test

3.1.4 MB does not affect the mortality of the *Anopheles* after being ingested during the membrane feeding.

Mosquito mortality was daily reported in order to detect any effect of MB on mosquito survivorship. There were no significant differences among MB treatment groups for mosquito mortality at 7-day [F (2.12) = 0.7838, p = 0.4787] compared to the control group. The mortality rates were similar in the 10 µM group [19.85% (SD = 2.917%), p = 0.3724], in the 5µM group [16.24 % (SD = 1.496%), p = 0.9835] compared to the control group [16.3% (SD = 2.371%)] (***Fig. 14***).

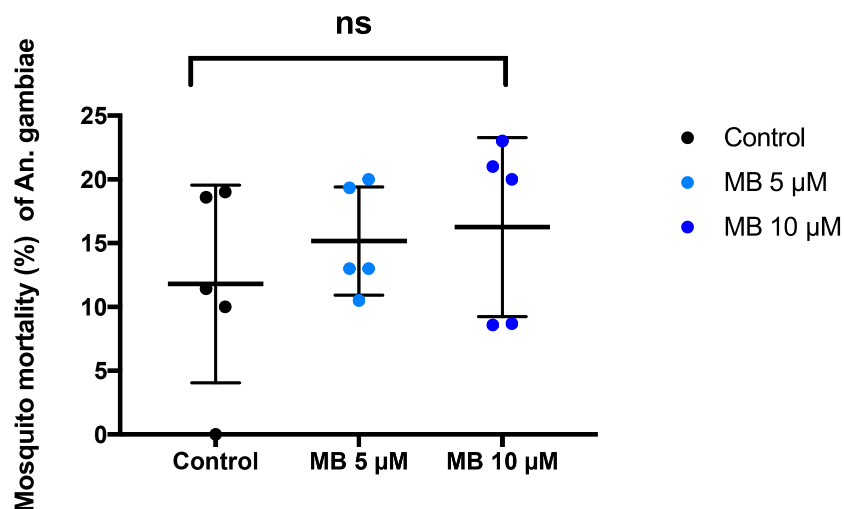

Fig. 14: Mosquito mortality rate post-feeding is presented as mean and standard deviation. Data from 5 independent experiments are presented. The black lines represent the mean and standard deviation. P=0.48 for comparisons of MB 5 µM vs control; P=0.23 for comparisons of MB 10 µM vs control.

3.2 *Ex vivo P. berghei* anti-mosquito stage assessment of MB.

3.2.1 Limited effect of MB on *P. berghei* mosquito stage development

In mosquitoes fed with an MB containing secondary blood meal on day 3 after being infected with *P. berghei*, mean prevalence of oocysts in midguts dropped from 84% in the control group (total 36 infected of 43 mosquitoes) to 54% (total 22 infected of 41 mosquitoes; $p<0.001$) and 76% (total 31 infected of 41 mosquitoes; $p=0.4$) in respectively the 100 and 50 mg/kg MB groups (***Table 2***). Likewise, infection intensity (***Fig. 15***) was reduced by the secondary blood meal containing MB (median 27 for 100 mg/ml and 74 oocysts per mosquito 50 mg/ml) compared to control (median 97 oocysts per mosquito; $p<0.001$ for comparisons of MB 100 mg/ml vs control). In terms of prevalence of infection and infection intensity in mosquitoes fed on day 6 post-infection, there was no significant difference between MB-treated groups vs control (***Table 2***). No significant difference was seen in the number of salivary gland sporozoites in MB-treated groups vs control (***Table 3***).In the mosquitoes fed with a secondary MB-containing blood meal on day 15 post 1st infection, no statistically significant effect on sporozoite numbers per mosquito versus control was observed (***Table 3***).

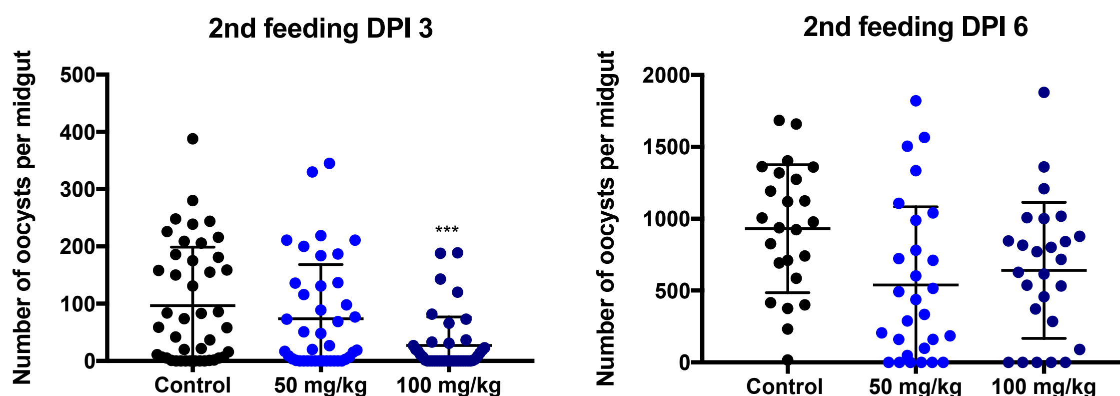

Fig. 15 *:* Exposure to MB during *Plasmodium* mosquito stage development shows moderate impact on oocyst numbers. Preliminary *P. berghei* infected

mosquitoes were divided into 3 groups which received secondary blood meals on anesthetized mice treated with 0 (control), 50 and 100 mg/kg MB 3 days (a) and 6 days (b) pi. The infection intensity is presented as the number of oocysts per midgut (black

Day post-infection	Methylene blue dose (mg/kg)	Ni (n)	Mean ± SD	Range	Prevalence %
DPI 3	0	43	97 ± 15.5	0 – 388	84
	50	41	74 ± 14.8	0 – 345	71
	100	41	27.17 ± 7.7	0 – 189	54
DPI 6	0	17	737.7 ± 90.9	17 – 1684	91
	50	21	476 ± 111.6	49 – 1821	75
	100	21	508.5 ± 89.1	1 – 1879	76

dots). The black lines represent the mean and standard deviation. Data are represented from 2 independents experiments. ***p=0.005 when compared to control by t-test.

Table 2 : The effect of MB administered in second blood meals to infected mosquitoes on oocyst development. Preliminary infected mosquitoes were divided into 3 groups, which received secondary blood meals dosed with 0 (control), 50 and 100 mg/kg MB 3- and 6-days pi. Infection intensity (Oocyst density) and infection rate (prevalence of oocyst) were estimated by counting the oocyst in the mosquito midguts. Ni Number of mosquitoes in each treatment group. Day 3; p=0.38 for mean oocysts comparisons of MB 50 mg/kg vs control; p=0.0005 for comparisons of MB 100 mg/kg vs control. Day 6; p=0.052 for comparisons of MB 50 mg/kg vs control; p=0.074 for comparisons of MB 100 mg/kg vs control.

Day post-infection	Methylene blue dose (mg/kg	Ni (n)	Arithmetic mean ± SE
DPI 6	0	29	63065± 23007
	50	30	48522 ± 4666
	100	14	35643 ± 10404
DP 15	0	30	37325 ± 3653
	50	31	35747 ± 25444

Table 3 : The effect of MB administered in second blood meals to infected mosquitoes on sporozoite development. Sporozoites intensity was estimated by counting the number of sporozoites per mosquito after salivary glands dissection D21 post-infection. Ni Number of mosquitoes in each treatment group. Day 6; p=0.24 for mean oocysts comparisons of MB 50 mg/kg vs control; p=0.18 for comparisons of MB 100 mg/kg vs control. Day 15; p=0.17 for comparisons of MB 50 mg/kg vs control.

3.3 *In vitro* and *in vivo* anti-sporozoite and anti-liver stage activity assessment of MB.

3.3.1 Pre-exposure of sporozoites with MB inhibits hepatocyte invasion

Freshly dissected sporozoites were incubated for 30 min with MB concentrations ranging from 1.25 to 50 µM. The effect of drug exposure on sporozoite invasion was evaluated by counting the number of EEFs 48h or 5 days after inoculation of the hepatocytes with the pre-incubated sporozoites of *P. berghei* and *P. falciparum,* respectively. Notably, MB exhibited significant hepatocyte invasion blocking activity (IC_{50} = 4.46 µM for *P. berghei*; IC_{50} = 4.7 µM for *P. falciparum*). No significant cytotoxicity towards hepatocytes was observed compared to the drug free control as measured by the number of hepatocytes nuclei (***Fig. 16***).

