Effect of fluorescent dyes on in-vitro differentiated, late-stage Plasmodium falciparum gametocytes

Running Title: Dyes against mature P. falciparum gametocytes

Tamirat Gebru\textsuperscript{a,c}, Benjamin Mordmüller\textsuperscript{a,b,c} and Jana Held\textsuperscript{a,b,c}\#

\textsuperscript{a}Institute of Tropical Medicine, Eberhard Karls University, Tübingen, Germany
\textsuperscript{b}Centre de Recherches Médicales de Lambaréné, Lambaréné, Gabon
\textsuperscript{c}German Centre for Infection Research

\#Corresponding author:
Jana Held

Present address:
Institute of Tropical Medicine
University of Tübingen
Wilhelmstraße 27
D-72074 Tübingen, Germany.
Tel: +49 7071 29 85569
Fax: +49 7071 29 5267
E-mail: janaheld@hotmail.de
Abstract:

Plasmodium falciparum gametocytes are not associated with clinical symptoms but they are responsible for transmitting the pathogen to mosquitoes. Therefore gametocytocidal interventions are important for malaria control and resistance containment but currently available drugs and vaccines are not well suited for that purpose. Several dyes have potent antimicrobial activity but their use against gametocytes has not been investigated systematically.

Gametocytocidal activity of nine synthetic dyes and four compounds with known activity was tested against stage V gametocytes of the laboratory strain 3D7 and three clinical isolates of P. falciparum with a bioluminescence assay.

Five of the fluorescent dyes had 50% inhibitory concentration (IC₅₀) values at sub-micromolar concentration against mature gametocytes. Three mitochondrial dyes: MitoRed, DiOC6 and rhodamine B were highly active (IC₅₀ < 200 nM). MitoRed showed the highest activity against gametocytes with an IC₅₀ of 70 nM against 3D7 and 120–210 nM against clinical isolates. All compounds were more active in the laboratory strain 3D7 in comparison with clinical isolates.

In particular, the endoperoxides artesunate and dihydroartemisinin showed a 10-fold higher activity in 3D7 compared to clinical isolates.

In contrast to all clinically used antimalarials, several fluorescent dyes had a surprisingly high in vitro activity against late stage gametocytes. Since they also act against asexual blood stages, they shall be considered as starting points for the development of new antimalarial lead compounds.
Introduction

Malaria leads to approximately one million deaths annually and remains a highly relevant public health problem in tropical and sub-tropical regions (1). *Plasmodium falciparum*, the most virulent species, is responsible for most of the malaria morbidity and accounts for almost all malaria mortality in Sub-Saharan Africa. Gametocytes are the stage responsible for transmission of the parasite from the human host to the mosquito. During the asexual blood stage, a small fraction of parasites differentiates into gametocytes. In *P. falciparum* differentiation occurs within 8-14 days in internal organs, mainly in bone marrow (2-4), from sequestered immature (stage I-IV) to free circulating mature stages (stage V). When a mosquito takes a blood meal it ingests stage V male and female gametocytes, which can further develop in the mosquito. In the mosquito gut, gametocytes develop into gametes that join to become a zygote which then matures into the ookinete followed by the oocyst stage. This stage gives rise to sporozoites, which are released from the oocyst and migrate to the salivary glands of the mosquito where they can be transmitted during a subsequent blood meal.

Even though gametocytes are essential for the spread of malaria, transmission-blocking agents have not been a priority in the search of antimalarial drugs for a long time. This has recently changed with the ambition to eradicate malaria (5). On the Malaria Eradication Research Agenda (malERA) development of a new generation of transmission blocking tools and therapies is a top priority since they are believed to be essential for the elimination and subsequent eradication of malaria (6,7).

