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Male and Female *Plasmodium falciparum* Mature Gametocytes Show Different Responses to Antimalarial Drugs

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It is the mature gametocytes of *Plasmodium* that are solely responsible for parasite transmission from the mammalian host to the mosquito. They are therefore a logical target for transmission-blocking antimalarial interventions, which aim to break the cycle of reinfection and reduce the prevalence of malaria cases. Gametocytes, however, are not a homogeneous cell population. They are sexually dimorphic, and both males and females are required for parasite transmission. Using two bioassays, we explored the effects of 20 antimalarials on the functional viability of both male and female mature gametocytes of *Plasmodium falciparum*. We show that mature male gametocytes (as reported by their ability to produce male gametes, i.e., to exflagellate) are sensitive to antifolates, some endoperoxides, methylene blue, and thiostrepton, with submicromolar 50% inhibitory concentrations (IC_{50}s), whereas female gametocytes (as reported by their ability to activate and form gametes expressing the marker Pfs25) are much less sensitive to antimalarial intervention, with only methylene blue and thiostrepton showing any significant activity. These findings show firstly that the antimalarial responses of male and female gametocytes differ and secondly that the mature male gametocyte should be considered a more vulnerable target than the female gametocyte for transmission-blocking drugs. Given the female-biased sex ratio of *Plasmodium falciparum* (~3 to 5 females:1 male), current gametocyte assays without a sex-specific readout are unlikely to identify male-targeted compounds and prioritize them for further development. Both assays reported here are being scaled up to at least medium throughput and will permit identification of key transmission-blocking molecules that have been overlooked by other screening campaigns.

Malaria is a disease of devastating economic and health burdens, with 216 million cases and 655,000 fatalities per year, among which most are either pregnant women or children of less than 5 years of age (1). The recent appreciation that local elimination and global eradication of malaria will require interventions that prevent parasite transmission from the human host to the vector (2) has revitalized the search for transmission-blocking drugs (3–7). One target of such drugs is the gametocyte, which is the parasite stage uniquely responsible for *Plasmodium* transmission to the mosquito.

Plasmodium asexual parasites form gametocytes at a low frequency (0.2 to 1%) (8), with sexually committed merozoites from one precommitted schizont all forming gametocytes of the same sex (9). In *Plasmodium falciparum*, gametocytes develop over a period of 12 days, during which they are initially susceptible to schizonticidal antimalarials (stages I to III), but for the final part of their maturation process (stages IV and V), they become broadly insensitive to most antimalarial drugs, except for primaquine and methylene blue (6, 10–13). Mature stage V gametocytes, when considered as a single population, are developmentally arrested, and current evidence suggests that they are minimally metabolically active (14). This greatly reduced metabolism likely accounts for their insensitivity to most schizonticidal drugs (10, 11) and suggests that they have reduced targetable biology for drug discovery. Although they are quiescent, mature gametocytes are "primed" for rapid and intricate development into gametes upon sensing a change in environment when taken up into the mosquito during a blood feed. In the gut of the mosquito, gamete formation is induced by a decrease in temperature and the presence of the gametocyte-activating factor xanthurenic acid (15). The process of male gamete formation and emergence from the red blood cell is called "exflagellation" and involves 3 rounds of DNA replication (16, 17), as well as axoneme assembly (18). This all occurs within ~20 min and results in the release of up to eight motile male gametes from each male gametocyte. Female gamete formation is less dramatic but involves activation and the translation of many transcripts which, up until this point, have been held under translational repression by the DDX6-class RNA helicase DOZI, as well as emergence from the red blood cell (19). Male gametes contact female gametes through cell-cell adhesion mediated by Pfs48/45-P230 (20), and membrane fusion occurs in a HAP2-dependent process to commence fertilization and ensure onward development (21).