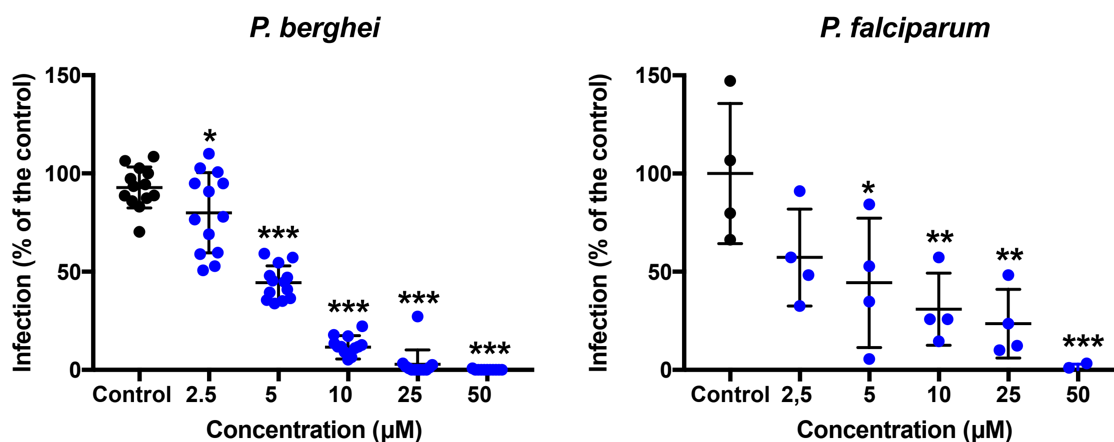

Fig. 16 : Exposure of freshly isolated sporozoites to MB inhibits hepatocyte invasion. (a) MB displayed an inhibition (IC_{50} = 4.46 µM) of *P. berghei* hepatocyte invasion after 1h pre-incubation. Data are presented from 4 independent experiments. (b) MB displayed a potent inhibition (IC_{50} = 4.7 µM) of *P. falciparum* hepatocyte invasion after 1h pre-incubation. *In vitro* sporozoite activity (infection scale, blue bars = EEFs). Data are presented from one representative experiment. The black lines represent the mean and standard deviation. *p < 0.05; **p < 0.01; ***p<0.001 when compared to control by t-test.

3.3.2 MB inhibits early *in vitro* liver stage development

MB activity against *P. berghei* liver stages was investigated at concentrations ranging from 1.25 to 100 µM. MB was either added simultaneously to the hepatocyte invasion or at 2h and 12h pi until parasite maturation 48 hours pi. Interestingly, we found that MB had a moderate activity on *P. berghei* liver stage development, with a more pronounced effect when MB was added at the same time as the parasites invasion (IC_{50} = 13.9 µM, 33.6 µM and 34.7 µM at 0h, 2h or 12h pi, respectively (***Fig. 17***). Moreover, MB showed also an effect on the parasite development as evidenced by the reduction of *P. berghei* EEFs size (***Fig. 18***).

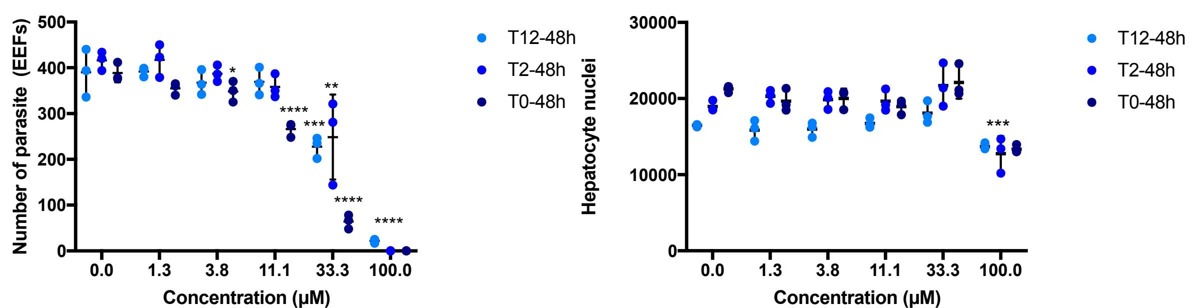

Fig. 17 : MB shows weak inhibition of liver stage infection *in vitro*. (a) MB activity against *P. berghei* liver stages was investigated at concentrations ranging from 1.25 to 100 µM. MB was added simultaneously to the hepatocyte invasion (0h), 2h or 12h pi. Activity is presented as the number of schizonts counted (blue bars = EEFs). (b) Toxicity to host cells is presented as the number of hepatocyte nuclei. Data are presented from one representative experiment. The black lines represent the mean and standard deviation. *p < 0.05; **p < 0.01; ***p < 0.001; ****p = 0.0001 when compared to control by t-test.

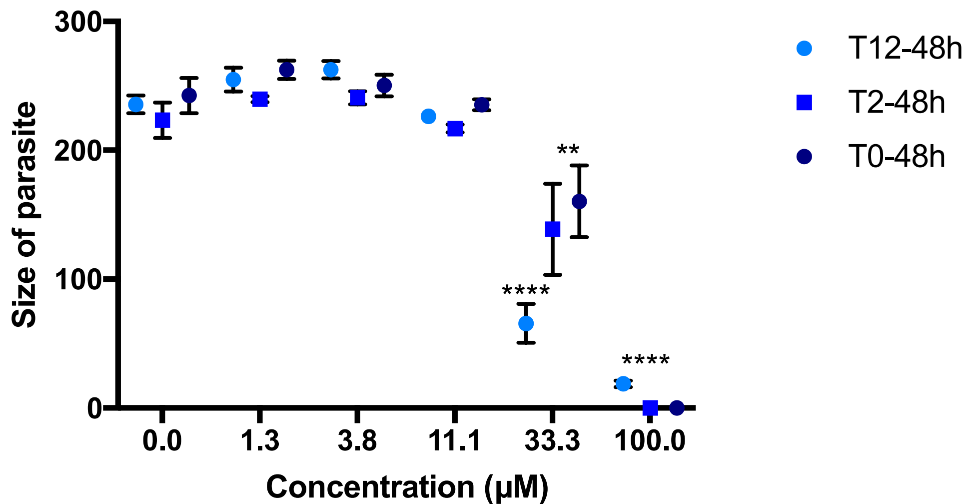

Fig. 18 : MB shows weak reduction on size of liver stage *in vitro*. MB activity against *P. berghei* liver stages was investigated at concentrations ranging from 1.25 to 100 µM. MB was added simultaneously to the hepatocyte invasion (0h) or 2h and 12h pi. Activity is presented as the size of schizonts counted (blue bars = EEF size). Data are mean, SD of triplicate measurements from one representative experiment. The black lines represent the mean and standard deviation. **p < 0.01; ****p = 0.0001 when compared to control by t-test.

3.3.3 Reduction of *in vivo* liver stage development by MB

Liver parasite load was significantly reduced in mice inoculated intravenously with *P. yoelii* sporozoites 30 min after 50 mg/kg MB treatment. However, complete blockade of parasite development was not achieved (***Fig. 19***).The mean luminescence (photons/sec) values measured at liver positions of mice treated with MB at 44h pi was 4.7×10^5 (SD, 4.6×10^5) vs 1.9×10^6 (SD, 3.5×10^6) for the untreated control group. In the *P. berghei* groups (***Fig. 20***) challenged by mosquito bite, blood stage parasitaemia was significantly reduced in the MB group [mean parasitaemia 0.112 ± 0.105, p = 0.0159 by comparison vs control] at day 6 compared to the control [mean parasitaemia 0.564 ± 0.242]. These results indicate that MB is moderately effective in preventing rodent malaria parasite liver and subsequent blood stage infections.