Most current antimalarial drugs show at most, only modest activity against early gametocytes but have either negligible or no effect on stage V gametocytes, although all are active against the asexual blood stage parasites (8-10) and some inhibit growth of pre-erythrocytic stages. Primaquine, an 8-aminoquinoline, is the only licensed drug that has proven gametocytocidal
activity in vivo. Its use is limited due to the risk of hemolysis in people with glucose-6-phosphate dehydrogenase (G6PD) deficiency, a common trait in malaria endemic areas. In addition, primaquine is a prodrug that requires activation by cytochrome P450 2D6 (CYP2D6), which has a variable phenotype that can lead to treatment failures in poor and intermediate metabolizers (11). Other drugs with activity against gametocytes are bulaquine, a newly registered compound in India (12) and tafenoquine, which is in late phase clinical development for more than a decade (13). Both drugs are 8-aminoquinolines and are likely to share safety and efficacy problems with primaquine in G6PD deficient individuals and CYP2D6 poor metabolizers, respectively (14). Dihydroartemisinin (DHA), the active metabolite of all artemisinin derivates, has been reported to be active against stage I to III gametocytes (15,16), but clinical relevant activity of DHA against late stage gametocytes is uncertain (8). Available evidence from clinical studies shows that transmission is only partially blocked following treatment with artemisinin based combination therapies (17,18), the current mainstay of antimalarial therapy in endemic areas.

In conclusion, there is no licensed drug available with a satisfactory profile that can be used to control transmission of P. falciparum. Some synthetic dyes have potent antimicrobial activity and have been successfully used as antimalarials since the late 19th century, when Guttmann and Ehrlich used the thiazine dye, methylene blue, for treatment of malaria (19). Since then several other dyes were tested for their antiplasmodial activity (20,21). We revived this idea in a previous study by testing a panel of synthetic dyes for their in vitro activity against asexual stages and identified several compounds with high in vitro activity (22). Methylene blue is known to show gametocytocidal activity (15,23-25), therefore we evaluated the activity of eight other antimalarial dyes against P. falciparum stage V gametocytes derived from the laboratory
strain 3D7 and three clinical isolates from Lambaréné, Gabon with an established bioluminescence assay (8).

**Materials and Methods**

**Cultivation of asexual parasites**

The *P. falciparum* strain 3D7 (Malaria Research and Reference Reagent Resource, ATCC, Virginia, USA) and clinical isolates obtained from Lambaréné, Gabon were kept in continuous culture as described earlier (26). Clinical isolates were from a study reported previously (27). Blood was sampled directly from patients with *P. falciparum* mono-infection and cryopreserved in glycerol at -150°C until culture for the experiment was initiated. The cultures of both 3D7 and clinical isolates were kept at 5% hematocrit in complete culture medium (RPMI 1640 supplemented with 25 mM HEPES, 28 mM NaHCO₃, 50 μg/mL gentamycin, 0.5% w/v Albumax II, 2.4 mM L-glutamine, and 0.14 mM hypoxanthine at 5% CO₂ and 5% O₂). For clinical isolates 5% human serum was added to the complete culture medium. Medium was replaced every 24 hours.

**Gametocyte culture and purification**

Gametocyte culture was performed as described previously (8) with some modifications. Cultures were started from asexual parasites kept in continuous culture with sorbitol synchronization twice weekly (28). Culture medium for gametocytes always contained 5% human serum. Initially, the culture was adjusted to 9% hematocrit and 0.5% parasitemia and kept at 5% O₂/CO₂ at 37°C with daily change of medium without parasite dilution for two weeks. In the second week, the volume of medium was doubled and cultures were treated with 50 mM N-acetyl-D-glucosamine (MP Biomedicals GmbH, Santa Ana, CA, USA) for four days beginning 11 days after initiation to remove asexual parasites. To enrich stage V gametocytes from approximately 2% to over 90% a two-step purification was performed on day 15 using a
NycoPrep 1.077 cushions density gradient (Axis-Shield PoC AS, Oslo, Norway) followed by magnetic separation with LD-MACS magnetic columns (Miltenyi Biotec, Gladbach, Germany) to remove remaining erythrocytes. Purification was done at 37°C to keep gametocytes viable and avoid exflagellation during the procedure.

Viability of mature gametocytes after separation
Viability of gametocytes after purification was assessed by microscopy (Figure 1) and the capacity of male gametocyte to exflagellate in exflagellation medium (complete culture medium with 5% human serum and 100 µM xanthurenic acid) for 20 minutes at room temperature as described earlier (25,29).