A number of potential screening assays have been reported in the literature as showing the ability to identify compounds that affect "late-stage" *P. falciparum* gametocytes, as reported by the expression of late-gametocyte-stage-specific reporter-driven transgenic parasites or metabolic ATP readouts (5–7). However, these assays report only on the number of reporter-expressing cells present or whether a gametocyte is metabolically active or

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inactive and do not additionally confirm precisely the maturity or functional viability of the gametocytes they assess. We contend that a more accurate readout for mature gametocyte viability is onward gamete formation, as only viable, mature stage V gametocytes are capable of forming gametes. A further confounding factor to "late-stage" gametocyte assays is that it has been reported that both in the laboratory and under field conditions, *P. falciparum* displays a female-biased sex ratio of approximately one male gametocyte to every three to five females (22, 23). Current gametocyte screening assays do not give a readout on the sex specificity of compound action. Therefore, it is highly likely that interventions specifically targeting the smaller population of male gametocytes may not be considered significant. We have standardized two gamete formation assays and show that male and female gametocytes respond differently to a range of current antimalarials. This finding is of critical importance to how the research community evaluates screening data from existing gametocyte assays. We are confident that these assays are amenable to scaling up and that they will be able to efficiently identify the most promising gametocyte-targeted antimalarial compounds.

MATERIALS AND METHODS

Gametocyte production. Asexual cultures of *P. falciparum* 3D7 parasites showing the ability to develop gametocytes and the capacity to be transmitted to mosquitoes were used to seed gametocyte cultures at 1% parasitemia and 4% hematocrit in a 10-ml total volume under 3% O_2–5% CO_2–92% N_2 gas. Culture medium (RPMI medium with 25 mM HEPES, 50 mg/liter hypoxanthine, 2 g/liter sodium bicarbonate, 10% human serum) was replaced daily for at least 14 days, with all medium, pipettes, and work surfaces heated to 37°C. Under these conditions, cultures follow a very reproducible progression, with asexual parasitemia rising to a peak and crashing at days 4 to 5, stage II gametocytes visible at day 7, stage III visible at day 9, stage IV visible at day 11, and stage V, with high levels of exflagellation, visible at day 14.

Exflagellation assay. On day 14 of culture, exflagellation was tested by withdrawing a 200-μl sample of gametocyte culture and briefly centrifuging the cells. The cell pellet was resuspended in 10 μl ookinete medium (RPMI medium with 25 mM HEPES, 50 mg/liter hypoxanthine, 2 g/liter sodium bicarbonate, 100 μM xanthurenic acid, 20% human serum) and then introduced into a chamber of a FastRead disposable hemocytometer slide (Immune Systems). After ~20 min, exflagellation was observed at a magnification of ×10, and the culture was deemed suitable for the assay if at least 30 exflagellation centers per field were counted in a preparation in which, following dilution, the erythrocytes formed a tight monolayer. To set up the assay, sterile 1.5-ml tubes containing 150 μl culture medium and compound to be tested (dissolved in dimethyl sulfoxide [DMSO] to a maximum assay DMSO concentration of 0.5%) were prewarmed to 37°C in a heater block. Once the cells of the gametocyte culture had settled on the bottom of the flask, half the gametocyte culture medium was then removed to concentrate the cells, which were then resuspended in the remaining medium. Fifty microliters of the cell suspension was quickly dispensed into each assay tube. Tubes were then quickly gassed (3% O_2–5% CO_2–92% N_2), sealed, and placed into a 37°C incubator. After 24 h, tubes were removed in small groups of no more than 7 and assessed in the exflagellation assay. The assay was performed with small batches, as exflagellation is a time-dependent event.

Female gametocyte activation assay. Gametocyte cultures were produced as described above, with the exception that day 16 gametocyte cultures were used. The reason for this is that, reportedly, female gametocytes mature slightly later than male gametocytes in culture (24). An anti-Pfs25 antibody (4B7; obtained from MR4) was coupled to Cy3 by use of an Amersham CyDye monoclonal antibody labeling kit (GE Healthcare) according to the manufacturer's recommendations. The labeled antibody was used at a 1:500 dilution of a 0.5-mg/ml IgG stock solution in the assay.

RESULTS

Semiautomated quantification of male gametocyte exflagellation. Male gametes are highly motile cells that are readily identified by light microscopy as they emerge from the infected erythrocyte. In a monolayer of erythrocytes, exflagellation locally disturbs surrounding cells while leaving distant cells motionless. We hypothesized that this could be exploited to form a simple criterion for computer-based identification and quantification of exflagellation centers at low magnification. Time-lapse movies of exflagellating cells in erythrocyte monolayers were captured at 4 frames per second for 5 s (Fig. 1A). An algorithm which evaluates each frame in turn and compares it to the preceding frame was written in the image manipulation software ImageJ. Pixel information that remained constant between the adjacent frames (i.e., motionless cells) was removed, while pixel information that was different between the two frames (i.e., cells disturbed by an exflagellation center) was retained. By combining the retained pixels for all 20 frames, it was possible to generate an image highlighting only exflagellation centers (Fig. 1B). After applying standard blurring and thresholding parameters to the processed image (Fig. 1C), it was then possible to enumerate exflagellation centers by using a standard particle counter function from within ImageJ.