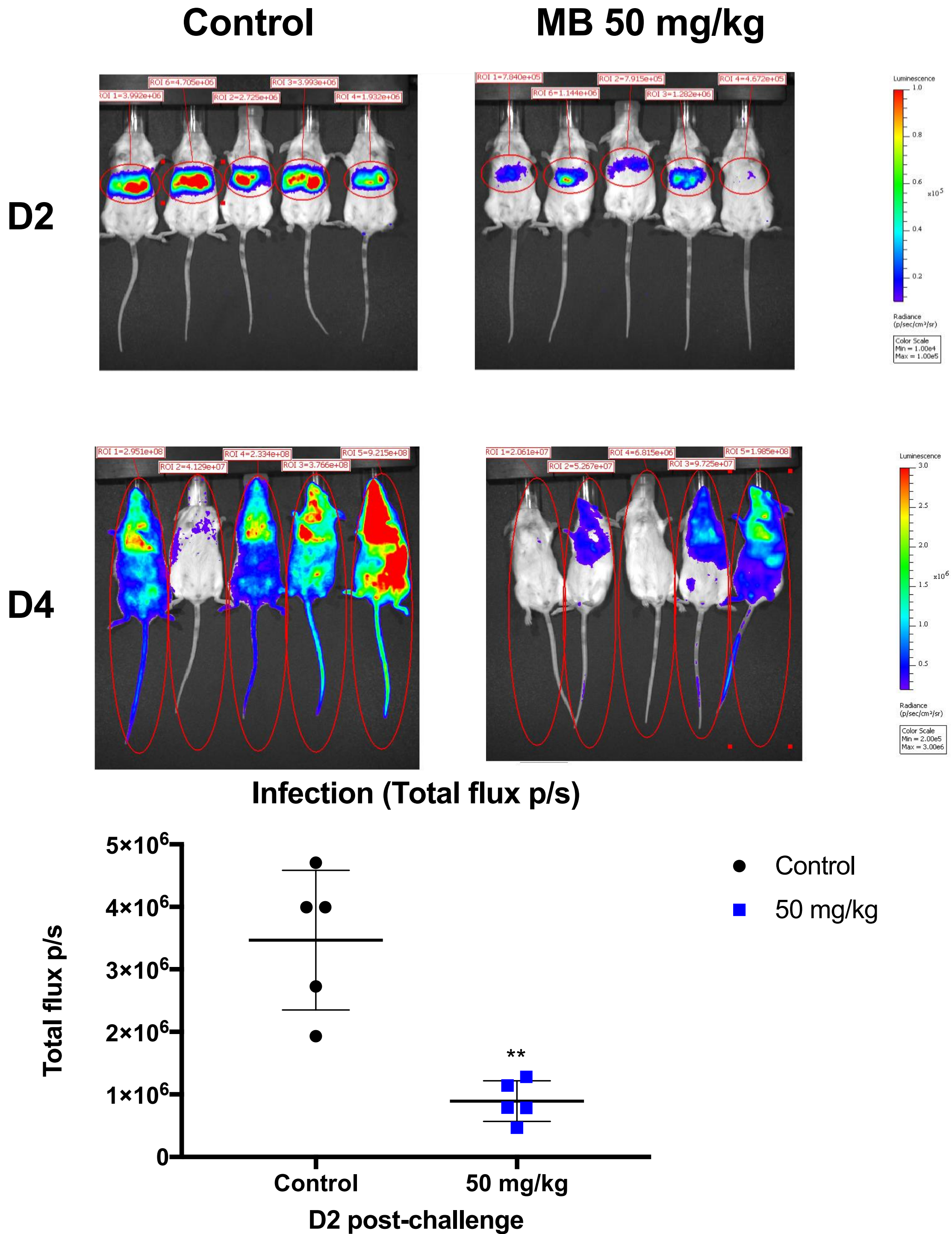

Fig. 19 : MB delayed and reduced the *P. yoelii* sporozoite infection in mice. Mice were first treated 30 min with MB (50 mg/kg) and were then challenged by retro-orbital injection of 5.000 sporozoites of *P. yoelii*. (a) In vivo images (IVIS) of luminescence reported at D2 and D4 post *P. yoelii*-infection. Rainbow images show the relative levels of luminescence ranging from low (blue), to medium (green), to high (yellow/red). (b) Mean luminescence levels (photons/s) for each group at 42h post-challenge. The black lines represent the mean and standard deviation. **p=001 when com-pared to control by t-test.

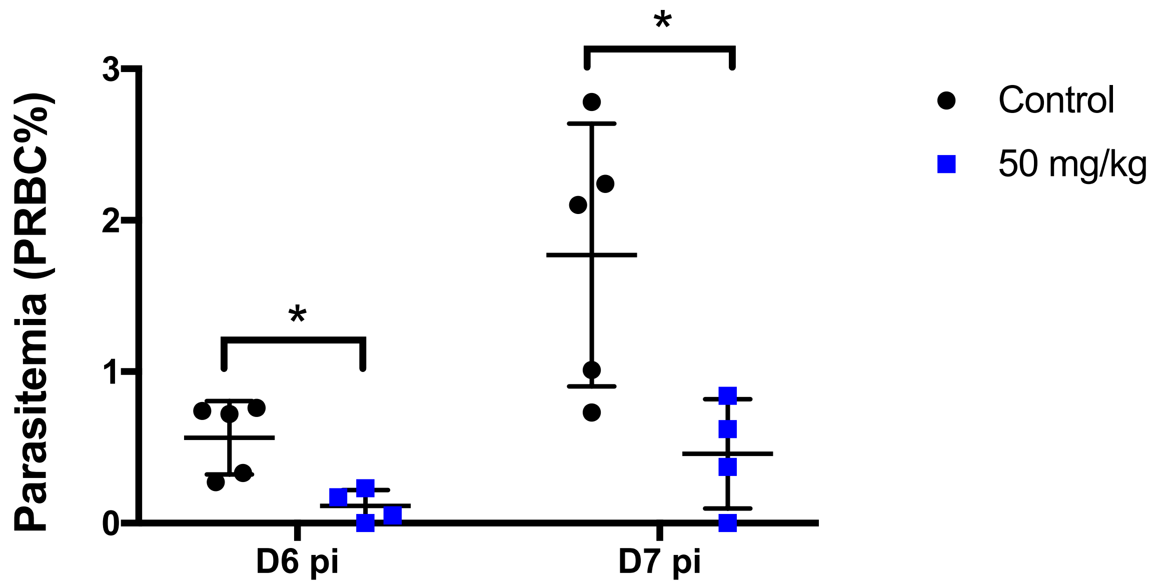

Fig. 20 : Single treatment of MB before challenge by mosquitoes bite reduces subsequent blood stage parasitemia. Mice were first treated with MB (50 mg/kg) 30 min before mosquito challenge and were then challenged by bite of *P. berghei*-infected mosquitoes. Blood stage parasitemia of *P. berghei* was monitored in mice by studying Giemsa-stained blood smears at day 6 and day 7 after the infection by mosquito bite. Data are presented from one representative experiment. The black lines represent the mean and standard deviation. Asterisks (*) indicate significant ($p < 0.05$) differences between each treatment group and control.

4 Discussion

Recent investigations on antimalarial activity of MB are rekindling the debate on its role for improved malaria control and eventually, eradication [68]. The WHO report even called for more research on the effects of MB on the transmission of the parasite in order to find among others a candidate to replace primaquine which poses many safety issues [54]. In this study, I provide new evidence of the effects of MB on these stages of the *Plasmodium* lifecycle.

4.1 *Ex vivo* membrane feeding assay

Gametocidal and transmission blocking property of MB have been freshly evaluated in the last decade and demonstrate a high inhibitory potency of *Plasmodium spp.* transmission. Gametocytes development is characterized by 5 morphologically distinct stages. In an *in vitro* drug exposure assay, the earliest developmental stages of gametocyte (stage I and II) were found high susceptible to MB. However, MB was less effective against stage IV and V gametocytes [76].

Potent inhibitory activity of *Plasmodium* transmission has also been reported *in vivo* in rodent model using *P. yoelii* after oral MB treatment (50 mg/kg 2 hrs before mosquito feeding) in mice. Reduction of infection intensity due to addition of MB was observed with *P. berghei* and *An. stephensi* mosquitoes but did not lead to a drastically reduction of the infection rate [77]. In addition, a recent large clinical trial in Mali [80] demonstrated its transmission blocking efficacy when used as additional dose (15 mg/kg per day MB for 3 days) in combination with artesunate-amodiaquine for the treatment of uncomplicated *P. falciparum* malaria. It has been shown that MB was able to block the transmission within two days after receiving the treatment. In Contrast, parasites were able to pass to the mosquitoes in participants who received ACT treatment without MB [80]. MB powerfully reduces gametocyte carriage rate in treated patients, blocking the transmission of the parasite to the mosquito. However, little information is available on the activity of MB against the subsequent development of *Plasmodium* parasites in the mosquito host. MB has been identified as a non-competitive inhibitor of *P. falciparum* glutathione reductase, which catalyses the reduction of glutathione disulphide using NADPH as a source of reducing equivalents

[65]. Experiments with *P. berghei* indicated that glutathione metabolism is essential for oocyst development in mosquitoes [101]. In terms of its clinical use, MB has been shown to exert highly potent action against asexual *Plasmodium* stages (IC_{50} = 4 nM) [69]. MB also kills gametocytes all stages, albeit at a somewhat higher rate with stage I-II gametocytes compared to more mature stages [76]. MB has not been shown to possess a significant activity against sporozoite motility [102] nor against hepatic stages of *P. falciparum*, *P. yoelii* and *P. cynomolgi* [77].