Compounds
We selected eight fluorescent dyes (see structures in Figure 2) based on their activity against asexual parasite stages evaluated previously (22) in addition to methylene blue and control compounds. The panel of fluorescent dyes consisted of three nucleic acid dyes: Hoechst 33342 (CAS number: 23491-52-3), acridine orange (CAS number: 65-61-2) and SYTO 9 (SYTO 9 green fluorescent nucleic acid stain; the structure is proprietary) and five mitochondrial dyes MitoRed (MitoTracker Red CMXRos; CAS number: 167095-09-2), MitoGreen (MitoTracker Green FM; CAS number: 201860-17-5), DiOC6 (CAS number: 53213-82-4), rhodamine 123 (MW: 380.83; CAS number: 62669-70-9) and rhodamine B (hexyl ester perchlorate; MW: 627.17; CAS number: 877933-92-1). We compared the activities of these dyes to methylene blue (MW: 319.86; CAS number: 61-73-4), epoxomicin (MW: 554.7; CAS number: 134381-21-8), DHA (dihydroartemisinin, MW: 284.35; CAS number: 71939-50-9), artesunate (MW: 384.4; CAS number: 88495-63-0) and primaquine (primaquine phosphate, MW: 455.33; CAS number: 63-45-6). Methylene blue was obtained from AppliChem. All other...
dyes were from Invitrogen. Epoxomicin and DHA were obtained from Calbiochem and Shinpoong, respectively. Artesunate and primaquine were from Santa Cruz Biotechnology. Methylene blue and acridine orange were dissolved in water, while all other tested dyes were dissolved in DMSO. Further dilutions were done with complete culture medium. Final DMSO concentrations were less than 0.5%, a concentration that does not interfere with gametocyte viability.

Epoxomicin was used as a positive control as it is a potent inhibitor of all clinically relevant parasite stages including gametocytes in vitro.

**ATP-based bioluminescence assay**

We used a bioluminescence assay based on the luciferin-luciferase adenosine triphosphate (ATP)-dependent reaction to assess activity of the different compounds against mature gametocytes.

The assay was performed as previously described with some modifications (8,30). In brief, compounds were three-fold serially diluted in a 96-well plate. After purification, gametocytes were counted microscopically using an Improved Neubauer Cell counting chamber and \(5 \times 10^4\) purified stage V gametocytes were added to each well in a final volume of 100 µL. In order to validate the test protocol and determine the linear relationship between ATP content and number of gametocytes in culture medium, we generated a standard curve using a two-fold serial dilution of 50,000 purified mature gametocytes. On every plate a serial dilution of viable stage V gametocytes was added as a control.

ATP measurements were done using equal volumes (50 µL) of gametocyte culture and BacTiterGlo reagent (Promega, Mannheim, Germany) in white opaque-walled 96-well plates, incubated at room temperature in the dark for 5 min to allow cell lysis and stabilization of the
luminescent signal. Subsequently, the luminescence signal was recorded with a luminometer (LUMO, Anthos Microsysteme, Krefeld, Germany).

Stage V gametocytes were exposed to serial dilutions of the test compounds for 24 hours or 48 hours to assess the effect of incubation time on the activity. Experiments using 3D7 and 48 hours incubation time were performed at least three times independently.

**Statistical analysis**

We used the drc-package version 2.3.7 of R version 2.15.1 to calculate the inhibitory concentrations of compounds by non-linear regression analysis of log-concentration-response curves. The 50% inhibitory concentrations (IC$_{50}$) were determined for each compound. If more than two experiments were performed the mean and standard deviation is given. The coefficient of determination ($R^2$) was calculated to assess correlations.

**Ethical approval**

We used three clinical isolates described in a previous study (27). The study was approved by the ethics committee of the International Foundation for the Albert Schweitzer Hospital in Lambaréné and followed the principles of the Declaration of Helsinki (6th revision). The participating children and legal representatives gave assent and informed consent, respectively.

**Results**

**Viability of mature gametocytes and validation of luminescence**

As a first step, we evaluated first the viability of gametocytes after purification and validated the ATP read out. Gametocytes appeared non-compromised after purification with intact cytoplasm (Figure 1), male gametocytes showed exflagellation and stage V gametocytes consistently remained viable for approximately three weeks. ATP levels and number of gametocytes in the assay correlated closely, $R^2 = 0.99$.