Establishment of the exflagellation assay. Exflagellation is a highly time-dependent process (Fig. 1D), with *P. falciparum* male gametes reported to emerge from the infected erythrocyte approximately 15 to 20 min after induction of exflagellation (25). Upon emergence, they then migrate away from the gametocyte residual body within the exflagellation center by flagellar locomotion and continue moving until they either reach and fertilize a female gamete or expend their energy resources and become immobile (26). To ensure an accurate and comparable quantitation of exflagellation between different samples and experiments, firstly the time point giving maximal exflagellation and secondly a window of linearity were established. Samples of day 14 mature stage V gametocytes from cultures already showing the ability to give high levels of exflagellation were taken and divided into 200-μl "microcultures" containing culture medium including 0.5% DMSO at 37°C to replicate conditions required for a drug screening assay. After 24 h of incubation, the microcultures were analyzed in turn. Exflagellation was induced as described in Materials and Methods. The concentrated culture was then transferred to a well of a disposable counting chamber and allowed to settle and form a tight monolayer. A single field of view was selected at random, and time-lapse movies were taken every minute from 10 to 50 min postactivation, using a 10× objective. The whole time-lapse series was then analyzed by the exflagellation counting algorithm, and the number of identified exflagellation centers detected over time was calculated. For each of three independent cultures, this was repeated with five samples of the same culture, and the data were normalized to account for differing absolute exflagellation levels between cultures (13 to 50 exflagellation centers observed per field) before being compared (Fig. 1D). Observed exflagellation levels were always found to be lower than that of the parent culture used to set up the assay, suggesting that manipulation of the parasites reduced their viability, although no prematurely activated parasites were observed. Even though the maximal number of exflagellation centers recorded varied from culture to culture