In my study, the transmission of fresh *P. falciparum* isolates obtained from donors could be disrupted by MB even when added as briefly as only <5 min before membrane feeding. Although MB shows *in vitro* a modest activity against mature stage gametocytes [76], I demonstrated that MB reduces the oocyst infection and intensity of *P. falciparum*, when *An. gambiae* were fed with different concentrations of MB short added to the serum-replaced infected isolates. Oocyst development was completely inhibited in all five independent replicates. These results demonstrated a robust transmission blocking activity of MB and are very consistent with previously reported transmission blocking activity in the murine parasite model *P. yoelii* and *An. stephensi* mosquitoes [77]. Our study may be one of the first showing a robust inhibition of the *ex vivo* transmission of *P. falciparum* under field conditions and reinforce evidence of using MB as additional tool to reduce the parasite transmission. Interestingly, I observed this transmission suppression without prior incubation time in our case, which differs to prior results with *P. berghei* reported after a minimum of 2 hours incubation prior membrane feeding [76, 77]. Following oral or i.v. administration plasma levels could, however, well achieve the therapeutic doses in human [103]. These results suggest that either MB has a super-rapid action against gametocytes or also acts against the mosquito stages. To expend our prior results, I further investigate the role of MB in plasmodial sporogonic stages development. Unlike ivermectin [104], MB did not affect mosquito survival.

4.2 Anti-sporogony development activity

Very few molecules have been identified able to interfere the development of the parasite in the mosquito. Among others candidates such as Atovaquone [85] or ivermectin ([84],[105]) have already shown an little or moderate activity against plasmodial mosquito stage and interfere the mosquito stage development. However, MB activity against mosquito stages has not been until now evaluated.

A previous study reported the key role of glutathione (GSH) redox system for the mosquito oocyst stage development [101]. MB has been early identified as potential inhibitor of Glutathione reductase (GR), which lead to death of the parasite [65] . The essential role of GR in the development of plasmodial stages in the mosquito and the enzyme inhibitory potency of MB prompted us to investigation the role of MB in the development of the parasite in the mosquito. In our study, MB showed a moderate reduction in oocyst numbers and infection prevalence when ingested via a second blood meal to the already *Plasmodium* infected *Anopheles* mosquitoes 3 days pi. This observation could be explained with the ability of MB to interfere with the glutathione redox system, which is essential for the initiation of oocyst development [101]. Consistent with this model, MB did not significantly reduce the oocyst burden in *An. stephensi* when ingested 6 days after the infectious blood meal. Even though I did not compare MB head-to-head to other drug candidates like ivermectin or atovaquone, the potency of MB appears to be similar or weaker [85, 105, 106]. Regarding the development of combination therapy, an additional study under similar condition comparing each candidate could of course bring more comparative data. In addition, no effect on the sporozoites numbers and development has been detected. Sporozoites from second fed mosquitoes with MB have shown the same ability to infect the hepatocytes post-dissection.

These results suggest that MB is not able to interfere the development of the parasite in the mosquito and do not alter the formation of the peritrophic and the midgut microbiota. One explanation could be that the oocysts are no longer be reachable by antimalarials after a minimum of 6 days post-infection. Indeed, after being generated zygotes invade the midgut epithelium of the mosquito and develop into oocysts. The

mosquito midgut represents an important nest for the mosquito lifecycle of the parasite and could protect the oocysts from antimalarials or others anti-plasmodial agents.

4.3 Anti-sporozoite and anti-liver stage activity

MB has been investigated on the hepatic stage of *P. falciparum*, *P. yoelii* and *P. cynomolgi* and was little effective at the liver stage level *in vitro*. MB was not able to prevent the development and the spreading of the parasites into the entire blood circulation. In contrast, MB is highly effective against the blood stage and the gametocytes and has shown a complete clearance of asexual stages in the blood stream of MB-treated malaria patients.

Very few molecules have been tested against plasmodial sporozoites due to difficulty of obtaining sporozoites from mosquito infected salivary glands and subsequent infections of mosquitoes. Monensin, isolated from *Spretomyces cinnamonensis* has been identified as a potential candidate targeting *Plasmodium spp*. sporozoites development and inhibits the hepatocyte invasions *in vitro*, as well as murine hepatic model *in vivo* [107]. However, monensin and monovalent ionophores has shown neurotoxicity and cardiotoxicity in animals and could induce several fatal damages in high-dose ingestion in humans [108, 109]. Pharmacological and safety data in humans remain poor and slow down investigations on antimalarial potency of monovalent ionophores. In a previous study, MB has also been screened to test for *in vitro* sporozoite motility inhibition and did not affect the sporozoite movement [102].

In my study, I pre-incubated fresh *P. berghei* and *P. falciparum* sporozoites with different concentrations of MB for 30 min. Exposure of *P. berghei* and *P. falciparum* sporozoites to MB also inhibited the capacity of sporozoites to establish hepatocyte infections. No detectable toxic effects of the residual MB were recorded after sporozoite treatment. Although MB has been shown not interfering the sporozoite motility [102] and failed to inhibit the liver stage development [77], MB seems to affect the capacity of sporozoites for productive hepatocyte invasion. I could hypothesize that MB may induce morphological deformation such as described on gametocyte [78] leading to the incapacity of the sporozoite to invade the hepatocyte.

In order to expand on these results, I re- assayed the MB activity against *P. berghei liver* stage development at different time point post hepatocyte invasion. Various of methylene concentration was then added to hepatocytes cultures concomitantly, 2h and 12h post infection. Interestingly, I found that MB had the most impressive activity when it was added concomitantly with *P. berghei* sporozoites to hepatocyte cells suggesting that MB has rather an activity against sporozoite and early liver stages, which concord to our previous results with the pre-incubation of the sporozoites. In contrast to previous observed results from Bosson-Vanga et al. [77], I observed a moderate activity of MB against *P. berghei* liver stages. At 33 µM MB was already effective against liver stages even if MB was added 12 hours post hepatocyte infection and MB reduced a reduction of parasite size. However, at a dose of 100 µM, MB was toxic for the hepatocytes. However, not only the murine parasite species used, as well as the MB formulation provided by our supplier, were different in our study, but also the timeline of the treatment which was delivered at the same time of the sporozoite inoculation in our study whereas the treatment was initiated three hours post sporozoite inoculation in the Bosson-Vanga study [77].

4.4 Safety and tolerability of methylene blue

MB is considered safe for the treatment of malaria. The most important side effects observed during the treatment was the discoloration of urine. Other AEs like urethritis, vomiting or gastrointestinal disorders were reported with higher doses of MB. Depending of the formulation, vomiting was especially often observed in children.
However, severe adverse events (SAEs) associated with MB treatment were rarely observed in RCTs. The most feared severe adverse event in case of using the 8-aminoquinolines and MB to reduce the parasite transmission is the risk of developing haemolytic anaemia (AHA) in individuals who have phosphate dehydrogenase (G6PD) deficiency.

Safety issue as Haemolysis associated with G6PD deficiency particularly for 8-aminoquinolines such as primaquine (PQ) has been reported and remains a brake to expansion in development of antimalarials [81]. This adverse event plays a key role in

the development of new antimalarial treatment regimens and has been investigated in several studies conducted on sub-Saharan African populations, in the case of Lapdap development for instance. Haemolysis conducted to failure of a large drug development project (Lapdap) because of toxicity of one of its component (dapsone), which cause clinically significant haemolysis in G6PD – deficient African patients [110].

MB treatment of malaria in Africa is associated with slightly reduced haemoglobin values in children with a full G6PD defect compared to non-G6PD deficient children. However, haemoglobin reduction appears often after antimalarial treatment [111]. Although observed reduction in HB values among children in Burkina Faso, there was no evidence for an excess of severe anaemia due to MB treatment. MB treatment demonstrated a much lower haemolysis risk associated with MB treatment [111].

4.5 Clinical perspectives and further investigations

Studies clearly demonstrate the efficacity and safety of MB in the treatment of malaria. Strong antimalarial activity has already been reported against *P. falciparum*, in particular in impairment gametocytes development. Adding a single dose of MB to existing ACT regimens is potentially useful to reduce *P. falciparum* transmission intensity and reduce the risk for development and spread of malaria parasites resistant against ACT. MB appears to be a potential alternative for reducing post-treatment infectivity in *P. falciparum* infections and could become a valuable component in using mass drug administration in areas of low endemicity in order to eliminate malaria.