**Activity of fluorescent dyes against mature stage V gametocytes**
Five of the eight fluorescent dyes in our assay showed IC₅₀ values below 1 μM (Table 1). Among the dyes tested, the three mitochondrial dyes: MitoRed, DiOC₆ and rhodamine B showed the best activity with IC₅₀ values below 200 nM; even lower than the control dye methylene blue (770 nM). Among all dyes, MitoRed was the most active compound against the laboratory strain 3D7 as well as against clinical isolates. Activities of the different dyes as well as the control drugs were consistently higher in the laboratory strain 3D7 than in clinical isolates (2–10 fold). Particularly, artemisinate and DHA had considerably higher IC₅₀ values in clinical isolates compared to 3D7. As expected for a prodrug, primaquine did not inhibit gametocytes at in vivo-achievable concentrations. Epoxomicin served as our internal positive control and was highly active against the 3D7 laboratory strain and clinical isolates. IC₅₀ values of the single compounds against asexual blood stages are given for comparison (Table 1). All compounds except epoxomicin are less active against gametocytes compared to asexual blood stages.

**Activities of compounds after different incubation times (24 hours and 48 hours) with mature gametocytes.**

*In vitro* gametocyte assays are poorly standardized and use different incubation times to test gametocytocidal compounds (8,30). To assess the influence of the incubation time on the antiparasitic activity, we incubated fluorescent dyes and standard antimalarial drugs for either 24 hours or 48 hours with stage V gametocytes of 3D7 parasites and clinical isolates. For most compounds the incubation time had no significant effect (Table 2). However, MitoRed was 3-fold more active when used over 48 hours compared to 24 hours. This effect was even more pronounced for epoxomicin and artemisinate incubations, with a 10-fold increase in activity when parasites were exposed for 48 hours.

**Discussion**
The primary aim of antimalarial treatment is to remove asexual blood stage parasites in order to resolve the illness and prevent or treat severe forms of malaria. In contrast, late stage gametocytes do not cause significant harm to the infected individual and appear in the circulation after a lag period due to sequestration. Most of the current antimalarials act against asexual infection but have no significant effect on sexual stages, which remain in the circulation for extended periods after treatment and could therefore be transmitted (31,32). To achieve sustained malaria control and contain resistance development, an efficient gametocytocidal drug is fundamental. We identified five synthetic dyes with good gametocytocidal activity. When compared to methylene blue, a drug with known gametocytocidal activity (15,23-25), these five dyes were more active, particularly, the three mitochondrial dyes MitoRed, DiOC6 and rhodamine B. Cytotoxicity of the mitochondrial dyes was higher than 2.5 µM when evaluated against HeLa cells in our previous publication (22). MitoRed was the most active compound against gametocytes. Interestingly, we had previously selected it as a promising candidate for development as an antimalarial due to its high activity against asexual blood stage parasites and its favorable selectivity index when tested against HeLa cells. It belongs to the rhodamine dyes. Some of its derivatives are used in the cosmetic industry where toxicity has been more extensively studied (33,34). However, no further toxicity data on MitoRed are available. The results from the current gametocyte assay underline the interesting properties of mitochondrial dyes as starting points for new antimalarials and further toxicity studies of the most promising compounds should be performed. Interestingly, all compounds except epoxomicin showed higher activity against asexual blood stages than against gametocytes. This is likely due to the less active metabolism during the late gametocyte stage. Dyes are selectively targeted to certain organelles in the organism presumably inhibiting the targeted organelle. Interestingly, atovaquone, a potent inhibitor of the cytochrome bc1 complex of the mitochondrion, does not
affect gametocyte development (8). Nevertheless, mitochondrial dyes are highly active which is most likely because they do not share the same mechanism of action.

In addition to *P. falciparum* 3D7 gametocytes, we used three clinical isolates from Lambaréné, Gabon, an area of high drug pressure and widespread chloroquine and folate inhibitor resistance (35,36). Gametocytes derived from the laboratory strain 3D7 seem to be more sensitive to most compounds than the tested clinical isolates. This finding has important implications for larger screening programs since hits achieved in laboratory isolates should be considered to be validated in clinical isolates. Primaquine presented one exception to this rule because it is more active against clinical isolates than against 3D7. However, the *in vitro* activity is likely not a good surrogate for the relevant activity of primaquine *in vivo*, as it has to be metabolized by CYP2D6 (37,38) and MAO-A (38) to be active. Therefore primaquine shows rather unspecific activity *in vitro* and can only be used as a comparator between assays, showing that our results are in line with results obtained by others (8,30,39).