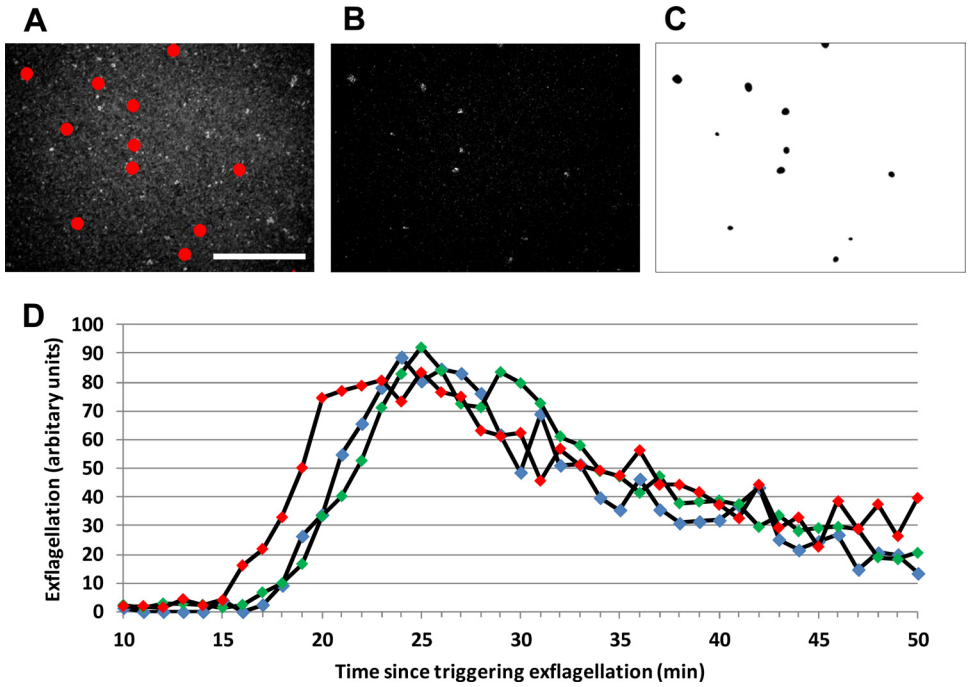

FIG 1 Exflagellation can be detected and quantified by computer-aided identification of moving exflagellation centers. Exflagellation of day 14 gametocyte cultures was triggered by incubation with ookinete medium containing 100 μM xanthurenic acid and a temperature decrease to room temperature for 20 min in a disposable hemocytometer. (A) Under these conditions, exflagellation centers can be identified manually and clearly as clusters of vigorous movement (highlighted by red circles) by using bright-field illumination and a 10× objective. Bar = 400 μm. (B) By recording a 4-s, 20-frame time-lapse image, subtracting each frame from the previous, and then combining the subtracted images, it is possible to identify exflagellation centers as intense white dots (regions of movement) on a black background (regions that are still). (C) Applying standard image processing to images permits exflagellation centers to be identified and counted. (D) By imaging the same field of view over time, the progression of exflagellation was recorded for three independent cultures (data shown in red, green, and blue), with each culture being sampled five times. Exflagellation progressed with a reproducible pattern, first being observed at 16 to 18 min posttriggering and reaching a maximum at 24 to 25 min. It then immediately diminished, with a half-life of ~12 min, until 40 min posttriggering, after which exflagellation reduced by an inconsistent rate.

(likely a product of culture gametocytemia and variations in the density of the erythrocyte monolayer between preparations), under the defined conditions of the assay the kinetics of exflagellation were strikingly similar. At the earliest time point recorded, 10 min postinduction, no exflagellation centers were detected. Depending on the culture, the first exflagellation centers were detected between 16 and 18 min postinduction and rapidly peaked thereafter, at 24 to 25 min postinduction. Upon reaching maximal activity, exflagellation levels almost immediately began to decrease, in a linear fashion, from 25 to 40 min, with an approximate half-life of 12 min. Thereafter, reductions in exflagellation/movement were unpredictable. Because maximum exflagellation activity was found not to be sustained, we decided to standardize the observations for the assay by recording data for 22 to 27 min postinduction, as this represents the region in which exflagellation was 80 to 100% of the maximum. By selecting this 5-min time window for standard analyses, some reduction of resolution of the assay was accepted for the opportunity to record a greater number of observations within a sample. However, the assay resolution remained sufficient to construct dose-response curves and calculate 50% inhibitory concentrations (IC_{50}s) (see Fig. S1A in the supplemental material).

Establishment of the female gametocyte activation assay. Female gamete formation requires the translation of DOZI-repressed mRNAs that are stored within the female gametocyte. *pfs25* is one such well-characterized translationally repressed gene (19), and the Pfs25 protein is an important transmission-blocking vaccine candidate (27). We hypothesized that Pfs25 expression on the surface of the plasma membrane would be a suitable marker for female gamete formation, as the related and similarly expressed P28 protein has been used for the same purpose in *P. berghei* (28). Gametocyte cultures were taken, activated by a temperature decrease and the addition of ookinete medium at a 1:20 dilution, and incubated at 27°C. After 2 h, a sample of the culture was removed and stained live with an anti-Pfs25–Cy3 antibody for 30 min before being observed by fluorescence microscopy. At this time point, cell surface staining could be observed faintly with a 100× objective for a subset of round cells—the activated female gametes (Fig. 2A). The culture was further sampled at 6 and 24 h postactivation, and staining was observed to increase markedly in intensity (Fig. 2A), indicating protein accumulation of Pfs25 over time. At the 24-h time point, fluorescence staining was intense enough to be observed using a 10× objective (Fig. 2B), and under the experimental conditions described, most Pfs25-positive cells were "round forms," with occasional retort forms observed. Due to the enhanced detection of activated gametes at low magnification (and hence more cells observed), 24 h was selected as the endpoint to measure drug effects on female gametocyte activation. An elevated intensity of Pfs25 expression was not linked to female gamete fertilization, as evidenced by the selective activity of antifolates (Table 1; see below). To aid in data analysis, a simple ImageJ algorithm was constructed to identify and count the fluo-