Appearance and development of resistant strains is a major public health threat. Resistant parasites have been identified against chloroquine, sulfadoxine, pyrimethamine, quinine and mefloquine and now against artemisinin [112]. Most of artemisinin resistant parasites have particularly developed in Southeast Asia and compromise the clinical efficacy of artemisinin-derivatives treatment. Resistance mechanism of *P. falciparum* against artemisinin has been identified and caused by a polymorphism of the K13 gene of the parasite, which lead to an upregulated protein production reducing the pro-oxidant activity of artemisinin. Parasites have been identified resistant if their clearance half-life exceeds 5 hours following a treatment of

ACT or Artesunate. Artemisinin resistance is widespread across the Greater Mekong Subregion but has now been reported in Africa. In Uwimana et al. [113], researchers found evidence of slowly parasite clearance following ACT treatment and identified K13 gene in children. Emergence of artemisinin resistance in Rwanda is alarming. Indeed, ACTs remain the most efficient and safe treatment of malaria in Africa. Artemisinin resistance could lead to loss of clinical efficacy of artemisinin-based therapy.

There is an urgent need to reduce the malaria transmission, especially in regions where evidences of artemisinin resistant parasites were found. Despite variation of gametocyte density between replicates, solid dose dependent results were obtained, establishing the transmission blocking activity of MB against the human parasite *P. falciparum* and are in excellent agreement with the previously reported activity in the murine parasite model *P. berghei* and *An. stephensi* mosquitoes. Adding a single dose of MB could kill mature gametocytes and block their transmission.

4.6 Conclusion

My study expands our knowledge of the activity of MB as the oldest synthetic anti-infective against the different stages of the complex life cycle of malaria parasites. I demonstrated an extremely rapid activity of MB on *P. falciparum* transmission to *An. gambiae* mosquitoes. Moreover, I found that MB has a moderate activity on oocyst development, reducing the oocyst burden and mosquito infectivity. Importantly, exposure of sporozoites to MB revealed a prominent *in vitro* inhibitory activity of hepatocyte invasion and I also observed a reduced parasite liver stage load upon exposure of early intra-hepatocyte parasite development to MB *in vivo*. Facing emergence and development of malaria resistance, my study thus provides further impetus for the clinical use of MB for instance in antimalarial combination therapy[3].

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[3] Reproduced from Saison, N., et al., *Rapid and Specific Action of Methylene Blue against Plasmodium Transmission Stages.* Pharmaceutics, 2022. **14**(12).

5 Summary

Background: Preventing the transmission of plasmodium parasite to the mosquito plays an essential role in the process of malaria eradication. MB, the oldest synthetic antimalarial, has been reinvestigated through the last decades because of its high potency, in particularly against the sexual stage of *Plasmodium*. This study aimed to investigate possible additional targets and activities of MB against the life cycle stages responsible for parasite transmission in humans and mosquitoes.

Methods: A field study using *P. falciparum* field isolates was carried out to assess transmission blocking potency using an *ex vivo* direct membrane feeding assay (DMFA) in Lambaréné (Gabon). Further investigations exploring anti-mosquito stage activity were carried out at CIMI (Paris). Three, 6 or 15 days of after infection with *P. berghei An. stephensi* mosquitoes were provided a second blood meal from mice treated with MB. Oocyst and sporozoite prevalence and intensity were assessed by microscopic examination of dissected midguts and salivary glands, respectively. To explore and characterise the effect of exposure of sporozoites and liver stages to MB the number and size of parasite schizonts in the hepatocytes was determined. Lastly, we evaluated the relevance of our findings *in vivo* by monitoring the development of liver stages after a single dose of MB in mice using both *P. yoelii* GFP-luciferase using *in vivo* imaging and wild-type *P. berghei* using microscopic assessment of asexual parasite appearance in the blood circulation.

Results: A robust inhibition of *P. falciparum* transmission was seen in *An. gambiae* in 5 independents DMFA experiments using *P. falciparum* field isolates, even when MB was added less than 5 minutes prior to membrane feeding. However, MB showed only a moderate effect on *P. berghei* oocyst development when MB was ingested 3 and 6 days after infection. MB did also not appear to have any effect on the formation of *P. berghei* sporozoites in *An. stephensi*. The exposure of *P. berghei* and *P. falciparum* sporozoites to MB inhibited hepatocyte invasion but MB exposure of liver stages led only to partial inhibition. Lastly, a reduction of the liver stage load was also observed in mice pre-treated with MB prior to inoculation with *P. yoelii* and *P. berghei* sporozoites.

Conclusion: The results confirm and provide new evidence of the capacity of MB to block the transmission of *P. falciparum* in *An. gambiae*. Most notably, transmission was blocked after only a brief incubation of field isolates prior to membrane feeding. These results should provide new impulses for the evaluation of MB in clinical studies designed to reduce and prevent malaria transmission.

6 Zusammenfassung

Hintergrund: Die Verhinderung der Übertragung des Plasmodium-Parasiten auf die Stechmücke spielt eine wesentliche Rolle bei der Ausrottung der Malaria. MB, eines der ältesten Malariamittel, wurde in den letzten Jahrzehnten aufgrund seiner hohen Wirksamkeit, insbesondere gegen das Sexualstadium von Plasmodium, erneut untersucht. Ziel dieser Studie war es, mögliche zusätzliche Angriffspunkte und Aktivitäten von MB gegen die für die Parasitenübertragung von Menschen zu den Mücken verantwortlichen Stadien zu untersuchen.

Methoden: In Lambaréné (Gabun) wurde eine Feldstudie mit *P. falciparum*-Feldisolaten durchgeführt, um die übertragungshemmende Wirkung mithilfe eines *Ex-vivo*-Direktmembranfütterungstests (DMFA) zu bewerten. Weitere Untersuchungen zur Erforschung der Aktivität gegen das Mückenstadium des Parasiten wurden am CIMI (Paris) durchgeführt. Eine zweite Blutmahlzeit von MB-behandelten Mäusen wurde 3, 6 und 15 Tage nach der Infektion an die zuvor mit *P. berghei* infizierten *An. stephensi*-Mücken verabreicht. Die Prävalenz und Intensität von Oozysten und Sporozoiten wurde durch mikroskopische Untersuchung der Magen und Speicheldrüsen ermittelt. Um die Wirkung von MB auf die Entwicklung von Sporozoiten und Leberstadien zu untersuchen und zu charakterisieren, wurde die Anzahl und Größe der Parasitenschizonten in den Hepatozyten nach der Behandlung mit Sporozoiten und Hepatozyten bestimmt. Schließlich wurde die Relevanz unserer Ergebnisse *in vivo* bewertet, indem wir die Entwicklung von Leberstadien nach einer einmaligen MB-Behandlung mit Mäusen sowohl mit *P. yoelii* GFP luc durch *In-vivo*-Bildgebung als auch durch mikroskopische Beurteilung des Auftretens von Parasiten mit *P. berghei* überwachten.

Ergebnisse: Eine robuste Hemmung der *P. falciparum*-Übertragung wurde bei *An. gambiae in* 5 unabhängigen DMFA-Experimenten mit *P. falciparum*-Gametozyten-Feldisolaten beobachtet, selbst wenn MB weniger als 5 Minuten vor der Fütterung der Mückenmembran zugesetzt wurde. MB zeigte jedoch nur eine mäßige Wirkung auf die Entwicklung von *P. berghei*-Oozysten, wenn MB 3 und 6 Tage nach der Infektion eingenommen wurde. MB scheint auch keine Auswirkungen auf die Bildung von *P. berghei* Sporozoiten in *An. stephensi* zu haben. Was die Aktivität von MB gegen

berghei Sporozoiten in *An. stephensi* zu haben. Was die Aktivität von MB gegen Sporozoiten und Leberstadien anbelangt, so hemmte die Exposition von *P. berghei* und *P. falciparum* Sporozoiten die Hepatozyteninvasion, aber MB zeigte eine mäßige Aktivität gegen Leberstadien nach der Hepatozyteninvasion. Schließlich wurde auch eine Verringerung der Anzahlen der Leberstadien von Parasiten beobachtet, wenn *P. yoelii* und *P. berghei* Sporozoiten und frühe Leberstadien nach einer einmaligen MB-Behandlung vor der Infektion von Mäusen *in vivo* mit MB behandelt wurden.

Schlussfolgerung: Die Ergebnisse bestätigen und liefern neue Beweise für die Wirksamkeit von MB bei der Blockierung der Übertragung von *P. falciparum* in *An. Gambiae* und zeigten eine beeindruckend schnelle Wirkung, nachdem es Feldisolaten vor der Membranfütterung zugesetzt wurde. Allerdings zeigte MB eine geringe Wirkung auf die Entwicklung von Oozysten und Sporozoiten, wenn es bereits infizierten Mücken verabreicht wurde. Darüber hinaus zeigte die Exposition von Sporozoiten mit MB eine deutliche Hemmung der Hepatozyteninvasion *in vitro* und eine signifikante Verringerung der Leberstadienanzahl *in vivo*, wenn Sporozoiten und Leberstadien durch eine einzige MB-Behandlung vor der Infektion von Mäusen exponiert wurden. Zusammenfassend kann gesagt werden, dass verschiedene mögliche therapeutische Zielstrukturen von Plasmodium untersucht und spezifische Aktivitäten von MB identifiziert wurden, die somit weitere Impulse für den Einsatz von MB in klinischen Studien zur Reduzierung und Verhinderung der Malariaübertragung geben.

