



Gametocytes from K13 Propeller Mutant *Plasmodium falciparum* Clinical Isolates Demonstrate Reduced Susceptibility to Dihydroartemisinin in the Male Gamete Exflagellation Inhibition Assay

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ABSTRACT Mutations in the kelch propeller domain (K13 propeller) of *Plasmodium falciparum* parasites from Southeast Asia are associated with reduced susceptibility to artemisinin. We exposed *in vitro*-cultured stage V gametocytes from Cambodian K13 propeller mutant parasites to dihydroartemisinin and evaluated the inhibition of male gamete formation in an *in vitro* exflagellation inhibition assay (EIA). Gametocytes with the R539T and C580Y K13 propeller alleles were less susceptible to dihydroartemisinin and had significantly higher 50% inhibitory concentrations (IC₅₀s) than did gametocytes with wild-type alleles.

KEYWORDS DHA, antimalarial agents, artemisinin, exflagellation, gametocytes

Artemisinin-based combination therapies (ACTs) are recommended as a first-line treatment for uncomplicated *Plasmodium falciparum* malaria (1). ACTs comprise a fast-acting artemisinin derivative and a long-acting partner drug (e.g., lumefantrine, mefloquine, and piperaquine) and are key contributors to the recent successes of global malaria control (1). However, reduced susceptibility to artemisinin (as measured by a slow parasite clearance rate) and clinical failure of ACTs (recrudescences) have recently been reported from the Greater Mekong Subregion (2–5). A novel *in vitro* ring-stage survival assay (RSA), in which 0- to 3-h ring-stage parasites from culture-adapted isolates from Cambodian patients are exposed to a pharmacologically relevant dose of dihydroartemisinin (DHA) (700 nM for 6 h), showed increased survival in parasites from slow-clearing versus fast-clearing infections (6). Reduced susceptibility to artemisinin was associated with single nucleotide polymorphisms in the propeller domain of a *P. falciparum* kelch protein (K13 propeller) (7). Cambodian isolates with the C580Y, Y493H, and R539T K13 propeller mutations were associated with slow parasite clearance rates and higher RSA survival rates (7–9).

Mature sexual-stage parasites (gametocytes) in peripheral blood are necessary for transmission of *Plasmodium* spp. to mosquitoes. Artemisinins are effective against immature gametocytes (10, 11) but show reduced efficacy against mature gametocytes (12), which can persist for weeks after treatment and thus contribute to onward transmission (13, 14). The ability of gametocytes from K13 propeller mutant parasites to withstand artemisinin exposure and remain functionally viable in patients following ACT could additionally impact transmission dynamics in the Greater Mekong Subregion. Few studies have characterized gametocytes from fast- and slow-clearing Cambodian isolates. Ashley et al. (4) reported a higher incidence of gametocytemia in patients with slow-clearing K13 propeller mutant parasites. St. Laurent et al. (15)

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TABLE 1 Cambodian *P. falciparum* clinical isolates used in this study

Isolate	Parasite clearance half-life (h) ^a	K13 propeller allele ^b
959	4.96	WT
937	5.58	WT
845	8.05	C580Y
968	7.97	C580Y
957	6.87	R539T
851	6.72	R539T

^aParasite clearance half-life was calculated based on posttreatment 6-hourly parasite density values, using the parasite clearance estimator developed by the Worldwide Antimalarial Resistance Network (17).

^bK13 propeller allele was determined from whole-genome sequence data (8).

showed that *in vitro*-cultured gametocytes from these mutant parasites could infect highly diverse *Anopheles* species from Southeast Asia and Africa.