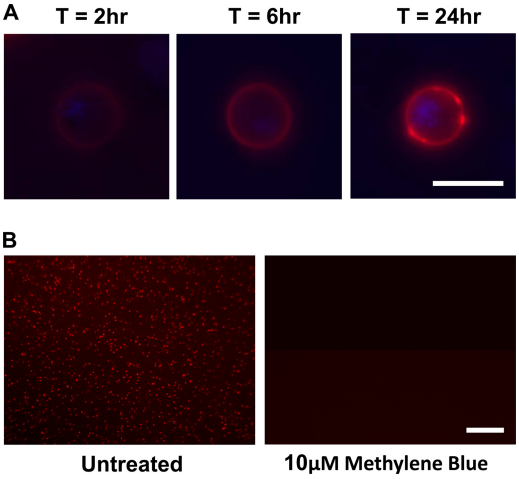

FIG 2 Female gamete production can be detected by surface Pfs25 expression. Activation of day 16 gametocyte cultures was triggered by addition of ookinete medium containing 100 μM xanthurenic acid to a final 1:20 dilution and a temperature decrease to room temperature. (A) The activated culture was sampled at 2, 6, and 24 h posttriggering and stained 1:200 with anti-Pfs25–Cy3 antibody (red) for 30 min. Cell nuclei were then counterstained with Hoechst dye (blue) and then visualized with a 100× objective. With increasing activation time, Pfs25 staining became progressively brighter. Bar = 10 μm. (B) At 24 h posttriggering, Pfs25 staining was intense enough to visualize activated female gametes in an untreated sample with a 10× objective, while no staining was observed in a sample treated with 10 μM methylene blue. Bar = 200 μm.

rescent cells in an automated fashion, using the particle counter function. As with the male assay, data resolution in the female assay was sufficient for dose-response analysis (Fig. S1B).

Antimalarial drug action against male and female gametogenesis. A panel of 20 current antimalarials were tested at a concentration of 1 μM in both the exflagellation and female gametocyte activation assays (Table 1). Both assays were performed in triplicate, with independent cultures for each replicate. For both assays, mature gametocytes were preincubated with compounds for 24 h before induction of gamete formation without removal of the compound. Therefore, both assays could detect compounds that killed the mature gametocyte, interfered directly during the process of gamete formation, or "sterilized" the mature gametocyte in such a way that it was metabolically viable but unable to form gametes.

The mean % inhibition values for male exflagellation and female activation for each compound were compared (Fig. 3). In support of previously published data (3), we observed that the endoperoxides artemisinin, artesunate, and dihydroartemisinin (DHA) inhibited exflagellation by 62.00 to 80.27% at 1 μM (Table 1). Another endoperoxide, artemether, showed less activity, with only 45.65% inhibition, which could possibly be accounted for by its poor solubility in aqueous solution compared to the other endoperoxides tested (29). We also confirmed the activities of the antifolate pyrimethamine and the antibiotic thiostrepton as antimalarials that inhibit exflagellation (3), giving 92.53% and 93.53% inhibition, respectively. In addition, we observed that another antifolate, cycloguanil, inhibited exflagellation by 96.57%. Methylene blue has been reported to kill gametocytes of all stages of development (6); although it has not been reported whether this effect is sex specific or sex independent, methylene blue at 1 μM inhibits exflagellation by 98.39%. While the above antimalarials showed strong activity against male gametocytes and exflagellation, we failed to identify any antimalarial in the screen that inhibited female gamete formation by more than 26.62% (Table 1). Given the fact that the cells in the female assay were exposed to the drugs for almost twice as long (~48 h for females versus ~24 h for males), this may reflect differences in underlying cell biology. In discovering that female gametocytes and female gamete formation appear to be less susceptible to antimalarials than male gametocytes and the process of exflagellation, the drugs were retested in the female assay at 10 μM in an attempt to detect any with weaker activity. At this concentration, only pyronaridine, thiostrepton, and methylene blue gave strong inhibition of female gamete formation (93.51%, 97.02%, and 98.13% inhibition, respectively). IC_{50}s in both assays were then determined by dose-response analysis of compounds that showed activity in either assay (Table 2).

Only pyronaridine demonstrated similar potencies in both the male and female assays (IC_{50}s of 2.7 μM and 1.8 μM, respectively), suggesting that it targets a biological pathway(s) common to gametocytes of both sexes. Both methylene blue and thiostrepton were strikingly more potent in the exflagellation assay than in the female gametocyte activation assay (4.7 and 14.9 times more potent, respectively). Interestingly, the antifolates pyrimethamine and cycloguanil potently inhibited exflagellation, with IC_{50}s of 8.7 and 124 nM, respectively, but showed essentially no activity in the female assay at 10 μM. With the exception of the slight activity of artemether, the endoperoxides that were active in the exflagellation assay revealed much less potent IC_{50}s (39 to 138 times less) than their reported IC_{50}s for killing asexual parasites (NF54 strain [3]). Nevertheless, the inhibition detected occurred at physiologically achievable concentrations (30, 31).