7 References

1. *World malaria report 2020*, in *20 years of global progress & challenges*. 2020, World health Organization: Geneva.

2. Samba, E., *The malaria burden and Africa.* Am J Trop Med Hyg, 2001. **64**(1-2 Suppl): p. ii.

3. Snow, R.W. and J.A. Omumbo, *Malaria*, in *Disease and Mortality in Sub-Saharan Africa*, nd, et al., Editors. 2006: Washington (DC).

4. Uneke, C.J., *Impact of placental Plasmodium falciparum malaria on pregnancy and perinatal outcome in sub-Saharan Africa: I: introduction to placental malaria.* Yale J Biol Med, 2007. **80**(2): p. 39-50.

5. *World malaria report*. 2017, World Health Organization, Geneva.

6. Howes, R.E., et al., *Global Epidemiology of Plasmodium vivax.* Am J Trop Med Hyg, 2016. **95**(6 Suppl): p. 15-34.

7. Singh, B. and C. Daneshvar, *Human infections and detection of Plasmodium knowlesi.* Clin Microbiol Rev, 2013. **26**(2): p. 165-84.

8. *Implications of insecticide resistance for malaria vector control* 2016, World Health Organization: Geneva.

9. Katz, F.F., *On the centenary of Sir Ronald Ross's discovery of the role of the mosquito in the life cycle of the malaria parasite.* J Med Biogr, 1997. **5**(4): p. 200-4.

10. Idro, R., et al., *Cerebral malaria: mechanisms of brain injury and strategies for improved neurocognitive outcome.* Pediatr Res, 2010. **68**(4): p. 267-74.

11. Babiker, H.A., P. Schneider, and S.E. Reece, *Gametocytes: insights gained during a decade of molecular monitoring.* Trends Parasitol, 2008. **24**(11): p. 525-30.

12. Sinden, R.E., *A biologist's perspective on malaria vaccine development.* Hum Vaccin, 2010. **6**(1): p. 3-11.

13. Sinden, R.E., *Gametocytogenesis of Plasmodium falciparum in vitro: an electron microscopic study.* Parasitology, 1982. **84**(1): p. 1-11.

14. N., H., *Effects of sublethal doses of pyrethroids on malaria vectors.*, in *School of Hygiene & Tropical Medicine*. 2002, University of London.

15. Prasittisuk, C. and C.F. Curtis, *Absence of effects of insecticides on susceptibility of anophelines to Plasmodium yoelii nigeriensis.* Southeast Asian J Trop Med Public Health, 1982. **13**(1): p. 127-32.

16. Sinden, R.E., et al., *Regulation of infectivity of Plasmodium to the mosquito vector.* Adv Parasitol, 1996. **38**: p. 53-117.

17. Billker, O., et al., *Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito.* Nature, 1998. **392**(6673): p. 289-92.

18. Meis, J.F., et al., *A scanning electron microscopic study of the sporogonic development of Plasmodium falciparum in Anopheles stephensi.* Acta Trop, 1992. **50**(3): p. 227-36.

19. Baton, L.A. and L.C. Ranford-Cartwright, *Plasmodium falciparum ookinete invasion of the midgut epithelium of Anopheles stephensi is consistent with the Time Bomb model.* Parasitology, 2004. **129**(Pt 6): p. 663-76.

20. Rosenberg, R. and J. Rungsiwongse, *The number of sporozoites produced by individual malaria oocysts.* Am J Trop Med Hyg, 1991. **45**(5): p. 574-7.

21. Angrisano, F., et al., *Malaria parasite colonisation of the mosquito midgut--placing the Plasmodium ookinete centre stage.* Int J Parasitol, 2012. **42**(6): p. 519-27.

22. Vaughan, J.A., *Population dynamics of Plasmodium sporogony.* Trends Parasitol, 2007. **23**(2): p. 63-70.

23. Warrell D. A. Clinical features of malaria. Essential Malariology.

24. Verhage, D.F., et al., *Clinical outcome of experimental human malaria induced by Plasmodium falciparum-infected mosquitoes.* Neth J Med, 2005. **63**(2): p. 52-8.

25. Roestenberg, M., et al., *Protection against a malaria challenge by sporozoite inoculation.* N Engl J Med, 2009. **361**(5): p. 468-77.

26. Marsh, K., et al., *Indicators of life-threatening malaria in African children.* N Engl J Med, 1995. **332**(21): p. 1399-404.

27. Dondorp, A., et al., *Artesunate versus quinine for treatment of severe falciparum malaria: a randomised trial.* Lancet, 2005. **366**(9487): p. 717-25.

28. Dondorp, A.M., et al., *Artemisinin resistance: current status and scenarios for containment.* Nat Rev Microbiol, 2010. **8**(4): p. 272-80.

29. Sinka, M.E., et al., *A new malaria vector in Africa: Predicting the expansion range of Anopheles stephensi and identifying the urban populations at risk.* Proc Natl Acad Sci U S A, 2020. **117**(40): p. 24900-24908.

30. Dorn, P.L., et al., *15 - Genetics of Major Insect Vectors*, in *Genetics and Evolution of Infectious Diseases (Second Edition)*, M. Tibayrenc, Editor. 2017, Elsevier: London. p. 341-382.

31. Sinka, M.E., et al., *The dominant Anopheles vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic precis.* Parasit Vectors, 2010. **3**: p. 117.

32. Gillies MT, d.M.B., *The Anophelinae of Africa south of the Sahara (Ethiopian Zoogeographical Region).* Publications of the South African Institute for Medical Research, 1968: p. 54: 1-343.

33. Foster WA, W.E., *Mosquitoes (Culicidae)*, in *Medical and veterinary entomology*. Academic Press: Burlington. p. (pp. 207-259)

34. Yaro, A.S., et al., *Dry season reproductive depression of Anopheles gambiae in the Sahel.* J Insect Physiol, 2012. **58**(8): p. 1050-9.

35. Bhatt, S., et al., *The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015.* Nature, 2015. **526**(7572): p. 207-211.

36. Strode, C., et al., *The impact of pyrethroid resistance on the efficacy of insecticide-treated bed nets against African anopheline mosquitoes: systematic review and meta-analysis.* PLoS Med, 2014. **11**(3): p. e1001619.

37. Namountougou, M., et al., *Multiple insecticide resistance in Anopheles gambiae s.l. populations from Burkina Faso, West Africa.* PLoS One, 2012. **7**(11): p. e48412.

38. Dambach, P., et al., *Nightly Biting Cycles of Anopheles Species in Rural Northwestern Burkina Faso.* J Med Entomol, 2018. **55**(4): p. 1027-1034.

39. L, M., *The epidemiology of malaria as an explanation of its distribution, including some implications for its control.*, in *Malaria: Principles and Practice of Malariology*, M. Wernsdorfer WH, I., Editor. 1988, Churchill Livingstone: Edinburgh. p. 913-998.

40. Tusting, L.S., et al., *Measuring changes in Plasmodium falciparum transmission: precision, accuracy and costs of metrics.* Adv Parasitol, 2014. **84**: p. 151-208.

41. Killeen, G.F., et al., *The potential impact of integrated malaria transmission control on entomologic inoculation rate in highly endemic areas.* Am J Trop Med Hyg, 2000. **62**(5): p. 545-51.

42. Kilama, M., et al., *Estimating the annual entomological inoculation rate for Plasmodium falciparum transmitted by Anopheles gambiae s.l. using three sampling methods in three sites in Uganda.* Malar J, 2014. **13**: p. 111.

43. Garrett-Jones, C., *The Human Blood Index of Malaria Vectors in Relation to Epidemiological Assessment.* Bull World Health Organ, 1964. **30**: p. 241-61.

44. Brady, O.J., et al., *Vectorial capacity and vector control: reconsidering sensitivity to parameters for malaria elimination.* Trans R Soc Trop Med Hyg, 2016. **110**(2): p. 107-17.

45. Christie, A.B., *Infectious Diseases: Epidemiology and Clinical Practice*. 1987, Edinburgh: Churchill Livingstone.

46. Phyo, A.P., et al., *Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study.* Lancet, 2012. **379**(9830): p. 1960-6.

47. Djimde, A.A., et al., *Gametocyte clearance dynamics following oral artesunate treatment of uncomplicated falciparum malaria in Malian children.* Parasite, 2016. **23**: p. 3.