The difference in activities between clinical isolates and 3D7 was particularly high for artemisinin derivatives. Whether or not this is due to drug pressure may only be hypothesized. Artemisinin combination therapy is the recommended first line therapy since 2003 in Gabon (40). Since then local parasite populations may have adapted to the presence of artemisinin derivatives. It would be very interesting to test parasite isolates obtained before 2003. Unfortunately, such isolates were not available.

A prolonged incubation time influenced the activity of the drugs. In particular, the most active compounds and artemisinin derivatives were more active when incubated for 48 hours instead of 24 hours. There are two possible explanations for this: i) the drug needs a longer time to exert its effect or ii) the drug acts early but the read-out only detects late changes in metabolism of the gametocytes. Since artemisinin derivatives have a very short half life (41),
scenario i) will result in very low in vivo activity, whereas scenario ii) is more likely. When we compare the results to our previous assays in which we used serum supplemented with Albumax instead of human serum (42), we can also see an approximately 10-fold increase of the IC$_{50}$ for artesunate and DHA for the Albumax supplemented medium. In vitro activity of artemisinin derivatives against late stage gametocytes is highly variable and depends on the assay used. Commonly used methods differ in the number of gametocytes, parasite strain, incubation times, serum supplementation and read out. This results in inhibitory concentrations between 3 nM and 11 µM for the same compound (8,30,43-45). These differences demonstrate that better standardization and validation of assays is required in order to compare results and identify compounds with promising activities. One method of independent validation would be the use of standard membrane feeding assays. Interestingly, most reports show modest (24,44) or no effect (46) of artemisinins on oocyst counts following membrane feeding.

In conclusion, fluorescent dyes, especially MitoRed, show high in vitro gametocytocidal activities and should be considered as starting points for the development new antimalarial compounds with transmission blocking potential. In addition to laboratory strains, gametocytes of clinical isolates should be considered for the screening of new compounds, as they have a different genetic background and might show a different resistance profile.

Acknowledgements

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References


Figures and Tables

Figure 1: Giemsa-stained mature gametocyte culture on day 15; 1A) before and 1B) after purification.

Figure 2: Chemical structure of tested synthetic dyes. The structure of SYTO 9 is proprietary.
Table 1: *In vitro* activities of standard antimalarial drugs and dyes against stage V gametocytes of *P. falciparum* 3D7 laboratory strain and clinical isolates after 48 h incubation.

<table>
<thead>
<tr>
<th>Activity against 3D7 gametocytes</th>
<th>Activity against clinical isolates (gametocytes)</th>
<th>Activity against 3D7 asexual blood stages</th>
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<td>Activity against clinical isolates (gametocytes)</td>
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<td></td>
<td>Isolate 1</td>
<td>Isolate 2</td>
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**Standard compounds**

- **Epoxomicin**: 0.0016 ± 0.0007
- **DHA**: 0.20 ± 0.07
- **Artesunate**: 0.38 ± 0.12
- **Primaquine**: 20.05 ± 5.67
- **Methylene Blue**: 0.77 ± 0.68

**Dyes (IC₅₀ < 300 nM for 3D7 gametocytes)**

- **MitoRed**: 0.07 ± 0.04
- **DiOC6**: 0.14 ± 0.03
- **Rhodamine B**: 0.18 ± 0.08
- **Hoechst 33342**: 0.23 ± 0.06
- **Syto9**: 0.26 ± 0.07

**Dyes (IC₅₀ > 1µM for 3D7 gametocytes)**

- **MitoGreen**: 1.02 ± 0.23
- **Acridine Orange**: 3.04 ± 1.07
- **Rhodamine 123**: 7.13 ± 2.97

Activities of evaluated compounds against mature gametocytes of 3D7 and three different clinical isolates of *P. falciparum* (measured after 48 h of incubation in serum supplemented medium). Results of 3D7 gametocytes are the mean of three independent experiments.

*: For comparison, the activity of dyes against asexual blood stages evaluated by HRP2-ELISA is given (results from Joanny et al. (22)). Activities of epoxomicin, artesunate and DHA were evaluated with the same method in our laboratory.
Table 2: *In-vitro* activities of compounds against mature gametocytes of 3D7 and clinical isolates of *P. falciparum* after 24 h or 48 h incubation.

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<tr>
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<td>24 h incubation</td>
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<td><strong>Standard compounds</strong></td>
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*: Mean of three independent experiments (48 hour assay of 3D7 laboratory strains).

Individual values are presented for all other results.
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