TABLE 1 Inhibition achieved by 20 antimalarial drugs in male and female gamete assays compared to untreated controls[a]

Drug	Male gametocyte exflagellation at 1 μM		Female gametocyte activation at 1 μM		Female gametocyte activation at 10 μM	
	Mean % inhibition	SEM	Mean % inhibition	SEM	Mean % inhibition	SEM
Amodiaquine*	40.37	10.68	−1.42	1.14	27.25	4.50
Artemether**	45.65	6.78	1.16	4.71	9.97	11.48
Dihydroartemisinin***	80.27	5.87	5.63	2.85	9.36	4.26
Artesunate**	66.95	9.19	6.81	5.56	10.30	2.30
Artemisinin***	62.00	0.43	−5.74	4.02	11.44	8.23
Azithromycin*	39.78	11.87	−7.29	5.09	12.95	6.92
Doxycycline^{NS}	32.10	11.59	4.09	8.37	45.41	3.18
Halofantrine*	22.15	3.12	−5.62	7.46	−0.89	9.63
Mefloquine (racemic)*	51.45	10.37	0.31	11.13	34.20	9.21
Mefloquine (+RS)*	38.22	7.34	0.92	8.56	30.57	4.65
Pamaquine^{NS}	43.90	9.50	−4.62	10.19	12.36	6.68
Primaquine^{NS}	14.55	9.94	−7.64	4.24	17.77	11.22
Pyrimethamine***	93.53	3.93	−7.72	4.90	7.43	6.52
Pyronaridine^{NS}	14.03	6.64	26.62	14.04	95.56	1.17
Sulfadiazine^{NS}	−13.21	28.86	1.20	11.72	2.65	6.93
Thiostrepton*	92.53	4.95	23.36	22.91	97.85	0.46
Riboflavin^{NS}	15.28	30.82	2.07	12.09	−0.17	8.84
Atovaquone^{NS}	12.02	3.74	12.31	5.36	28.05	11.33
Cycloguanil***	96.57	1.74	−0.76	9.53	17.63	5.27
Methylene blue***	98.39	1.61	10.69	3.92	95.82	1.99

[a] Data are mean % inhibition values with respect to DMSO carrier controls ($n = 3$) ± standard errors of the means (SEM). Asterisks beside compound names indicate the statistical significance of differences in % inhibition at 1 μM in both male and female assays as measured by unpaired Student's t test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, no significant difference.