We sought to explore the effect of DHA on *in vitro*-cultured mature stage V gametocytes from K13 propeller mutant and wild-type (WT) Cambodian clinical isolates in an exflagellation inhibition assay (EIA). The EIA is based on the ability of functionally viable mature male gametocytes to undergo a time- and temperature-dependent release of exflagellating male gametes, and it measures the ability of a compound to inhibit the number of exflagellation centers per microscopic field in drug-treated gametocyte cultures compared to dimethyl sulfoxide (DMSO)-treated controls. We recently demonstrated the ability of this assay to predict the transmission-blocking behavior of antimalarials in mosquitoes infected with *P. falciparum* in the standard membrane feeding assay (16). In the present study, we compared the DHA susceptibilities of mature gametocytes from slow- and fast-clearing Cambodian isolates (17) (Table 1) by estimating their 50% inhibitory concentration (IC₅₀) in EIA. (All methods in this study were carried out in accordance with GlaxoSmithKline guidelines and regulations. Human red blood cells were obtained from Biobank of Castilla y León, BST, and the Centro de Transfusiones de Madrid, and the relevant ethics committee approvals were obtained from Autonomous University of Madrid [CEI-45-890] to enable the use of these samples from blood donors. All use of human biological samples in this study was in accord with the terms of the informed consents given by the sample donors.)

Gametocyte cultures were generated as described previously (16). Five-milliliter cultures containing 1% to 3% stage V gametocytes were incubated with increasing concentrations of DHA for 6 h (short exposure) or 48 h (long exposure). Controls comprising gametocytes exposed to the same final concentration of DMSO (0.1%) were processed in parallel. After the 6-h incubation, short-exposure cultures were centrifuged at 500 × *g* for 5 min, and the supernatant with the remaining DHA was removed by aspiration, replenished with fresh medium, and further incubated for a total duration of 48 h. Percent exflagellation inhibition (EI) was determined at 6 and 48 h. DHA was not removed for long-exposure treatments, and the EI was measured at 48 h. For EI determination, 100 μl of DHA-exposed and DMSO-exposed cultures were centrifuged at 500 × *g* for 30 s, and the packed cells were resuspended in 15 μl of prewarmed (37°C) medium, introduced into a hemocytometer, and incubated at room temperature (16). Movement of exflagellation centers was recorded between minutes 18 and 22 by video microscopy and quantified using a semiautomated method, and the EI was calculated for each treatment as previously described (16, 18), using the equation EI = [(E_C - E_T)/E_C] × 100, where E_C and E_T are the number of exflagellation centers per field in the control and the DHA-treated sample, respectively. EI versus DHA concentration was fitted to a normalized sigmoidal dose-response curve (19) with two parameters (log 50% inhibitory concentration [logIC₅₀] and Hill slope). The *F* test performed on the sum of squares to compare fits and implemented in GraphPad Prism version 6.07 (19) was used to compute statistical significance, comparing the estimated (best-fit) logIC₅₀ parameter for isolates with the R539T and C580Y versus WT alleles.

With two isolates per allelic group, the DHA IC₅₀ for gametocytes from K13 propeller mutant isolates was significantly higher than that for the WT isolates (Fig. 1). Inhibition