48. Sawa, P., et al., *Malaria transmission after artemether-lumefantrine and dihydroartemisinin-piperaquine: a randomized trial.* J Infect Dis, 2013. **207**(11): p. 1637-45.

49. Huho, B.J., et al., *Artemisinin-based combination therapy does not measurably reduce human infectiousness to vectors in a setting of intense malaria transmission.* Malar J, 2012. **11**: p. 118.

50. Ouedraogo, A.L., et al., *The plasticity of Plasmodium falciparum gametocytaemia in relation to age in Burkina Faso.* Malar J, 2010. **9**: p. 281.

51. Kumar, N. and H. Zheng, *Stage-specific gametocytocidal effect in vitro of the antimalaria drug qinghaosu on Plasmodium falciparum.* Parasitol Res, 1990. **76**(3): p. 214-8.

52. Lelievre, J., et al., *Activity of clinically relevant antimalarial drugs on Plasmodium falciparum mature gametocytes in an ATP bioluminescence "transmission blocking" assay.* PLoS One, 2012. **7**(4): p. e35019.

53. *Updated WHO policy recommendation: Single dose primaquine as a gametocytocide in Plasmodium falciparum malaria*. October 2012: Geneva.

54. *WHO Evidence Review Group: The Safety and Effectiveness of Single Dose Primaquine as a P. falciparum gametocytocide* 2012: Pullman Hotel,Bangkok, Thailand.

55. Butcher, G.A., *Antimalarial drugs and the mosquito transmission of Plasmodium.* Int J Parasitol, 1997. **27**(9): p. 975-87.

56. Kobylinski, K.C., B.D. Foy, and J.H. Richardson, *Ivermectin inhibits the sporogony of Plasmodium falciparum in Anopheles gambiae.* Malar J, 2012. **11**: p. 381.

57. Kobylinski, K.C., et al., *Ivermectin susceptibility, sporontocidal effect, and inhibition of time to re-feed in the Amazonian malaria vector Anopheles darlingi.* Malar J, 2017. **16**(1): p. 474.

58. Stowers, A. and R. Carter, *Current developments in malaria transmission-blocking vaccines.* Expert Opin Biol Ther, 2001. **1**(4): p. 619-28.

59. Gwadz, R.W., *Successful immunization against the sexual stages of Plasmodium gallinaceum.* Science, 1976. **193**(4258): p. 1150-1.

60. Mendis, K.N. and G.A. Targett, *Immunisation against gametes and asexual erythrocytic stages of a rodent malaria parasite.* Nature, 1979. **277**(5695): p. 389-91.

61. Gwadz, R.W. and I. Green, *Malaria immunization in Rhesus monkeys. A vaccine effective against both the sexual and asexual stages of Plasmodium knowlesi.* J Exp Med, 1978. **148**(5): p. 1311-23.

62. Ponnudurai, T., et al., *The production of mature gametocytes of Plasmodium falciparum in continuous cultures of different isolates infective to mosquitoes.* Trans R Soc Trop Med Hyg, 1982. **76**(2): p. 242-50.

63. Rener, J., et al., *Target antigens of transmission-blocking immunity on gametes of plasmodium falciparum.* J Exp Med, 1983. **158**(3): p. 976-81.

64. Guttmann P., E.P., *Ueber die Wirkung des methylenblau bei Malaria.* Berlin Klin. Wochenschr. , 1891. **28**: p. 953-956.

65. Schirmer, R.H., et al., *Methylene blue as an antimalarial agent.* Redox Rep, 2003. **8**(5): p. 272-5.

66. Schirmer, R.H., et al., *"Lest we forget you--methylene blue...".* Neurobiol Aging, 2011. **32**(12): p. 2325 e7-16.

67. Krafts, K., E. Hempelmann, and A. Skorska-Stania, *From methylene blue to chloroquine: a brief review of the development of an antimalarial therapy.* Parasitol Res, 2012. **111**(1): p. 1-6.

68. Lu, G., et al., *Efficacy and safety of methylene blue in the treatment of malaria: a systematic review.* BMC Med, 2018. **16**(1): p. 59.

69. Vennerstrom, J.L., et al., *Antimalarial dyes revisited: xanthenes, azines, oxazines, and thiazines.* Antimicrob Agents Chemother, 1995. **39**(12): p. 2671-7.

70. Atamna, H., et al., *Mode of antimalarial effect of methylene blue and some of its analogues on Plasmodium falciparum in culture and their inhibition of P. vinckei petteri and P. yoelii nigeriensis in vivo.* Biochem Pharmacol, 1996. **51**(5): p. 693-700.

71. Akompong, T., N. Ghori, and K. Haldar, *In vitro activity of riboflavin against the human malaria parasite Plasmodium falciparum.* Antimicrob Agents Chemother, 2000. **44**(1): p. 88-96.

72. Anstey, N.M., et al., *Elevated levels of methaemoglobin in Tanzanian children with severe and uncomplicated malaria.* Trans R Soc Trop Med Hyg, 1996. **90**(2): p. 147-51.

73. Blank, O., E. Davioud-Charvet, and M. Elhabiri, *Interactions of the antimalarial drug methylene blue with methemoglobin and heme targets in Plasmodium falciparum: a physico-biochemical study.* Antioxid Redox Signal, 2012. **17**(4): p. 544-54.

74. Arora, K. and A.K. Srivastava, *Antimalarial efficacy of methylene blue and menadione and their effect on glutathione metabolism of Plasmodium yoelii-infected albino mice.* Parasitol Res, 2005. **97**(6): p. 521-6.

75. Kelner, M.J. and N.M. Alexander, *Methylene blue directly oxidizes glutathione without the intermediate formation of hydrogen peroxide.* J Biol Chem, 1985. **260**(28): p. 15168-71.

76. Adjalley, S.H., et al., *Quantitative assessment of Plasmodium falciparum sexual development reveals potent transmission-blocking activity by methylene blue.* Proc Natl Acad Sci U S A, 2011. **108**(47): p. E1214-23.

77. Bosson-Vanga, H., et al., *Differential activity of methylene blue against erythrocytic and hepatic stages of Plasmodium.* Malar J, 2018. **17**(1): p. 143.

78. Wadi, I., et al., *Methylene blue induced morphological deformations in Plasmodium falciparum gametocytes: implications for transmission-blocking.* Malar J, 2018. **17**(1): p. 11.

79. Bradley, J., et al., *Transmission-blocking Effects of Primaquine and Methylene Blue Suggest Plasmodium falciparum Gametocyte Sterilization Rather Than Effects on Sex Ratio.* Clin Infect Dis, 2019. **69**(8): p. 1436-1439.

80. Dicko, A., et al., *Efficacy and safety of primaquine and methylene blue for prevention of Plasmodium falciparum transmission in Mali: a phase 2, single-blind, randomised controlled trial.* Lancet Infect Dis, 2018. **18**(6): p. 627-639.

81. Youngster, I., et al., *Medications and glucose-6-phosphate dehydrogenase deficiency: an evidence-based review.* Drug Saf, 2010. **33**(9): p. 713-26.

82. Muller, O., P. Meissner, and U. Mansmann, *Glucose-6-phosphate dehydrogenase deficiency and safety of methylene blue.* Drug Saf, 2012. **35**(1): p. 85; author reply 85-6.

83. Tsuchido, Y., et al., *Development of Delayed Hemolytic Anemia After Treatment with Oral Artemether-Lumefantrine in Two Patients with Severe Falciparum Malaria.* Am J Trop Med Hyg, 2017. **96**(5): p. 1185-1189.

84. Pinilla, Y.T., et al., *Promising approach to reducing Malaria transmission by ivermectin: Sporontocidal effect against Plasmodium vivax in the South American vectors Anopheles aquasalis and Anopheles darlingi.* PLoS Negl Trop Dis, 2018. **12**(2): p. e0006221.

85. Fowler, R.E., R.E. Sinden, and M. Pudney, *Inhibitory activity of the anti-malarial atovaquone (566C80) against ookinetes, oocysts, and sporozoites of Plasmodium berghei.* J Parasitol, 1995. **81**(3): p. 452-8.

86. Gimnig, J.E., et al., *Density-dependent development of Anopheles gambiae (Diptera: Culicidae) larvae in artificial habitats.* J Med Entomol, 2002. **39**(1): p. 162-72.

87. Ouédraogo, A.L., et al., *Supplement material_A protocol for membrane feeding assay to determine the infectiousness of P. falciparum naturally infected individuals to Anopheles gambiae*. 2013.

88. Delves, M.J. and R.E. Sinden, *A semi-automated method for counting fluorescent malaria oocysts increases the throughput of transmission blocking studies.* Malar J, 2010. **9**: p. 35.