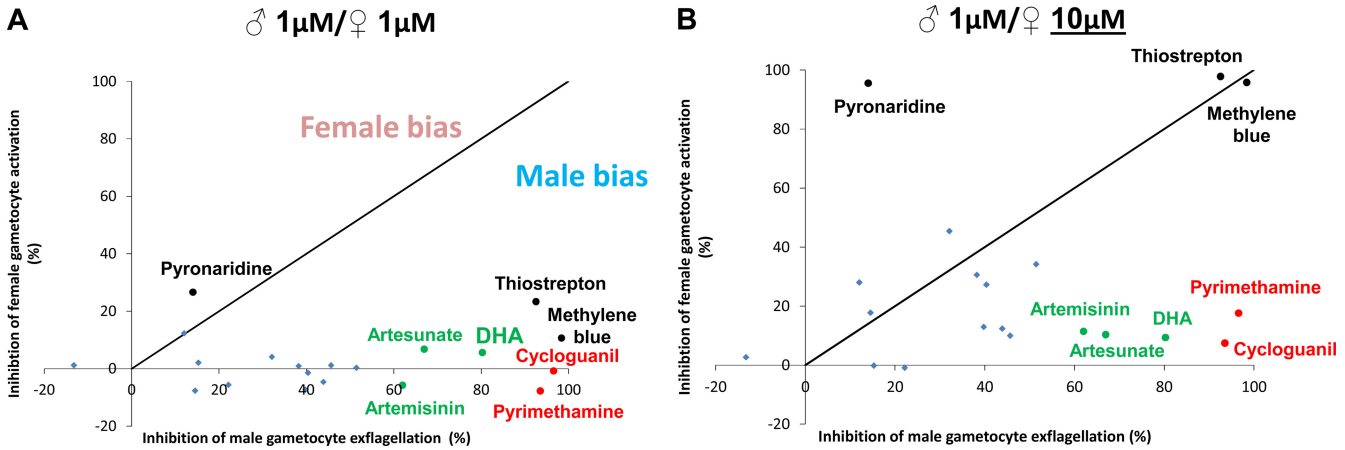

FIG 3 Response of a panel of antimalarial drugs in both male and female assays. (A) Twenty antimalarial drugs were evaluated at 1 μM in the male and female assays, in triplicate experiments (see compounds evaluated and corresponding percentages of inhibition in Table 1). Drug activities in both assays were compared by plotting male activity along the *x* axis and female activity along the *y* axis. At 1 μM, no drug showed appreciable activity in the female assay, while thiostrepton, methylene blue, pyrimethamine, cycloguanil, artesunate, artemisinin, and DHA all showed activity in the male assay. (B) The 20 antimalarial drugs were retested at 10 μM in the female assay and compared again to the 1 μM male assay data. At 10 μM, only pyronaridine, thiostrepton, and methylene blue showed activity.

DISCUSSION

The goal of eradication of malaria requires the development of effective transmission-blocking therapies (2). Here we present two assays for separate evaluations of the functional maturity and viability of male and female gametocytes as reported by their ability to undergo gamete formation. To maximize the parasite biology targeted by both assays, mature gametocytes were preincubated with compounds of interest for 24 h before gamete formation was induced. Therefore, both assays identified compounds that act upon the mature gametocyte directly and also those that inhibit processes unique to gamete formation (14). Using a panel of established antimalarials, we show for the first time that mature male and female gametocytes respond very differently to current antimalarials. Our observations are consistent with the hypothesis that mature male gametocytes are sensitive to a wider range of drugs than is the case for mature female gametocytes.

Mature gametocytes of both sexes are in cell cycle arrest and are effectively quiescent in the peripheral blood, awaiting triggers to induce gamete formation caused by the change in environment upon uptake in the mosquito blood meal (14). Therefore, by the time a gametocyte has become fully mature and infectious, its metabolic activity is likely reduced to "housekeeping" functions such as ATP production (5) and general redox activity (7). During male gametocyte development, the cell prepares for gamete formation by reducing the endoplasmic reticulum and ribosome content, enlarging its nucleus, and aggregating kinetochores close to a microtubule organizing center in preparation for rapid mitosis during exflagellation (32). Upon induction of exflagellation, the mature male gametocyte undergoes explosive development. Within 15 to 20 min, it has performed *de novo* synthesis of DNA (17), undergone three rounds of mitosis, assembled the component parts of eight axonemes, and escaped the red blood cell (33). While occurring rapidly, all of these processes must be choreographed carefully for exflagellation to be successful (16). Perhaps herein lies the sensitivity of the male gametocyte to perturbation by antimalarial drug action. It is logical to deduce that the male-specific activity of the antifolates tested (pyrimethamine and cycloguanil) is due to their inhibition of the folate-mediated pyrimidine synthesis required for DNA replication during exflagellation. Female gametogenesis does not require DNA synthesis, which would account for the lack of activity of antifolates in the female assay.

The specific activity of the endoperoxides in the male assay only, with no clear female activity, is a surprising finding. The mode of action of endoperoxides is thought to require heme-mediated activation of the endoperoxide bridge, generating cytotoxic metabolites that cause oxidative damage to the cell (34, 35). An alternative hypothesis that has been suggested is that endoperoxides interfere with calcium homeostasis within the parasite by a direct interaction with PfATP6 (36). When mature, gametocytes of both sexes have metabolized the erythrocyte hemoglobin, which is the likely causative agent underpinning the high sensitivity of asexual and early gametocytes to endoperoxides. Endoperoxide activity against mature male gametocytes could therefore represent a residual male-specific source of heme, an alternative mode of action, a differential in the uptake of endoperoxides favoring uptake into mature male gametocytes over mature female gametocytes, or simply a by-product of the relative fragility of the male gametocyte. Given the widespread use of artemisinin com-

TABLE 2 IC_{50}s of drugs showing activity in the assays compared to their reported IC_{50}s against asexual parasites[a]

Drug	IC_{50} (nM)		
	Male assay	Female assay	Asexual parasites
Pyronaridine	2,700	1,831	4.9
Methylene blue	201	941	3.62
Thiostrepton	96	1,427	387.7
Pyrimethamine	8.7	>10,000	17
Cycloguanil	124	>10,000	4.5
Artemisinin	224	>10,000	5.8
Dihydroartemisinin	85	>10,000	1.1
Artesunate	483	>10,000	3.5