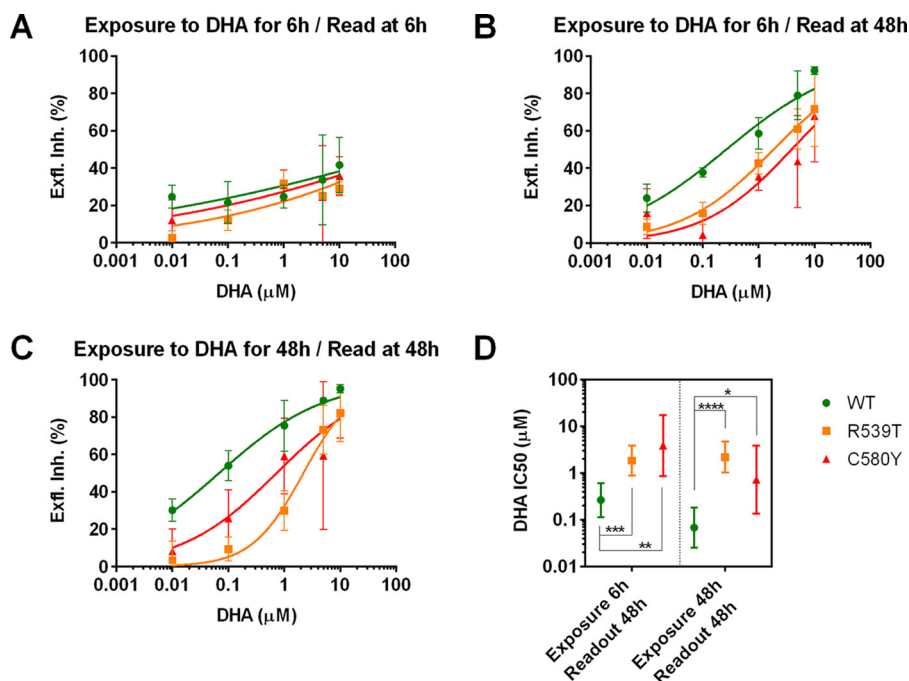


FIG 1 Dose response of exflagellation inhibition (Exfl. Inh.) (%) relative to control versus DHA concentration grouped by K13 propeller allele (WT in green, R539T in orange, and C580Y in red) for short exposure (6 h) and read at 6 h (A) or 48 h (B) and for long exposure (48 h) read at 48 h (C). The points represent the mean of the measured values, the error bars represent the standard error of the means, and the colored lines represent the corresponding best fit curves (normalized sigmoidal function with two parameters). (D) EI IC_{50} of WT, R539T, and C580Y isolates for different exposures. The bars represent the 95% confidence interval for each estimate. For each treatment, the numbers of independent replicates per isolate were as follows: isolate 959, 3; isolate 937, 2; isolate 845, 2; isolate 968, 2; isolate 957, 2; and isolate 851, 7. *, $P \leq 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

of male gamete formation by short exposure of gametocytes to DHA did not permit EI IC_{50} estimation at 6 h in the dose range used (Fig. 1A). However, when the same 6-h DHA-exposed gametocytes were subjected to EIA at 48 h, the IC_{50} could be estimated (Fig. 1B), suggesting that short exposure to DHA results in a delayed impairment of their ability to form male gametes. The IC_{50} s of R539T and C580Y gametocytes were significantly higher than that of the WT, showing that gametocytes from K13 propeller mutant slow-clearing isolates were better able to tolerate DHA exposure than were the WT fast-clearing isolates (Fig. 1D). Given the short plasma half-life of DHA, the short exposure time of 6 h mimics *in vivo* conditions, as it does in the RSA. We explored the effect of a long exposure time of 48 h in the EIA (Fig. 1C), which again showed significantly higher IC_{50} s for the K13 propeller mutants than for the WT gametocytes. These results indicate that measuring the EI at 48 h in both short and long exposures can differentiate between gametocyte susceptibility profiles to DHA. Long exposure to DHA resulted in ~ 4 -fold and 5-fold reductions in the IC_{50} s of WT (0.27 and 0.068 μM , respectively) and C580Y (3.9 and 0.73 μM , respectively) gametocytes, but the IC_{50} s of R539T gametocytes were similar (1.9 and 2.2 μM , respectively) regardless of the duration of exposure. C580Y gametocytes displayed high variability in EI between independent replicates, as seen by the 95% confidence intervals (Fig. 1D). Despite this variability, highly significant differences could be observed using two isolates per K13 propeller group (Table 1), with a minimum of two replicates per isolate. Importantly, gametocytes from K13 propeller mutant isolates formed male gametes after exposure to DHA concentrations much higher than the physiologically relevant dose (700 nM) (6), suggesting that these could have a fitness advantage over WT gametocytes that enables them to persist in peripheral blood and remain functionally viable despite artemisinin treatment. Phenotyping of additional isolates in the EIA and determining whether K13

propeller mutant gametocytes are better able to form oocysts and sporozoites in the mosquito will further complement these observations and hypotheses.

To our knowledge, this is the first study demonstrating the effect of DHA on gametocytes produced *in vitro* from culture-adapted K13 propeller mutant Cambodian isolates. We have successfully used EIA to show that gametocytes from K13 propeller mutant slow-clearing isolates were more resistant to DHA than gametocytes from WT fast-clearing isolates, thereby providing a reliable assay with the potential to screen for antimalarials against mature gametocytes from drug-resistant clinical isolates.

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