89. Sylla, E.H., J.F. Kun, and P.G. Kremsner, *Mosquito distribution and entomological inoculation rates in three malaria-endemic areas in Gabon.* Trans R Soc Trop Med Hyg, 2000. **94**(6): p. 652-6.

90. Grobusch, M.P., et al., *Intermittent preventive treatment against malaria in infants in Gabon--a randomized, double-blind, placebo-controlled trial.* J Infect Dis, 2007. **196**(11): p. 1595-602.

91. Ouédraogo, A.L., et al., *A protocol for membrane feeding assays to determine the infectiousness of P. falciparum naturally infected individuals to Anopheles gambiae*. 2013.

92. Williams, J. and J. Pinto, *Training Manual on Malaria Entomology*. 2012.

93. Vincke, I.H. and M. Lips, *A new plasmodium from a wild Congo rodent Plasmodium berghei n. sp.* Ann Soc Belg Med Trop (1920), 1948. **28**(1): p. 97-104.

94. Cox, F.E., *History of the discovery of the malaria parasites and their vectors.* Parasit Vectors, 2010. **3**(1): p. 5.

95. Sinden, R.E., *Sexual development of malarial parasites.* Adv Parasitol, 1983. **22**: p. 153-216.

96. Billker, O., A.J. Miller, and R.E. Sinden, *Determination of mosquito bloodmeal pH in situ by ion-selective microelectrode measurement: implications for the regulation of malarial gametogenesis.* Parasitology, 2000. **120 (Pt 6)**: p. 547-51.

97. Arai, M., et al., *Both mosquito-derived xanthurenic acid and a host blood-derived factor regulate gametogenesis of Plasmodium in the midgut of the mosquito.* Mol Biochem Parasitol, 2001. **116**(1): p. 17-24.

98. Saison, N., et al., *Rapid and Specific Action of Methylene Blue against Plasmodium Transmission Stages.* Pharmaceutics, 2022. **14**(12).

99. Manzoni, G., et al., *A rapid and robust selection procedure for generating drug-selectable marker-free recombinant malaria parasites.* Sci Rep, 2014. **4**: p. 4760.

100. Churcher, T.S., et al., *Measuring the blockade of malaria transmission--an analysis of the Standard Membrane Feeding Assay.* Int J Parasitol, 2012. **42**(11): p. 1037-44.

101. Pastrana-Mena, R., et al., *Glutathione reductase-null malaria parasites have normal blood stage growth but arrest during development in the mosquito.* J Biol Chem, 2010. **285**(35): p. 27045-27056.

102. Douglas, R.G., et al., *Screening for potential prophylactics targeting sporozoite motility through the skin.* Malar J, 2018. **17**(1): p. 319.

103. Peter, C., et al., *Pharmacokinetics and organ distribution of intravenous and oral methylene blue.* Eur J Clin Pharmacol, 2000. **56**(3): p. 247-50.

104. Chaccour, C.J., et al., *Ivermectin to reduce malaria transmission: a research agenda for a promising new tool for elimination.* Malar J, 2013. **12**: p. 153.

105. Kobylinski, K.C., et al., *Ivermectin susceptibility and sporontocidal effect in Greater Mekong Subregion Anopheles.* Malar J, 2017. **16**(1): p. 280.

106. Paton, D.G., et al., *Exposing Anopheles mosquitoes to antimalarials blocks Plasmodium parasite transmission.* Nature, 2019. **567**(7747): p. 239-243.

107. Leitao, R. and A. Rodriguez, *Inhibition of Plasmodium sporozoites infection by targeting the host cell.* Exp Parasitol, 2010. **126**(2): p. 273-7.

108. Aleman, M., et al., *Salinomycin toxicosis in horses.* J Am Vet Med Assoc, 2007. **230**(12): p. 1822-6.

109. Kouyoumdjian, J.A., et al., *Fatal rhabdomyolysis after acute sodium monensin (Rumensin) toxicity: case report.* Arq Neuropsiquiatr, 2001. **59**(3-A): p. 596-8.

110. Luzzatto, L., *The rise and fall of the antimalarial Lapdap: a lesson in pharmacogenetics.* Lancet, 2010. **376**(9742): p. 739-41.

111. Muller, O., et al., *Haemolysis risk in methylene blue treatment of G6PD-sufficient and G6PD-deficient West-African children with uncomplicated falciparum malaria: a synopsis of four RCTs.* Pharmacoepidemiol Drug Saf, 2013. **22**(4): p. 376-85.

112. Fairhurst, R.M. and A.M. Dondorp, *Artemisinin-Resistant Plasmodium falciparum Malaria.* Microbiol Spectr, 2016. **4**(3).

113. Uwimana, A., et al., *Emergence and clonal expansion of in vitro artemisinin-resistant Plasmodium falciparum kelch13 R561H mutant parasites in Rwanda.* Nat Med, 2020. **26**(10): p. 1602-1608.

8 Ehrenerklärung

Ich erkläre, dass ich die der medizinischen Fakultät der Eberhard Karls Universität Tübingen zur Promotion eingereichte Dissertation mit dem Titel „ Rapid and specific action of methylene blue against *Plasmodium* transmission stages“ im Centre de Recherches Médicales de Lambaréné und im Centre d’Immunologie et des maladies infectieuses (Sorbonne Paris) mit Unterstützung der Arbeitsgruppe um Prof. Dr. Steffen Borrmann und Prof. Dominique Mazier ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die aufgeführten Hilfsmittel benutzt habe.

Die vorliegende Arbeit wurde selbstständig mit Hilfe von Prof. Dominique Mazier, Prof. Dr. Steffen Borrmann, Dr. Jean-François Franetich und Dr. Yudi Tatiana Röckl-Pinilla konzipiert. Zusammen mit Prof. Dr. Steffen Borrmann und Prof. Dominique Mazier wurden die Versuche und die Arbeit betreut.

In Zusammenarbeit mit ärztlichen Mitarbeitern vom CERMEL und der AG von Prof. Dr. Borrmann wurden die Probanden rekrutiert sowie die Blutproben entnommen. Die mikroskopische Diagnostik erfolgte durch Mitarbeiter des CHU am CERMEL.
Die Transmission Blocking Experimenten und die anschließende statistische Auswertung wurde durch mich, Nathanaël Saison mit Hilfe von dem AG von Prof. Dr. Borrmann in Lambaréné durchgeführt. Die *in vitro* Experimenten sowie Tierexperimenten und die anschließende statistische Auswertung wurde durch mich, Nathanaël Saison mit Hilfe von Dr. Jean - François Franetich, in Paris durchgeführt. Die vorliegende Arbeit wurde in der Zeitschrift *Pharmaceutics* MDPI angenommen und veröffentlicht am 14.12.2022.

Die vorliegende Arbeit wurde in der Zeitschrift *Pharmaceutics* MDPI angenommen und veröffentlicht am 14.12.2022.

Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden. Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht.

Tübingen, den 11. März 2023

Nathanaël François Louis Saison

9 Veröffentlichungen

Parts of this dissertation have already been published in the following publication:

Saison, N.; Franetich, J.-F.; Pinilla, Y.T.; Hoffmann, A.; Boussougou-Sambe, S.T.; Ngossanga, B.; Tefit, M.; Ashraf, K.; Amanzougaghene, N.; Tajeri, S.; Adegnika, A.A.; Mazier, D.; Borrmann, S. Rapid and Specific Action of Methylene Blue against *Plasmodium* Transmission Stages. *Pharmaceutics* **2022**, 14, 2794. https://doi.org/10.3390/pharmaceutics14122794

10 Acknowledgement

To begin, I would like to thank Prof. Dr. Steffen Borrmann to supervise me on my project and Prof. Dominique Mazier for giving me the opportunity to work at CIMI (Paris). Thank you both for placing your trust in me and giving precious time to move forward as far as possible in the various ambitious projects of our collaboration.

Second, I want to thank Prof. Dr. Ayola Akim Adegnika for giving me the opportunity to work at Insectary of CERMEL in Lambaréné. I want to thank all patients and healthy volunteers participating to the ex vivo study, staff members from the insectary of Centre de Recherche Médicale de Lambaréné, Lambaréné, Gabon.

Moreover, I would like then to thank the Team of Prof. Mazier at CIMI for having welcomed me, even if the conditions of the pandemic slowed down our experiments' progress. Many thanks to Dr. Jean-François Franetich who helped me to conceptualise and design the procedures and to show a large number of experiments. I am truly grateful for your time and support in the various phases and steps of the project.

This project in Lambaréné has allowed me to develop great friendships, in particular I would like to thank Anton Hoffmann for his support and kindness. With you, I could overcome many pitfalls and sometimes deceptions.