[a] Asexual data were all obtained from NF54 parasites as reported by Delves et al. (3), except the IC_{50} of methylene blue, which represents the geometric mean for 23 *P. falciparum* isolates as reported by Pascual et al. (44).

bination therapies (ACTs) around the world for treating malaria, further study should be given to these findings to determine if they are clinically relevant. To date, field data suggest that ACTs have only a moderate impact on transmission, which is caused by reductions in the time of gametocyte carriage and in the proportion of infected mosquitoes after feeding on an infected individual (37). While reductions in gametocyte carriage by endoperoxides are likely caused by the downstream effects of the elimination of asexual parasitemia and early-stage gametocytes from the afflicted individual, our data suggest that it may be plausible that the immediate reduction in the proportion of infected mosquitoes is caused by direct endoperoxide-mediated inhibition of exflagellation.

During female gametocyte development, the primary role of the female gametocyte is to prepare for fertilization and onward mosquito development. Approximately 370 transcripts are held in translational repression controlled by DOZI, awaiting female gamete formation and fertilization (19). Therefore, upon induction, a female gamete must egress from the red blood cell, withdraw translational repression, and commence translation of the repressed mRNA transcripts. It would seem, then, within the biological range investigated by the female activation assay, that the only (currently known) targetable biological pathways that are active are general housekeeping functions, cell egress, and translation. This perhaps does not provide a rich resource of targets for antimalarial drug discovery, as evidenced by the few active compounds identified in the panel, their low potencies compared to those for both exflagellation and asexual parasites, and the absence of female-specific compounds.

Methylene blue has been reported to kill gametocytes potently, with an IC_{50} of 490 nM against "late-stage" gametocytes (5, 6, 38), which is comparable to the 201 nM male and 941 nM female IC_{50}s we determined in our assays (Table 2). The mode of action of methylene blue against *Plasmodium* is not fully characterized, but it is thought to inhibit the activity of the cellular antioxidant glutathione reductase (39). It has not been reported whether male gametocytes are more sensitive to oxidative damage than female gametocytes; however, this hypothesis could be supported by our observation that endoperoxides were inhibitory in the exflagellation assay and not in the female activation assay (Table 1). Thiostrepton is an antimalarial antibiotic targeting apicoplast protein synthesis (40) and the *Plasmodium* proteasome, and it has been reported to be active against early-stage gametocytes (41). Here we showed activity against both male and female gametocytes, as expected for a compound targeting such universal biological pathways. Interestingly, the male gametocyte is 15 times more sensitive to thiostrepton than the female (and 4 times more sensitive than asexual parasites [3]), even though the apicoplast remains in the residual body of the gametocyte and is not present in the newly formed male gametes (42). The role of the proteasome in exflagellation has never been investigated; however, another proteasome inhibitor, epoxomycin, has been shown to have potent activity against "late-stage" gametocytes (5). Therefore, it seems likely that thiostrepton acts directly upon the mature gametocyte and that male gametocytes are more sensitive. The 4-aminoquinoline pyronaridine was found to be similarly active against both male and female gametocytes in our assays. The low micromolar IC_{50}s agree well with the reported IC_{50} of 3.25 μM against "late-stage" gametocytes (5). This potency is much lower than the reported IC_{50} of 4.9 nM against NF54 asexual parasites (3) and is close to the reported cytotoxic level against HepG2 cells (5), suggesting that the activity of pyronaridine in both assays is not clinically relevant.

Screening for *Plasmodium* transmission-blocking drugs is both time-consuming and very expensive. Consequently, any drug screening assays developed must be sensitive enough to interrogate the maximum amount of clinically relevant cell biology and extract as much useful information as possible. Here we present evidence that the male gametocyte is likely a "richer" target for drug discovery than the female. Given the high female bias of gametocyte sex ratios both in laboratory strains and in the field, previous gametocyte drug screening assays without a sex-specific readout may have failed to recognize a significant proportion of potential transmission-blocking compounds that specifically target the male (43), because global inhibition of gametocytes would not have fallen within levels considered "significant." In the current absence of a *P. falciparum* drug screening assay with dual sex-specific readouts or an integrated reporter (i.e., fertilization or ookinete formation), we conclude that transmission-stage drug screening campaigns would be more efficient if they were focused upon the mature male gametocyte. Development of a high-throughput male gamete formation assay or a dual male-female reporter assay should be prioritized.

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